Astrocytes Increase the Functional Expression of P-Glycoprotein in an *In Vitro* Model of the Blood–Brain Barrier

Pieter Jaap Gaillard,[1] Inez Cornelia Johanna van der Sandt,[1] Levina Helena Voorwinden,[1] Dung Vu,[1] Jette Lyngholm Nielsen,[1] Albertus Gerrit de Boer,[1,2] and Douwe Durk Breimer[1]

Received May 2, 2000; accepted July 2, 2000

Purpose. To investigate the influence of astrocytes on P-glycoprotein (Pgp) expression and intracellular accumulation of Pgp substrates, separate from their net transcellular transport across the blood–brain barrier (BBB).

Methods. An *in vitro* BBB model was used, comprising of brain capillary endothelial cells (BCEC) monolayers or BCEC co-cultured with astrocytes.

Results. BCEC+astrocyte co-cultures seemed to express a higher level of Pgp compared to BCEC monolayers. Inhibition of Pgp results in an increased intracellular accumulation of Pgp substrates in both BCEC monolayers and BCEC+astrocyte co-cultures, and increased the sensitivity for vinblastine mediated disruption of the *in vitro* BBB (called the vinblastine exclusion assay). BCEC monolayers were more sensitive to vinblastine mediated disruption compared to BCEC+astrocyte co-cultures. In the latter, but not in BCEC monolayers, an inhibitable polar transport of Pgp substrates was only found from the brain to the blood side of the filter.

Conclusions. Astrocytes increase the functional expression of Pgp in our *in vitro* BBB model. These results also illustrate that an important role for Pgp on the BBB is to protect the barrier against intracellular accumulation of cytotoxic BBB disrupting compounds.

KEY WORDS: blood–brain barrier; brain capillary endothelial cells; astrocytes; P-glycoprotein; vinblastine exclusion assay; multidrug resistance.

INTRODUCTION

P-glycoprotein (Pgp) is considered to restrict the access of certain compounds to the central nervous system (CNS), thereby protecting the CNS against the entry and accumulation of potentially damaging Pgp substrates (1). At the cellular level, Pgp restricts the intracellular accumulation of Pgp substrates in brain capillary endothelial cells (BCEC), which constitute the blood–brain barrier (BBB) (2). From a pharmaceutical point of view, however, Pgp limits the CNS entry of Pgp substrate drugs, which may potentially be beneficial for the treatment of CNS related diseases (including brain tumors). For successful therapeutic drug treatment, it may be desirable for certain drugs to inhibit Pgp functionality with (non)-competitive inhibitors of Pgp. This may, however, interfere with the physiological role of Pgp expressed at the BBB (3).

In vitro models of the BBB, with a functionally expressed Pgp, facilitate screening studies for new Pgp substrates, Pgp inhibitors or Pgp inducers (4). Moreover, it allows studies on the physiological function of Pgp at the level of the BBB. Although astrocytes are known to induce and maintain BBB properties in BCEC (5), their relevance on the functionality and expression of Pgp on the BBB is still poorly described (3). Moreover, the role of astrocytes and Pgp on the BBB is currently under debate (6,7).

In our *in vitro* model of the BBB, astrocytes induce and maintain several of the BBB properties, while they do not express Pgp themselves at detectable levels (in press). However their influence on the functional expression of Pgp has not yet been determined. In this paper, the influence of astrocytes on the functional expression of Pgp on BCEC was determined with our *in vitro* model of the BBB. This was in particular related to specific assays for intracellular drug accumulation and transcellular drug transport. The functional expression of Pgp in the *in vitro* BBB model was compared to MDR1 (the multidrug resistance gene encoding for Pgp) transfected and control LLC-PK1 cells and to a clone (PD-7) of (the Pgp overexpressing) Caco-2 human intestinal epithelial tumor cell-line.

METHODS

Preparation of the *In Vitro* BBB Model

The preparation of the *in vitro* BBB model has been described before (in press). Briefly, brain capillaries were isolated from cortices of brains of bovine origin. Calf brains were obtained at the slaughterhouse (Molendijk B.V., Nieuwerkerk a/d IJssel, The Netherlands). The capillary fraction was obtained after homogenization, trapped on nylon meshes and subsequently enzymatically digested. Astrocytes were isolated from cortices of brains of newborn Wistar rats (Harlan B.V., Zeist, The Netherlands) and used to prepare astrocyte conditioned medium and co-cultures. Brain capillary endothelial cells (BCEC) were cultured from brain capillaries on collagen and fibronectin coated culture flasks in 50% astrocyte conditioned medium. BCEC were passaged on collagen coated Transwell polycarbonate filters (surface area: 1 cm^2 or 0.33 cm^2, pore-size: 0.4 μm, Corning Costar) and cultured to tight monolayers in 50% astrocyte conditioned medium in 4 days. For BCEC+astrocyte co-cultures, astrocytes were seeded on the bottom of the filters 2 days before BCEC. A schematic drawing of the BCEC+astrocyte co-culture model is depicted in Figure 1.

Assessment of Pgp Expression

Pgp expression was determined by Western blot analysis. Briefly, cell material was isolated from MDR1 transfected

[1] Department of Pharmacology, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, The Netherlands.

[2] To whom correspondence should be addressed at Department of Pharmacology, Leiden/Amsterdam Center for Drug Research, (LACDR), Leiden University, P.O. Box 9503, 2300 RA Leiden, The Netherlands. (e-mail: B.Boer@LACDR.LeidenUniv.NL)

ABBREVIATIONS: Pgp, P-glycoprotein; CNS, central nervous system; BCEC, brain capillary endothelial cells; BBB, blood–brain barrier; MDR, multidrug resistance; PSC, SDZ-PSC833; HPLC, high performance liquid chromatography; TEER, transendothelial electrical resistance; FLU, sodium fluorescein.

0724-8741/00/1000-1198$18.00/0

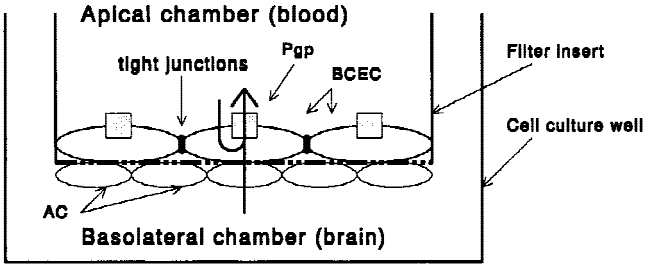

Fig. 1. Schematic drawing of the *in vitro* blood–brain barrier co-culture model. Abbreviations: Pgp (P-glycoprotein), BCEC (brain capillary endothelial cells) and AC (astrocytes).

and control LLC-PK1 cells, primary cultured newborn rat astrocytes, bovine brain capillaries, primary cultured bovine BCEC in plastic culture flasks, and BCEC isolated from filters with BCEC monolayers or BCEC+astrocyte co-cultures. Upon SDS-PAGE, the proteins were blotted on a nitrocellulose (PVDF) membrane (Immobilon-P, Millipore Corporation, Bedford, MA, USA). Specific immunoreactivity for Pgp was detected with C219 murine monoclonal antibody and visualized by chemiluminescence.

Assessment of Pgp Related Intracellular Drug Accumulation and Efflux

Accumulation and Release Assay

Pgp mediated drug efflux from BCEC was determined by the accumulation and release method. Briefly, confluent monolayers of BCEC, cultured in collagen coated plastic 24-wells cell culture plates, were loaded with rhodamine 123 (1 μM) for 3 h and washed 3 times with drug free DMEM. The accumulation was performed in the presence of the potent Pgp inhibitor SDZ-PSC833 (PSC; 0.5, 1 and 2 μM (8)) or vehicle (>1000 times dilution of PGEG, which is a mixture of 75% (v/v) polyethylene glycol 200, 10% (v/v) ethanol and 15% (v/v) 5% (w/v) D-glucose. The amount of released rhodamine 123 was determined by high performance liquid chromatography (HPLC) and expressed as a percentage (mean % ± S.D.) of the vehicle treated BCEC (9).

Vinblastine Exclusion Assay

Pgp mediated drug efflux from BCEC was determined by means of the sensitivity for vinblastine induced disruption of the *in vitro* BBB. In particular, vinblastine is a Pgp substrate that disrupts the cytoskeleton of a cell by binding intracellularly to microtubule ends (10). By these means, vinblastine disrupts the tight junctions between BCEC, because the functional units of the tight junctions (i.e. adhesion molecules like occludin and ZO-1, etc.) are functionally attached to cytoskeleton components (e.g. F-actin) (11). *In vitro* BBB disruption was monitored by transendothelial electrical resistance (TEER), which is a measure for the permeability of small ions through tight junctions between BCEC, by using an electrical resistance system (ERS) with a current-passing and voltage-measuring electrode (Millicell-ERS, Millipore Corporation, Bedford, MA, USA). TEER (Ωcm^2) was calculated from the displayed electrical resistance on the readout screen by subtraction of the electrical resistance of a collagen coated filter without cells and a correction for the filter surface area.Vinblastine (10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M in ethanol) was added to the *in vitro* BBB, and the change in TEER (% of vehicle group) was determined during 5 h. The final ethanol concentration in the culture medium on the cells was kept below 0.1%. Pgp was inhibited with 1 μM PSC, applied 1 h before the start of the vinblastine exclusion assay. Control filters were treated with vehicle of PSC (>1000 times dilution of PGEG).

Assessment of Pgp Related Transcellular Drug Transport

Polar Transport Assay

Pgp mediated active transcellular transport across the *in vitro* BBB was determined by the polar transport assay. Briefly, the transported amount after 6 h of the Pgp substrates doxorubicin and rhodamine 123 from the basolateral to the apical side of the filters was determined and expressed as a percentage (mean % ± S.D.) of the transported amount from the apical to the basolateral side of the (vehicle treated) filters, both on BCEC monolayers and BCEC+astrocyte co-cultures. Identical experiments were performed with Pgp inhibited transport on BCEC+astrocyte co-cultures. Pgp was inhibited with 1 μM PSC, applied 1 h before the start of the transport experiment. Control filters were treated with vehicle of PSC (>1000 times dilution of PGEG). The initial concentration in the donor chamber of the filter of doxorubicin and rhodamine 123 was 3 μM and 1 μM, respectively. For this specific assay, the *in vitro* BBB model was prepared in such a way that the volume of the apical and basolateral side of the filter was equal (i.e. 750 or 230 μl on each side for filters with a surface area of 1 cm^2 or 0.33 cm^2, respectively). By these means, putative differences in Pgp saturation were prevented, as the same concentration (and thus amount) of doxorubicin and rhodamine 123 was applied to each side of the filter. The concentration of doxorubicin and rhodamine 123 in the acceptor chamber was determined by means of HPLC (9).

A/b Ratio Assay

Pgp mediated active transcellular transport across the *in vitro* BBB was determined by the development and maximal capacity of the ratio between the apical and basolateral concentration (a/b ratio) of rhodamine 123. For comparison, the a/b ratio of sodium fluorescein (FLU) was determined, serving as a structurally related reference compound for Pgp independent (active) transport. Rhodamine 123 (50 nM) and FLU (25 ng/ml) were added to the culture medium and simultaneously applied to both sides of the filters (surface area: 0.33 cm^2) containing BCEC monolayers, BCEC+astrocyte co-cultures, MDR1 transfected LLC-PK1 cells and Caco-2 cells (PD-7 clone). LLC-PK1 and Caco-2 cells were cultured in similar cell culture conditions as the *in vitro* BBB model (9). After a given time, the concentration of rhodamine 123 and FLU in the apical and basolateral chamber was determined by HPLC, and expressed as the a/b ratio (9).

Materials

Disposable sterile plasticware was purchased from Corning Costar (Cambridge, MA, USA), Micronic (Lelystad, The Netherlands) and Greiner (Alphen a/d Rijn, The Netherlands). Instant sterile endotoxin free cell culture media, supplements and PBS were obtained at BioWhittaker Europe (Verviers, Belgium). Collagen, vinblastine, rhodamine 123 and sodium fluorescein were obtained at Sigma Chemicals (St.Louis, MO, USA). Fetal calf serum (from P.A. Biologicals Corporation PTY Ltd (Sydney, Australia)) was obtained at Greiner (Alphen a/d Rijn, The Netherlands), fibronectin at Boehringer Mannheim B.V. (Almere, The Netherlands) and doxorubicin at the Hospital Pharmacy (Leiden University Medical Centre, Leiden, The Netherlands). Inorganic salts and all other reagents were of analytical grade. PSC was a gift from Novartis Pharmaceuticals (Basel, Switzerland).

RESULTS

Pgp Expression

The level of Pgp expression in brain capillaries, astrocytes and BCEC isolated from culture flasks and BCEC isolated from filters with or without the direct influence of astrocytes was compared. MDR1 transfected and control LLC-PK1 cell lines were used as positive and negative control for Pgp expression, respectively. In Figure 2, a photograph of Pgp immunoreactivity is displayed. No Pgp expression was detected in control LLC-PK1 cells and astrocytes. Strong Pgp expression was detected in the MDR1 transfected LLC-PK1 cell line, brain capillaries and BCEC isolated from culture flasks. Weak Pgp expression was detected in BCEC isolated from filters with BCEC monolayers, whereas a slightly higher level of Pgp expression appeared to be expressed in BCEC isolated from BCEC+astrocyte co-cultures.

Accumulation and Release Assay

A significantly higher amount (142 ± 25, 156 ± 30, 144 ± 14 (mean % of vehicle ± S.D.), $p<0.05$ unpaired t-test) of rhodamine 123 was released from BCEC which were preloaded in the presence of the Pgp inhibitor PSC (0.5, 1 and 2 μM, respectively) compared to the vehicle treated BCEC.

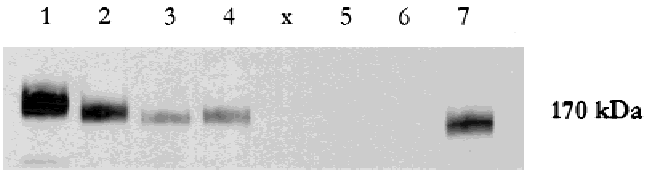

Fig. 2. Photograph of the immunoreactivity of Pgp (by the C219 monoclonal antibody) in 10 μg protein of brain capillaries (lane 1), primary cultures of BCEC isolated from culture flasks (lane 2), BCEC monolayers isolated from filters (lane 3), BCEC isolated from filters co-cultured with astrocytes (lane 4), primary cultures of astrocytes (lane 5), LLC-PK1 cells (lane 6, negative control) and MDR1 transfected LLC-PK1 cells (lane 7, positive control). Lane x represents a pre-stained protein marker.

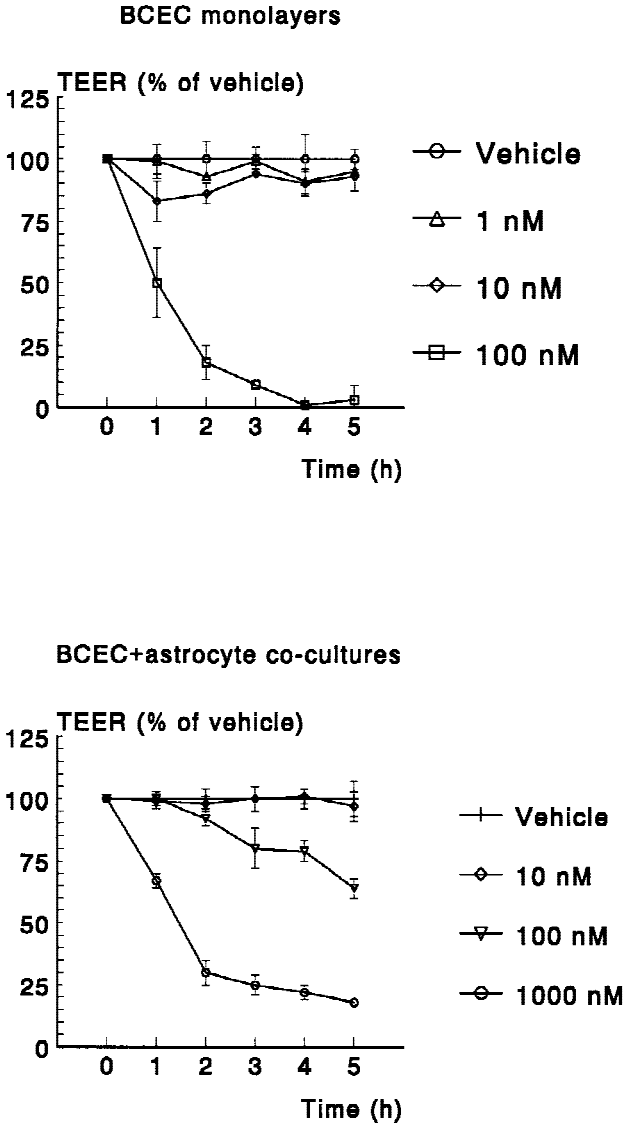

Fig. 3. Vinblastine exclusion assay. Concentration-dependent vinblastine mediated decrease of TEER (mean % of vehicle group ± S.D.) in filters with BCEC monolayers (top panel) and BCEC+astrocyte co-cultures (bottom panel). Note the difference in the sensitivity of vinblastine mediated BBB disruption, i.e. BCEC+astrocyte co-cultures are disrupted by 1000 nM vinblastine, whereas BCEC monolayers are already disrupted by 100 nM vinblastine.

Vinblastine Exclusion Assay

Vinblastine induced a concentration-dependent, irreversible decrease in TEER (mean % of vehicle ± S.D.) across BCEC monolayers and BCEC+astrocyte co-cultures (Figure 3). BCEC monolayers were more sensitive to the vinblastine mediated decrease in TEER (disruption at 100 nM vinblastine), compared to BCEC+astrocyte co-cultures (disruption at 1000 nM vinblastine).

Inhibition of Pgp with PSC, resulted in enhanced sensitivity for the vinblastine mediated decrease in TEER in both BCEC monolayers (disruption at 10 nM vinblastine) and BCEC+astrocyte co-cultures (disruption at 100 nM vinblastine) (Figure 4).

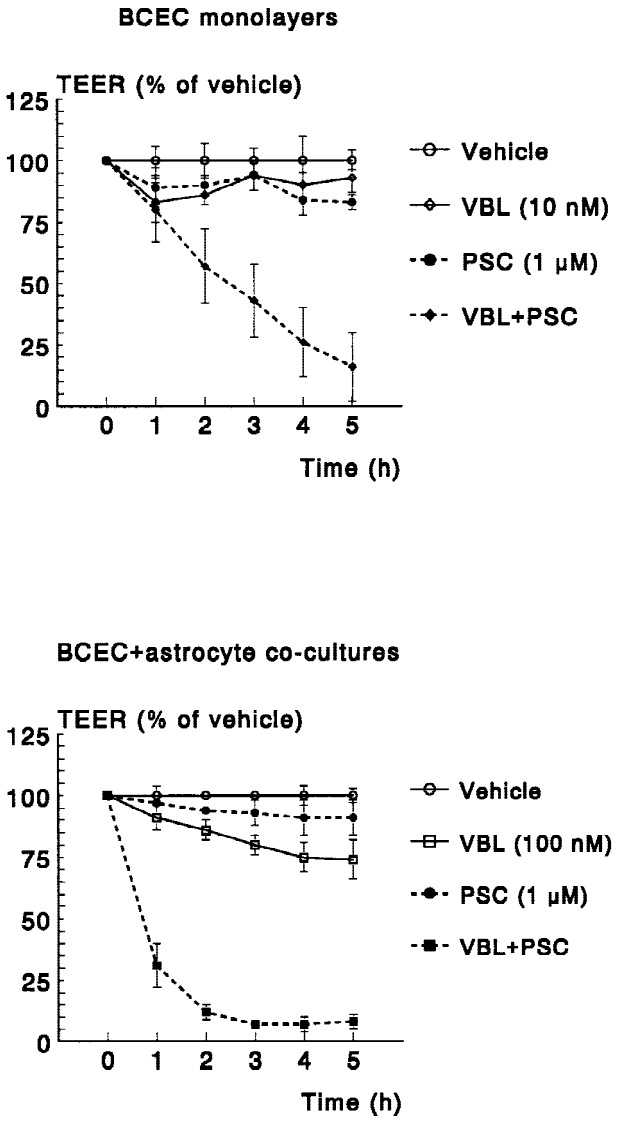

Fig. 4. Vinblastine exclusion assay. Increased sensitivity for the vinblastine mediated decrease of TEER (mean % of vehicle group ± S.D.) in filters with BCEC monolayers (top panel) and BCEC+astrocyte co-cultures (bottom panel) in which Pgp is inhibited by PSC (1 μM), i.e. BCEC+astrocyte co-cultures are now disrupted by 100 nM vinblastine, and BCEC monolayers are now disrupted by 10 nM vinblastine.

Polar Transport Assay

No significant polar transport across BCEC monolayers for doxorubicin and rhodamine 123 was observed (Figure 5, $p>0.05$ unpaired t-test). In contrast, significant polar transport across BCEC+astrocyte co-cultures for doxorubicin and rhodamine 123 was observed (Figure 5, $p<0.05$ unpaired t-test).

Inhibition of Pgp, did not significantly enhance the penetration of doxorubicin and rhodamine 123 across BCEC+astrocyte co-cultures from the apical to the basolateral side of the filter, compared to vehicle treated groups (Figure 6, $p>0.05$ unpaired t-test). On the other hand, inhibition of Pgp reversed the polar transport of doxorubicin and rhodamine 123 across BCEC+astrocyte co-cultures to approximately the same level of transport across the vehicle treated BCEC+astrocyte co-cultures from the apical to the basolateral side of the filter (Figure 6). The experimental procedures (i.e. the addition of doxorubicin, rhodamine 123, PSC and vehicle) had no effect on TEER across BCEC monolayers and BCEC+astrocyte co-cultures (not shown).

A/b Ratio Assay

At the start of the assay, the a/b ratio (mean ± S.D.) was found to be 1.01 ± 0.01 and 0.98 ± 0.03 for rhodamine 123 and FLU, respectively, when measured on filters with BCEC. After 48 h, BCEC monolayers displayed a ratio which was just, but significantly, above 1 for rhodamine 123 (1.03 ± 0.02, $p<0.05$ unpaired t-test, compared to the a/b ratio for rhodamine 123 at 0 h, Figure 7). The a/b ratio for rhodamine 123 after 48 h was found to be 1.20 ± 0.05 in BCEC+astrocyte co-cultures (Figure 7), while after two quick washes it was found to recover to only 0.99 ± 0.02 and 1.03 ± 0.02 after 5 h and 24 h, respectively. In contrast, the a/b ratio for rhodamine 123 after 48 h in MDR1 transfected LLC-PK1 cells was found to be 1.63 ± 0.13 (Figure 7). In addition, the a/b ratio for rhodamine 123 after 48 h in Caco-2 cells was found to be 10.0 ± 0.6 (Figure 7), while after two quick washes it was found to recover to 7.2 ± 0.2 in 3 h. During all procedures (except in MDR1 transfected LLC-PK1 cells), the a/b ratio for FLU was determined as an internal control, and found to stay close to 1 (see also Figure 7).

DISCUSSION

The aim of these investigations was to assess the influence of astrocytes on the functional expression of Pgp on BCEC and to relate this to the physiological role of Pgp at the BBB. For this purpose, Pgp expression and intracellular accumulation of Pgp substrates, separate from their net transcellular transport across the *in vitro* BBB was determined.

The brain capillaries displayed a strong level of Pgp expression with a varying molecular size (i.e. ranging from 170 up to 190 kDa), when compared to the cultured BCEC or MDR1 transfected cells (i.e. a single band of 170 kDa, Figure 2), a phenomenon observed by others as well (3,12,13). This larger and variable molecular size of Pgp in the brain capillaries could be the result of different levels of *in vivo* glycosylation, which is lost in the culture of BCEC (12), or by cross-reactivity of the C219 antibody with a 190 kDa protein in brain capillaries (13) or other Pgp expressing cell types still present in the brain capillaries (like lymphocytes (14)). The primary cultured BCEC displayed a stronger level of Pgp expression compared to BCEC which were passaged to filters, despite the fact that they were cultured in 50% astrocyte conditioned medium. However, when BCEC were passaged to filters with astrocytes on the bottom, the level of Pgp expression seemed to be somewhat increased (Figure 2). This would indicate that the expression of Pgp in BCEC was maintained (or (re-)induced) by the direct influence of astrocytes, as was suggested before (3). In contrast to the suggestion of Pardridge and colleagues that Pgp was expressed on astrocytes and not on endothelial cells (6), we present evidence that Pgp is expressed on BCEC and not on the primary cultures of newborn rat astrocytes (Figure 2). Co-localization of discontinuous Pgp expression and astrocyte foot process markers, as was described by Pardridge (6), may possibly be

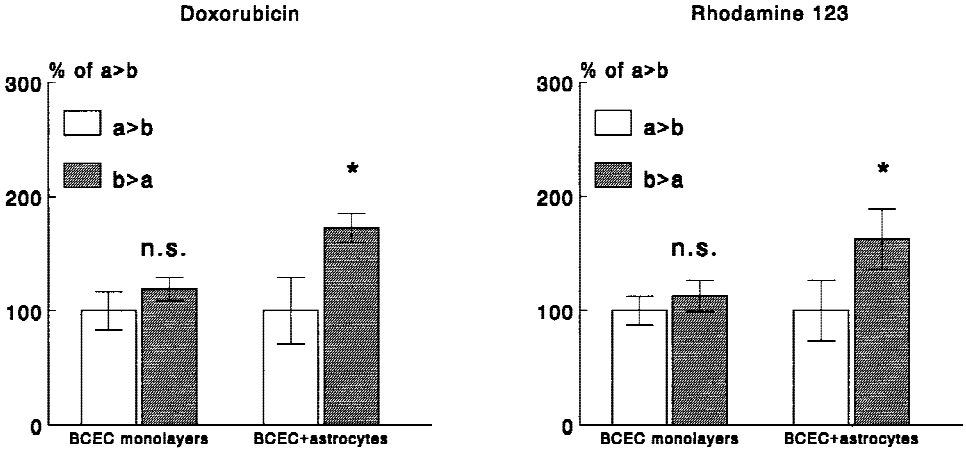

Fig. 5. Polar transport assay. Polar transport of doxorubicin (left panel) and rhodamine 123 (right panel) across filters with BCEC monolayers and BCEC+astrocyte co-cultures. The basolateral to apical (b>a) transport is expressed as a percentage of the apical to basolateral (a>b) transport (mean ± S.D., after 6 h). Note that a significant polar transport of doxorubicin (left panel) and rhodamine 123 (right panel) was only observed across filters with BCEC+astrocyte co-cultures. * indicates a significant difference between the groups ($p<0.05$, unpaired t-test). n.s. indicates no significant difference between the groups ($p>0.05$, unpaired t-test).

explained by the finding that astrocytes increase (i.e. locally enhance) expression of Pgp on BCEC.

In this paper, two new applications of the *in vitro* BBB model were described. First, by combining two specific characteristics of vinblastine (i.e. tight junction disruption and Pgp substrate), a new detection method for the functional expression of Pgp was developed (called the vinblastine exclusion assay). The major advantage of this assay is that no analytical detection method for intracellular accumulation or active transport of Pgp substrates (e.g. HPLC or radiolabeling of drugs) and Pgp expression (e.g. PCR, Western blot analysis or in situ hybridization) was needed. The vinblastine exclusion assay is based on the concept that intracellular accumulation of vinblastine leads to a concentration-dependent disruption of tight junctions between BCEC (10), which is readily determined by changes in TEER (15). The intracellular accumulation of vinblastine is normally reduced by Pgp. Therefore, changes in the functional expression of Pgp will be reflected in a change in sensitivity of the concentration-dependent effect of vinblastine. The functional expression of Pgp may be experimentally modified by changes in cell culture conditions (i.e. co-culture with astrocytes (Figure 4)), exposure to inhibitors or inducers of Pgp expression or by competition with other Pgp substrates or inhibitors (i.e. PSC (Figure 5)). The vinblastine mediated acute disruption of the tight junctions described here is not a general Pgp substrate characteristic, but probably unique to cytoskeleton disrupting substrates, like vinca alkaloids (10,16). In fact, other Pgp substrates in-

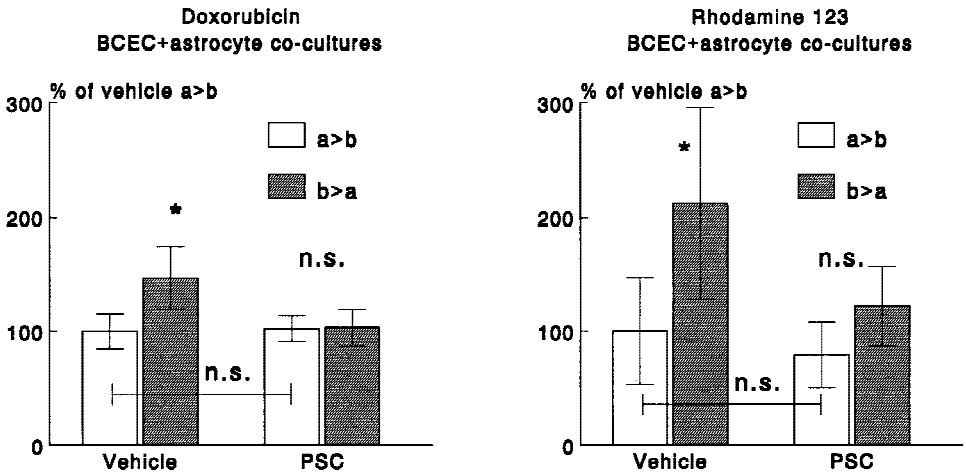

Fig. 6. Polar transport assay. Effect of Pgp inhibition with 1 μM PSC on the polar transport of doxorubicin (left panel) and rhodamine 123 (right panel) across filters with BCEC+astrocyte co-cultures. The transport for each group is expressed as a percentage of the apical to basolateral (a>b) transport of the vehicle group (mean ± S.D., after 6 h). Note that a significant inhibition of the transport was only observed in the basolateral to apical direction and that the opposite direction was not significantly enhanced. * indicates a significant difference between the groups ($p<0.05$, unpaired t-test). n.s. indicates no significant difference between the groups ($p>0.05$, unpaired t-test).

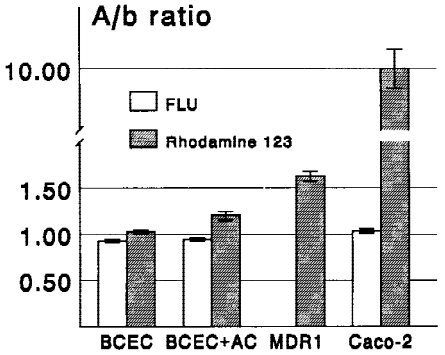

Fig. 7. A/b ratio assay. Apical/basolateral concentration ratio's (a/b ratio, mean ± S.D.) for FLU and rhodamine 123 after 48 h on filters with BCEC monolayers (BCEC), BCEC+astrocyte co-cultures (BCEC+AC), MDR1 transfected LLC-PK1 cells (MDR1, a/b ratio for FLU was not determined) and Caco-2 cells. Note the low Pgp mediated transcellular active transport capacity in BCEC monolayers and BCEC+astrocyte co-cultures, compared to the MDR1 transfected LLC-PK1 cells and the Pgp overexpressing Caco-2 cells.

cluding etoposide, doxorubicin, dexamethasone, cortisol, progesterone, quinidine, verapamil and rhodamine 123, but also non Pgp substrates like FLU and indomethacin (applied at micromolar concentrations, alone or in the presence of PSC), did not exhibit a disrupting effect in our *in vitro* BBB model. Second, a qualitative assay for active transcellular drug transport across the *in vitro* BBB was developed (called the a/b ratio assay). The a/b ratio assay is based on the concept that polarized expression of transporters on BCEC can actively transport and maintain a concentration difference of its substrates across the *in vitro* BBB. Addition of equal amounts of substrate for an active transporter (e.g. rhodamine 123 for Pgp) to both chambers of a filter (Figure 1), will lead to a polarized accumulation of the substrate to one side of the filter. By sampling both chambers simultaneously in time, the kinetics and maximal capacity for the concentration difference is obtained (expressed as ratio of the concentration in the apical and basolateral chamber). The major advantage of this assay is that putative interference from passive (paracellular) transport routes was ruled out (in contrast to the polar transport assay). In addition, changes in active transport are determined within 1 filter, and not between groups of filters (like in the polar transport assay), which reduced intra-assay variability considerably.

Inhibition of Pgp increased the accumulation of rhodamine 123 in BCEC, which suggests that Pgp was capable of reducing the intracellular accumulation of rhodamine 123 in BCEC monolayers. Moreover, inhibition of Pgp increased the sensitivity for vinblastine induced *in vitro* BBB disruption, which convincingly demonstrated that Pgp is functionally expressed in both BCEC monolayers and BCEC+astrocyte co-cultures (Figure 4). In addition, Pgp functionality is enhanced by astrocytes, as the sensitivity for the vinblastine induced BBB disruption in BCEC+astrocyte co-cultures is smaller when compared to BCEC monolayers (Figure 3). We demonstrated that Pgp actively transports Pgp substrates from the basolateral (CNS) side to the apical (blood) side in our *in vitro* BBB model (Figure 5, 6, and 7). However, this active transcellular transport was only convincingly demonstrated in the presence of astrocytes, despite the fact that Pgp was expressed in BCEC monolayers. Inhibition of Pgp in BCEC+astrocyte co-cultures, did not lead to an enhanced CNS penetration of the Pgp substrates doxorubicin and rhodamine 123 (Figure 6). It did, however, diminish the active transport from the basolateral (CNS) side to the apical (blood) side (Figure 6). In addition, the active transcellular transport of rhodamine 123 was most likely mediated by Pgp since in the same experiment an active transcellular transport of FLU across the *in vitro* BBB and the Caco-2 cells was absent (Figure 7). When compared to the MDR1 transfected LLC-PK1 cells and the Pgp overexpressing Caco-2 cells, the capacity of Pgp mediated transcellular transport across the *in vitro* BBB was low (Figure 7). This was probably not the result of a lack of barrier formation of the *in vitro* BBB, because the LLC-PK1 and Caco-2 monolayers displayed a 2-5 times lower transepithelial electrical resistance than BCEC±astrocyte cultures (not shown).

The physiological role of Pgp at the BBB could be considered from two different angles. The first angle is that Pgp actively prevents the entry of Pgp substrate drugs into the CNS and transports these out of the CNS, across the BBB. This view is based on the concept that lipophilic drugs cross the BBB either from the blood or from the CNS side, driven by a concentration gradient by passive diffusion. Subsequently, the luminally located Pgp actively expels the Pgp substrates (back) into the blood stream (depicted in a schematic drawing in Figure 8A). This is in agreement with the pharmacological observation that Pgp substrates have a lower CNS penetration than would be expected on the basis of their

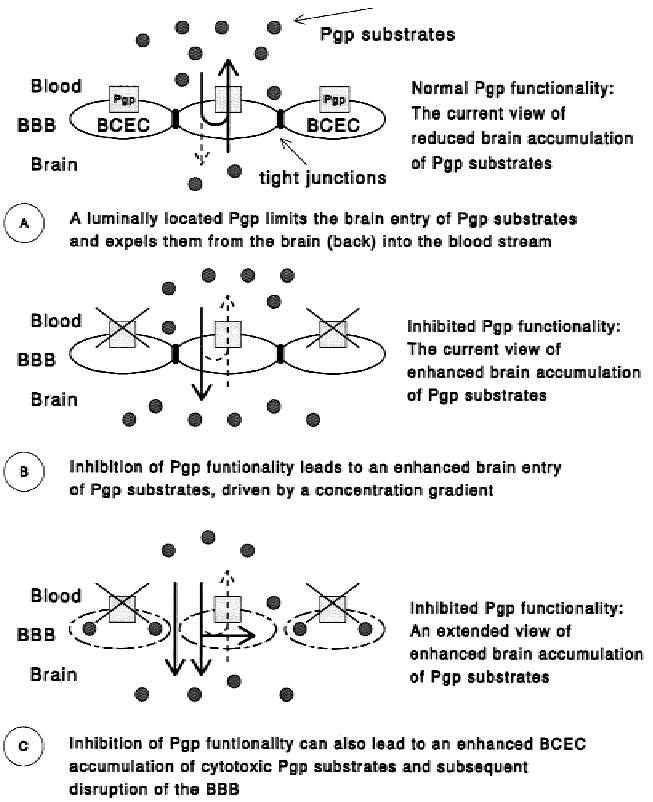

Fig. 8. Schematic drawing of the primary role of Pgp on the blood–brain barrier (8A), followed by two scenario's of the effect of Pgp inhibition and simultaneous exposure to (cytotoxic) Pgp substrates (8B and 8C). Abbreviations: Pgp (P-glycoprotein), BBB (blood–brain barrier), BCEC (brain capillary endothelial cells).

lipophilicity (17). Hence, Pgp inhibition will result in increased CNS accumulation of Pgp substrates (including drugs and endogenous substrates, depicted in a schematic drawing in Figure 8B). Indeed, in mice with disrupted Pgp genes and in rats treated with Pgp inhibitors, the CNS accumulation of Pgp substrates was found to be considerably enhanced (18–21). The second angle to look at the physiological role of Pgp at the BBB is that analogous to the role of Pgp on single tumor cells (i.e. to limit the intracellular accumulation of (cytotoxic) compounds that are harmful for BCEC). Hence, Pgp indirectly protects the CNS by protecting the BBB, because damage to BCEC will lead to a decreased barrier function. In particular, the luminally located Pgp actively expels Pgp substrates (entered from either the blood or the CNS, driven by a concentration gradient by passive diffusion) out of the cell membrane or cytosol of BCEC (see also Figure 8A). This will also result in a concentration difference of the Pgp substrate between the blood and the CNS. Again, this is in agreement with the pharmacological observation that Pgp substrates have a lower CNS accumulation than would be expected on the basis of their lipophilicity and that Pgp inhibition leads to an enhanced CNS accumulation of Pgp substrates. Although the two angles are based on the same underlying mechanism of Pgp, the latter is based on the prevention of an intracellular accumulation of potentially harmful compounds for BCEC. If this is the case, Pgp inhibition will potentially result in the disruption of the BBB (depicted in a schematic drawing in Figure 8C). Our results support the latter physiological role of Pgp at the BBB. The vinblastine exclusion assay, in the presence and absence of Pgp inhibitors, exemplified the relevance of Pgp mediated drug efflux from BCEC for the maintenance of the integrity of the BBB. In studies with rats, high concentrations of members of several classes of anticancer Pgp substrate drugs (e.g. vinorelbine, teniposide, 5-fluorouracil and etoposide) have been reported to cause a disruption of the BBB, already without the co-administration of Pgp inhibitors (16,22,23). Moreover, in clinical trials the co-administration of both etoposide and doxorubicin with cyclosporin A, increased the incidence of nausea and vomiting considerably (24,25), which is indicative for a disrupted BBB (26). Similar results have been obtained with combinations of tamoxifen and vinblastine, but also following PSC treatment alone (26). The finding in animal studies that inhibition of Pgp does not significantly alter the pharmacokinetics (but rather the distribution) of Pgp substrates, further underlines the relatively minor role of Pgp in active drug clearance (19,20). Indeed, it can be interpreted that Pgp protects the eliminating organs against a putative intracellular accumulation of (cytotoxic) compounds (27). Pgp mediated transcellular drug transport across the *in vitro* BBB from the CNS to the blood side of the filter has a limited capacity and could only convincingly be demonstrated in the presence of astrocytes. Furthermore, inhibition of Pgp did not lead to an enhanced transcellular drug transport across the *in vitro* BBB from the blood to the CNS side of the filter for doxorubicin and rhodamine 123. On the other hand, the Pgp mediated restriction of intracellular drug accumulation in the cells of the BBB was already convincingly demonstrated in BCEC monolayers, and astrocytes were merely found to enhance the Pgp mediated extrusion of the intracellularly accumulated drugs from BCEC. Based on these findings, we postulate that an important role of Pgp on the BBB is to protect the barrier against the accumulation of cytotoxic BBB disrupting agents (see Figure 8C). By these means, the multidrug resistant BBB protects the brain against neurotoxic Pgp substrates and, furthermore, it enables the maintenance of all other BBB properties by protecting the tight junction functionality (28). Therefore, the use of Pgp inhibitors for an enhanced drug delivery of Pgp substrates across the BBB should be applied with caution. In this respect, the integrity of Pgp expressing tissue barriers (including the BBB) should be monitored preferably in an early stage of drug development. Moreover, these results illustrate that effects on BBB integrity should be monitored in the presence of the Pgp substrate in studies where Pgp inhibitors, or animals with disrupted Pgp genes, are being tested, an exertion which is often omitted in *in vivo* studies.

In conclusion, astrocytes influence the functional expression of Pgp in our *in vitro* BBB model. Furthermore, a physiological role of Pgp on the BBB is to protect the barrier against the accumulation of cytotoxic BBB disrupting agents, in addition to the pharmacological concept that Pgp is expressed on the BBB in order to restrict the access of exogenous compounds to the CNS.

REFERENCES

1. C. Cordon-Cardo, J. P. O'Brien, D. Casals, L. Rittman-Grauer, J. L. Biedler, M. R. Melamed, and J. R. Bertino. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc. Natl. Acad. Sci. U.S.A.* **86**:695–698 (1989).
2. A. Tsuji, T. Terasaki, Y. Takabatake, Y. Tenda, I. Tamai, T. Yamashima, S. Moritani, T. Tsuruo, and J. Yamashita. P-glycoprotein as the drug efflux pump in primary cultured bovine brain capillary endothelial cells. *Life Sci.* **51**:1427–1437 (1992).
3. L. Fenart, V. Buee-Scherrer, L. Descamps, C. Duhem, M. G. Poullain, R. Cecchelli, and M. P. Dehouck. Inhibition of P-glycoprotein: rapid assessment of its implication in blood-brain barrier integrity and drug transport to the brain by an *in vitro* model of the blood-brain barrier. *Pharm. Res.* **15**:993–1000 (1998).
4. D. J. Begley, D. Lechardeur, Z. D. Chen, C. Rollinson, M. Bardoul, F. Roux, D. Scherman, and N. J. Abbott. Functional expression of P-glycoprotein in an immortalised cell line of rat brain endothelial cells, RBE4. *J. Neurochem* **67**:988–995 (1996).
5. R. C. Janzer and M. C. Raff. Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature* **325**:253–257 (1987).
6. W. M. Pardridge, P. L. Golden, Y. S. Kang, and U. Bickel. Brain microvascular and astrocyte localization of P-glycoprotein. *J. Neurochem.* **68**:1278–1285 (1997).
7. B. Joly, V. Lecureur, C. Puozzo, A. Guillouzo, and O. Fardel. Involvement of P-glycoprotein in an *in vitro* blood-brain barrier model. *Int. J. Oncology* **9**:1029–1033 (1996).
8. A. Seelig. A general pattern for substrate recognition by P-glycoprotein. *Eur. J. Biochem.* **251**:252–261 (1998).
9. I. C. J. van der Sandt, M. C. Blom-Roosemalen, A. G. de Boer, and D. D. Breimer. Specificity of doxorubicin versus thodamine-123 in assessing P-glycoprotein functionality in the LLC-PK1, LLC-PK1:MDR1 and Caco-2 cell lines. *Eur. J. Pharm. Sci.* (in press).
10. S. Nag. Role of the endothelial cytoskeleton in blood-brain-barrier permeability to protein. *Acta Neuropathol. Berl.* **90**:454–460 (1995).
11. L. L. Mitic and J. M. Anderson. Molecular architecture of tight junctions. *Annu. Rev. Physiol.* **60**:121–142 (1998).
12. D. Lechardeur and D. Scherman. Functional expression of the P-glycoprotein *mdr* in primary cultures of bovine cerebral capillary endothelial cells. *Cell Biol. Toxicol.* **11**:283–293 (1995).
13. E. Beaulieu, M. Demeule, J. F. Pouliot, D. A. Averill Bates, G. F.

Murphy, and R. Beliveau. P-glycoprotein of blood brain barrier: Cross-reactivity of Mab C219 with a 190 kDa protein in bovine and rat isolated brain capillaries. *Biochim. Biophys. Acta* **1233**: 27–32 (1995).
14. U. Bommhardt, J. C. Cerottini, and H. R. MacDonald. Heterogeneity in P-glycoprotein (multidrug resistance) activity among murine peripheral T cells: Correlation with surface phenotype and effector function. *Eur. J. Immunol.* **24**:2974–2981 (1994).
15. J. L. Madara. Regulation of the movement of solutes across tight junctions. *Annu. Rev. Physiol.* **60**:143–159 (1998).
16. C. Mouchard-Delmas, B. Gourdier, R. Vistelle, and M. Wiczewski. Modification of the blood-brain barrier permeability by vinorelbine: Effect of intracarotid infusion compared with intravenous infusion. *Anticancer Drugs* **7**:213–219 (1996).
17. V. A. Levin. Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. *J. Med. Chem.* **23**:682–684 (1980).
18. A. H. Schinkel, E. Wagenaar, C. A. Mol, and L. van Deemter. 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 (1996).
19. D. Leveque and F. Jehl. P-glycoprotein and pharmacokinetics. *Anticancer Res.* **15**:331–336 (1995).
20. E. C. de Lange, G. de Bock, A. H. Schinkel, A. G. de Boer, and D. D. Breimer. BBB transport and P-glycoprotein functionality using MDR1A (-/-) and wild-type mice. Total brain versus microdialysis concentration profiles of rhodamine-123. *Pharm. Res.* **15**:1657–1665 (1998).
21. O. C. Meijer, E. C. de Lange, D. D. Breimer, A. G. de Boer, J. O. Workel, and E. R. de Kloet. Penetration of dexamethasone into brain glucocorticoid targets is enhanced in mdr1A P-glycoprotein knockout mice. *Endocrinology* **139**:1789–1793 (1998).
22. M. K. Spigelman, R. A. Zappulla, J. Johnson, S. J. Goldsmith, L. I. Malis, and J. F. Holland. Etoposide-induced blood-brain barrier disruption. Effect of drug compared with that of solvents. *J. Neurosurg.* **61**:674–678 (1984).
23. L. A. MacDonell, P. E. Potter, and R. A. Leslie. Localized changes in blood-brain barrier permeability following the administration of antineoplastic drugs. *Cancer Res.* **38**:2930–2934 (1978).
24. A. M. Yahanda, K. M. Alder, G. A. Fisher, N. A. Brophy, J. Halsey, R. I. Hardy, M. P. Gosland, B. L. Lum, and B. I. Sikic. Phase I trial of etoposide with cyclosporine as a modulator of multidrug resistance. *J. Clin. Oncol.* **10**:1624–1634 (1992).
25. N. L. Bartlett, B. L. Lum, G. A. Fisher, N. A. Brophy, M. N. Ehsan, J. Halsey, and B. I. Sikic. Phase I trial of doxorubicin with cyclosporine as a modulator of multidrug resistance. *J. Clin. Oncol.* **12**:835–842 (1994).
26. B. L. Lum and M. P. Gosland. MDR expression in normal tissues. Pharmacologic implications for the clinical use of P-glycoprotein inhibitors. *Hematol. Oncol. Clin. North Am.* **9**:319–336 (1995).
27. J. Wijnholds, G. L. Scheffer, M. van der Valk, P. van der Valk, J. H. Beijnen, R. J. Scheper, and P. Borst. Multidrug resistance protein 1 protects the oropharyngeal mucosal layer and the testicular tubules against drug-induced damage. *J. Exp. Med.* **188**: 797–808 (1998).
28. M. Cereijido, J. Valdes, L. Shoshani, and R. G. Contreras. Role of tight junctions in establishing and maintaining cell polarity. *Annu. Rev. Physiol.* **60**:161–177 (1998).