A Comparison of Drug Transport through Cultured Monolayers of Bovine Brain Capillary and Bovine Aortic Endothelial Cells

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Received March 7, 1994; accepted June 11, 1994

To examine how efficient primary cultured brain capillary endothelial cells are as an in vitro blood-brain barrier (BBB) system with regard to drug transport, we compared bovine brain capillary endothelial cells (BBCEC) with bovine aortic endothelial cells (BAEC). Paracellular and transcellular transport were examined. [14C]Sucrose and fluorescein isothiocyanate-dextran (FITC-dextran) (average molecular weight about 4400 and 71200) were used as indices of paracellular transport. The permeability coefficients of [14C]Sucrose and FITC-dextran through BBCEC monolayers were about 3 and 30–40 fold lower than those through BAEC monolayers, respectively. Transcellular transport was examined by means of [3H]leucine and [3H]alanine uptake studies. The K_m value (Michaelis constant) of the amino acid uptake was lower in BBCEC than in BAEC. BBCEC had higher alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GTP) activity than BAEC when cultured in vitro. These results suggest that BBCEC are suitable for drug transport studies across the BBB in vitro.

Keywords endothelial cell; blood-brain barrier; paracellular transport; transcellular transport; alkaline phosphatase; γ-glutamyl transpeptidase

Endothelial cells play an important role in the regulation of vascular permeability, and the mechanism of its regulation has been examined in detail using cultured bovine and porcine aortic endothelial cells, as well as human umbilical vein endothelial cells (HUVEC). Recently a number of investigators have studied drug transport across the blood-brain barrier (BBB) with primary cultured brain capillary endothelial cells in vitro. This in vitro BBB system possesses many specialized properties of brain capillary endothelial cells in vivo; that is, tight junctions, a transport system for nutrients, glucose, and amino acids, and enzyme activities such as alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GTP).

However, brain capillary endothelial cells rapidly lose ALP and γ-GTP activity when cultured in vitro. Partridge et al. have reported that the permeability coefficients (P) in this in vitro BBB system were about 150-fold greater than that of in vivo BBB measured by means of internal carotid perfusion/capillary depletion. Primary cultured brain capillary endothelial cells do not perfectly reflect the conditions of in vivo BBB.

Even if primary cultured brain capillary endothelial cells are used, they are unsuitable for use as an in vitro BBB system unless they mimic in vivo conditions. In drug transport studies, if primary cultured brain capillary endothelial cells are used, they are unsuitable for use as a substitute for brain capillary endothelial cells. The isolation of brain capillary endothelial cells is much more difficult than that of aortic endothelial cells or HUVEC. So far, no comparison between cultured endothelial cells in the BBB and those in other organs has been reported with regard to drug transport studies. The efficacy of primary cultured brain capillary endothelial cells as an in vitro BBB system has not been examined.

In this study, we examined whether primary cultured bovine brain capillary endothelial cells (BBCEC) were useful as an in vitro BBB system with regard to paracellular and transcellular transport compared with primary cultured bovine aortic endothelial cells (BAEC). The paracellular transport studies were performed using a permeation chamber (Transwell™). [14C]Sucrose and fluorescein isothiocyanate-dextran (FITC-dextran) (average molecular weight about 4400 and 71200) were used as model substrates for the paracellular transport studies through the endothelial monolayers. Transcellular transport was examined by means of amino acid uptake. [3H]Leucine and [3H]Alanine were used as model substrates for the uptake studies. The essential neutral amino acids that are required for cerebral metabolism are transported into the brain from the plasma by a specific transport system in brain capillary endothelial cells. The affinity of the transport system in brain capillary endothelial cells for neutral amino acids, including leucine and alanine, is unusually high compared with other organs. We examined whether primary cultured BBCEC had high affinity for leucine and alanine for the transport system, as they do in vivo. In addition, the affinities of primary cultured BBCEC and BAEC were compared.

MATERIALS AND METHODS

Materials Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Nissui, Tokyo, Japan. Fetal calf serum (FCS) was obtained from Filtron, Brooklyn, NY, USA. FITC-dextran was obtained from Sigma Chemical Co., St. Louis, MO, USA. [14C(U)]Sucrose was obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO, USA. t-[4,5,3H(N)]Leucine and L-3H]Alanine were obtained from Du Pont/New England Nuclear, Boston, MA, USA. Transwell™ (pore
size 0.4 μm) was obtained from Costar, Cambridge, MA, U.S.A. Rat tail collagen I was obtained from Collaborative Research, Inc., Massachusetts, U.S.A. Fibronectin (from human plasma) was obtained from Boehringer Mannheim, Tokyo, Japan. Rhodamine-phalloidin was obtained from Molecular Probes, Eugene, OR, U.S.A. 3,5-Diaminobenzoic acid dihydrochloride was obtained from Nacalai Tesque, Kyoto, Japan. All other chemicals were of reagent grade and commercially available.

**Cell Culture** BBCEC were isolated by the method of Audus and Borchartd. BAEC were isolated by mechanical scraping. To obtain single cells, the vesicle fragments were suspended in 1 mg/ml collagenase (30 min) and trypsin-EDTA (2.5, 0.2 mg/ml, 10 min). The digested preparation was then passed over a nylon sieve (300 μm mesh) and rinsed with DMEM supplemented with 10% FCS. Isolated BBCEC and BAEC were seeded into Transwell™, 12-wells or 35-mm dishes coated with collagen I (3 mg/ml) and fibronectin (10 μg/ml). BBCEC and BAEC were cultured with DMEM supplemented with 10% FCS, 100 μg/ml penicillin G, 100 μg/ml streptomycin, 2.5 μg/ml amphoterin B, 20 units/ml heparin, and 10 ng/ml epidermal growth factor.

**Paracellular Transport** Primary cultured BBCEC and 3rd—5th passage BAEC were seeded into Transwell™ (pore size 0.4 μm) coated with collagen I (3 mg/ml) and fibronectin (10 μg/ml) and cultured for 4–8d. The permeability of [14C]sucrose and FITC-dextran (average molecular weight about 4400 and 71200) through endothelial monolayers was investigated by the method of Shasby et al. with a minor modification. FITC-dextran and sucrose are reported to be transported through the intercellular spaces of the endothelial junction. HEPES-buffered Ringer's solution (141 mM NaCl, 4 mM KCl, 2.8 mM CaCl2, 10 mM HEPES, 1 mM MgSO4, 10 mM d-glucose, pH 7.4) was added to the abluminal compartment and [14C]sucrose (0.2 μCi/ml, 50 μm) and FITC-dextran (50 μM) in HEPES-buffered Ringer's solution was added to the luminal compartment. The experiments were performed at 37 °C in a 95% air–5% CO2 humidified atmosphere with gentle shaking. Aliquots were removed from the abluminal compartment at various times. The radioactivity of [14C]sucrose was counted with a liquid scintillation counter. FITC-dextran was quantified using a fluorescence spectrophotometer (excitation 495 nm; emission 550 nm).

The permeability experiments were performed on day 4, 6 and 8. The permeability of [14C]sucrose and FITC-dextran reached plateau levels at day 6 in both BBCEC and BAEC. The data from day 6 are shown in Fig. 1 and Table I. In preliminary studies, the permeability of FITC-dextran (average molecular weight about 71200) through 3rd—5th passage BAEC was as much as that through primary cultured BAEC.

After the transport studies, the cells on the membrane were detached and the cellular DNA contents were determined by means of a fluorometric assay using diaminobenzoic acid. The cell number on the membrane was determined according to the cellular DNA content.

**Permeability Coefficients** The permeability coefficients (Pc) of [14C]sucrose and FITC-dextran through cultured monolayers were calculated from the following equation.4)

\[ 1/P_{EC} = 1/P_{total} + 1/P_{memb} \]

where \( P_{EC} \), \( P_{total} \) and \( P_{memb} \) represent the \( P_c \) of the endothelial monolayer alone, the endothelial monolayer on the membrane and the membrane alone, respectively. The permeability coefficients in Table I mean \( P_{EC} \).

**Rhodamine-Phalloidin Stain** F-actin was visualized as described by Phillips and Tsan with some modifications. Briefly, BBCEC and BAEC were seeded into 35-mm dishes coated with collagen I and fibronectin. Thereafter, confluent monolayers of BBCEC and BAEC were washed with phosphate-buffered saline (PBS, pH 7.4) and fixed with 10% formalin in PBS (pH 7.4) for 20 min. They were then exposed to 0.1% TritonX-100 for 4 min, washed with PBS, and stained with rhodamine-phalloidin (3.3 \( \times 10^{-8} \) M) for 20 min. The cells were viewed under a fluorescence microscope. Photomicrographs were taken with Neopan 400 Presto film (Fuji, Tokyo, Japan) and processed to an ISO rating of 1600. The film was developed with Pandol (Fuji, Tokyo, Japan).

**Transcellular Transport (Amino Acid Uptake)** Primary cultured BBCEC and primary cultured BAEC were seeded into 12-wells coated with collagen I and fibronectin. After reaching confluence, amino acid uptake was studied according to Hughes and Lantos10 with a minor modification. Briefly, the confluent monolayer was first washed 3 times with 1 ml of incubation solution (HEPES-buffered Ringer's solution containing 0.1% bovine serum albumin (BSA)) at 37 °C. The time course studies of uptake were initiated by adding 400 μl of incubation solution containing [3H]leucine and [3H]alanine (0.4 μCi/ml, 100 μM), and were incubated for various times at 37 °C. The initial influx of leucine into both BBCEC and BAEC was rapid and the curve had begun to turn over by 3 min (data not shown). On the other hand, the initial influx of alanine was linear for at least 3 min. These results reflect the fact that the tissue volume of leucine is smaller than that of alanine.10 From these studies, we selected an incubation time for the saturation studies of 1 min for leucine and 3 min for alanine, before efflux began.

The saturation studies of uptake were initiated by adding 400 μl of the incubation solution containing the desired concentrations of amino acid plus tracer, and the mixture was incubated at 37 °C. [14C]Sucrose was used as the extracellular space marker. Uptake studies were stopped with ice-cold incubation buffer, then the cells were washed 3 times with incubation buffer and solubilized with 0.5 N NaOH overnight at 37 °C. Aliquots were removed to measure the levels of radioactivity. The protein content was measured with a Bio-Rad assay kit using BSA as the standard.

**Parameter Estimation** To estimate the kinetic parameters of [3H]leucine and [3H]alanine uptake in BBCEC and BAEC, the uptake rate (V), which was corrected for extracellular space using [14C]sucrose, was fitted to the following equation, consisting of both a saturable term and a nonsaturable linear term, by using the nonlinear least-squares regression analysis program MULTI:27)

\[ V = V_{max} \cdot S/(K_m + S) + K_0 \cdot S \]
where \( V_{\text{max}} \) is the maximal uptake rate of the saturable component, \( S \) is the concentration of substrate, \( K_m \) is the half-saturation constant of the saturable component (Michaelis constant) and \( K_d \) is the constant for the nonsaturable uptake.

**Enzyme Assay** BBCEC and BAEC were primarily cultured in 35-mm dishes coated with collagen I and fibronectin. The cells were washed 3 times with alkaline buffer (0.1 M glycine, 0.001 M MgCl₂, pH 10.5) to measure ALP activity or with Tris buffer (0.1 M, pH 9.0) to measure \( \gamma \)-GTP activity. The cells were scraped from the dishes with a rubber policeman and the cell suspension was sonicated. The levels of ALP, \( \gamma \)-GTP and DNA levels were measured in aliquots of the sonicate. The level of ALP was determined using p-nitrophenylphosphate as the substrate. One unit of ALP activity was defined as the formation of 1 \( \mu \)mol p-nitrophenol per min at 37 °C. The level of \( \gamma \)-GTP was determined using L-glutamyl-p-nitroanilide as the substrate and glycyl-glycine as the acceptor. One unit of \( \gamma \)-GTP activity was defined as the amount that liberated 1 nmol of p-nitroaniline per min at 25 °C. The activity was expressed in terms of the cell number, which was determined according to the cellular DNA content.

**RESULTS AND DISCUSSION**

**Paracellular Transport** Time course studies of the paracellular transport of \(^{14}\)C-sucrose through the cultured monolayers of BBCEC and BAEC were performed using the permeation chamber, Transwell™ (Fig. 1). \(^{14}\)C-Sucrose passed freely through the polycarbonate membrane, which was coated with collagen I and fibronectin. The transport rate of \(^{14}\)C-sucrose through BBCEC monolayer was less than that through BAEC monolayer.

The \( P_e \) of \(^{14}\)C-sucrose and FITC-dextran (average molecular weight about 4400 and 71200) in BBCEC and BAEC were estimated (Table I). The \( P_e \) in both BBCEC and BAEC decreased with an increase in molecular weight. The \( P_e \) in BBCEC was much lower than that in BAEC. Especially, in case of FITC-dextran the \( P_e \) in BBCEC was 30—40 fold lower than that in BAEC. The difference in the permeability between BBCEC and BAEC did not depend on cell density because the number of BBCEC was a little less than that of BAEC (Fig. 2). The \( P_e \) of \(^{14}\)C-sucrose in BBCEC, 69.58 ± 10⁻³ cm/h, was as much as the \( \text{in vitro} \) \( P_e \) described by Dehouck et al., and 1 and 2 log orders smaller than the \( \text{in vitro} \) \( P_e \) described by Pardridge et al. and Smith et al., respectively. Pardridge et al. have reported that the \( \text{in vitro} \) \( P_e \) was an average of 150-fold greater than that of the \( \text{in vivo} \) BBB. Taking account of the reports for their \( \text{in vitro} \) \( P_e \), our \( \text{in vitro} \) results are also thought to be leakier than the \( \text{in vivo} \) conditions. However, in BBCEC the paracellular permeability of \(^{14}\)C-sucrose and FITC-dextran was more limited compared with that in BAEC (Fig. 1, Table I). This \( \text{in vitro} \) BBB system is considered sufficient, since it reflects \( \text{in vivo} \) conditions to some degree, with regard to the paracellular pathway.

Many investigators have demonstrated parallel changes in paracellular permeability and the structure of the peripheral actin filaments that underlie the tight junction at the level of the adhering junction or belt desmosomes, using cultured monolayers of epithelial cells or endothelial cells. To determine whether BBCEC differ from BAEC with regard to cytoskeletal structures, confluent monolayers of BBCEC and BAEC were stained with rhodamine-phalloidin (Fig. 3). Phalloidin is a fungal toxin that tightly binds to actin filaments. Phase contrast microscopy showed that BBCEC and BAEC were confluent (Fig. 3A, B). The rhodamine-phalloidin which stained actin filaments in BBCEC were

<table>
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<tr>
<th>Compound</th>
<th>Permeability coefficients (×10⁻³ cm/h)</th>
<th>BBCEC</th>
<th>BAEC</th>
</tr>
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<tbody>
<tr>
<td>(^{14})C-Sucrose</td>
<td>69.6 ± 4.3</td>
<td>270 ± 6</td>
<td>215 ± 35</td>
</tr>
<tr>
<td>FITC-dextran 4400</td>
<td>5.73 ± 0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FITC-dextran 71200</td>
<td>1.59 ± 0.25</td>
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</table>

Values are means ± S.D. of three or four experiments.

**Fig. 1.** Time Course of \(^{14}\)C-Sucrose Transport through Cultured Monolayers of BBCEC (●) and BAEC (○) and a Polycarbonate Membrane (■).

\(^{14}\)C-Sucrose samples were introduced into the luminal compartment, and their transport through endothelial monolayers was examined after gentle shaking at 37 °C. Each point represents the mean ± S.D. of three or four experiments. All symbols and error bars are indicated in the figure, but somewhere error bars are not seen because they are so small.

**Fig. 2.** The Cell Number of BBCEC and BAEC on the Membrane.

The cells were detached from the membrane after paracellular transport studies were performed. The cells were counted according to the cellular DNA content. Data are expressed as means ± S.D. of five or six experiments. a) \( p < 0.005 \) significantly different from BBCEC.
arranged as dense peripheral bands (Fig. 3C). On the other hand, the peripheral actin bands in BAEC were weakly stained (Fig. 3D). That the cells did not reach confluence did not cause this weak stain. This staining profile was observed over 2 weeks after the cells reached confluence. Therefore, it seems that the formation of a tight junction in BAEC is not as strong as that in BBCEC. We consider that the decrease in permeability through the BBCEC monolayer compared with that of BAEC is not due to the cell density on the membrane increasing because of more cells, but to junctional formation in itself.

**Transcellular Transport** Transcellular transport was studied by means of [3H]leucine and [3H]alanine uptake using confluent monolayers of primary cultured BBCEC and primary cultured BAEC. The concentration dependence of [3H]leucine and [3H]alanine uptake in BBCEC and BAEC was examined (Fig. 4) and is summarized in Table II. The $K_m$ value for leucine transport into BBCEC was similar to that of the rat in vivo as reported by Smith et al. (29 μM)\(^4\) and Miller et al. (87 μM).\(^4\) These results seem to demonstrate the presence of a neutral amino acid transport system in BBCEC.

The $K_m$ and $V_{max}$ values in BBCEC were lower than those in BAEC. The $K_m$ value of neutral amino acid transport through the BBB are 1—2 log orders less than that into nonbrain tissues such as intestinal epithelia, renal tubule, red blood cells, liver and so on.\(^4\) There are no reports describing the affinity of the transport of amino acids in cultured endothelial cells in other organs. Our results suggest that BBCEC have a higher affinity amino acid transport system than BAEC, and that BBCEC have a specialized amino acid transport system. This *in vitro* BBB system, using primary cultured BBCEC, seems to have transport functions similar to those of *in vivo* BBB with regard to the transcellular pathway.

**Enzyme Activity** ALP and γ-GTP activity in BBCEC were examined when BBCEC were isolated from bovine brain and when BBCEC reached confluence in a primary culture (Table III). ALP and γ-GTP activity in primary cultured BAEC at confluence were also measured. These activities in confluent BBCEC were lower than those just after isolation, but were much higher than those in BAEC. These results suggest that even if primary cultured BBCEC reduced ALP and γ-GTP activity when cultured *in vitro*, their levels were still much higher than those in primary cultured BAEC.

Several investigators have studied the mechanism of BBB transport for solutes using primary cultured BBCEC.
Fig. 4. Concentration Dependence of $[^3]$HLeucine (A, C) and $[^3]$HAlanine (R, D) Uptake into BBCEC (A, B) and BAEC (C, D)

The cells were incubated with various concentrations of $[^3]$HLeucine for 1 min and $[^3]$HAlanine for 3 min. The uptake of $[^3]$HLeucine and $[^3]$HAlanine was measured. Each point represents the mean ± S.D. of three experiments. All symbols and error bars are indicated in the figure, but somewhere error bars are not seen because they are so small.


<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Cells</th>
<th>$K_{m}$ (μM)</th>
<th>$V_{max}$ (nmol/mg protein/min)</th>
<th>$K_{d}$ (pL/mg protein/min)</th>
</tr>
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<tbody>
<tr>
<td>Leucine</td>
<td>BBCEC</td>
<td>55.5 ± 5.2</td>
<td>4.71 ± 0.47</td>
<td>1.52 ± 0.70</td>
</tr>
<tr>
<td></td>
<td>BAEC</td>
<td>70.8 ± 0.7</td>
<td>1.88 ± 0.33</td>
<td>1.22 ± 0.44</td>
</tr>
<tr>
<td>Alanine</td>
<td>BBCEC</td>
<td>145 ± 58</td>
<td>9.46 ± 2.91</td>
<td>3.36 ± 1.24</td>
</tr>
<tr>
<td></td>
<td>BAEC</td>
<td>632 ± 73</td>
<td>4.85 ± 0.50</td>
<td>0.60 ± 0.10</td>
</tr>
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Parameters represent means ± S.D. obtained from the nonlinear least-squares regression analysis.

**TABLE III.** ALP and γ-GTP Activity in BBCEC and BAEC

<table>
<thead>
<tr>
<th>Cells</th>
<th>ALP activity (U/10^6 cells)</th>
<th>γ-GTP activity (U/10^6 cells)</th>
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<tbody>
<tr>
<td>BBCEC</td>
<td>109 ± 16</td>
<td>6.63 ± 0.76</td>
</tr>
<tr>
<td>BBCEC</td>
<td>65.0 ± 13.7</td>
<td>1.30 ± 0.17</td>
</tr>
<tr>
<td>BAEC</td>
<td>1.89 ± 0.21</td>
<td>0.07 ± 0.02</td>
</tr>
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</table>

ALP and γ-GTP activity in BBCEC were measured when BBCEC were isolated from bovine brain and when BBCEC reached confluence in primary culture. ALP and γ-GTP activity in confluent monolayers of primary cultured BAEC were measured. Data are expressed as means ± S.D. of three or four experiments.

There are no published comparisons of the BBB properties between primary cultured BBCEC and endothelial cells in other organs. Our studies suggest that primary cultured BBCEC maintain several specialized properties of the BBB in vivo, such as tight junctions and an amino acid transport system, as well as ALP and γ-GTP activities, compared with primary cultured BAEC. Primary cultured BBCEC would be better as an in vitro BBB system for studying drug transport and enzymes.

However, the paracellular permeability of solutes through the BBCEC monolayer is leaky even in primary cultures in vitro compared with in vivo, and ALP and γ-GTP activities decrease when cultured in vitro. Previously, we reported that glial extracellular matrix elevated γ-GTP activity in BBCEC and BAEC. Many investigators have reported that ALP and γ-GTP activities and the formation of tight junctions in brain capillary endothelial cells were induced by co-culture with glial cells, or by culture with glial conditioned medium. These procedures would increase the efficiency of primary cultured brain capillary endothelial cells as an in vitro BBB system.

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