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APPLICATION OF CULTURED ENDOTHELIAL CELLS OF THE BRAIN MICROVASCULATURE IN THE STUDY OF THE BLOOD-BRAIN BARRIER

Donald W. Miller, Kenneth L. Audus, and Ronald T. Borchardt[1]

Department of Pharmaceutical Chemistry, School of Pharmacy, University of Kansas, Lawrence, Kansas 66045

SUMMARY: The endothelial cells of the brain microvasculature provide both physical and enzymatic barriers to the exchange of molecules between the extracellular fluid environment of the brain and the systemic circulation. To better understand these barrier properties and the factors that influence them at the cellular level, an in vitro model of the blood-brain barrier has been developed using primary cultures of bovine brain microvessel endothelial cells. The focus of the present paper is on the procedures associated with the isolation, culturing, and maintenance of bovine brain microvessel endothelial cells. Experimental applications of the cell culture model in studying the blood-brain barrier and efforts directed at improving the current cell culture model are also discussed.

Key words: brain endothelial cells; blood-brain barrier.

INTRODUCTION

The exchange of molecules between the extracellular fluid compartment of the brain and the systemic circulation is restricted by what is commonly referred to as the blood-brain barrier (BBB). Although other cell types may contribute to the integrity of the BBB, the endothelial cells of the brain microvasculature provide the primary limitation to the passage of molecules from the blood to the brain. The barrier properties of brain capillary endothelial cells are attributable, in part, to the formation of tight intercellular junctions which limit the paracellular diffusion of large macromolecules (greater than 20 angstroms) such as peptides and proteins (43). In addition, brain capillary endothelial cells have no fenestra and a reduced amount of pinocytotic activity, thereby reducing the amount of intracellular passage of molecules between the blood and brain (19). Finally, endothelial cells of the brain microvasculature have a variety of enzymatic systems which metabolize compounds before and during intracellular transport (61). Together these morphologic and biochemical characteristics of endothelial cells in the brain microvasculature restrict the bidirectional passage of polar, ionized, and hydrophilic compounds from the blood and the brain.

For many years the BBB was believed to be a static barrier. However, recent studies have indicated that the BBB is a dynamic cellular system that can be modified under various physiologic (30) and pathophysiologic conditions (9,25,30). In addition to maintaining the extracellular fluid environment essential for normal central nervous system function, the BBB also presents a formidable obstacle in the delivery of therapeutic agents from the systemic circulation to the brain. In this regard, current interest has centered around the identification and characterization of carrier systems that exist within the BBB for transporting nutrients and specific macromolecules and the possible use of such systems in the delivery of therapeutic agents (*see* 8, 9, 55 for reviews). Therefore, examining the cellular events that regulate BBB function may not only lead to a better understanding of physiologic and pathophysiologic conditions affecting the integrity of the BBB but also aid in the development of improved systems for the delivery of therapeutic agents to the central nervous system.

To gain a better understanding of the BBB at the cellular level, an in vitro model using cultured brain microvasculature endothelial cells has been developed (4,5,18,31). Such a model allows for the mechanistic study of the metabolic and transport systems present in brain capillary endothelial cells, as well as the factors that influence or modify permeability in these cells. The following is a detailed description of the methods used for the isolation and culturing of brain endothelial cells and a brief presentation of data supporting the utility of cultured brain endothelial cells in the study of the BBB.

METHODS

Isolation of brain endothelial cells. The procedure for the isolation of bovine brain endothelial cells (BBEC) is a modification of the techniques first described by Bowman and colleagues (16,17) for the isolation of brain microvessel endothelial cells. Although the protocol described below and summarized in Table 1 has been developed for isolation of microvasculature endothelial cells from two bovine brains (approximately 250 to 300 g of

[1] To whom correspondence should be addressed.

wet brain matter), similar approaches have been used in the isolation of microvasculature endothelial cells from both canine and primate brains in our laboratory. In addition, the isolation and culturing of human brain microvessel endothelial cells has been reported recently (23). The steps involved in the isolation of BBEC can be divided into three general categories: collection of gray matter, enzymatic isolation of crude brain microvessels, and centrifugal separation of endothelial cells from other cellular components. Each of the three categories are discussed in greater detail below.

Fresh bovine brains are transported from the meat processing plant to the laboratory in approximately 300 ml of sterile, ice-cold, minimum essential medium (MEM; 9.5 mg/ml) containing 11.92 mg/ml HEPES, 50 μg/ml polymixin B and gentamicin, and 2.5 μg/ml amphotericin B and adjusted to a pH of 7.4. For best results, the brains should be obtained as close to the time of slaughter as possible. Under aseptic conditions the brain stem and lower brain regions are separated from the cortex and discarded. The remaining brain material is bathed in cold phosphate buffered saline containing 300 μg/ml of penicillin G and streptomycin (PBS3X). Using sterile curved forceps, the outer blood vessels and meninges are removed and the brain matter is placed in a separate container of cold PBS3X. After removing the outer blood vessels and meninges, the brain matter should be firm to the touch and light-pink in appearance. Portions of the brain matter which are placid or discolored are removed with a sterile scalpel or knife. Once the outer blood vessels and meninges have been removed, the gray matter is collected. This can be done by aspirating the gray matter into collection containers (modified 150-ml plastic syringe cylinders connected to a vacuum pump) using a 1-ml plastic pipette tip with 3 to 4 mm cut off the end. Alternatively, the outer gray matter can be removed by scraping with a sterile scalpel. During both the removal of the meninges and the collection of the gray matter the brain material is kept at 4° C.

The enzymatic digestion of the gray matter is a two-step process requiring both dispase (a neutral protease) and collagenase/dispase enzyme digestions. The first digestion with dispase separates microvessel segments from neuronal tissue, while the second enzyme digestion with collagenase/dispase removes the basement membrane and pericytes leaving crude microvessel endothelial cells. Dispase (125 mg/ml; Boehringer-Mannheim, Indianapolis, IN, or Sigma Chemical Company, St. Louis, MO) and collagenase/dispase (5 mg/ml; Boehringer-Mannheim) are prepared in MEM (pH 7.4) and incubated in a shaker bath for 30 min at 37° C. The dispase solution is then centrifuged for 30

TABLE 1

PROTOCOL FOR THE ISOLATION OF MICROVASCULATURE ENDOTHELIAL CELLS FROM BOVINE BRAINS

Section I Isolation of gray matter	Remove surface vessels and meninges using sterile curved forceps: (brain matter bathed in PBS at 4°C) ↓ Aspirate cerebral gray matter: collect in 150-ml containers ↓ Pass gray matter through 1000-μm screen: collect in preweighed 500-ml bottles with screw-top lids ↓
Section II Dispase and collagenase/dispase enzyme digestions	Add 4 ml dispase solution (125 mg/ml) per 50 g of brain matter and incubate mixture for 30 min at 37°C in shaker bath (100 to 120 oscillations/min) ↓ Add MEM, pH 9.4, in volume equal to the weight of the collected brain matter and incubate mixture under same conditions for an additional 2.0 h ↓ Centrifuge suspension for 10 min at 1000 ×*g* ↓ Resuspend pellet in 500 ml of 13% dextran solution (avg. MW 70 000) and centrifuge for 10 min at 5800 ×*g* ↓ Resuspend crude microvessel pellet (dark red in color) in 20 ml collagenase/dispase solution (1 mg/ml) and incubate for 2 h at 37°C in shaker bath ↓
Section III Gradient separation of microvessel endothelial cells	Centrifuge suspension for 10 min at 1000 ×*g* ↓ Resuspend microvessels in 8 ml MEM, pH 7.4, and gently layer over 50% Percoll gradient ↓ Centrifuge cell suspension in Percoll gradient for 10 min at 1000 ×*g* ↓ Collect microvessel endothelial cells in 50-ml centrifuge tubes (cloudy, pnkish, diffuse layer directly below the band of cell debris found at the top of gradient) ↓ Wash endothelial cells (twice) by adding up to 50 ml of culture media and centrifuging for 10 min at 1000 ×*g* ↓ Resuspend endothelial cells in culture media containing 20% equine serum and 10% DMSO: Aliquot into 2-ml cyrotubes and store at −70°C

min at 1000 $\times g$. Both enzyme solutions are sterile filtered and stored at −20° C until use. For best results the enzyme solutions should be prepared the day before or the morning of the BBEC isolation. While the total amount of enzyme solution necessary is dependent on the amount of gray matter collected, 25 ml of dispase and 6.5 ml of collagenase/dispase is sufficient for isolating BBEC from two brains.

The collected gray matter is prepared for the first digestion by passing through a 1000-μm screen into sterile, preweighed screw-top bottles. Alternatively, the gray matter can be minced with a sterile scalpel into 1- to 2-mm cubes and collected in preweighed screw-top bottles. Dispase solution is added (4 ml dispase solution for every 50 g of brain material) and the resulting mixture is incubated in a shaker bath (100 to 120 oscillations/min) for 20 to 30 min at 37° C. After the initial 20- to 30-min incubation period, a volume of MEM (same as described above except HEPES is replaced with 6.05 mg/ml tris base and pH is adjusted to 9.5) equal to the initial weight of the collected grey matter is added and the incubation is continued under the same conditions for an additional 2 h. The addition of MEM (pH 9.5) is important because disruption of the cellular organelles during the initial 20 to 30 min of the dispase enzyme digestion decreases the pH of the suspension and thus decreases the activity of the neutral protease. The actual amount of MEM (pH 9.5) necessary can be determined by sampling a known quantity of the cell suspension, centrifuging for 10 min at 1000 $\times g$, and determining the volume of MEM (pH 9.5) required to adjust the pH of the supernatant to 7.4. For most isolations, the addition of MEM (pH 9.5) in a 1:1 volume-to-weight ratio as described above is sufficient to return the pH to a neutral range and ensure a proper yield during this digestion. However, if the color of the suspension changes from a pinkish to yellow tint during the course of the incubation, the pH is too acidic and sterile NaOH should be added.

After the dispase digestion, the brain matter is centrifuged for 10 min at 1000 $\times g$. The result of this centrifugation is a dark red pellet and a larger layer of semisolid material (light color) with a layer of liquid (dark color) on the top. The dark liquid layer is removed and the remaining pellet and semisolid layer containing the microvessel segments are resuspended in a sterile 13% dextran solution and centrifuged for 10 min at 5800 $\times g$ to further separate the microvessel segments from neuronal cell debris. The sterile dextran solution is prepared by dissolving 65 g of dextran in 422 ml of distilled water. This mixture is autoclaved, and after cooling, sterile stock solutions of gentamicin, polymixin B, and amphotericin B are added to give final concentrations of 40.0, 50.0, and 2.5 μg/ml, respectively. The volume of the dextran solution is adjusted to the final volume of 500 ml by adding 50 ml of sterile MEM10X and 25 ml of 1 *M* HEPES. After the dextran centrifugation, there should be a dark red pellet and a lighter colored liquid supernatant with a yellow semisolid layer on the very top. The semisolid layer on the top is broken up and poured off along with the liquid supernatant. The remaining dark red pellet containing the microvessel segments is resuspended in collagenase/dispase solution using a sterile 5-ml pipette tip to disperse the pellet in the enzyme solution. The cell suspension is then adjusted up to a volume of 20 ml using MEM (pH 7.4) and incubated in a water shaker bath (100 to 120 oscillations/min) at 37° C for 4 h.

After the 4-h collagenase/dispase digestion, the volume of the cell suspension is adjusted to 50 ml with MEM (pH 7.4) and centrifuged for 10 min at 1000 $\times g$. The supernatant is discarded and the remaining pellet containing the crude microvessel endothelial cells is resuspended in approximately 8 ml of MEM (pH 7.4), distributed evenly over a Percoll gradient, and centrifuged for 10 min at 1700 $\times g$. The Percoll solution is prepared by adding 15 ml of MEM10X, 7.5 ml of 1 *M* HEPES, and 52 ml of deionized water to 75 ml of Percoll (Sigma Chemical Company). Gentamicin, polymixin B, and amphotericin B are added to give final concentrations of 40, 53, and 2.5 μg/ml, respectively. The Percoll gradient is generated by centrifuging the Percoll solution for 1 h at 4° C and 26 000 $\times g$. For optimal separation of BBEC from other cellular components, generation of the Percoll gradient should be initiated during the last 45 min of the collagenase/dispase digestion. After the Percoll gradient centrifugation, three layers can be observed; a white solid layer of cell debris is found on the top, a diffuse layer slightly red in color containing the isolated endothelial cells is located directly below, and a solid layer of red blood cells is found at the bottom. Using a 5-ml syringe with a sterile 18-gauge needle, the entire middle layer containing the endothelial cells is removed. A high density of endothelial cells is often found directly below the top white layer, and to collect these cells some of the top cell debris layer may also be collected.

To provide a clean isolation, MEM:F12 is added to the collected BBEC and the suspension is centrifuged for 10 min at 1000 $\times g$. The supernatant is discarded and the pellet is once again resuspended in MEM:F12 and centrifuged for 10 min at 1000 $\times g$. The BBEC are then prepared for storage by resuspending the pellet from the final centrifugation in freezing medium. The freezing medium is a solution that is 70% by volume MEM:F12, 20% by volume equine serum, and 10% by volume dimethyl sulfoxide (DMSO). In a typical BBEC isolation the cells are resuspended in approximately 38 ml of freezing media. Aliquots from the cell suspension are placed in cryotubes and stored at −70° C for up to 2 mo. The isolated endothelial cells can be stored for longer periods of time in liquid nitrogen or at −135° C.

Culturing of primary BBEC. Primary BBEC have been successfully cultured on both solid plastic surfaces (35 to 100-mm dishes; 6, 24, and 96-well culture plates) and filter membranes (polycarbonate filters, regenerated cellulose membranes and various commercial membrane inserts such as Transwell and Millicell). The procedure required for preparing the surfaces for culturing of BBEC is essentially the same for both solid and membrane surfaces and is illustrated in Table 2. Because endothelial cells in general do not grow very well on plastic surfaces, the culture plates and membrane filters are coated with collagen and fibronectin before seeding. Rat-tail collagen (3 mg/ml in 0.1% acetic acid solution) is added to the growing surfaces in a volume of 0.1 ml/cm^2. After a short period (1 to 5 min), the excess collagen is removed and the growing surfaces are exposed to ammonia fumes for 20 to 30 min to promote cross-linking of the collagen. The collagen-coated surfaces are sterilized by exposure to UV light for 60 to 90 min. Fibronectin (human or bovine; 0.04 mg/ml; Sigma Chemical Company) is also added in excess (0.1 ml/cm^2) to the collagen-coated growing surfaces. After a period of 30 min the excess fibronectin is

removed and BBEC that have been thawed and washed with culture media are seeded onto the growing surfaces at a density of 50 000 cells/cm^2. It should be noted that other laboratories have reported successful culturing of brain microvasculature endothelial cells using commercially available extracellular matrices (36) or fibronectin alone (18). For the first 3 days, the BBEC are cultured in MEM:F12 supplemented with 50 μg/ml polymixin B and 100 μg/ml heparin sulfate, to reduce microbial contamination and to promote endothelial cell growth, respectively. After the Day 3, polymixin B is no longer necessary and can be omitted. The culture medium is changed every other day until the BBEC become a confluent monolayer (10 to 12 days). Endothelial cell growth factor (ECGF; Sigma Chemical Company) can be added to the culture medium when necessary to stimulate or accelerate the formation of a confluent monolayer.

Experimental applications of cultured BBEC. Primary cultures of BBEC can be used to examine many different aspects of BBB function. Careful consideration of the conditions of the experiment, including the culturing surface, is required during the design of each experiment. Cells cultured on solid plastic surfaces are best suited for metabolism and receptor characterization studies. To identify cellular enzyme systems, confluent monolayers of BBEC are washed with PBS and the BBEC are scraped from the culture dishes, collected in a centrifuge tube, and centrifuged for 10 min at 1000 ×*g*. The pellet is resuspended in PBS and homogenized. The homogenate is centrifuged for 15 min at 15 000 ×*g*, and the supernatant from this centrifugation is assayed for enzymatic activity. In contrast to lysing the cells and assaying enzyme activity, specific substrates can be incubated with intact monolayers of BBEC and metabolism monitored under various experimental conditions. Although metabolism studies using cell extracts are helpful in identifying enzyme systems present in cultured BBEC, the use of intact monolayers is necessary to determine the susceptibility of a specific compound to metabolism by BBEC. Studies by Baranczyk-Kuzma et al. (15) and Scriba and Borchardt (49) provide a more comprehensive description of the experimental procedures involved in both types of metabolism studies.

Identification of various ligand receptors can also be accomplished using BBEC cultured on solid plastic surfaces. For receptor characterization studies, the BBEC monolayers are exposed to a specific labeled ligand. After incubating, the nonbound ligand is washed away with cold PBS and the cells are dissolved by adding 1 ml of 0.2 N NaOH to the monolayer. After 8- to 12-h, the NaOH solution is collected and the amount of labeled ligand is determined and a protein assay is performed. By varying the experimental conditions (e.g., temperature, incubation time, concentration of unlabeled ligand, exposure to acid wash solutions, etc.) important information necessary for understanding the kinetics of the receptor is compiled. Experiments by Smith et al. (54) are representative of the types of receptor studies possible using cultured brain endothelial cells.

Although transcellular transport studies with BBEC cultured on solid plastic surfaces are not possible, both the uptake and

TABLE 2

PREPARATION AND MAINTENANCE OF PRIMARY CULTURES OF BOVINE BRAIN CAPILLARY ENDOTHELIAL CELLS

Excess rat tail collagen (3 mg/ml) is added to growth surfaces

↓

Excess collagen is removed and growth surfaces are placed in ammonia fumes for 20 to 30 min

↓

Collagen-coated surfaces are sterilized by exposure to UV light for a minimum of 60 min

↓

Growth surfaces are wetted with human fibronectin (0.04 mg/ml) for 30 min

↓

Excess fibronectin is removed and BBEC are seeded at a density of 50 000 cells/cm^2 using the following culture media[a]

45% MEM
45% Hams F12 nutrient mix
10% plasma-derived horse serum
13 m*M* sodium bicarbonate
10 m*M* HEPES
50 μg/ml gentamicin
2.5 μg/ml amphotericin B
50 μg/ml polymyxin B
100 μg/ml heparin sulfate

↓

Medium is changed every 2 days until confluent monoolayer is obtained (10 to 12 days) using following culture media[a]

45% MEM
45% Hams F12 nutrient mix
10% plasma-derived horse serum
13 m*M* sodium bicarbonate
10 m*M* HEPES
50 μg/ml gentamicin
2.5 μg/ml amphotericin B
100 μg/ml heparin sulfate

[a] Culture medium is adjusted to a pH of 7.4 and sterile filtered.

efflux of various compounds can be examined (*see* 29, 40, 42, 56 for detailed descriptions). To evaluate the uptake or transport of compounds into cultured BBEC, the cell monolayers are exposed to the compound for a time, after which unbound compound is removed by washing with cold buffer solution. The amount of compound remaining associated with the cells is then assayed to determine uptake. Efflux experiments are similar in protocol to uptake experiments in that the monolayer is exposed to a specific compound for a time, after which the unbound compound is removed by washing the BBEC with cold PBS. However, instead of assaying the amount of compound internalized by the cells, assay buffer is added and the efflux of the compound from the cells to the extracellular compartment is monitored over time. At the end of the experiment, the cells are removed from the culture surfaces and analyzed for the amount of compound still present in the cells and protein content. Both qualitative and quantitative assessment of the degradation of the compound remaining within the endothelial cells and released into the extracellular compartment provide additional information about the endocytic process.

The same type of experiments described above for BBEC cultured on solid plastic surfaces are also applicable to cells cultured on filters or membrane inserts. However, the use of BBEC cultured on filters or membrane inserts gives an added advantage in that cell polarity with respect to metabolism or receptor distribution can be examined. The polarity of BBEC in the uptake and efflux of internalized molecules can also be evaluated using cells cultured on filters or membrane inserts. The use of BBEC cultured on filters or membrane inserts is required for determining the transcellular transport or permeability of a compound across the monolayer. In transport/flux studies, a known amount of compound is added to either the apical (luminal) or basolateral (abluminal) side. The appearance of the compound in the opposite compartment is monitored for a time by withdrawing samples and replacing with an equal volume of assay buffer. Active transport, unlike passive diffusion, is saturable, self-inhibiting, and temperature and energy dependent. Studies examining active transport systems are normally carried out in the presence of a "nonpermeable" compound of similar molecular weight to access the leakiness of the monolayer. More detailed descriptions of the procedures used to examine both the passive diffusion and active transport of compounds across BBEC monolayers can be found in the studies of Shah et al. (50), Audus and Borchardt (6), Raub and Newton (42), and Raub et al. (41).

RESULTS AND DISCUSSION

The utility of cultured BBEC as an in vitro model for the BBB depends on the extent to which cultured BBEC retain the characteristics of brain capillary endothelial cells found in vivo. Using Factor VIII as a specific marker for endothelial cells, Guillot et al. (29) determined that more than 99% of the cells in the primary cultures of BBEC stained positive for the Factor VIII antigen. Additional biochemical studies indicate that enzyme markers specific for the BBB (gamma-glutamyl transpeptidase and alkaline phosphatase) are also detected in cultured BBEC (6,37). Morphologic studies performed at the electron microscope level show primary cultured BBEC resemble noncultured BBEC in that there are no fenestra, few pinocytotic vesicles, tight intercellular junctions, and an abundance of mitochondria (5,6,29). Furthermore, lectin binding studies indicate that the surface composition of primary cultures of BBEC is similar to that previously reported for brain microvascular endothelial cells both in vivo and in vitro in a variety of species including humans (40,62). Primary cultures of BBEC are also functionally polarized (27,40,42). Several other enzymes, such as monoamine oxidases, catechol *O*-methyltransferase, angiotensin converting enzyme as well as aminopeptidases and acid hydrolases, have also been observed in primary cultures of BBEC (12–15,33,48,49). Together, these studies suggest that the typical morphologic structure and enzymatic activity of brain capillary endothelial cells are retained in primary cultures.

An important characteristic of brain capillary endothelial cells is the formation of tight intercellular junctions. Two methods commonly used to evaluate tight intercellular junctions in cultured BBEC are transendothelial electrical resistance (TEER) and nonpermeable marker diffusion. Although TEER is a more convenient index of tight intercellular junction formation, it seems to be a less reliable method for evaluating tight junctions in endothelial cells than is the use of nonpermeable markers (35). As shown in Table 3, primary cultures of brain endothelial cells have higher electrical resistances and lower permeability values than cultured endothelial cells from other regions of the body, indicating the presence of more developed tight junctions in cultured brain endothelial cells. However, compared to in vivo values of electrical resistance and marker permeability, the tight junctions in primary cultured brain endothelial cells are not as developed as those in vivo (Table 3). Consequently, while the permeation of compounds across confluent primary BBEC monolayers are similar with respect to lipophilicity and molecular weight (MW), the actual values are several orders of magnitude higher than has been reported in vivo. This is exemplified in the transport and diffusion studies using primary BBEC cultured on polycarbonate filters (50), regenerated cellulose membranes (3,60), and Millipore membrane inserts (47). Despite these permeability differences in the BBB and the culture model, active transport of compounds can be evaluated in primary cultures of BBEC by correcting for the "leakage" of the monolayer using a nonpermeable marker of similar molecular weight (e.g., mannitol, sucrose, inulin, dextrans). By using such a method, active transport systems have been identified in primary BBEC cultures for glucose (56), amino acids (7), choline (59), and nucleosides (51).

Primary cultures of BBEC have been very useful in the identification and characterization of peptide and protein receptors within the brain microvasculature. Receptors for proteins such as ricin and transferrin have been demonstrated in cultured BBEC (40,42). In contrast to the reduced level of fluid phase endocytosis observed in cultured BBEC (29), both ricin and transferrin undergo more extensive but slower internalization through the receptor-mediated endocytic process. Experiments using BBEC cultured on Transwell membrane inserts indicate that the receptor-mediated endocytosis of both ricin and transferrin is polarized to the apical side (35,37,54). Receptors for several other peptides and proteins such as insulin (34) insulin growth factor I (44), atrial natriuretic factor (54), and angiotensin II (28) have also been identified in primary cultures of brain

TABLE 3

ASSESSMENT OF TIGHT INTERCELLULAR JUNCTIONS IN ENDOTHELIAL CELLS FROM VARIOUS ORGANS USING BOTH ELECTRICAL RESISTANCE AND MARKER FLUX AS INDICES

Cell Type[a]	Electrical Resistance, ohms cm^2	Solute Permeability, $cm/s \times 10^{-7}$		Reference
		Sucrose	Albumin	
Cultured brain endothelial cells	61–783	0.8, 37.7, 245	0.009, 1.3	22,41,45,46,53,60
Cultured aortic endothelial cells	13–21	260	0.36, 0.52	1,38,39,46,57
Cultured pulmonary endothelial cells	6		4.78, 7.35	24,52
Brain capillary endothelial, noncultured	1900	0.25–0.93[b]	0.005–0.01[b]	2,11,20,21,32

[a] Studies cited are not restricted to a particular species, and cultured endothelial cells may be either primary or passaged cell lines.
[b] Permeability values for sucrose and albumin in noncultured brain capillary endothelial cells were determined using 1.35 cm^2 as the capillary surface area in 100 g of brain tissue as indicated in Crone and Levitt (20).

microvasculature endothelial cells. Although the precise functions of these receptors are currently unknown, each macromolecule is internalized through receptor-mediated processes and, therefore, may represent potential systems for the transport of the various peptides across the BBB.

Modification of BBB permeability by environmental, pathophysiologic, and cellular factors have been well documented. However, the mechanisms responsible for such alterations in BBB permeability are for the most part unknown. Consistent with the available in vivo data, permeability of primary cultured BBEC has been altered by exposure to aluminum (10), arabinose (6,17), or angiotensin II (27). Aluminum increased the permeability of cultured BBEC as demonstrated by the increased diffusion of fluorescein. The mechanism for the permeability increases observed with aluminum seems to be related to the neutralization of the cell surface because membrane fluidity was unaffected and pinocytosis, as evaluated by the internalization of lucifer yellow, was decreased (10). The utility of cultured brain endothelial cells in examining the cellular mechanisms responsible for permeability changes in the BBB is best illustrated for the permeability changes produced by angiotensin II. As has been demonstrated in vivo, exposure of confluent BBEC monolayers to angiotensin II resulted in a 20 to 30% increase in the pinocytotic activity as measured with lucifer yellow (27). The increased pinocytosis observed with angiotensin II was receptor mediated as the angiotensin II receptor antagonist, sarathrin, prevented the effects of the peptide. Furthermore, the increased pinocytosis produced by angiotensin II, but not bradykinin or phorbol myristate acetate, was abolished by prior exposure to indomethacin, an inhibitor of prostaglandin synthesis (27). Together these studies suggest the specific receptor-mediated effects of angiotensin II on the pinocytotic activity of cultured BBEC is most likely mediated by prostaglandins. In contrast to the effects of angiotensin II on pinocytosis, the passive diffusion of dextran across BBEC cultured on polycarbonate filters was reduced by 80% (3,26). In addition, the effects of angiotensin II on permeability were polarized to the apical side as addition of the peptide to the basolateral side had no effect on the diffusion of dextran.

The major limitation of cultured BBEC as a model for the BBB is the increased permeability of cultured BBEC due to leakage through intercellular junctions. Recently, attempts have been made to enhance the formation of tight junctions and enzymatic activity in cultured BBEC using factors released by astrocytes and neurons (22,41,45,58). The results of these experiments seem promising because permeability of primary cultured BBEC monolayers decreased substantially when co-cultured with astrocytes or exposed to astrocyte-conditioned media. The decrease in permeability observed in the above studies (as much as twofold as seen with sucrose) was attributed to an enhanced development of tight junctions. A further enhancement of tight junction formation in primary cultured BBEC has been reported by Rubin and colleagues (45) by using astrocyte conditioned media in conjunction with compounds that increase cellular concentrations of cAMP. In this study, electrical resistance was increased 10-fold by using astrocyte conditioned media and various cAMP analogues. These studies suggest that the current culture system for BBEC may be improved through the addition of cellular factors or second messengers or both, thus further increasing its utility as a model for the in vivo BBB.

Examining the dynamics of the BBB is important in both the understanding of conditions that influence BBB integrity and the development of improved strategies for the delivery of therapeutic agents to the central nervous system. Despite its limitations, the application of cultured BBEC in the study of BBB functions presents the opportunity to examine many features of the BBB at a cellular level not possible using in vivo techniques or isolated brain capillaries. Furthermore, the use and identification of cellular factors that enhance the formation of tight intercellular junctions in cultured brain endothelial cells provide the potential to even more closely model the BBB.

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