Brain Microvessel Endothelial Cells in Tissue Culture: A Model for Study of Blood-Brain Barrier Permeability

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utes of death, were transported to the laboratory in iced tissue culture medium. Cortical tissue was cleaned of meninges and superficial blood vessels. Two hundred grams of brain tissue was minced to 1 to 2 mm cubes, incubated for 3 hours at 37°C in 500 ml of medium containing 0.5% dispase (Boehringer Mannheim, Indianapolis, IN), and collected by centrifugation at 1,000 g for 10 minutes. The pellets were then suspended in 500 ml of medium, now containing 13% dextran (Sigma, St Louis, MO; average molecular weight, 60,000). Microvessels were separated from other brain tissue by centrifugation of the suspension at 8,500 g for 10 minutes. A further 9- to 12-hour treatment of the microvessels with 1 mg/ml of collagenase/dispase (Boehringer Mannheim) in 20 ml of medium removed the basement membrane and most pericytes. Microvessels were pelleted at 1,000 g for 10 minutes and suspended in 8 ml of medium. Two milliliters of this suspension was placed on each of four 50 ml Percoll gradients, prepared as described previously [1].

Centrifugation at 1,000 g for 10 minutes separated the endothelial cells from contaminating debris. The band containing clumps of endothelial cells was removed, diluted with medium, and collected by centrifugation. The cells exhibited 80 to 90% viability by trypan blue exclusion, and the yield was approximately 2 x 10^6 viable cells. The cells were seeded onto a fibronectin-coated substrate as previously described [2] and grown in a tissue culture medium consisting of equal parts of nutrient mixture F-12 (GIBCO) and MEM containing 10 mM Hepes (pH 7.4) and 13 mM sodium bicarbonate with 10% porcine plasma-derived serum (Sterile Systems, Logan, UT). Plasma-derived serum was used to prevent growth of cells not containing FVIII/vWF-AG [2].

Examination
Identification of cells possessing FVIII/vWF-AG was performed essentially as described by Jaife and colleagues [9]. Rabbit antiserum to human factor VIII-associated protein was obtained from Behring Diagnostics (La Jolla, CA), and fluorescein-conjugated goat antirabbit IgG was purchased from Cappel Laboratories (Cocranville, PA). This antiserum to human FVIII/vWF-AG cross reacts with bovine antigen and is used to identify endothelial cells derived from bovine aorta [19] and pulmonary artery [20].

For examination with the phase-contrast microscope, 25 mm round plastic coverslips (Lux Scientific, Elkart, IN) were coated with fibronectin to promote cell adhesion, placed in 35 mm mult-wells (Lux Scientific), and seeded with 2 x 10^5 cells per square centimeter. After the cells achieved confluence (about one week), the coverslips were placed in Sykes-Moore chambers (Bellco, Vineland, NJ). Twenty-one-gauge needles connected to syringes with PE-160 tubing containing 10 mM Hepes (pH 7.4) and 13 mM sodium bicarbonate were glued (Silastic, Dow Corning, Midland, MI) to an 8 mm x 16 mm, forming a sealed chamber. The nylon screen was coated with rat tail collagen prepared as described by Michelopoulos and Piot [11] and fixed with 4% glutaraldehyde as described by Cereijido and colleagues [5]. After three washes with 50 ml of sterile PBS, the collagen was treated with 10 μg/cm² human fibronectin for 10 minutes and the collagen-fibronectin matrix was washed an additional 10 times with 50 ml of PBS. Bovine brain endothelial cells were seeded at 4 x 10^4 cells compared to a control medium (MEM) with a calcium concentration of 1.8 mM.

For scanning electron microscopy the cells were then fixed by treatment with the same medium, now containing glutaraldehyde in a final concentration of 2.5%. After being kept 1 hour at room temperature and overnight at 4°C, the cells were rinsed three times with phosphate-buffered saline (PBS, pH 7.2), postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.0) for 30 minutes, rinsed an additional three times with distilled water, and air dried. The specimens were then coated with 20 nm of gold and observed in a scanning electron microscope at 15 kV. Although air drying may produce surface artifacts, it ensures the integrity of the lateral associations of cells [3].

For transmission electron microscopy endothelial cells grown on collagen-coated coverslips were rinsed twice with 5 ml of PBS (pH 7.2) and then fixed with 2.5% glutaraldehyde in PBS for 15 minutes followed by 2.5% glutaraldehyde containing 3% tannic acid (adjusted to pH 7.0 with 5 N sodium hydroxide). After 45 minutes the cell layer was washed three times with PBS and postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.0) for 1 hour. The cell layer was rinsed three times with 0.1 M cacodylate, dehydrated through ethanol, and embedded in Polybed/Araldite (Polysciences, Warrington, PA). Sections were stained with uranyl acetate and lead citrate and viewed in a Philips 400 electron microscope.

For freeze-fracture etching, monolayers grown on fibronectin-coated coverslips were fixed in 2.5% glutaraldehyde in PBS (pH 7.2) for 1 hour and washed in three changes of PBS. The samples were then placed in 20% glycerol in PBS for 1 hour. Four mm discs of the plastic coverslips were cut and placed on specimen carriers [24]. The plastic discs and specimen carriers were frozen rapidly in a slurry of liquid nitrogen, placed in a double-replicating device, and transferred to liquid nitrogen for storage until placed in the freeze-fracture unit. The monolayers were fractured in a Balzers high vacuum freeze-etch unit at −115°C. Platinum-shadowed carbon replicas of the fractured faces were coated with 5% colloidin in amyl acetate and cleaned in Clorox bleach for 24 hours. Subsequently the replicas were rinsed in six changes of double distilled water, collected on HEX-460 grids (Polysciences), dipped in amyl acetate for 15 minutes to remove the collodion, and examined in a Philips 400 electron microscope at 60 kV.

To carry out transendothelial tracer studies, a support for the cells was fabricated from a 22.2 x 1.6 mm Plexiglas disc in which a 9.5 mm diameter hole had been cut. The hole was beveled to a diameter of 12.7 mm. Nylon mesh (118 μ; Terto, Elmsford, NY) was attached across the smaller (bottom) side of the hole. The tops of the Plexiglas discs then were glued (Silastic, Dow Corning, Midland, MI) to an 8 mm piece of polycarbonate tubing (19 x 16 mm), forming a sealed chamber. The nylon screen was coated with rat tail collagen prepared as described by Michelopoulos and Piot and fixed with 4% glutaraldehyde as described by Cereijido and colleagues [5]. After three washes with 50 ml of sterile PBS, the collagen was treated with 10 μg/cm² human fibronectin for 10 minutes and the collagen-fibronectin matrix was washed an additional 10 times with 50 ml of PBS. Bovine brain endothelial cells were seeded at 4.0 x 10^4 cells

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per square centimeter and allowed to grow and cover the surface for 10 days. Immediately prior to a transport experiment, the chamber containing the cells was affixed to a second piece of polycarbonate tubing with vacuum grease, forming a double-sided chamber. The chamber was then placed in a 35 mm Petri dish and 10 ml of MEM, containing 29 mM Hepes (pH 7.4), 4.2 mM sodium bicarbonate, and 1.8 mM calcium, was added to the dish. The cells were covered with 1.2 ml of the same medium, and a 5-minute equilibration period was begun. The fluid in the bottom chamber was in free communication with the surrounding medium, with mixing facilitated by a stirring bar placed in the bottom chamber. After the equilibration period, the medium covering the cells was replaced with an equal volume of medium containing 5 μCi/ml sucrose labeled with carbon 14 (New England Nuclear, Boston, MA). Samples of 0.2 ml were taken from the bottom chamber at various times for measurement of radioactivity by liquid scintillation spectrometry. The volume of the bottom chamber was returned to 10 ml by addition of 0.2 ml of medium after removal of each sample.

The effect of exposure to low calcium on the movement of 14C-sucrose across the endothelial cell monolayer was studied by adding the calcium-free medium during the 5-minute equilibration period. The effect of an osmotic stress was studied by exposure of the cells for the 5-minute equilibration period to medium containing 1.6 M arabinose. Transcellular movement of 14C-sucrose was then measured using normal medium.

Results

Figure 1A illustrates the structure of confluent bovine brain endothelial cells cultured in 10% porcine plasma-derived serum. The endothelial origin of these cells is supported by the immunofluorescent demonstration of FVIII/vWF-AG (Fig 1B). This antigen is found exclusively in endothelial cells and is widely used as a marker for endothelial cells in culture [8]. As did the endothelial cells from rat brain in our previous cultures [1], the bovine endothelial cells contain angiotensin-converting enzyme and form a nonthrombogenic surface (results not shown). Figure 1C illustrates the structure of cultured brain endothelial cells as observed in the scanning electron microscope. The formation of a continuous sheet of cells with overlap of membranes is evident. That these areas of cell overlap contain junctional complexes was confirmed by transmission electron microscopy (Fig 1D). The nature of these junctions was investigated further by the freeze-fracture technique. Figure 1E illustrates that the region between endothelial cells is characterized by a continuous network of complex anastomosing membrane particle arrays. This pattern is similar to that seen in the tight junctions of brain capillaries in vivo [21]. In addition, the endothelial cells in culture have few pinocytotic vesicles, a further similarity to their in vivo counterpart (see Fig 1D).

Satisfied that primary cultures of brain microvessel cells possess many of the properties that are known to have important barrier functions in vivo, we investigated the barrier formed in vitro by a monolayer of these cells. To observe the effects on structure of experimental manipulation, cells were grown to confluence on fibronectin-coated coverslips, mounted in a perfusion chamber, and observed by phase microscopy. After the cells were observed and photographed under normal conditions, test media were perfused through the chamber and serial observations of structural changes were documented. In each case morphological changes developed rapidly and were maximal after less than 5 minutes of exposure to the test medium. The chambers were then reperfused with normal culture medium and the cultures returned to the 37°C incubator. Observations were made for the next 24 hours to document reversibility of the cellular changes produced by the treatment. Separate coverslips were incubated under control and test conditions and then prepared for scanning electron microscopy either after the exposure to experimental conditions or 6 hours after return to normal culture conditions.

Figure 2A illustrates the effect on endothelial cultures of a 2-minute exposure to calcium-free medium. The retraction of cells from one another is apparent. Scanning electron microscopic examination (Fig 2B) revealed a loss of the ability to form continuous sheets. That these cells survived this treatment and reestablished contact after being returned to a calcium-containing medium is illustrated by scanning electron microscopy in Figure 2C (6 hours after treatment).

The effect of 1.6 M arabinose on the cultured endothelial cell monolayer at the phase microscopic level is revealed in Figure 2D. A reduction in phase contrast imaging indicates removal of water from the cells. Because this treatment does not result in cell separation, the junctional complexes must be relatively intact. Scanning electron microscopy revealed craters in or

Fig 1. Characteristics of cultured brain microvessel endothelial cells. (A) Phase contrast micrograph of confluent bovine brain endothelial cells. (× 525 before 10% reduction.) (B) Immunofluorescent demonstration of factor VIII/von Willebrand antigen in cultured bovine brain microvessel endothelial cells. (× 1,200 before 10% reduction.) (C) Scanning electron micrograph of cultured bovine brain microvessel endothelial cells, demonstrating close apposition of cells. (× 700 before 5% reduction.) (D) Transmission electron micrograph of cultured bovine brain microvessel endothelial cells, revealing overlap and a junctional complex between two cells. Only a few pinocytotic vesicles are visible. (× 41,000 before 10% reduction.) (E) Transmission electron micrograph of platinum-carbon replica of freeze-fractured endothelial monolayer. Arrows denote the borders of tight junctions between cells. Complex anastomosing linear arrays of membrane particles are apparent. (× 35,000 before 5% reduction.)

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Fig 2. Morphological effects of calcium removal or arabinose treatment on cultured brain microvessel endothelial cells. (A) Phase contrast micrograph of cells treated for 2 minutes with Ca$^{2+}$-free medium containing 5 mM EGTA, demonstrating retraction of cells from one another. (x 600 before 10% reduction.) (B) Scanning electron micrograph of cells after calcium removal, revealing a substantial separation of the cells and exposure of the underlying coverslip. (x 740 before 10% reduction.) (C) Scanning electron micrograph of cells 6 hours after their return to calcium-containing medium, demonstrating the restoration of cell contacts. (x 850 before 10% reduction.) (D) Phase contrast micrograph of cells treated for 2 minutes with 1.6 M arabinose, revealing regions of clearing in or near the junctional contacts. In addition, nuclear and cytoplasmic structures are indistinct. (x 625 before 10% reduction.) (E) Scanning electron micrograph of arabinose-treated cells, showing craters in or near the junctional contacts between cells. (x 780 before 10% reduction.) (F) Scanning electron micrograph of arabinose-treated cells 6 hours after return to an isotonic medium, demonstrating restoration of normal structure. (x 850 before 10% reduction.)
The ability to isolate and culture endothelial cells derived from brain microvessels provides a new way to investigate cellular properties and metabolic reactions important to the function of the blood-brain barrier [1, 6, 14, 16, 22]. The cells used in this investigation were prepared from capillaries isolated from bovine brain. Special care was taken to purify the endothelial cells from possible contaminating cells such as pericytes, smooth muscle cells, and glial cells. Because of the potential for contamination by these other cell types, we established the presence of several specific markers of endothelium in our cultured cells. The markers included FVIII/vWF-AG, angiotensin-converting enzyme, and the production of a nonthrombogenic surface. In addition to these general endothelial properties, the cultured cells also formed frequent tight junctions and contained few pinocytotic vesicles (see Fig 1). These results indicate that the cells in vitro retain features responsible for formation of the blood-brain barrier in vivo.

In this investigation we studied the effect of two experimental manipulations on the integrity of the barrier formed by cultured brain endothelial cells. Removal of ionized calcium from the extracellular fluid is known to result in separation of tight junctions in epithelial tissues [10]. We found nearly complete separation and retraction of the cultured endothelial cells under these conditions (see Fig 2). Consistent with the wide separation of the endothelial cells, there was a marked increase in the movement of sucrose across the monolayer (see Fig 3). As in the epithelial tissues, junctional contact was reestablished by return of the cells to a physiological concentration of calcium.

The permeability of the blood-brain barrier in vivo is enhanced for several hours by brief infusion of hypertonic arabinose through the carotid artery [17]. This technique is used to increase the uptake of polar substances that normally are excluded in brain and is of considerable interest as a method for permitting the entry of drugs, proteins, and other organic molecules [12, 13]. We found a distinctive change in the structure of brain capillary endothelial cells treated with arabinose at the concentration used in vivo (see Fig 2). Unlike the major disruption of barrier continuity found after removal of calcium, a more limited change in perijunctional structure was produced by the hypertonic treatment. Not surprisingly, this treatment resulted in a smaller increase in the transcellular movement of sucrose than did the exposure to calcium-free medium (see Fig 3). Again, the morphological reaction was reversible, and the continuity of the cell layer was reestablished within 6 hours of return to normal culture medium. No lasting toxic effect was noted from this brief hypertonic treatment.

Brain microvessel endothelial cells in tissue culture form a barrier that responds to experimental manipulation. The integrity and permeability of this barrier can be monitored using morphological and tracer tech-
niques. This new system should provide a useful model for study of blood-brain barrier function and reaction to injury.

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References
2. Bowman PD, Betz AL, Goldstein GW: Primary culture of microvascular endothelial cells from bovine retina. In Vitro 18:626-632, 1982
24. Yee AG, Fischbach GD, Karnovsky MJ: Clusters of intramembranous particles on cultured myotubes at sites that are highly sensitive to acetylcholine. Neurobiology 75:3004-3008, 1978