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

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Endothelial cells were prepared from bovine brain microvessels and grown in tissue culture. They contained factor VIII/von Willebrand antigen, the most specific marker available for determination of the endothelial origin of cells in culture. The cultured cells formed complex tight junctions and contained few pinocytotic vessels. These properties are responsible for formation of the blood-brain barrier in vivo. When monolayers of the endothelial cells were exposed briefly to a calcium-free solution or treated with 1.6 M arabinose, distinctive morphological changes occurred in the intercellular contacts. In either case, a normal structure was reestablished following return to control medium. To assess the effect of these treatments on transcellular permeability, we measured the movement of sucrose labeled with carbon 14 across a monolayer of endothelial cells cultured on a collagen-coated nylon mesh. Removal of external calcium increased the rate of sucrose movement by 120%; the arabinose treatment increased transcellular flux by 40%.

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Defects in brain microvessel function occur in a wide variety of neurological diseases. The resulting breakdown of the blood-brain barrier may produce edema and result in further brain injury from increased intracranial pressure or inadequate tissue perfusion. The cellular mechanisms responsible for such changes in brain microvessel permeability are not well understood.

Under normal conditions the endothelial cells in brain microvessels are sealed together by continuous tight junctions that limit the movement of polar molecules across the vessel wall [18]. In addition, the endothelial cells contain only a few pinocytotic vesicles, and these do not appear to carry material across the cell from lumen to interstitial fluid [4]. After injury caused by acute hypertension [23] or ischemia [15], more pinocytotic vesicles are found in the endothelial cells, and these are thought to act as a carrier system to transfer plasma into the brain. Alternately, separation of tight junctions between endothelial cells may provide a paracellular pathway for the abnormal movement of plasma constituents into brain. Changes in tight junction permeability are reported after infusion of hypertonic solutions through the cerebrovascular bed [17].

To study formation and disruption of the barrier in more detail, we developed a method to isolate microvessels from bovine brain and then release and grow the endothelial cells in tissue culture. The cultured cells contain factor VIII/von Willebrand antigen (FVIII/vWF-AG), the most specific marker for endothelial cells. Ultrastructural examination revealed frequent tight junctional complexes and few pinocytotic vesicles, suggesting a resemblance of the cultured cells to their in vivo counterpart. Alterations in the integrity of this in vitro barrier were produced by removal of extracellular calcium or treatment with high concentrations of arabinose and studied by histological observation and measurement of transcellular tracer movement across a monolayer of the endothelial cells grown on a collagen-coated nylon mesh. A portion of this work appeared in a preliminary report [7].

Methods

Isolation of Microvessels and Culture of Endothelial Cells

Endothelial cell cultures were prepared by a modification of our previously described method for rat brain [1]. The tissue culture medium used for cell isolation consisted of minimal essential media (MEM; GIBCO, Grand Island, NY) with 50 mM Hepes buffer (pH 7.4), 100 μg/ml of penicillin, 100 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B. Bovine brains, obtained from a local slaughterhouse within 30 min-

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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 5,800 *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 2×10^8 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 Jaffe 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 (Cochranville, 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].

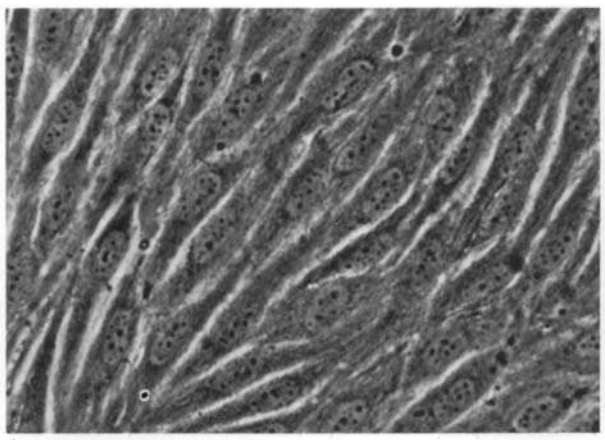
For examination with the phase contrast microscope, 25 mm round plastic coverslips (Lux Scientific, Elkhart, IN) were coated with fibronectin to promote cell adhesion, placed in 35 mm multiwells (Lux Scientific), and seeded with 2×10^4 cells per square centimeter. After the cells achieved confluence (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 were used to pierce the O-ring seal and provide for exchange of media while under observation. A calcium-free medium was prepared by addition of ethyleneglycol-*bis*-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) (Sigma) to 5 mM in Spinner's formulation of MEM (GIBCO), which lacks Ca^{++}. This solution was buffered to pH 7.4 with 29 mM Hepes. A hypertonic medium was prepared by addition of arabinose (Sigma) to 1.6 M in autoclavable MEM (Flow Labs, Rockville, MD) buffered to pH 7.4 with 29 mM Hepes. Results were 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% collodion 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 × 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; Tetko, 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 × 16 mm), forming a sealed chamber. The nylon screen was coated with rat tail collagen prepared as described by Michelopoulis and Pitot [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 $\mu g/cm^2$ 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 4.0×10^4 cells



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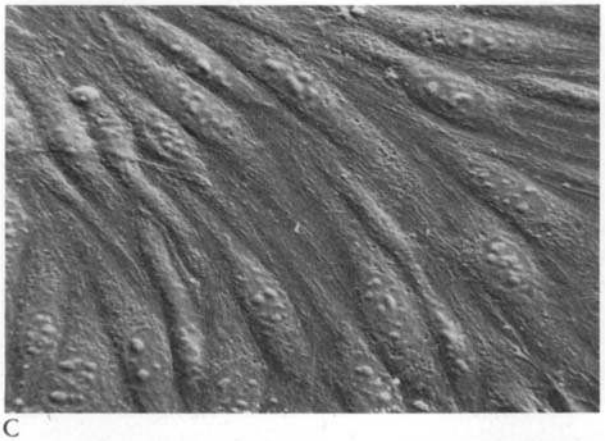

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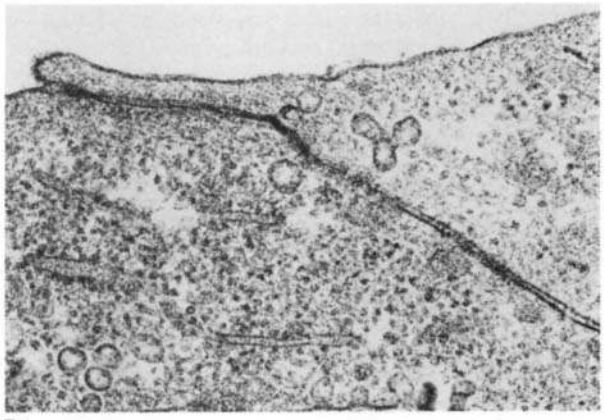

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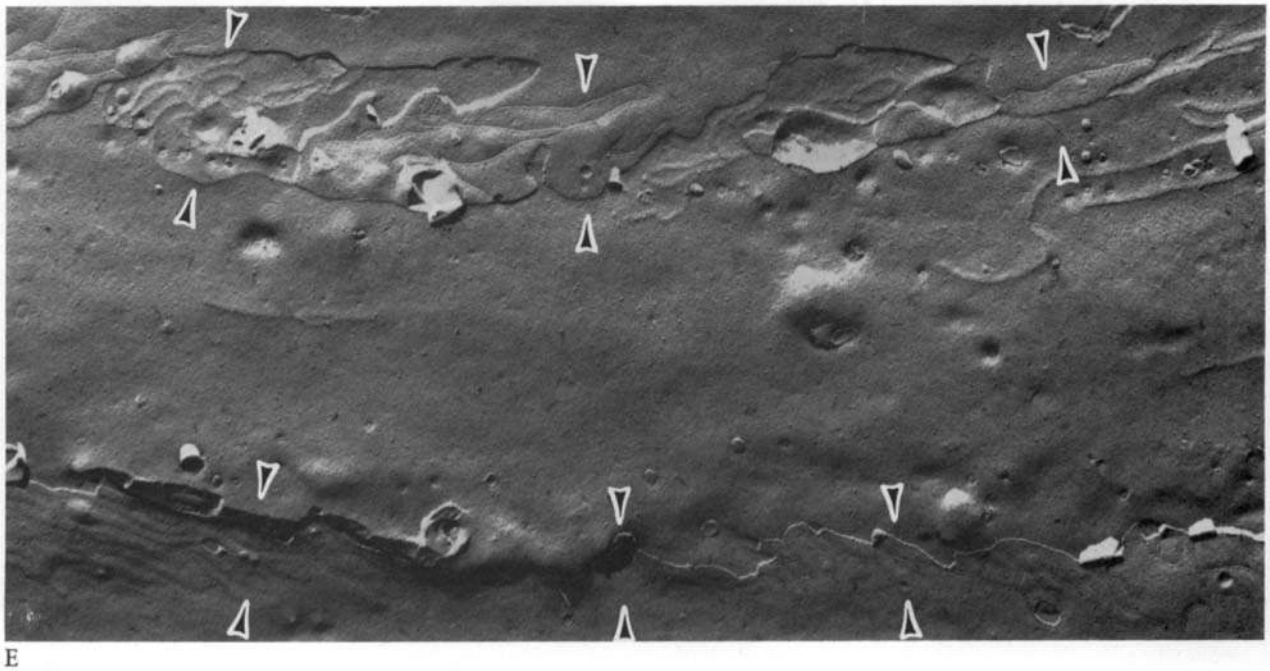

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per square centimeter and allowed to grow and cover the surface for 10 days. Immediately prior to a transport experiment, the chamber containing the cells was affixed to a second piece of polycarbonate tubing with vacuum grease, forming a double-sided 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 ^{14}C-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 ^{14}C-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 demarcate the borders of tight junctions between cells. Complex anastomosing linear arrays of membrane particles are apparent. (×35,000 before 5% reduction.)

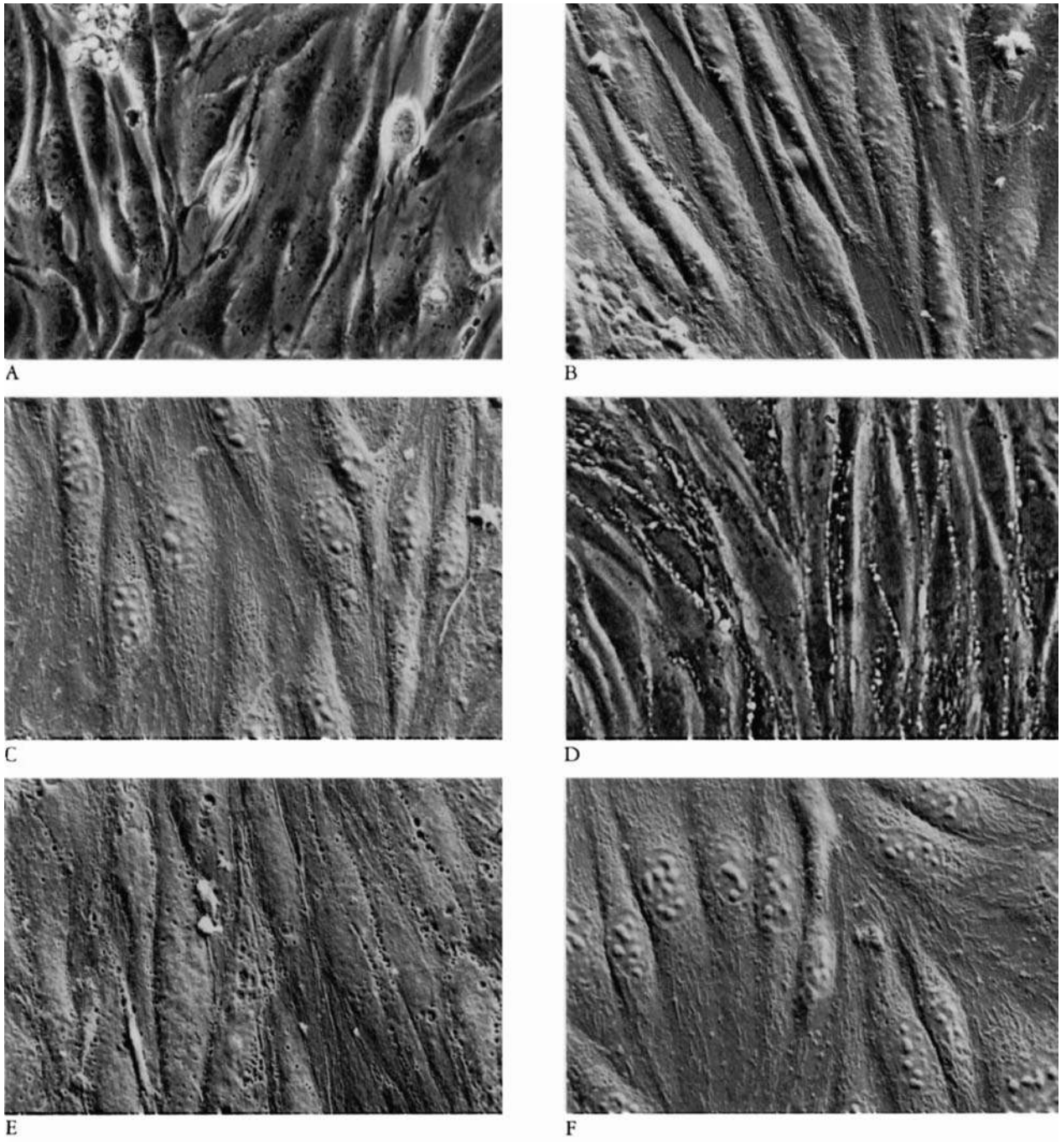

Fig 2. Morphological effects of calcium removal or arabinose treatment on cultured brain microvessel endothelial cells. (A) Phase contrast micrograph of cells treated for 2 minutes with Ca^{++}-free medium containing 5 mM EGTA, demonstrating retraction of cells from one another. (×600 before 10% reduction.) (B) Scanning electron micrograph of cells after calcium removal, revealing a substantial separation of the cells and exposure of the underlying coverslip. (×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. (×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. (×625 before 10% reduction.) (E) Scanning electron micrograph of arabinose-treated cells, showing craters in or near the junctional contacts between cells. (×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. (×850 before 10% reduction.)

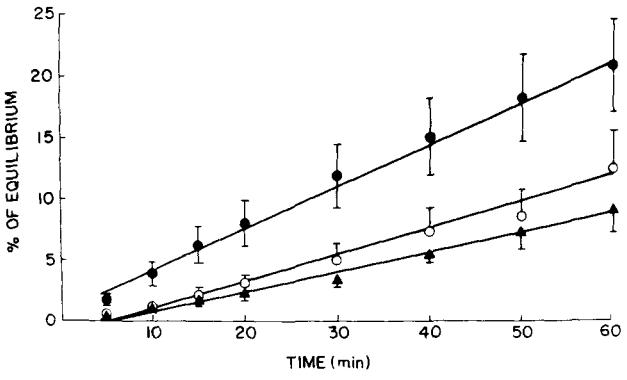

Fig 3. Permeability effects of calcium removal or arabinose treatment on cultured brain microvessel endothelial cells. The amount of labeled sucrose that has moved across the monolayer is shown as a percentage of the calculated equilibrium value. Points represent the means ± standard errors of the mean for four to six experiments at each time point. The lines drawn through the experimental points were obtained using linear regression analysis of all data within the control (triangles), *calcium-free* (filled circles) *or arabinose-treated* (open circles) *groups. To compare the effect of treatment on the rate of transcellular sucrose movement, the slopes of the experimental groups were compared to the slope of the control group using a single-tailed* t *test. Values obtained (control, 0.160 ± 0.017; calcium free, 0.347 ± 0.044; arabinose treated, 0.221 ± 0.029) indicate a highly significant* ($p < 0.005$) *increase in the rate of sucrose movement following calcium removal and a more modest increase* ($p < 0.05$) *following arabinose treatment.*

near the junctional complexes (Fig 2E). Although these sites may provide a pathway for the movement of sucrose across the monolayer (to be discussed), scanning electron microscopy does not permit a determination of whether they represent true transcellular channels or, possibly, fluid-filled vesicles that collapsed during air drying. Figure 2F demonstrates that endothelial cells treated for 5 minutes with 1.6 M arabinose regain normal structure 6 hours after return to normal medium.

We examined the effects of these experimental manipulations on the movement of an extracellular marker across cultured brain capillary endothelium. Figure 3 presents the findings concerning the movement of ^{14}C-sucrose across a monolayer of endothelial cells grown on collagen-coated nylon mesh. The monolayer of cells provides a barrier to the diffusion of sucrose, as indicated by the control findings. Exposure of the monolayer to calcium-free media for 5 minutes increased the rate of movement of sucrose by 120%. In contrast, exposure of the monolayer to 1.6 M arabinose for 5 minutes increased sucrose movement by only 40%.

Discussion

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 by 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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