Long-term Culture of Microvascular Endothelial Cells Derived From Mongolian Gerbil Brain

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A method for long-term culture of microvascular endothelial cells from Mongolian gerbil brain and their biologic properties in vitro are described. Microvessels were isolated from Mongolian gerbil brain by a combination of enzymatic treatment, filtration, and centrifugation and were seeded onto a gelatin-coated dish. A morphologically homogeneous cell plaque showing a cobblestone appearance was removed 2 to 3 weeks after the seeding, and the cells were subcultured. The cultured cells grew as monolayers of flat polygonal cells and were carried for more than 20 passages without morphologic change. These cells synthesized prostacyclin and retained an endothelial specific marker, factor VIII-related antigen. When the cells were cultured in a collagen gel, they rapidly formed capillarylike tubular structures without endothelial cell growth factor or special substrata. Long-term culture of purified microvascular endothelial cells derived from Mongolian gerbil brain will facilitate the study of the function of microvascular endothelial cells in human brain under normal and pathologic conditions. (Stroke 1989;20:947-951)

It is well recognized that vascular endothelial cells are important in maintaining physiologic homeostasis and permeability of blood vessels. The development of culture techniques for large-vessel endothelium has stimulated the study of endothelial functions, including the production of nonthrombogenic surface, mediation of exchanges between blood and tissue, and metabolic participation in circulatory homeostasis.1 Investigation of microvascular endothelial cell biology has also been spurred by recent advances in culture techniques of microvascular endothelial cells from a variety of sources.2-5 A large number of cultured endothelial cells is needed to investigate their biologic properties. However, recent techniques for the culture of cerebral microvascular endothelium have shown problems, such as long-term culture and contamination from other cell types.6-9 This study presents a method for the long-term culture of microvascular endothelial cells derived from Mongolian gerbil brain without contaminating cell types, followed by a description of their properties in vitro.

Materials and Methods

We isolated microvessels from 20 young adult Mongolian gerbil brains by enzymatic treatment and centrifugation, similar to the method described by Bowman et al.7 A filtration technique was added to their procedures in order to reject large vessels. Ten aseptically removed brains were cleaned of meninges, minced, and incubated for 3 hours at 37° C in 5 ml of a medium containing 0.5% dispase (Boehringer Mannheim Corp., Indianapolis, Indiana). The medium consisted of minimal essential medium (MEM) (Nissui, Tokyo, Japan) with 50 mM N-(2-hydroxyethyl)piperazine-2'-ethanesulfonic acid (HEPES) buffer. After enzymatic treatment, brain tissue was suspended in 20 ml of a medium containing 13% dextran (Sigma Chemical Co., St. Louis, Missouri) (average molecular weight, 60,000). The vessels, consisting mainly of microvessels, were separated from other brain tissue by centrifugation at 37° C in 5 ml of a medium containing 0.5% dispase (Boehringer Mannheim Corp., Indianapolis, Indiana). The medium consisted of minimal essential medium (MEM) (Nissui, Tokyo, Japan) with 50 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) buffer. After enzymatic treatment, brain tissue was suspended in 20 ml of a medium containing 13% dextran (Sigma Chemical Co., St. Louis, Missouri) (average molecular weight, 60,000). The vessels, consisting mainly of microvessels, were separated from other brain tissue by centrifugation at 5,800g for 10 minutes, passed through a 300-µm nylon mesh, and treated with 1 mg/ml collagenase/dispase (Boehringer Mannheim) in 2 ml medium for 6 hours. Clumps of cells, which were collected by centrifugation after enzymatic treatment, were layered over 7 ml Percoll (Sigma) gradients and banded by centrifugation at 1,000g. Percoll gradients containing 50% Percoll in MEM were established by centrifugation at 26,000g for 50 minutes. The band containing mainly endothelial cells, located in the upper one third of the gradients,9 was removed and seeded onto a gelatin-coated dish (25 cm², Falcon, Lincoln Park, New Jersey) in MEM with 20% fetal bovine serum (FBS).
Two to 3 weeks after seeding, a plaque of homogenous cells, which showed a characteristic cobblestone pattern, was mechanically removed with an 18-gauge needle and subcultured. Serial passage was accomplished by a brief exposure to 0.25% trypsin with 0.01% ethylenediaminetetraacetic acid (EDTA) at a subculture ratio of 1:2. The culture medium, which consisted of MEM with 20% FBS, was replaced with fresh medium every 2–3 days.

Cultured endothelial cells seeded on gelatin-coated Lab-Tek (Miles Laboratories, Naperville, Illinois) slides were used for this study. Immunohistochemical staining for factor VIII–related antigen (FVIIIIRAg) was carried out using the peroxidase-antiperoxidase (PAP) method. Rabbit immunoglobulin G (IgG) anti-human FVIIIIRAg (Dakopatts Corp., Santa Barbara, California), goat serum anti-rabbit IgG (Tago Inc., Burlingame, California), and rabbit PAP (Dakopatts Corp.) were used. In the controls, nonimmunized rabbit serum was used instead of antiserum. The antiserum to human FVIIIIRAg cross-reacts with the antigen of Mongolian gerbil and was used to identify endothelial cells of Mongolian gerbil.

Endothelial cells grown on collagen (Type 1, Nitta, Osaka) coated Lab-Tek slides were fixed with 2.5% glutaraldehyde in Millonig’s phosphate buffer for 30 minutes at 4° C. The slides were rinsed three times with Millonig phosphate buffer and fixed with 1% osmium tetroxide at 4° C. After 30 minutes, the cell layer was washed twice with distilled water, dehydrated in ethanol, and embedded in Epon 812 resin (TAAB Laboratories, Aldermaston). Sections were stained with uranyl acetate and lead citrate and viewed in an Akashi LEM-2000 electron microscope.

We used cells from early (within 10 passages) and late (after 40 passages) cultures in our study. Gels of reconstituted collagen fibers were achieved by mixing 8 vol cold collagen solution (Type 1, Nitta, Osaka) with 1 vol 10X MEM and 1 vol sodium bicarbonate (22 mg/ml) containing 20 mM HEPES buffer. The freshly trypsinized cells were cultured in collagen gels using two different methods. In the first method, the cells were seeded on collagen-coated dishes and allowed to grow for 24 hours to obtain a subconfluent monolayer. The culture medium was then removed, and the collagen gels were poured on top of the first gel and polymerized for 10 minutes at 37° C. In the second method, the cells were directly suspended in gelling cold collagen solutions, and the cold collagen was allowed to polymerize for 10 minutes at 37° C. In both methods, fresh medium was added and replaced at 48-hour intervals. The reorganization of the endothelial cells was examined with light and transmission electron microscopy.

Culture bottles (25 cm², Falcon) at confluence were washed twice with warm Dulbecco’s phosphate-buffered saline (PBS), (37° C, pH 7.3–7.65) and incubated with 4 ml of MEM containing 20% FBS. These cell layers were incubated for 2 hours at 37°C in 5% CO₂ in air, and the supernatant fluids were then removed and kept at −80°C for subsequent bioassay. The release of prostacyclin in the supernatant fluids was measured by the radioimmunoassay of its stable degradation product, 6-keto-PGF₁α, with a commercial radioimmunoassay kit (New England Nuclear, Boston, Massachusetts). The quantity of 6-keto-PGF₁α in the culture medium was also measured by radioimmunoassay, and the value was subtracted from the total amount.

Results

Primary cultures of endothelial cells emerging from microvessel isolates 2–3 weeks after seeding had a characteristic appearance that distinguished them from other cell types scattered around the colonies. They had the typical appearance of endothelium in a monolayer: polygonal cells with a large round nucleus and very little overlapping of adjacent cells. One of the colonies that consisted of 100 or more cells was mechanically removed, and the cells were subcultured. This technique was tried 40 times, and 12 cell lines were successfully isolated. Each cell line was subcultured for as many as 20 passages without any significant change in the growth rate or morphology (Figure 1). Two cell lines (GBME 30 and 33) were subcultured for more than 50 passages without morphologic changes. The attachment and growth rate of the endothelial cells were enhanced by coating the culture flask with gelatin. The doubling time was 48–72 hours, and the confluent culture density at subculture was about 3×10⁶ cells/25 cm². Without this treatment, cell lines could not be maintained for more than 10 passages.

Positive staining for FVIIIIRAg was observed in almost all cultured cells in early and late passages. Staining was localized in the cytoplasm and concentrated in the perinuclear space (Figure 2). On the other hand, no positive staining was observed in the control.
Cultured endothelial cells were investigated in early and late passages by transmission electron microscopy. Ultrastructurally, these cultured cells at confluence revealed close proximity with one another in vivo, but the junctions seemed to be gap junctions. Their cytoplasm was rich in mitochondria and rough endoplasmic reticula. Pinocytotic vesicles, which were present in moderate numbers, were localized in subplasmalemmal regions. No fenestration was observed in their cytoplasm. Basement membranelike structures were present between the cultured cells and collagen matrix (Figure 3). Tubelike structures were sometimes observed above the monolayer surface on the gelatin-coated dish. When the cells were cultured in a collagen gel, they formed a network of branching and anastomosing cords of cells not only by the reorganization of preformed monolayers (Method 1, Figure 4), but also by resuspending freshly trypsinized cells in gelling collagen solutions (Method 2, Figure 5). The cross section of these cords revealed tubular structures resembling blood capillaries, and microvilli were sometimes observed on the luminal surface (Figures 6, 7).

Production of prostacyclin by cultured endothelial cells was investigated in one cell line (GBME 18) subcultured for as long as 25 passages before senescence and in another cell line (GBME 30) cultured for more than 50 passages. The production of 6-keto-PGF$_{1\alpha}$, the stable degradation product of prostacyclin, at four, six and nine passages of GBME 18 and 44 passages of GBME 30 was 11.4±2.7, 10.7±2.1, 14.1±2.5, and 8.2±1.0 ng/3x10$^6$ cells/2 hr (n=6), respectively. Confluent monolayers at 44 passages of GBME 30 were also incubated in PBS(+), with and without 100 µg of arachidonic acid (sodium salt, Sigma). The production of 6-keto-PGF$_{1\alpha}$ in PBS(+) was 49.6±8.0 and 1.9±0.6 ng/3x10$^6$ cells/2 hr, respectively (Figure 8).

**Discussion**

Cultured endothelial cells derived from cerebral microvessels provide a valuable model in vitro for the study of the unique properties of these impor-
tant cells. Several investigators have reported methods of culturing microvascular endothelial cells isolated from various brains, including rat, mouse, and bovine brains. Debault et al. established an endothelial cell line from a primary culture of cerebral microvessels from mice. These cultured endothelial cells have retained several of their unique properties. Other authors have failed to obtain long-term cultures, and some of their cultures were impure.

The Mongolian gerbil is widely used as an experimental model for cerebral ischemia. Unilateral occlusion of a common carotid artery results in cerebral infarction in about 30% of gerbils. In this study, a method was developed for the long-term culture of microvascular endothelial cells from gerbil brain, and two cell lines were maintained for more than 50 passages without morphologic change. Each cell line originated from one colony of a primary culture, and no contaminating cells were observed. Neither tumor-conditioned medium nor endothelial cell growth factor was needed for serial cultivation. Positive reactivity for FVIIIRAg was also observed in cultured cells, even in late passage. Ultrastructurally these cultured cells resembled the capillary endothelium in vivo. Although the junctions seemed to be gap junctions, the cells revealed close proximity, and no fenestration was observed in the cytoplasm. The cultured cells in both early and late passages formed capillarylike tubular structures in collagen gel, and microvilli were sometimes observed in the luminal surface. Formation of tubular structures has been observed in cultured capillary endothelial cells derived from other organs, but not in cells from brain. Prostacyclin has been shown to be produced by cultured endothelial cells.

FIGURE 6. Transmission-electron micrographs of sections perpendicular to layer of endothelial cells in collagen gel (Method 1). Endothelial cells form capillarylike tube with narrow lumen. Note microvilli extending into lumen. Original magnification, ×1,800. Bar=10 μm.

FIGURE 7. Cross section of capillarylike tubular structures grown in collagen gel (Method 2). Tube was formed by four endothelial cells. Original magnification, ×4,300. Bar=5 μm.

FIGURE 8. Synthesis of prostacyclin by cultured cerebral microvascular endothelial cells. PBS, phosphate-buffered saline (+); AA, 100 μg arachidonic acid.
from large vessels.\textsuperscript{17} Cultured capillary endothelial cells also produce prostacyclin, but only a few reports have investigated their ability to do so.\textsuperscript{17} The cell lines derived from gerbil brain produce prostacyclin in large quantities, and this ability was unchanged by cultivation.

The method mentioned above could provide a large amount of microvascular endothelial cells that retain some important biologic characteristics of microvascular endothelial brain cells. Capillary endothelial cells of the brain play an important role in the occurrence of ischemic brain injury. By using these cultured cells as a model for brain capillary function, we could further investigate the mechanism of ischemic brain injury.

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\textbf{KEY WORDS} • cerebral ischemia • endothelium • gerbils
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