Primary Culture and Characterization of Microvascular Endothelial Cells From Macaca Monkey Retina

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Purpose. To develop methods for the culture of microvascular endothelial cells (EC) from Macaca monkey retina and to investigate their propagation and survival in vitro.

Methods. Endothelial cells from capillary fragments were cultured on fibronectin-coated dishes in QB-58 serum-free medium containing 20 μl/ml bovine retinal extract, 90 μg/ml heparin, 10% fetal bovine serum, and 10% monkey serum. Non-EC were removed manually. Endothelial cell-specific properties were assessed by endocytosis of acetylated low-density lipoprotein (ac-LDL) and by immunocytochemical staining. The response to growth factors was assayed by 3H-thymidine incorporation. The synthesis of matrix macromolecules was studied by metabolic labeling with 3H-proline and identification by sodium dodecyl sulfate-polyacrylamide gel electrophoresis-immunoblotting.

Results. Under these culture conditions, migrating cells emerged from capillary fragments after 1 to 2 days and formed large colonies by 1 week. Cells exhibited a mean doubling time of 44.5 hours during the first 3 to 5 days of culture and 23 hours at 6 to 8 days in culture, and they formed a confluent monolayer by 12 to 14 days. These cells demonstrated uptake of ac-LDL, expressed von Willebrand factor and the cell adhesion protein CD31, and did not contain smooth muscle α-actin. Before purification, 92% of the cells in primary cultures were identified as EC. The EC could be maintained in vitro for more than 1 month without the addition of growth factors; however, basic fibroblast growth factor and vascular endothelial growth factor each stimulated cell replication. Secreted extracellular proteins included fibronectin, collagen types I and IV, laminin, and SPARC (secreted protein, acidic, and rich in cysteine).

Conclusions. This study is the first description of the culture and propagation of purified retinal EC from Macaca monkey, a widely accepted model for the human retina. These cultures will be highly relevant to studies of abnormal vascular disease in the human eye. Investigative Ophthalmology & Visual Science. 1996;37:2185–2194.

It is now appreciated that retinal microvascular endothelium is an integral component in the development of diabetic retinopathy and retinopathy of prematurity, which are major causes of blindness worldwide. Although endothelial cells (EC) have a common topography throughout the vasculature, fundamental differences are found among EC derived from different species and from different organs or vascular beds.1–5 Macaca monkey retina is a widely accepted model for the human retina,6,7 and the monkey has been used to develop a model of retinal neovascularization in vivo8 that might be more relevant to human disease than the extant rat or mouse models.9,10 A dependable method for the isolation and culture of primate retinal microvascular EC would improve our understanding of the factors regulating retinal EC, particularly because cultures of retinal EC from various animals have demonstrated that significant differences exist among species.11–13

The availability of cultured monkey retinal microvascular EC provides a potentially important tool in the study of retinal neovascularization and the underlying mechanisms controlling EC growth in a primate. In this article, we provide the first detailed description...
of the isolation and culture of Macaca monkey EC and analyze some of the factors and parameters of their propagation.

MATERIALS AND METHODS

Isolation and Culture of Retinal Endothelial Cells

Macaca eyes were obtained from the Tissue Program of the Regional Primate Research Center at the University of Washington (Seattle) in accordance with the approval protocols consistent with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For each cell preparation, both eyes were removed from one deeply anesthetized Macaca nemestrina subsp. fascicularis (2.5 to 8 years old) and were placed in ice-cold calcium-free and magnesium-free Hank’s balanced salt solution containing 10 mM HEPES (pH 7.4), 0.1% wt/vol bovine serum albumin, 100 U/ml penicillin G, and 100 µg/ml streptomycin SO4 (preparation buffer). With the aid of a dissecting microscope, an incision was made in each eye posterior to the ciliary body and was extended circumferentially. The anterior half of the eye and the lens were discarded. The vitreous was removed, and the retina was pulled gently away from the retinal pigment epithelial layer. The retina was washed in preparation buffer and was minced finely with ophthalmic scissors. Subsequently, the tissue was transferred to a 15 ml centrifuge tube, washed by a brief centrifugation, and resuspended in 10 ml of 0.3% collagenase-dispase (Boehringer Mannheim Biochemica, Indianapolis, IN) containing 20 U/ml DNase I (Sigma Chemical, St. Louis, MO) at 37°C with gentle, constant agitation. The tissue fragments were washed twice with preparation buffer and were resuspended in 10 ml of 0.3% collagenase-dispase (Boehringer Mannheim Biochemica, Indianapolis, IN) containing 20 U/ml DNase I (Sigma Chemical, St. Louis, MO) at 37°C with gentle, constant agitation. The fragments were washed twice with the same medium, resuspended in 10 ml DMEM containing 20% FBS, and plated at a density of 1000 cells/35 mm dish or 1 to 5 cells/well in 96-well plates precoated with fibronectin in complete culture medium. Basic fibroblastic growth factor (bFGF; Biomedical Technologies, Stoughton, MA) (25 ng/ml) was added to some culture wells.

Cloning. The EC colonies were isolated and dissociated with trypsin in cloning cylinders. Suspended cells were plated at a density of 1000 cells/35 mm dish or 1 to 5 cells/well in 96-well plates precoated with fibronectin in complete culture medium. Basic fibroblastic growth factor (bFGF; Biomedical Technologies, Stoughton, MA) (25 ng/ml) was added to some culture wells.

Fluorescence-Activated Cell Sorting. Cells were prelabeled with 1,1’ diocadecyl-3,3,3’,3’-tetramethyl-indocarbocyanine-tagged, perchlorate-acetylated, low-density lipoprotein (DiI-ac-LDL) (Biomedical Technologies) at 2 µg/ml in culture medium by incubation overnight. Cells were washed in calcium- and magnesium-free PBS and 0.02 M EDTA, and were incubated with nonenzymatic cell dissociation solution. Then they were washed in DMEM containing 10% FBS and were resuspended in the same medium at 10⁶ cells/ml. Mixing was carried out at an excitation wavelength of 514 nm and an emission wavelength above 550 nm (FACStar, Becton Dickinson).

Immunocytochemistry

The EC cultures were fixed in 100% cold methanol for 10 minutes at 4°C, washed with PBS, incubated with 70% methanol containing 3% H2O2 for 30 minutes to inactivate endogenous peroxidases, and
Assay for $^3$H-Thymidine Incorporation

The effects of different culture media and growth factors on EC were studied by $^3$H-thymidine incorporation. Endothelial cells were seeded in 24-well plates at a density of 5000 cells/well in complete culture medium and were grown for 20 hours. Cultures were rinsed several times with DMEM without serum and were incubated in DMEM with 2% FBS for 16 hours. The medium was changed as described in the figure legends. Control cultures were grown in DMEM containing 2% FBS. Cultures were incubated for 48 hours and were exposed to $2 \mu$Ci/ml [methyl-$^3$H]-thymidine (81.3 Ci/mmol, 1 mCi/ml; New England Nuclear, Boston, MA) for the last 4 hours. They were washed three times with PBS, incubated in ice-cold 10% trichloroacetic acid for 1 hour, washed twice with anhydrous ethanol at $-20^\circ$C, and air dried. Three hundred microilters of NaOH (0.2 N) was added to each well for 30 to 40 minutes at 68°C. The cpm incorporated into DNA were quantified by liquid scintillation counting in 3 ml Ecolume (ICN, Irvine, CA). Data were expressed as means ± SD of three experiments performed in triplicate.

Metabolic Labeling and Analysis of Secreted Proteins

Endothelial cells cultured on fibronectin-coated dishes were radiolabeled at approximately 90% confluence. The cells were rinsed five times with serum-free DMEM and were incubated for 1 hour in serum-free DMEM supplemented with 50 μg/ml sodium ascorbate and 64 μg/ml β-aminopropionitrile fumarate. The medium was aspirated, and cultures were incubated for 20 hours in the same DMEM containing 20 μCi/ml L-[2,3,4,5-$^3$H] proline (100 Ci/mol; Amersham, Arlington Heights, IL). At the end of the incubation, the medium was collected and was centrifuged briefly to remove cells and debris. The following proteinase inhibitors were added to the final concentrations indicated: 0.2 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, 2.5 mM EDTA, and 0.5 μg/ml pepstatin A (Peninsula Laboratories, San Carlos, CA). The medium was dialyzed against 0.1 N acetic acid and was finally lyophilized.

Lyophilized proteins were solubilized in 2 × SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) sample buffer and incorporated cpm were measured by scintillation counting. Samples equivalent to 30,000 cpm were reduced with 50 mM dithiothreitol and were denatured by heating at 100°C for 3 to 5 minutes in sample buffer. Proteins were examined by discontinuous SDS–PAGE on horizontal slab gels. Protein molecular weight standards included myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), α-chymotrypsinogen (27.5 kDa), and lysozyme (14.3 kDa). Proteins were stained with Coomassie brilliant blue R-250, destained, and incubated in amplifier (DuPont, Boston, MA). Dried gels were exposed to RP X-Omat film (Eastman Kodak, Rochester, NY) at −70°C.

Western Blot Analysis

Culture media proteins, prepared as described above but without radiolabel, were subjected to SDS–PAGE and were transferred to 0.4 μm nitrocellulose membranes at 100 V for 1 hour at 4°C. The membranes were stained with amido black to assess the efficiency of transfer and were blocked in 5% nonfat dried milk and 0.05% Tween 20 for 2 hours before incubation with antibodies for 16 hours at 4°C. Primary antibodies were mouse anti-human cellular fibronectin (Sigma), rabbit anti-mouse collagen IV (1:5000 dilution of a 5
mg/ml stock solution; Collaborative Biomedical Products, Bedford, MA), rabbit anti-mouse laminin (1:1000 dilution of a 1 mg/ml stock solution; Collaborative Biomedical Products), rabbit anti-mouse SPARC immunoglobulin G (directed against a synthetic C-terminal peptide of SPARC) at 9 μg/ml, and an antiserum produced in guinea pig against rat type I collagen prepared in our laboratory. The titer and specificity of the anti-type I collagen immunoglobulin G antibody were determined by enzyme-linked immunosorbent assays and Western blot analyses of purified collagen and conditioned media from cultured fibroblasts. Blots were washed in PBS and were incubated in 125 I-protein A at 0.5 μCi/ml (New England Nuclear). After several washes, the blots were exposed to x-ray film.

RESULTS

In this study, we observed the growth of monkey endothelium derived from retinal microvasculature. The microvascular fragments, composed of 3 to 10 cells each, adhered to fibronectin-coated dishes after 2 to 3 hours (Fig. 1A). Migration of cells from these fragments began 1 to 2 days after plating (Fig. 1B), and, after 4 days in culture, the cells formed contiguous islands of spindle-shaped cells that were closely opposed to one another (Fig. 1C). After 1 week, a marked proliferation occurred that resulted in the formation of large colonies (Fig. 1D). We confirmed that these colonies were composed of EC through the use of EC markers (see below). The major contaminating cells were smooth muscle cells, which grew into dense colonies of multilayered, small, elongated cells that could be distinguished readily from EC colonies under phase-contrast microscopy (Fig. 1E). In addition, these cells, stained for smooth muscle α-actin, did not contain von Willebrand factor or CD31 and did not bind Dil-Ac-LDL (data not shown). Although there is no unique marker to date for the identification of pericytes, these cells can be recognized by characteristic morphologic features and occasionally were seen among endothelial colonies. The non-EC were removed from cultures as described in Materials and Methods. After 12 to 14 days in culture, EC colonies contacted each other and formed a confluent monolayer (Fig. 1F). At confluence, proliferation ceased, and the cells did not overgrow into multilayered aggregates. Some cells cultured at saturation density for 2 to 3 weeks maintained a uniform monolayer configuration. In contrast, some of the EC colonies produced a sprouting pattern after the monolayer was formed (Fig. 1G). The sprouting cells were elongated and associated with other EC to form long, cord-like structures (Fig. 1H).

To confirm the identity of the cultured EC, we used several EC markers. Monkey EC produced von Willebrand factor (Fig. 2A). Punctate fluorescence was observed after incubation in Dil-ac-LDL (Fig. 2B). Antibody against CD31, a protein highly specific to EC, stained the cell surface, especially regions of intercellular contacts (Fig. 2C). These cells did not express smooth muscle α-actin (data not shown). The Ulex europaeus agglutinin-1 (UEA-1) binds to α-L-fucosyl residues of glycoproteins present on the surface of human EC and has been used as a marker for human capillary EC. Figure 2 also shows double immunofluorescence labeling for CD31 (Fig. 2C) and UEA-1 (Fig. 2D). The arrow in Figure 2D indicates a cell that was labeled by UEA at a significantly higher level than the adjacent EC (shown in Fig. 2C as reactive

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933414/)
FIGURE 2. Characterization of endothelial cells (EC) cultured from monkey retinal microvasculature. (A) Monolayer of EC immunocytochemically labeled for von Willebrand factor by the avidin-biotin-peroxidase technique. The cytoplasm is stained, but the nucleus is not. (B) Accumulation of Dil-ac-LDL (10 \( \mu \)g/ml) by cultured EC after a 12-hour incubation. (C,D) Double-labeling immunofluorescence of cultured microvascular cells with anti-CD31 antibody and UEA-1 lectin. Staining by anti-CD31 antibody conjugated to Texas red (C) is localized to EC cell membranes, especially at intercellular junctions. An unlabeled cell is indicated by an arrow. UEA-1 conjugated to fluorescein isothiocyanate (D) labeled both the nonendothelial cell (arrow) and EC. Magnifications, \( \times 134 \) (A,B); \( \times 209 \) (C,D).

with anti-CD-31 antibody). Therefore, UEA is not a specific marker for monkey EC in vitro. VCAM-1 was not detected in unstimulated retinal monkey EC, and staining with anti-CD34 antibody was also negative (data not shown).

To assess the purity of primary cultures, we performed flow cytometry on cells labeled with Dil-Ac-LDL. Cultured retinal vascular smooth muscle cells served as a negative control to set the range of fluorescence intensity for positive cells, as indicated by the bars in Figure 3. Primary cultures contained approximately 90% EC (Fig. 3B). After fluorescence-activated cell sorting of the labeled cells, approximately 99% EC were obtained (Fig. 3C).

Plasma-derived serum (PDS) has been used for the selective growth of retinal EC in culture. In our system, PDS did not inhibit selectively the growth of purportedly non-EC; more important, it did not support the growth of monkey retinal EC. Cultures grown in PDS initially exhibited a colony size similar to that of EC grown in FBS. However, after 1 week in PDS, the cells failed to proliferate and became enlarged and attenuated, lost characteristic endothelial morphology, and often deteriorated after 14 days (Fig. 4). After testing different culture conditions for the growth of monkey retinal EC, we arrived at an optimal culture medium, as described in this article, for long-term propagation of EC. Endothelial cells incubated in QBSF-58-complete culture medium gave rise to larger and denser colonies than the EC in DMEM-complete culture medium. Potential differences in the proliferation of cells grown in DMEM versus QBSF media, both of which contained FBS, retinal extract, and heparin at the same concentrations, were assessed by the incorporation of \(^3\)H-thymidine (Fig. 5). Results substantiated the microscopic observations: QBSF culture medium was superior to DMEM for stimulation of endothelial cell proliferation (\( P < 0.01 \)). In addition, retinal extract was superior to endothelial cell growth supplement (Sigma) for the proliferation of monkey EC (data not shown).

By counting the cells growing within colonies, we ascertained that EC grew in complete culture medium, with a mean doubling time of 44.5 hours from days 3 to 5; the doubling time decreased to 23 hours from days 6 to 8. After 1 week, the size of the endothelial colonies enlarged rapidly, colonies contacted each other, and a uniform population of EC formed a monolayer by 12 to 14 days in culture. Monkey EC could be passaged only at high density.
cloned the EC at low density, most cells did not attach; the few attached EC were spread but vacuolated, failed to divide, and eventually detached. A few attached EC grew slowly into a small colony in the presence of high doses of bFGF (25 ng/ml). The EC sorted by fluorescence-activated cell sorting grew poorly on fibronectin-coated dishes, and by 1 week they exhibited signs of senescence or degeneration—the cells were large, flattened, and vacuolated. Eventually, most of the cells lifted off the substrate and died. We found that the standard procedure of trypsin treatment and centrifugation decreased the plating efficiency significantly. With nonenzymatic cell dissociation and no centrifugation, 95% of the EC adhered and grew on fibronectin-coated substrates. Passaged EC could be maintained in culture for more than 1 month and could be cultured to passage four in complete culture medium without the addition of growth factors such as bFGF or vascular endothelial growth factor (VEGF). After 1 month, EC grew slowly and required additional growth factors to traverse the cell cycle.

To achieve quiescence, we seeded EC in 2% FBS in DMEM. The addition of VEGF and bFGF improved DNA synthesis (Fig. 6). The maximum stimulation of thymidine incorporation was 30 ng/ml of VEGF and 10 ng/ml bFGF in 2% FBS–DMEM. Although the simultaneous addition of bFGF and VEGF resulted in an enhanced mitogenic effect (Fig. 6C), synergy between these two growth factors was not observed.

For analysis of proteins secreted by monkey EC, subconfluent cultures were labeled metabolically with [3H]-proline, and newly synthesized proteins secreted into the culture medium were characterized by SDS-PAGE (Fig. 7). [3H]-proline-labeled proteins secreted by subconfluent, nongrowing retinal primary or passage 3 EC were visualized by autoradiography (Fig. 7, lanes 1 to 2). Fibronectin, SPARC, laminin, and collagen types I and IV could be identified as prominent secretory products of primary and passaged subconfluent cultures. The identity of these proteins was confirmed by Western blot analysis (Fig. 7, lanes 3 to 7).

DISCUSSION

To the best of our knowledge, this is the first description of the culture and characterization of monkey retinal EC. Numerous techniques and culture conditions have been developed for bovine retinal EC,18,20,23-25 and pure human retinal EC have been obtained.
by cell sorting. However, previously published methods could not be adapted readily to monkey EC. By using modified EC culture techniques, we obtained pure monkey retinal EC cultures that retained many of their phenotypic properties. These monkey EC cultures should be valuable for experiments designed to probe the intrinsic properties of primate retinal EC.

Many lines of evidence support the identification of these cultures as EC. At confluence, the cultures showed contact inhibition of growth, a characteristic of vascular endothelium. Immunocytochemical studies provided further evidence for the endothelial origin of these cultures. CD31, a highly specific EC marker not present on any other cell type except leukocytes, might be involved in homotypic intercellular interactions. Staining for CD31 showed that monkey EC deposited this antigen at points of cell–cell contact. The monkey cultures also produced von Willibrand factor, a widely accepted marker for EC, and exhibited uptake of Dil-ac-LDL, a property used for EC identification and isolation. Although UEA has been described as a marker of human EC in vitro and in vivo and UEA-coated Dynabeads have been used for the isolation of human EC, this lectin was not a specific ligand for monkey EC in vitro. Similar results have been described in vivo. In fact, in our mixed cultures, UEA reacted preferentially with nonendothelial cells. Human cerebral EC in primary culture constitutively expressed low levels of VCAM-1, an EC-membrane glycoprotein, which was increased after treatment with inflammatory mediators. However, monkey retinal EC did not express detectible levels of VCAM-1 under basal conditions in vitro. It is also possible that the lack of immunostaining with anti-VCAM-1 and anti-CD34 resulted from species incompatibility of the antibodies, as opposed to the absence of Macaca antigen.

A critical step for the isolation of large numbers of EC was minimization of the growth of other cell types. The microvessel isolation procedure was designed to limit the number of non-EC plated on the dish. A high density of 92% EC was generated in the primary cultures, a higher percentage than reported for human retinal EC. Cell sorting produced a higher purity of 99%, but this procedure shortened the life span of the EC. Although the manual removal of contaminants surrounding EC colonies is tedious and time consuming, mechanical weeding during the early days of culture isolated pure EC colonies and ensured that a uniform population of EC in the cultures was available for subsequent analysis. In the impure cultures, the progressive increase in the number of smooth muscle cells and pericytes coincided with the arrest of EC proliferation. This arrest might be mediated by the activation of transforming growth factor-β.

Monkey retinal EC were sensitive to factors in their culture medium. PDS, which lacks platelet-de-
vascular EC derived from human adipose tissue ex-13'23'24 However, the value of derived products, has been used for the growth of retinal EC from other species. In summary, we have shown that monkey retinal EC can be isolated, cultured, and characterized. The availability of these cells allows an in-depth analysis of their growth parameters. This approach should provide important new information on EC properties that could lead to a better understanding of defects or alterations in the processes underlying pathologic retinal neovascularization.

Unlike bovine retinal EC, which grew rapidly, survival repeated passages, and could be cloned, monkey retinal EC were much less hardy. We did not succeed in cloning these cells. They could be passaged for more than 1 month, but thereafter they exhibited a reduction in growth rate. A plating efficiency of <50% was obtained when trypsin was used to isolate cells for passage. Treatment of cells with trypsin has been shown to cause a loss of membrane lipid, inhibit angiotensin-converting enzyme, and suppress bradykinin receptors. Nonenzymatically treated monkey EC survived freezing, storage, and thawing.

Previous studies of bovine EC have indicated that these cells synthesized and secreted growth factors such as bFGF and VEGF that were involved in the autocrine regulation of EC growth. Exogenous VEGF and bFGF each induced the proliferation of monkey EC. The mitogenic effects of these growth factors on EC were tested in 2% rather than 10% serum to minimize the mitogenic effects of serum on EC and to ensure a low basal level of DNA synthesis. Our data show that bFGF is a more potent mitogen than VEGF. The effect of VEGF on retinal EC growth stimulation measured by DNA synthesis was at best twofold; however, the mitogenic effect of VEGF on retinal EC was elevated to three to four times under hypoxic conditions (Yan Q, et al, unpublished data, 1996). Although Goto et al observed a synergistic effect of VEGF and bFGF on the proliferation of bovine capillary EC within collagen gels, we were unable to confirm this result with monkey EC in the two-dimensional culture system used in our study.

A dynamic and reciprocal relationship exists between capillary EC and the extracellular matrix that can influence cellular phenotype, particularly several properties relevant to angiogenesis. Our laboratory has demonstrated that angiogenesis in vitro is associated with changes in several matrix proteins, including SPARC and type I collagen in bovine aortic EC. In our experiments with monkey retinal EC, some of the contact-inhibited monolayers subsequently displayed a "sprouting" growth pattern that resulted in cordlike or protocapillary structures; monkey retinal microvascular EC, therefore, have the capacity to form capillary-like structures in vitro. This property could be advantageous for studies on the regulation of growth factors and the synthesis and degradation of matrix proteins during primate angiogenesis in vitro. In this study, we examined the synthesis of secreted proteins by cultured, nonsprouting EC and showed that fibronectin, SPARC, laminin, and collagen types I and IV were the principal products. The changes in matrix protein biosynthesis when these cells undergo angiogenesis in vitro (as shown in Fig. 1H) are currently under investigation.

In summary, we have shown that monkey retinal EC can be isolated, cultured, and characterized. The availability of these cells allows an in-depth analysis of their growth parameters. This approach should provide important new information on EC properties that could lead to a better understanding of defects or alterations in the processes underlying pathologic retinal neovascularization.
Key Words
extracellular matrix, microvasculature, monkey, retinal endothelial cells, SPARC (secreted protein, acidic, and rich in cysteine)

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