Simplified Methods for Consistent and Selective Culture of Bovine Retinal Endothelial Cells and Pericytes

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Different matrix components, in combination with various media and serum supplements, were evaluated for their ability to promote selectively the growth of bovine retinal endothelial cells in primary culture. The optimal setting for the selective growth of retinal endothelial cells was a fibronectin/hyaluronic acid matrix, Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% pooled human serum and 100 μg/ml heparin. These conditions consistently yielded virtually homogeneous cultures of endothelial cells, assessed using specific endothelial markers. Thus obtained, the retinal endothelial cells could be subcultured and maintained in phenotypically stable long-term serial cultivation. Homogeneous cultures of retinal pericytes were obtained when microvessel isolates were seeded to uncoated or gelatin-coated culture dishes and grown in DMEM supplemented with 20% fetal bovine serum. The retinal pericytes could also be subcultured and cultivated for numerous population doublings. Additionally, observations from this study suggest that two populations of pericytes may be obtained in culture and distinguished on the basis of their relative size and antigenic properties.


Many investigators have experienced difficulties in the primary culture of retinal microvascular endothelial cells. These difficulties are related to the pericytes that accompany and eventually predominate cultures of endothelial cells. Currently published methods for elimination of the retinal pericytes have included cloning, pericyte weeding, and use of plasma-derived serum. However, none of these procedures for pericyte removal yield homogeneous cultures of endothelial cells consistently. Since even minor contamination with retinal pericytes or other cell types could conceivably alter metabolic, proliferative, and other characteristics of endothelial cells, improvements in the methods used for obtaining homogeneous cultures of retinal endothelial cells are necessary.

Matrix components have been used successfully in culture of endothelial cells from various sources. In this study we evaluated the effects of some matrix components and serum supplements (fetal bovine versus pooled human serum) on the growth of retinal endothelial cells and pericytes in primary culture. We report selective media and matrix combinations which promote the growth of endothelial cells and pericytes from the bovine retinal circulation.

Materials and Methods

Isolation of Retinal Microvessels

Bovine eyes were obtained from a local slaughterhouse and transported on ice to the laboratory. They were cut circumferentially 3 mm posterior to the limbus, the vitreous humor was removed, and the retina was exposed and transferred to Dulbecco's phosphate-buffered saline (PBS) containing Ca²⁺ and Mg²⁺ (GIBCO, Grand Island, NY). Retinas were homogenized by two gentle up/down strokes in a 15-ml Dounce homogenizer (type A pestle). The homogenate was filtered over an 88-μm sieve (Tetko, Elmsford, NY), and large vessels were removed with forceps from the retentate. The remaining retentate was digested in 0.066% collagenase (142 units/mg, Worthington, CLS, Cooper, Freehold, NJ) and 0.033% bovine serum albumin in PBS for 45 min at 37°C. The homogenate was filtered over an 88-μm sieve (Tetko, Elmsford, NY), and large vessels were removed with forceps from the retentate. The remaining retentate was digested in 0.066% collagenase (142 units/mg, Worthington, CLS, Cooper, Freehold, NJ) and 0.033% bovine serum albumin in PBS for 45 min at 37°C. The homogenate was subjected to centrifugation (1000 × g, 2 min), and the pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% fetal bovine serum (FBS), and transferred to culture dishes coated with the indicated components. After allowing 2–3 hr for cell attachment, the media were removed and replaced with the indicated culture media.

Matrix and Media Components

Fibronectin was obtained from several commercial sources (Sigma, St. Louis, MO; Calbiochem, La Jolla, CA; and New York Blood Bank, New York, NY) and...
was also prepared from human plasma as previously described. All preparations of fibronectin were used at concentrations of 100 μg/ml and yielded similar results. Hyaluronic acid (grade I; Calbiochem) was used at a concentration of 100 μg/ml. Experiments with the combination of fibronectin and hyaluronic acid used 100 μg/ml of each component. Gelatin (Difco, Grand Island, NY) and pig skin gelatin (Eastman Kodak, Rochester, NY) were used at concentrations of 0.2%. Falcon six-well plates were coated with these components and incubated 30 min at 23°C, and residual matrix solutions were removed. Heparin (grade I, porcine mucosa; Sigma) was used at a concentration of 100 μg/ml. Experiments at 100 μg/ml (equivalent to 7 units/ml). The culture media (DMEM) were supplemented with either 20% fresh pooled human serum (PHS) or FBS. Endothelial cell growth factor (ECGF) was prepared as previously described or, in some experiments, purchased from Advanced Magnetics (Cambridge, MA). All culture materials, unless otherwise indicated, were purchased from GIBCO.

Microvascular Adhesion and Counting of Adherent Cells

Retinal microvessels were seeded to culture dishes coated with various matrix components in DMEM supplemented with 20% FBS. After 3 hr, the media and debris were removed and fresh media added. Microvessels were counted in five random fields per 35-mm dish (100X). For counts of adherent cells, cells were initially identified by their morphology (later confirmed by selective staining). Endothelial cells grew as colonies of closely apposed cells, whereas pericytes, at least in early primary culture, exhibited an irregular morphology and minimal cell-cell contact. The number of individual pericytes was also determined in five random fields as described. It was difficult to count the number of individual endothelial cells due to their close proximity and greater density. Additionally, reduced viability and cell detachment occurred with endothelium exposed to lower temperatures and the altered gas environment during the counting procedure. Therefore, the number of colonies of endothelial cells was used as an approximation for relative endothelial cell growth. A typical endothelial cell colony in early primary (1–3 days) culture was 30 ± 20 μm in diameter (long axis) and contained 12 ± 4 cells (n = 4). In later primary (4–7 days), a typical colony was 100 ± 50 μm (long axis) in diameter and contained 50 ± 5 cells (n = 4).

Cell Identification

Endothelial cells were identified using two specific markers, ie, Factor VIII-related antigen and acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (Dil-Ac-LDL), using published methods. Pericytes were identified by two specific markers, ie, positive staining for the α isoform of smooth-muscle actin and for the monoclonal antibody-defined ganglioside antigen, 3G5, as described previously. Anti-human Factor VIII-related antigen was obtained from ICN (Lisle, IL), monoclonal anti-α-smooth muscle actin was from Sigma, monoclonal anti-3G5 was kindly supplied by Dr. R. Nayak, Joslin Diabetes Center, Boston), and Dil-Ac-LDL was from Biomedical Technologies (Staughton, MA). Rhodamine-conjugated antisera were obtained from ICN. For all determinations with antisera, parallel controls were run using nonimmune serum at similar dilutions for the first incubation.

Cell Sorting

Before cell sorting, cells were labeled with Dil-Ac-LDL (10 μg/ml) for 4 hr at 37°C. The cells were washed with PBS and trypsinized to produce a suspension of single cells. The cells were washed with DMEM containing 10% FBS, then fixed in 10% buffered formalin and transported to the fluorescent-activated cell-sorting facility of the Department of Pharmacology, Boehringer Ingelheim (Ridgefield, CT). Cells were sorted with a Becton Dickinson Model 440 fluorescent-activated cell sorter (Mountain View, CA), essentially as described previously by Voyta and co-workers.

Subculture of Endothelial Cells and Pericytes

Endothelium and pericytes were washed once with Ca2+, Mg2+-PBS, then incubated in 0.015% and 0.03% trypsin ethylenediaminetetraacetic acid in Versene (GIBCO) respectively for 5 min at 37°C. The cell suspensions were transferred to DMEM supplemented with 10% FBS and pelleted by centrifugation at 100 x g for 3 min. The cell pellet was resuspended in DMEM with 10% FBS and seeded to fibronectin-coated culture dishes. After allowing cell adhesion and spreading (2–6 hr), the media were removed and replaced with DMEM containing 10% PHS (endothelial cells) or FBS (pericytes). Culture media were replaced every 48 hr. The growth rate of subcultured cells was determined by cell number as a function of time. For growth experiments, cells were seeded at an initial density of 104cells/17-mm well. At the required times, cells were detached with trypsin, diluted in Isoton (Coulter, Hialeah, FL), and cell numbers were measured by flow cytometry using a Coulter counter Model Zr.
Data Analysis

All experimental protocols were repeated at least three times, and the n values, where provided, denote the number of experiments. In each experiment each experimental group was tested in quadruplicate. All data are presented as the mean ± one standard deviation of the mean. Data were analyzed by one-way analysis of variance followed by Bonferonni's modification of Student's t-test for multiple comparisons. Significance was accepted at $P < 0.05$.

Results

Counts of numbers of microvessels per 100X field, performed 3 hr after plating to allow for adhesion, showed that vessels plated on uncoated plastic or on dishes coated with hyaluronic acid or heparin, yielded 0.6 ± 0.5 adherent vessels per field, and vessels plated on dishes coated with gelatin, fibronectin, fibronectin plus hyaluronic acid, or fibronectin plus heparin plus hyaluronic acid yielded an average of 1.8–2.2 ± 0.8 microvessels per field (n ≥ 4). The larger values all differed significantly from the lower ones. Migration of cells from the microvessels was observed within 24–48 hr and gave rise to mixed cultures of endothelial cells growing in cobblestone-like patches and physically separated, irregularly shaped pericytes (Fig. 1). Eventually the two cell types became more closely associated, and cells in these mixed cultures could not be distinguished on the basis of their different morphologies. Mixed cultures were observed from all matrix settings, suggesting that matrix setting alone would not be sufficient for the selective culture of endothelial cells.

To evaluate the capacity of matrix and serum combinations to facilitate endothelial growth while concomitantly diminishing pericyte growth, the ratio of endothelial colonies:individual pericytes was determined. Seeding microvessels to uncoated, gelatin-, or fibronectin-coated plastic and culture in DMEM with 20% FBS or PHS yielded endothelial colony:pericyte ratios of less than one at both early (4–7 days) and late (9–12 days) primary culture. In contrast, fibronectin, in combination with heparin and/or hyaluronic acid, significantly increased, relative to all other matrix settings, the ratio of endothelial cell colonies to pericytes when the culture media were supplemented with 20% PHS but not FBS (Figs. 2A-B). Although the combination of hyaluronic acid, fibronectin, and heparin with 20% PHS did not significantly affect the endothelial colony:pericyte ratio at early primary culture compared with cultures similarly prepared without hyaluronic acid, the addition of hyaluronic acid promoted the formation of larger (>150 μm, long axis) endothelial colonies. Therefore, although the ratio of endothelial colonies to pericytes did not change in later primary cultures (compared with early primary), the number of endothelial cells was markedly increased. Supplementation of the culture media with ECGF (10–100 μg/ml) did not significantly alter the endothelial colony:pericyte ratio under any of the experimental conditions tested (data not shown). The reproducibility of the culture conditions of DMEM with 20% PHS supplemented with heparin and a fibronectin/hyaluronic-acid matrix for selective culture of endothelial cells was demonstrated in all 40 primary cultures in which homogeneous or near homogeneous (>98%) populations of retinal endothelial cells were obtained. After 7–9 days in primary culture, the endothelial cells formed a typical cobblestone monolayer, stained positive for Factor VIII-related antigen (Fig. 3) which incorporated Dil-Ac-LDL. A secondary sprouting layer of cells was observed in primary and subcultured endothelial cells. The sprouting cells stained for Factor VIII-related antigen, but they did not incorporate Dil-Ac-LDL. Additionally, the sprouting cells, but not the underlying monolayer, stained for α-smooth-muscle actin (Figs. 3, 4). None of the endothelial cell cultures could be stained with the anti-3G5 antisera.

To obtain pure cultures of pericytes, microvessel isolates were cultured in DMEM supplemented with 20% FBS on plastic. Two populations of pericytes were initially identified, based on their dimension in

Fig. 1. (A) Phase micrograph of retinal vascular cells in primary culture. (EC) endothelial cell colony (P) pericytes. Size marker 10 μm. (B) Same field as (A) demonstrating uptake of the fluorescent Dil-Ac-LDL in the endothelial colony but not the outlying pericytes.
A.

![BRE Colonies: BPR Cells](chart)

B.

![BRE Colonies: BPR Cells](chart)

**Fig. 2.** Ratio of endothelial colonies (BRE) to pericytes at early (A) (5–7 days) and late (B) (9–12 days) primary culture. At 5 days the average number of endothelial cells/colony was 12 ± 4, at 9 days the average number of endothelial cells/colony was 40 ± 5, except for the FN + H + HYL setting in which the average number of endothelial cells/colony at late primary exceeded 150. *Denotes significantly different from cells cultured in media supplemented with 20% PHS on uncoated plastic. Data are expressed as the mean ± one standard deviation, n = 5. Abbreviations: P, plastic, G, gelatin, PSG, pig skin gelatin, FN fibronectin, HYL hyaluronic acid, H heparin. Open bars are DME supplemented with 20% PHS, hatched bars DME supplemented with 20% FBS.

the long axis. “Small” pericytes, typically less than 30 μm in length, stained uniformly for α-smooth-muscle actin and 3G5. In contrast, “large” pericytes, typically greater than 30 μm in length, contained many stress fibers staining intensely for α-smooth-muscle actin but stained sporadically for the 3G5 antigen (Figs. 5, 6). The small and large pericytes could be distinguished in primary cultures, although the large pericytes were more common in subcultured preparations. Pericytes did not incorporate Dil-Ac-LDL or stain for Factor VIII-related antigen (data not shown).

Fluorescent-activated cell sorting was used as an additional analysis to evaluate the purity of the primary cultures obtained using the selective culture conditions described above. Monolayer cultures of endothelial cells and pericytes were incubated with Dil-Ac-LDL as described, and the population distribution of cells with positive fluorescence determined. As shown in Figure 7, no cells from the pericyte cultures were found to be fluorescent, but all of the cells from the endothelial cultures were fluorescent. The fluorescence intensity observed for the pericytes was identical to that of unstained chicken red blood cell controls (data not shown). The somewhat wide distribution of fluorescence for the endothelial cells probably was the result of variation in the amount of the labeled LDL incorporated by the cells under the experimental conditions. When the two cell types were mixed together, this procedure could clearly distinguish the two populations (Fig. 7).

We compared the growth rates of endothelial cells and pericytes when subcultured under identical conditions, ie, on a fibronectin matrix in DMEM supplemented with 10% PHS. Under these conditions, subcultured endothelial cells (initial seeding density of 10^3 cells/17-mm well) formed a confluent monolayer at 6–8 days with an average number of 1.0 ± 0.2 × 10^5 cells/17-mm well (n = 20). However, this did not result in contact inhibition because the endothelial cells continued to divide forming a secondary sprouting layer of cells, eventually attaining a maximal density of 2–4 × 10^5 cells/17-mm well. In many of the cultures, the final phase of subculture was characterized by the formation of tube-like structures (observed at 28–35 days) although some of the culture spontaneously detached from the dishes before this phase. The cells forming the sprouting layer and tubes had somewhat different phenotypic properties, staining positive for Factor VIII-related antigen and α-smooth-muscle actin, but not incorporating Dil-Ac-LDL (similar to Fig. 4). The sprouting and tube-forming cells did not stain for 3G5 (data not shown).

Retinal pericytes subcultured and seeded at an initial density of 10^3 cells/well grew more slowly, attaining a maximal density of 3–5 × 10^4 cells/35-mm well. At no time were multicellular layers of tubelike structures observed, although at maximal densities the edges of the cells appeared to overlap. Similar growth rates and cell morphologies were observed when pericytes were subcultured to plastic and grown in DMEM supplemented with 10 or 20% FBS (data not shown).

**Discussion**

Long-term serial cultivation of retinal microvascular endothelial cells has been complicated by variable contamination with pericytes. It has been reported...
Fig. 3. Retinal endothelial cells in primary culture. (A, C) Phase micrographs. (B) Same field as (A), showing incorporation of DiI-Ac-LDL. (C) Showing positive staining for Factor VIII related antigen. Sprouting cells (arrow) stain for factor VIII but do not incorporate DiI-Ac-LDL. Size marker 10 μm.

Fig. 4. (A) Phase micrograph of retinal endothelial cells in primary culture. (B) Same field as (A) stained for α-smooth muscle actin. Note that sprouting cells, but not underlying monolayer, stain for α-smooth muscle actin. Size marker 10 μm.

Fig. 5. (A) Phase micrograph of retinal pericytes. (B) Same field as A stained for α-smooth muscle actin. Size marker 10 μm. Note both large and small pericytes stain positive for α-smooth muscle actin.

Fig. 6. (A) Phase micrograph of retinal pericytes. (B) Same field as (A) stained with the 3G5 antibody. Size marker 10 μm. Arrow indicates large pericycle which did not stain as intensely for 3G5 compared to adjacent smaller pericytes.
that pericytes can inhibit endothelial cell proliferation, and this and other properties of pericytes could significantly alter the outcome of experiments designed to evaluate characteristics of retinal endothelial cells. In this study we explored the capacity of different matrix settings to promote selectively the growth of endothelium over pericytes. The optimal conditions for selective and sustained endothelial cell growth were achieved using a matrix of fibronectin and hyaluronic acid, and DMEM supplemented with heparin and 20% PHS. The selection of these conditions was derived from analyses of culture conditions which promoted microvessel adhesion, reduced pericyte migration from adherent microvessels, and selectively promoted endothelial cell growth. Although gelatin- and fibronectin-coated culture dishes resulted in increased microvessel adhesion, these matrix settings did not selectively promote endothelial proliferation. Gelatin-coated or uncoated plastic yielded vigorous pericyte growth with sparse endothelial contamination (<2%) in the presence of 20% FBS, providing a simple method for the reproducible culture of near homogeneous pericytes. The DMEM supplemented with PHS in the presence of hyaluronic acid alone or in combination with fibronectin, and by heparin in combination with fibronectin, selectively favored endothelial cell growth. Optimal endothelial cell growth was independent of the yield of adherent microvessels but was dependent on the capacity of the culture conditions to sustain a high endothelial colony:pericyte ratio from early to late culture. The reproducibility of the endothelial cell culture conditions was demonstrated by the observation that all 40 primary cultures of retinal microvessels yielded homogeneous or near homogeneous cultures of retinal endothelial cells. The purity of the endothelial cell and pericyte cultures was confirmed by fluorescent-activated cell sorting of cells labeled with Dil-Ac-LDL. We also observed that the cells could be subcultured up to nine times (at a 1:2 split ratio) and maintain the phenotypic properties (ie, Factor VIII-related antigen, Dil-Ac-LDL uptake) described in this study.

The major advantages of the methods described here for the selective culture of retinal endothelial cells and pericytes are reproducibility and simplicity. There is no requirement for extensive measures previously described to remove or discourage pericytes (eg, weeding, cloning, Percoll gradients, or the use of plasma-derived serum). Virtually all of the primary cultures, initiated using the endothelial and pericyte-specific conditions described in this study, yield homogeneous cultures of the desired cell type. Primary cultures of endothelial cells were identified using the specific endothelial cell markers, Dil-Ac-LDL incorporation and positive staining for Factor VIII-related antigen. Morphologically, these cells were characterized by their spindle or polygonal shape and cobblestone appearance when a confluent monolayer was attained. Endothelial cells formed secondary sprouting layers and random tube-like networks after attaining a confluent monolayer, similar to that described previously for retinal and other types of microvascular endothelium. These sprouting cells were phenotypically distinguished from the monolayer by the lack of Dil-Ac-LDL incorporation and by positive staining for α-smooth muscle actin. The sprouting cells did stain for Factor VIII-related antigen, suggesting that these cells were an altered phenotype of endothelial cells. Kocher and Madri recently reported that treatment of rat fat-pad microvessel endothelial cells with transforming growth factor β resulted in the transient expression of
α-smooth-muscle actin, an observation supporting the concept that endothelial cells can be induced to express this actin isofrom.

Retinal pericytes did not express any of the endothelial cell-specific markers and were identified on the basis of their distinctive morphology, positive staining for the α-isofrom of smooth-muscle actin, and for the ganglioside antigen, 3G5. In many of the primary and subcultured preparations of pericytes, two populations of pericytes could be distinguished on the basis of their relative size and staining properties for α-smooth-muscle actin and 3G5. In the initial study describing the distribution of 3G5, Nayak et al.10 also described populations of cultured pericytes which stained poorly if at all for 3G5. At this time it is not clear if these observations suggest the possibility that there are more than one population of pericytes in vivo or if these populations are culture induced.

In conclusion, we established specific culture conditions which selectively provide homogeneous or near homogeneous cultures of retinal pericytes and endothelial cells in primary and subculture. Further studies will be required to elucidate the molecular mechanisms whereby these culture conditions result in selective growth of endothelial cells and pericytes. Nonetheless, these techniques will provide investigators with a relatively simple, but more importantly, a consistent method for the isolation and culture of homogeneous populations of retinal endothelial cells and pericytes for various metabolic, biochemical, and other determinations.

Key words: heparin, hyaluronate, endothelium, pericytes, retina

Acknowledgments

The authors thank Bob Mannix who made numerous trips to the local slaughterhouse to obtain the eyes for this study and to Dr. Julio A. Rimarachin for helpful discussions. The authors also wish to thank Dr. Randy Barton and Carol Stearns of Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, for their invaluable assistance in the fluorescent-activated cell-sorting analyses.

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