Retinal Vascular Endothelial Cells and Pericytes

Differential Growth Characteristics In Vitro

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Long-term cultures of retinal capillary endothelial cells (RCEC) and of pericytes were grown from collagenase-treated calf retinal vessels. By the use of mechanical separation and differential growth response in various media, pure cell lines were derived from cloned capillary cells as well as from multiple vessel fragments. RCEC and pericytes appeared different under phase contrast microscopy. In addition, RCEC produced factor VIII antigen and angiotensin-converting enzyme, and pericytes did not. RCEC preferred to grow in tumor-conditioned medium, whereas pericytes preferred a standard tissue culture medium supplemented with calf serum. Extracellular matrix, together with tubular structures, developed in postconfluent RCEC but not in pericytes. This study demonstrates that it is possible to grow long-term, pure cultures of RCEC and of pericytes that possess distinctive morphology, growth, and synthetic capabilities in vitro. Invest Ophthalmol Vis Sci 24:470-480, 1983

Tissue culture of vascular cells offers promising investigative inroads in the study of atherosclerosis, diabetes mellitus, inflammation, and neoplasia. The vast majority of studies to date have used endothelium and smooth muscle from large vessels, namely, aorta, umbilical cord, and pulmonary artery. The ease with which vascular cells can be obtained from these sources facilitates experiments using large numbers of cells at early passage. However, the data may not accurately reflect the response of the capillary vasculature, which is the site of major pathophysiology in such conditions as diabetic retinopathy and tumor-induced neovascularization. For example, proper evaluation of vasoactive agents isolated from such sources as tumor, vitreous, and retina may require assay upon their natural target tissue.

Retinal capillaries consist of endothelial cells and pericytes (mural cells, intramural pericytes). Endothelial cells line the lumen, whereas pericytes surround the vessel circumferentially. A basement membrane completely envelops the pericyte and separates them from endothelial cells except at occasional points of intercellular contact. Retinal capillary cells are of special interest because, in diabetic retinopathy, pericytes degenerate with endothelial cell sparing and proliferation.

Growth of retinal vascular capillary endothelial cells (RCEC) has been previously demonstrated in tissue culture from fetal calf, kitten, rabbit, and human eyes. Retinal vascular pericyte growth was abundant from fetal calf and adult monkey, but RCEC proliferation in these cultures was limited. Recent studies demonstrating long-term growth of capillary endothelium from adrenal gland, brain, and foreskin have used specialized techniques such as precoated culture dishes, "cell sweeping," and Percoll gradient centrifugation. These methods permit both isolation and optimal growth of pure capillary cell lines derived from very limited amounts of tissue. Using the techniques outlined by Folkman et al., we have succeeded in establishing long-term, pure cultures of calf RCEC and pericytes.

Our studies demonstrate for the first time the long-term growth of cloned and pure cultures of RCEC. We report that RCEC and pericytes differ as to appearance by phase contrast and electron microscopy, the presence of the identifying cell markers factor VIII antigen and angiotensin-converting enzyme, and the growth response to tumor-conditioned medium.

Materials and Methods

Media

Dulbecco's modified Eagle medium (DMEM) was prepared from powder (GIBCO, Grand Island, NY) and titrated to pH 7.4. Penicillin, 200 units/ml, and streptomycin, 200 µg/ml (GIBCO) were added prior to culture at a final concentration of 100 units/ml and 200 µg/ml, respectively.
to sterilization, which was performed using a Millipore GSWP filter and Millipore APZO pre-filter. DMEM-10, formulated by adding 10% heat-inactivated calf serum (GIBCO) to DMEM, was used to feed pericytes and mouse sarcoma 180 cells. Some experiments used DMEM with 20% calf serum (DMEM-20).

Tumor-conditioned medium (TCM) consisted of DMEM aspirated from growing cultures of mouse sarcoma 180 cells. Cellular material was removed and medium stored as described by Folkman et al. Before use, an equal volume of DMEM was added and then 10% calf serum was added to the final mixture.

Isolation of Retinal Vascular Cells

Calf eyes from a local abattoir were processed as previously described; retinal capillary fragments were retained on 88-/µm and 53-/µm nylon sieves. Following harvest, the vessels from each sieve were incubated in phosphate-buffered saline, pH 7.4, containing 0.75% collagenase (Worthington, Freehold, NJ) and 0.5% bovine serum albumin (Sigma, St. Louis, MO), for 30 min at 37 C. After centrifugation at 800 g for 10 min, the pellets were resuspended in either DMEM or TCM and pipetted into 60-mm gelatin-coated tissue culture dishes (Falcon, Oxnard, CA). Cultures were incubated in a mixture of 95% air, 5% CO₂ at 37 C. Medium was changed three times per week. Fibronectin (Collaborative Research, Waltham, MA) was used to coat culture dishes in some instances.

Cloning of RCEC and pericytes: Cultures were observed daily by phase-contrast microscopy (Zeiss). By day 2 following harvest it was usually possible to identify individual cells and small colonies of two or three endothelial cells or pericytes. Under direct observation using phase-contrast microscopy within a laminar flow hood, adjoining cells of the unwanted type were swept away with a finely tapered Pasteur pipette attached to a micromanipulator (Brinkmann, Westbury, NY) as described by Folkman et al; this procedure is called weeding. The culture was then rinsed twice with Earle’s balanced salt solution (EBSS), pH 7.4, (GIBCO), and fed with its original tissue culture medium. Weeding was repeated daily, if necessary, to maintain purity of the cell colony, until the desired RCEC or pericyte colony contained approximately 200 cells, at which time all other cells were swept from the plate with a Teflon policeman. After aspirating the culture medium, colonies of endothelial cells were exposed to 1.0 ml of 0.05% trypsin-0.06% EDTA. First-passage cells were transferred to a gelatin-coated, 16-mm culture well (Falcon) and maintained in either DMEM or TCM. The same technique was used to transfer cells successively to 35-, 60-, and 100-mm tissue culture dishes.

Pure cultures of RCEC and pericytes from multiple vessel fragments: An alternative method for obtaining pure cultures of RCEC and pericytes allowed cells to grow from multiple vessel fragments and/or multiple individual vascular cells for 5–7 days. At that time, spot-weeding was used to remove unwanted endothelial cells or pericytes from semi-confluent primary or first-passage cultures. Subsequent passages were examined frequently for rare nonendothelial cells or nonpericytes, which were removed by weeding. This technique employed TCM to foster growth of RCEC and DMEM-10 for proliferation of pericytes.

Cell Growth Measurements

RCEC and pericytes were assayed for their growth response in DMEM-10, DMEM-20, or TCM. Confluent cultures were exposed to trypsin and suspended in their original culture medium. Gelatin-coated, 16-mm multiwell dishes (Costar, Cambridge, MA) were seeded with 10⁴ cells per well, after which an additional 1 ml of their original culture medium was added. Following a 4-hr incubation, plating efficiency, which averaged 41% for RCEC and 23% for pericytes, was determined, and seeding medium was replaced with test media. Cultures received fresh test media every other day. Cells to be counted were rinsed with EBSS and released from the bottom of each well with trypsin, suspended in Hematall (Fisher, Pittsburgh, PA), and counted in a Coulter Counter.

Immunofluorescence

Cultured RCEC and pericytes were examined for the presence of factor VIII using indirect immunofluorescence as previously described. A monospecific bovine factor VIII antibody was a gift of J. Brown, University of California, San Diego. Cover slips were examined using a Zeiss photomicroscope III with xenon epifluorescence illumination.

Angiotensin-converting Enzyme (ACE)

ACE activity in cultured cells was determined using a radioassay (Ventrex, Portland, ME). Confluent cultures were rinsed eight times with EBSS and exposed to 1% Triton-X 100 in DMEM at 37 C. Cells were harvested with a Teflon policeman, sonicated, and incubated with the tritiated substrate, (3H)benzoyl-Phe-Ala-Pro. Aliquots of the cell suspension were then extracted with Ventrex Scintillation Cocktail #2,
Fig. 1. Phase contrast photomicrographs of calf retinal vascular cells in tissue culture. A, capillary fragment following isolation and treatment with collagenase; B, early RCEC colony, day 2; C, early pericyte colony, day 9; D, confluent, cloned RCEC, day 30; E, confluent, cloned pericytes, day 22. B–E, same magnification. Arrow = residual vessel fragment.
and the reaction product, (3H)benzoyl-phenylalanine, was measured with a Beckman scintillation counter. The ACE inhibitor SQ 20,881 (E. R. Squibb, Princeton, NJ) was used to control for dipeptide cleavage due to the action of ACE.

Ultrastructure

Sixteen-millimeter discs cut from clear polyester transparency sheets were sterilized by soaking in absolute ethanol for 3 hr. Cultures of RCEC or pericytes were grown on gelatin-coated discs in 16-mm multiwell dishes (Costar). After confluence, cells were fixed in buffered 2.5% glutaraldehyde, postfixed in 2% osmium tetroxide, dehydrated in graded alcohols, and embedded in Medcast epoxy resin. Thin sections were made on a Reichert ultramicrotome and examined with a Philips 200 transmission electron microscope.

Results

Morphology

Following treatment with collagenase, the basement membrane envelope of the capillary segments appeared partially disrupted with increased prominence of the endothelial cells and pericytes. The enclosed cells looked like beads on a chain (Fig. 1A). Vessel fragments attached to the plate and produced growth of cells earlier after collagenase treatment than previously noted. Numerous individual vascular cells, fully released after treatment of the harvested vessel fragments with collagenase, were also found to attach to the plate after this enzymatic treatment. By day 2, colonies containing up to four endothelial cells or pericytes were found to have grown onto the plate from either vessel fragments or isolated vascular cells (Figs. 1B, 1C). After 7 days, mixed colonies of both cell types could be found growing from a vessel fragment.

Endothelial cells were small, curved, and spindle-shaped, with a centrally placed nucleus and one to three nucleoli. Cells clustered as a tightly packed whorl with a circumscribed serpiginous border. As cultures approached confluence, the monolayer assumed a regular, faceted appearance, characteristic of vascular endothelium from both large and small blood vessels (Fig. 1D).

Endothelial cells could undergo as many as nine passages (3 to 4 months) with cultures split 1:10 before exhibiting marked pleomorphism. Postconfluent endothelial cell cultures always displayed both a second cell layer and elongated tubular structures after 7 to 10 days. These characteristics were observed in both cloned and uncloned cultures. Trypsinization and passaging of such cell cultures failed to demonstrate a second cell type using the criteria outlined in this study.
As previously described, pericytes grew as angular, loosely apposed, larger cells that tended to migrate singly from the colony. The ends of the somewhat rectangular cells had a scalloped edge, often with long processes. At confluence the pericytes formed an irregular sheet of cells with occasional multilayered foci (Fig. 1E). Pericytes maintained their growth characteristics through a minimum of ten passages (4 months) with cultures split 1:4.

Endothelial cells required a gelatin surface for attachment and consistent growth, whereas pericytes regularly proliferated on uncoated as well as gelatin-coated plastic. There was no appreciable difference in endothelial cell growth on fibronectin-coated plastic as compared with gelatin-coated plastic (data not shown).

Immunofluorescence

After incubation with factor VIII antiserum, both RCEC and pericytes grown in TCM exhibited prominent, clump-like fluorescence. This nonspecific fluorescence was eliminated after adsorption of factor VIII antiserum with a suspension of mouse sarcoma 180 cells. Typical granular, cytoplasmic immunoreactive factor VIII staining was found in RCEC but not in pericytes (Fig. 2).

Angiotensin-converting Enzyme

Retinal capillary endothelial cells consistently displayed ACE activity significantly above the amount of product released in the presence of the inhibitor SQ 20,881. This activity increased over a 20- to 60-min period, whereas reaction product did not increase in samples containing the ACE inhibitor. ACE activity in RCEC was determined to be $11.02 \pm 2.51$ units/mg at 30 min ($n = 3$ experiments); one unit is defined as the quantity of enzyme required to hydrolyze substrate at an initial rate of 1%/min at 37°C. Pericyte cultures demonstrated little or no enzyme activity above that seen in the presence of the inhibitor; ACE activity was $1.32 \pm 1.25$ units/mg at 30 min ($n = 2$). Furthermore, ACE activity in pericytes failed to increase with time.

Growth Characteristics

Primary cloned cell colonies incubated in TCM regularly demonstrated large numbers of proliferating RCEC and few pericyte colonies. In contrast, primary cultures grown in DMEM-10 or DMEM-20 produced many pericyte colonies but small numbers of endothelial cells. Cultures weeded so as to retain endo-
Retinal vascular endothelial cells grew well in TCM but not in DMEM. RCEC within primary or early-passage plates grew slowly in DMEM-10, with cells acquiring a large, flattened appearance. At later passages and using high seeding density, growth in DMEM-10 could be sustained for several passages. Pure cultures of pericytes proliferated in DMEM and in TCM, but cells appeared more compact and generally “healthier” in DMEM.

Use of the differential growth response of RCEC and pericytes to the various media as well as resistance of pericytes to lift from the plate following treatment with the more dilute trypsin solution created new, noncloned cell lines more efficiently than did the single colony/weeding technique. Thus, the relatively few pericytes growing in primary and early-passage cultures incubated in TCM could be identified and removed, leaving pure RCEC cultures. The opposite was true for cultures incubated in DMEM-10. At each passage, the resulting cell lines were inspected for rare contaminating cell types, which were wiped easily off the plate.

Cell-counting experiments substantiated these observations. When grown in TCM, RCEC had more than twice the number of cells after 12 days than in DMEM-10 (Fig. 3A). Generally, pericytes grew better in DMEM-10 than in TCM after 12 to 15 days, but the difference was not always significant (Fig. 3B). Pericytes grown and maintained in either DMEM-10 or TCM responded similarly when evaluated by cell-counting experiments.

Inasmuch as half of TCM is 10% tumor-depleted serum plus 10% fresh serum, DMEM-20 was also used as a test medium. RCEC grew poorly in DMEM-20 (Fig. 4A), whereas pericytes were stimulated by this medium (Fig. 4B).

No difference in appearance or growth characteristics were noted between cloned and uncloned pure cultures of their respective cell type.

**Ultrastructure**

Transmission electron microscopy of RCEC and pericytes demonstrated numerous organelles typical of metabolically active cells. Junctional complexes, microtubules, and pinocytotic vesicles were seen routinely in RCEC and pericyte cultures.

The two types could be distinguished, however, by the appearance of their extracellular matrix and the pattern of their multilayered growth. Minimal amounts of extracellular material were found beneath monolayers of RCEC and pericytes. When permitted to grow beyond confluence, a second cell layer formed with a thick extracellular matrix between layers of RCEC (Figs. 5, 6). RCEC cultures more than two cells thick were never observed. In contrast, both pre- and post-confluent pericyte cultures displayed areas with as many as five cells superimposed one upon
Fig. 5. Postconfluent culture of RCEC day 13 after seventh passage. A, electron micrograph showing bilayer and extracellular matrix having parallel layers of fibrillar material. L = lumen, XM = extracellular matrix, arrow = junctional complex. B, phase contrast photomicrograph illustrating tube formation at early (single arrowhead) and late (two arrowheads) stages.

Another. A scant extracellular matrix was found between layers of pericytes (Fig. 7). The ultrastructural appearance of pericytes remained the same whether the culture media was DMEM-10 or TCM. The ability of RCEC and pericytes to form multilayers was seen in both early- and late-passage cells.
Tubular structures formed by three or four RCEC and joined by junctional complexes were noted within the lower layer of postconfluent pure cultures (Figs. 5, 6). Amorphous debris was found occasionally inside the lumen. Similar lumina were not observed in RCEC as a monolayer or among pericytes at any time. Preliminary results indicate that RCEC plated at high density will grow as multilayers, accumulate intercellular matrix, and form tubes in medium such as DMEM-10 that is devoid of tumor factors.

**Discussion**

We have established long-term cloned and uncloned pure cultures of RCEC and pericytes with consistent morphologic and growth patterns through multiple passages. Each cell type can be characterized by a typical appearance with phase contrast microscopy, the presence or absence of certain metabolic markers, growth response in several tissue culture media, and ultrastructural characteristics.

Although cloned cell lines ensure maximum uniformity of cell type as well as response to in vitro testing, we found that our method of growing pure cultures from multiple vessel fragments was equally effective and far more efficient. Using the criteria outlined in this study, cloned cultures of RCEC and pericytes could not be distinguished from their respective multiple-vessel pure cultures. Furthermore, although capillary cells were derived from multiple vessel fragments—presumably a sampling of vessels from various parts of the retina—they provided cultures of uniform appearance and growth characteristics.

The whorl-like appearance assumed by RCEC shortly after isolation, followed by a cuboidal pattern at confluence and subsequent passages, is typical of endothelium from large vessels and from microvasculature. The linear processes seen between endothelial cells in late passages using phase contrast microscopy and presumably observed as luminal structures in our transmission electron micrographs may be rudimentary forms of the tubular networks recently described in capillary endothelium by Folkman and Haudenschild. Our calf pericytes resemble the appearance and growth characteristics noted in pericytes from fetal calf and adult monkey in their angular shape, loose apposition of cells and ability to form multilayers.

Although not found solely in vascular endothelium, factor VIII antigen and ACE are cell markers that have been routinely found in cultured vascular endothelium; their combined presence in our RCEC strongly supports the endothelial origin of the cell line. The absence of factor VIII in retinal pericytes, also demonstrated previously, is consistent with studies done with pericytes from adrenal and brain capillaries as well as aortic smooth muscle. Simi-
Fig. 7. Post-confluent culture of retinal vascular pericytes day 13 after fourth passage. A, electron micrograph showing an irregular multilayer with scattered fibrillar extracellular material. XM = extracellular material, P = culture dish. B, phase contrast photomicrograph of multilayered aggregate of cells (arrow).

larly, relatively little or no ACE activity in our retinal pericytes corroborates Frank's findings in pericytes from calf, as well as the work of others on pericytes in rat brain and aortic smooth muscle.

The primary ultrastructural marker for vascular endothelial cells is the Weibel-Palade body, a rod-shaped organelle. These structures have been noted in vertebrate retinal capillary endothelium in vivo and are routinely seen in cultured endothelium from human large vessels and capillaries; they are absent in bovine endothelial cells and were therefore not observed in our cells.

Minimal amounts of morphologically apparent extracellular material from capillary cells in culture are a feature apparently common to these cells as monolayers. Multilayered cultures of endothelial cells have been described in cultured endothelium from adrenal gland grown in TCM and from fetal...
aorta grown in nonconditioned medium (B. M. Glaser, personal communication, May 1982). The rather abundant accumulation of intercellular matrix within multilayered cultures of RCEC has not been described previously in cultured capillary endothelium from other sources. Although it is difficult to extrapolate in vitro results to actual disease processes, our data suggest that retinal vascular endothelium could play an important role in both normal basement membrane synthesis and vessel wall thickening in diabetic retinopathy.\(^{26}\) It must be stated, however, that inasmuch as supplemental ascorbic acid, which is necessary for optimal collagen synthesis,\(^{27}\) was not added to our culture media, synthetic capabilities in vitro of RCEC and pericytes cannot properly be compared at this time.

The difference between large- and small-vessel environment is underscored by substratum and nutritional requirements. Large-vessel endothelium and smooth muscle grow very well on tissue culture plastic, but RCEC required a coating of either gelatin or fibronectin. Capillary endothelium from adrenal gland,\(^{16}\) brain,\(^{17}\) and skin\(^ {18}\) have similar prerequisites. In contrast, retinal vascular pericytes will grow on either a coated surface or plastic. Perhaps, as suggested by Archer and Gardiner,\(^ {28}\) endothelial cells in vivo require a similarly "prepared" surface or scaffold prior to migration or neovascularization. Such a concept is, in part, supported by our observation that the formation of the tubular-like structures in RCEC cultures occurred in association with a substantial intercellular matrix.

Our studies demonstrate that RCEC are stimulated to divide by medium conditioned with sarcoma cells. The conditioning effect was exclusive of a possibly increased serum concentration, as shown by the poor growth response of RCEC to DMEM-20. Pericytes, in sharp contrast, appeared to prefer the standard medium over TCM, yet 20% serum greatly stimulated their growth. At this time we cannot say whether a medium conditioned with nontumor cells would have a similar growth effect on RCEC. It is tempting to speculate that RCEC may proliferate in response to a tumor angiogenesis factor(s) or a cell density factor in the conditioned medium and pericytes do not. Equally, if not more important, may be the extent to which the two cell types migrate in response to TCM and conditioned medium. Zetter\(^ {29}\) has shown that adrenal capillary endothelial cells grow well in either TCM or aortic endothelial-conditioned medium, but only TCM causes the cells to migrate. Experiments are currently in progress to address this point as well as to study interactions of RCEC and pericytes in vitro and the effect of TCM upon their morphologic and synthetic capabilities.

An understanding of the dynamics of retinal capillary cell function and interaction is vital to progress in the management of retinal vascular disease and diabetic retinopathy. We anticipate that in vitro models of vascular retinal capillary cell proliferation will further this goal.

**Key words:** vascular endothelium, pericytes, retina, tissue culture, angiogenesis, diabetic retinopathy

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