
Tissue culture of retinal vessels from fetal calf eyes produced colonies of endothelium, pericytes, and smooth muscle. Identification of endothelial cells was based upon culture morphology, \[^{3}H\] thymidine labeling of isolated vessels, and factor VIII immunofluorescence. Thimerosal added to the culture medium destroyed pericytes and muscle cells, leaving only endothelial cell colonies in the primary cultures. This tissue culture system may be useful in the study of retinal vascular cell biochemistry and pathophysiology.

The retinal microvasculature is lined with endothelium, which serves to maintain the blood-retinal barrier and to facilitate nutrient exchange. Mural cells or intramural pericytes are embedded in the vascular basement membrane of the capillaries.\(^1\) Arteries, arterioles, and large veins all possess small numbers of vascular smooth muscle cells.\(^2\) The specific contributions of each vascular cell type to the integrity of the retinal circulation remain speculative,\(^3\) but an understanding of their physicologic and biochemical functions is of prime importance in the understanding and treatment of retinal vascular disease. For example, preferential loss of pericytes, endothelial cell proliferation, capillary microaneurysms, and large vessel sclerosis are typical findings in diabetic retinopathy,\(^4\) but the initiating mechanisms and the consequences of these changes have so far eluded investigation.

Recent studies have demonstrated the growth of retinal vessel pericytes from adult animals in vitro and the presence of the sorbitol pathway within these cells.\(^5\) Endothelial cell growth was not apparent in those preparations, nor has tissue culture of intraretinal vascular endothelial cells been reported to date. This report describes the proliferation of fetal calf retinal vascular endothelium in vitro and confirms the identity of these cells with the use of a known endothelial cell marker.

Materials and methods

Tissue culture. Retinal vessels free of contaminating neural tissue were harvested from fetal calf eyes of varying gestation on 210, 88, and 53 \(\mu\)m nylon sieves in succession by the homogenization technique of Meezan and co-workers.\(^7\) Eyes were processed in groups of eight to 20 at a time within 8 hr after death. Vessels were maintained in Coon's modified Ham's F-12M tissue culture medium as previously described\(^8\) except for supplementation with 20% fetal calf serum (GIBCO, Grand Island, N.Y.). Following 7 days of incubation, cultures demonstrating presumed endothelial cells were exposed to the above medium containing 0.25 \(\mu\)g/ml sodium ethyl mercurithiosalicylate (thimerosal, Sigma Chemical Co., St. Louis, Mo.), as described by Wagner and Matthews.\(^8\) After exposure to thimerosal for a total of 3 to 5 days, the cultures were again placed in F-12 medium. Cultures received fresh medium with or without thimerosal every other day. In some experiments Dulbecco's modified Eagle medium (DMEM; GIBCO) or Medium 199 (GIBCO) was used in place of F-12M.

Autoradiography. Freshly isolated vessels were incubated in medium containing 20 \(\mu\)Ci/ml \[^{3}H\] thymidine (55 Ci/mmol; New England Nuclear, Boston, Mass.) for 18 hr. Specimens were fixed in methanol, prestained, and prepared for scintillation autoradiography.\(^3\) After the specimens had been exposed for 1 week at \(-88^\circ\) C, the autoradiographs were developed and poststained.

Immunofluorescence. With nonspecific bovine factor VIII antibody obtained from Dr. E. Kibby, Temple University, our cultured retinal vascular cells were examined for indirect immunofluorescence. Cells grown on glass cover slips and in Labtek flasks were washed three times with phosphate-buffered saline (PBS), \(pH\) 7.4, fixed in acetone at \(4^\circ\) C for 10 min, air-dried, and stored desiccated at \(4^\circ\) C until used. Specimens were incubated with factor VIII antibody diluted 1:10 at room temperature for 30 min. Rabbit IgG (Cappel, Cochranville, Pa.) diluted 1:10 was used as a control. Antibodies were rinsed off with three 10 min washes of PBS, and the cells were then exposed to fluorescein-conjugated goat anti-rabbit globulin (FITC; Cappel), diluted 1:10 for 30 min at room temperature. In order to remove nonspecific fluorescence, both factor VIII antibody and IgG were pretreated with cell suspensions of cultured bovine skin fibroblasts; for similar reasons FITC was adsorbed with beef liver acetone powder (Sigma). As an additional control, cells were exposed to FITC without factor VIII or IgG preincubations. Cover slips were mounted with 10% glycerol in PBS and examined with a Leitz Orthoplan fluorescence microscope.

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Fig. 1. Phase-contrast photomicrographs of retinal vascular colonies. A, Mixed mural and endothelial cells, day 11. B, Endothelial cells, day 11. C, Endothelial cells after exposure to thimerosal and acetone fixation, 3 months. D, Mural cells after acetone fixation, day 5. Arrows surround endothelial cells. Calibration bar in A applies to all.

Ultrastructure. Tissue-cultured cells were prepared for transmission electron microscopy as previously described.6

Results. Colonies of polygonal, angulated, and somewhat spindle-shaped cells were noted radiating from both capillaries and larger vessel fragments 2 to 5 days after vessel isolation (Fig. 1, D). These cells grew to an average size of 40 by 180 μm with a morphology and growth pattern similar to those of both cultured adult retinal vascular pericytes and umbilical vein smooth muscle cells.5 Inasmuch as these pericytes and smooth muscle cells from capillaries and from larger retinal vessels, respectively, appeared to have identical characteristics in culture, herein they will be collectively termed mural cells. By day 7, islands of polygonal epithelioid cells adjacent to the original vessel fragment could be seen within some of these colonies (Fig. 1, A). This second cell type contained a centrally placed nucleus and grew as a densely packed, regular mosaic of 40 by 45 μm cells in a monolayer (Fig. 1, B) resembling cultured endothelial cells from umbilical cord and aorta, especially since the latter appear during the first several days following isolation.11,12 The faster-growing mural cells crowded the epithelioid cell clusters without overgrowing them, although mural cells formed multilayered aggregates elsewhere on the plate.

Treatment of cultured retinal vascular cells with medium containing thimerosal resulted in degeneration of mural cells. Dead mural cells detached from the plate, leaving scattered endothelial cell colonies. The flat epithelioid cells thus isolated grew very slowly and in most instances became senescent by 3 months (Fig. 1, C). Occasionally, however, endothelial cell colonies grew to confluence, but only when accompanied by viable mural cells which had survived previous thimerosal treatment. Substitution of DMEM for F-12M

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Fig. 2. A to C Light micrographs of retinal vessels exposed to [3H]thymidine 12 to 48 hr following isolation, stained with periodic acid-Schiff-hematoxylin. e, Endothelial cell; m, mural cell. A and B, Capillaries. C, Precapillary vessel. D and E, Indirect immunofluorescence. D, Positive staining for factor VIII antigen in retinal endothelial cells; E, Background immunofluorescence in retinal mural cells.

did not alter endothelial or mural cell growth patterns either before or after thimerosal treatment. Endothelial cells were not observed in instances where vessels were incubated in Medium 199.

Autoradiographs of freshly isolated retinal vessels demonstrated incorporation of tritiated thymidine in 44% of the 4037 vessel fragments counted. Of these, 3% displayed a labeled endothelial cell. The remaining vessels displayed at least one positive mural cell (Fig. 2, A to C).

Endothelial cells incubated with bovine factor VIII antibody exhibited characteristic granular fluorescence in the cytoplasm (Fig. 2, D). When incubated in IgG or FITC alone, endothelial cells displayed faint nonspecific fluorescence. Immunofluorescent staining for factor VIII was not evident in mural cells (Fig. 2, E).

Exhaustive attempts to identify specific endothelial organelles or Weibel-Palade bodies (WPBs) by electron microscopy were unsuccessful. Rare tubular organelles and membrane-bound rod-shaped bodies were noted, but they nonetheless failed to satisfy all the necessary requisites.11, 12 Typical ultrastructural findings in our endothelial cells included junctional complexes, microtubules, microfilaments, myofilaments, rough and smooth endoplasmic reticulum, and Golgi (Fig. 3). All endothelial cells examined appeared healthy and metabolically active. It was not possible to distinguish morphologically a toxic effect due to thimerosal. Cultured mural cells also failed to display WPBs.

Discussion. The presence of factor VIII antigen within our second cell type establishes these cells as endothelial in origin. This is further supported by their epithelioid morphology and evidence for endothelial cell mitotic activity within vessel fragments at the time of isolation. Absence of WPBs in our cells is consistent with reports of cultured microvascular endothelium,8, 14 the lack of these organelles specifically in bovine tissue,15 and a paucity within capillaries in situ.16

Thimerosal reversibly complexes with sulfhydryl enzymes and is thought to have a toxic effect...
on rapidly mitosing cells while sparing slower growing endothelium. The mechanism for its differential effect on mural cells and endothelium and the implications for understanding their respective cell functions are unknown at this time. It is quite possible, however, that thimerosal has a definite but less devastating toxic effect on retinal endothelial cells than on mural cells. This would account for poor endothelial cell growth following treatment. On the other hand, the observation of continued endothelial cell proliferation in association with viable mural cells may imply a requirement by our endothelium for a conditioned environment. To this end experiments using feeder layers, tumor-conditioned medium, and added growth factors are currently in progress. Traditional cloning techniques, which have failed in preliminary experiments to re-establish retinal endothelial cell growth following thimerosal treatment, may be necessary to sort endothelial from mural cells soon after vessel isolation.

Although originating from a common mesenchymal stem cell, endothelium and pericytes appear to respond differently when challenged by ischemia, aging, and diabetes. The absence of factor VIII antigen in cultured pericytes perhaps reflects this functional disparity. Factor VIII antigen is a glycoprotein synthesized by endothelium both in vivo and in vitro and is involved in blood coagulation and platelet function. Vascular smooth muscle cells from large vessels lack this antigenic marker, as is also the case in our cultured mural cells.

Smooth muscle appears to differentiate from primitive pericytes in the course of fetal retinal vascular development and functional adaptation. In culture these two cell types appear to have similar properties. This finding is intriguing inasmuch as a muscular or sphincterlike function of intramural pericytes has been subject to considerable debate. It is interesting that intramural pericytes grow more abundantly in our culture system than do endothelial cells and that pericytes seem to foster endothelial proliferation, in view of what is found in diabetic retinopathy; i.e., endothelial cell proliferation and pericyte degeneration. In the following report, Frank et al. describe similar limitations in culturing retinal capillary endothelium from kitten. Their primary cultures, however,
displayed a relative paucity of pericytes. Retinal neovascularization no doubt reflects the interplay of multiple local and systemic processes; initial attempts to understand these phenomena must begin with a basic understanding of the biochemistry and growth characteristics of retinal vascular cells. Our tissue culture model and that of Frank et al. may be quite useful toward achieving this goal.

Using our present culture conditions, we regularly observed endothelial cell growth from fetal calf eyes but rarely from adult eyes; pericyte and smooth muscle cells grew easily from young and mature tissues. Accordingly, consideration must be given to conditions and agents capable of stimulating endothelial growth from adult retinal vessels in vitro and the implications such means may have in understanding retinal vascular pathophysiology.

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In vitro proliferation of endothelial cells from kitten retinal capillaries. ROBERT N. FRANK, V. EVERETT KINSEY,† KARIN W. FRANK, KEVIN P. MIKUS AND ANN RANDOLPH.

When microvessels (predominantly capillary) fragments freshly isolated from the retinas of young kittens are in-