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Retinal Capillaries: Proliferation of Mural Cells in vitro

Abstract. *Capillaries from bovine, monkey, and human retinas maintained in tissue culture produced a monolayer of cells. Autoradiographic and electron microscopic evidence indicated that the mural cells (intramural pericytes) were the cells that proliferated. Since intramural pericytes are damaged selectively in diabetes mellitus, their availability in culture will be useful in seeking means to control diabetic retinopathy.*

Vertebrate retinal capillaries contain two cell types: endothelial cells and mural cells, or intramural pericytes (*1, 2*). Endothelial cells line the vessel lumen while mural cells are embedded in the vascular basement membrane and surround the vessel with extensive cytoplasmic processes. Details of the physiological roles of these cells are largely unknown (*3, 4*); however, abnormalities in their biochemistry and function may be critical factors in the pathogenesis of several retinal vascular diseases which are common causes of blindness. For example, in diabetic retinopathy mural cells are affected early and degenerate, leaving capillaries with a preponderance of endothelial cells (*3, 5*). The biochemical events that precede these anatomical changes are unknown. Their elucidation would be an important step toward the understanding and eventual treatment of many retinal vascular diseases.

The capability of maintaining cells in tissue culture greatly facilitates studies of their biochemistry, growth, and function. While long-term culture of endothelial cells from human umbilical vein has been reported by several investigators (*6, 7*), to the best of our knowledge such a system of sustained growth has not been described for retinal vascular cells (*8*). We now report methods whereby cellular proliferation from bovine, rhesus monkey, and human retinal capillaries can be obtained regularly. Our observations indicate that the cells which grow under the imposed culture conditions originate from the mural cells of the retinal capillaries.

Eyes from rhesus monkeys, cattle, and humans were usually processed for culture 4 to 8 hours after enucleation (*9*). Eyes were cleansed in a physiologic saline solution and rinsed in tincture of benzalkonium chloride (0.13 percent) before dissection. Retinas were removed from the eyes, minced in modified Earle's salt solution, homogenized, and filtered as described by Meezan and co-workers (*10*), using sterile technique. Vessels remaining on the 85-μm sieve were washed into sterile plastic petri plates with Ham's F-12 medium containing 5 percent fetal calf serum; 2 m*M* glutamine; penicillin, 200 unit/ml; and streptomycin, 200 μg/ml. The plates were incubated at 37°C in an atmosphere of 95 percent air and 5 percent CO_2.

Isolated retinal capillaries could be identified by their size and structure (Figs. 1A and 2A). Endothelial cell nuclei were distinguished by their location within the capillary wall and their ellipsoidal shape, with the long axis parallel to the lumen. Mural cell nuclei had round profiles which protruded from the capillary wall like "bumps on a log." Occasionally blood cells were seen trapped within the vessels. Perivascular glia or neural cells were seen only rarely adherent to the isolated vessels. We never observed cellular proliferation from these infrequent contaminants.

Approximately 1 to 2 percent of the isolated vessels displayed growth. When placed in culture media, the vessels behaved as explants, producing an advancing sheet of polygonal cells (each about 35 by 70 μm) by day 8 to 12. The configuration of some vessels varied as loosely adherent branches shifted positions (Fig. 1B). During the first 2 to 4 days after isolation, the vessels often appeared to entwine upon themselves, making it difficult to distinguish cell types by their morphological appearance (Fig. 1, C to E). By day 9 large cells with ovoid nuclei and multiple nucleoli could be seen projecting from the closely packed cluster of vessel strands (Fig. 1F). An advancing sheet of cells was usually apparent by day 17 (Fig. 1G) and the establishment of a dense monolayer by day 30 (Fig. 1H). Rate of growth was slow, with several proliferating vessels requiring 2 to 4 months to fill a 60-mm culture plate. Older cultures generally displayed cells in monolayers of uniform density. However, some of these cultures contained cells of varying size and packing. Sometimes cells were found irregularly heaped on each other. Cells were never found to abut one another in either regular epithelioid or typical fibroblast patterns (*6, 7, 11*). Several cultures have been subcultured many times over an 8-month period without any change in morphological appearance or growth characteristics.

Electron microscopy of cells grown from rhesus monkey retinal capillaries for 30 days in culture revealed ultrastructural characteristics suggestive of metabolically active, somewhat specialized cells. Smooth endoplasmic reticulum, Golgi, and coated vesicles were prominent near the cell cen-

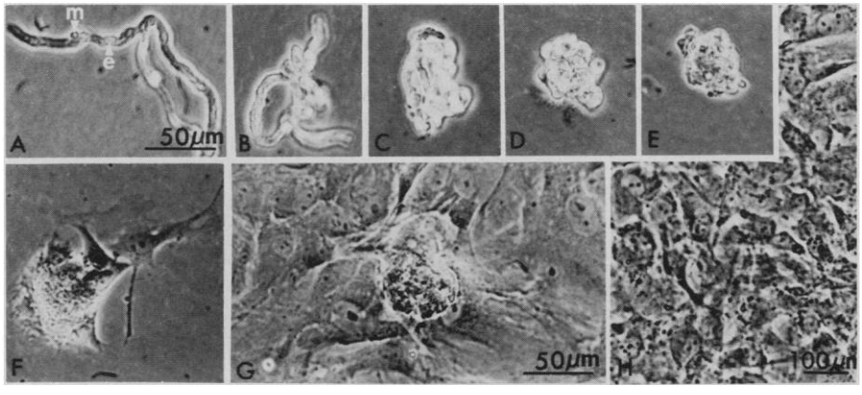

Fig. 1. Phase contrast photomicrographs of a single rhesus monkey retinal capillary observed in culture for more than 30 days: (A) day 0, (B) day 1, (C) day 2, (D) day 3, (E) day 6, (F) day 9, (G) day 17, and (H) day 30. Abbreviations: *e*, endothelial cell; *m*, mural cell (A to G, × 265; H, × 81).

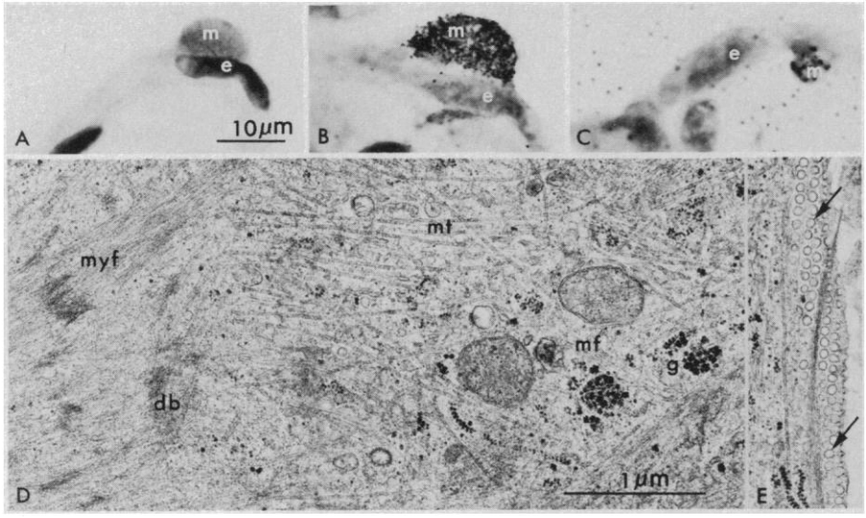

Fig. 2. (A to C) Light micrographs of specimens stained with periodic acid–Schiff–hematoxylin. (A) Monkey retinal capillary at time of isolation. (B) Bovine retinal capillary incubated with [^{3}H]thymidine for 24 hours beginning on day 2. (C) Monkey retinal capillary incubated with [^{3}H]thymidine for 12 hours beginning on day of isolation. Abbreviations: *e*, endothelial cell; *m*, mural cell (× 1,400). (D to E) Electron micrographs of cells in 30-day culture of monkey retinal capillaries, showing organelles and other features characteristic of mural cells in vivo. Abbreviations: *db*, dense bodies; *g*, glycogen; *mf*, microfilaments; arrows, micropinocytotic vesicles; *mt*, microtubules; *myf*, myofilaments. Fixed in 1.5 percent glutaraldehyde with 4 percent sucrose buffered at *p*H 7.2 with 50 m*M* sodium cacodylate, then embedded in Epon (× 29,000).

ter, intermixed with extensive rough endoplasmic reticulum. Micropinocytotic vesicles, microfilaments, and microtubules were abundant near the cell surface (Fig. 2E). Myofilaments occurred individually and in bundles associated with dense bodies (Fig. 2D). Glycogen was common and often aggregated in large deposits. Many narrow filopodia extended for undetermined lengths from the cell surface. Notably absent were the so-called specific endothelial organelles or Weibel-Palade bodies, which are thought to be characteristic of vascular endothelium both in vivo and in culture (*6, 7, 12*).

Autoradiography was performed by incubating vessels from retinas of rhesus monkeys and cattle in culture medium containing [^{3}H]thymidine at concentrations of 2 to 4 μc/ml. After incubation for 8 to 24 hours, the vessels were washed in phosphate-buffered saline, fixed, prestained, dipped in Kodak NTB-2 emulsion, and stored for 6 to 8 weeks at 4°C. The autoradiographs were then developed (*13*) and the specimens were poststained.

Inasmuch as capillaries lost both form and cellular characteristics after 3 days in culture, nuclei incorporating [^{3}H]thymidine before this time were easier to identify. Out of 2625 total nuclei counted, 20 (0.8 percent) were found to be labeled. Seventeen of 1207 mural cells (1.4 percent) were labeled (Fig. 2, B and C). We counted 1236 identifiable endothelial cells but none were labeled. Because of poor staining qualities or conformational changes, 182 nuclei could not be classified. Three of these (1.6 percent) were labeled.

Histological and ultrastructural evidence supports the contention that the mural cells, not the endothelial cells, are the elements which proliferate in our cultures. Glycogen occurs normally in mural cells (*2*) but not in mature endothelial cells (*14*). Unusual amounts of glycogen accumulated in mural cells while endothelial cells remained unaffected in a reported case of glycogen storage disease (*3*). Myofilaments have been found in mural cells by several investigators (*2, 15*). Moreover, it has been suggested that mural cells may be capable of differentiating into smooth muscle cells under certain in vivo conditions (*16*). Elaborate junctional complexes occur between the plasma membranes of endothelial cells in situ and in culture (*7, 17*), but they have not been observed between mural cells (*3, 4*). We found only slight modifications of membrane structure in regions of cell-to-cell contact in our retinal capillary cultures. The organelle content, which includes abundant micropinocytotic vesicles, lysosomes, and myofilament bundles with dense bodies, distinguishes the cells in our cultures from cultured neuroglia (*18*) and retinal neurons (*19*).

The demonstration that mural cells survive and proliferate in our cultures—precisely the opposite of the case in diabetic retinopathy—suggests that this preparation can be used to study the developmental and biochemical differences between mural cells and endothelial cells. Such studies have eluded investigation by other means. Although extrapolation of results obtained from cells grown in long-term culture to in vivo conditions must be done with caution, our preparation may be of considerable use for the study of retinal capillary physiology in both health and disease.

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