Histopathology of Experimental Preretinal Neovascularization

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Despite the morbidity resulting from abnormal retinal neovascularization, morphological events associated with its development have not been fully described. We therefore studied sequential morphologic events during preretinal neovascularization in an experimental model induced by injection of 250,000 homologous fibroblasts into the vitreous cavity of rabbits. Within 2 days following fibroblast injection, thickening of many venular and capillary endothelial cells resulted in partial obliteration of their lumina. ³H-thymidine incorporation occurred first in the nonvascular cells of the superficial medullary ray and thereafter in the preretinal vessels and extraretinal fibroblasts. Capillary budding was obvious within 3 days, with endothelial cells extending cytoplasmic processes into fragmented extracellular matrix (ECM). Endothelial cells, at the tips of budding vessels, and at more proximal sites in the parent vessel, incorporated ³H-thymidine and did not lose cell contact or migrate individually into the ECM. Lumina were present throughout the entire length of the buds and endothelial cells remained polarized. Neovascular events observed in this experimental model parallel those previously described in diabetic retinopathy and retinopathy of prematurity in humans. Invest Ophthalmol Vis Sci 30:1495–1503, 1989

Neovascularization is an important component of normal physiologic processes such as embryonic development, corpus luteum formation and wound healing.[1-4] Neovascularization is also part of pathologic processes such as solid tumor growth,[5] diabetic retinopathy,[6,7] retinopathy of prematurity,[8,9] neovascular glaucoma[10,11] and corneal vascularization.[12] Despite the morbidity associated with retinal neovascularization, the process of neovascularization as it develops in the retina has not been completely described.

Histopathologic studies of retinal neovascularization and of the blood retinal barrier in diseases such as proliferative diabetic retinopathy,[13,14] retinopathy of prematurity[8] and in an experimental model of retinal neovascularization[15] indicate that there may be differences between retinal and corneal neovascularization. Corneal neovascularization has been thoroughly described using a corneal pocket assay.[16]

The purpose of this study was to document the sequential events that occur during preretinal neovascularization in an experimental rabbit model. Although, the rabbit retinal vasculature differs from that of human in lying mostly anterior to the inner limiting membrane over the medullary rays rather than being intraretinal, the capillary endothelial cells have tight junctions that do not leak fluorescein or HRP under normal conditions.[1,5] Neovascularization was induced by the injection of homologous fibroblasts into the vitreous cavity of pigmented rabbits and evaluated at selected time intervals by autoradiography of ³H-thymidine uptake, and light and transmission electron microscopy of serial and adjacent sections.

Materials and Methods

Pigmented rabbits of both sexes weighing 2.5–3.5 kg were used in the experiments. The animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research. All animals, while anesthetized (Ketamine 30 mg/kg and Rompun 5 mg/kg [Xylazine], Mobay Corporation, Shawnee, KS), underwent examination by slit-lamp biomicroscopy and fundus ophthalmoscopy, and those that were judged to be normal were selected for experimental use. Four eyes received injection of phosphate buffered saline as controls. Twenty-eight eyes were injected with 250,000 homologous fibroblasts (in 0.1 ml of PBS) into their vitreous cavity.[17] The injection was made with a 27-gauge needle 4 mm behind the

limbus, under stereomicroscopic control. The cells were placed over the optic disc and the vascularized medullary wings, which extend temporally and nasally from the optic disc. After injection of the cells, the rabbits were placed on their backs for 30 min to allow the cells to settle on the posterior retina. The eyes were examined on days 1, 2, 3, 5, 7, 14 and 28 after injection. Fundus photography of all animals was performed on these days using a Zeiss fundus camera with a 30° field (Carl Zeiss, West Germany). Horseradish peroxidase (HRP, type II, Sigma Chemical Company, St. Louis, MO) at 0.25 g HRP/kg body weight, dissolved in 2 ml of PBS adjusted to ph 7.3, was injected into the marginal ear vein using a Harvard pump (Harvard Apparatus, Millis, MA) at 0.68 cc/min. The HRP was allowed to circulate for 30 min prior to euthanasia with an overdose of pentobarbital. In addition, 24 hr prior to the HRP injection, 32 rabbit eyes are injected with 10 μCi ^{3}H-thymidine (specific activity 20.0 Ci/mMol) (New England Nuclear Products, Boston, MA) using a 10 μl Hamilton syringe (Hamilton, Reno, NV).

Enucleations were performed on days 1, 2, 3, 5, 7 and 14 after fibroblast injection (n = 4 for each day). The enucleated eyes were placed in 4% paraformaldehyde and 5% gluteraldehyde in 0.05 M phosphate buffer. A 4 mm slit was made in the inferior portion of the eye to allow better access for the fixative. After 5 hr, the eyes were placed in 0.05 M phosphate buffer for two 10 min rinses, and placed overnight in fresh 0.05 M phosphate buffer at 26°C. Following dissection, retinal sections were placed in 3,3 diaminobenzidine-Tris Buffer (DMB) (Sigma) under low light conditions, and subsequently incubated with DMB in total darkness for 1 hr. Tissues were then incubated in fresh DMB containing 0.005% H_2O_2 in darkness for 1 hr. Subsequently, after washing three times with distilled water, they were placed in 1.3% osmium tetroxide, 1.5% ferrocyanide, 5% sucrose in 0.05 M phosphate buffer and incubated for 10 min in 0.05 M phosphate buffer containing 5% sucrose.

After the third wash, the tissues were dehydrated in graded concentrations of ethanol and embedded in a low-viscosity epoxy resin. The blocks were trimmed and serially sectioned for light (1 μm) and electron microscopy (60–90 nm), using a LKB ultramicrotome (LKB Instruments Inc., Rockville, MD). Serial sectioning of the blocks was also performed. Serial sections were taken in the following manner: a total of ten 1-μm thick serial sections were taken followed by multiple 60 to 90 nm thin sections. This process was repeated until capillary buds were sectioned through entirely.

Thin sections were stained with lead citrate and uranyl acetate and examined with a JEM 1200 EX

transmission electron microscope (JEOL Inc., Peabody, MA). Thick sections were stained with azure II and methylene blue.

Tritiated Thymidine Autoradiography

One micron thick sections were cut and placed on gelatin-coated slides. Slides were subsequently emersed in Kodak NTB-2 photographic emulsion (Kodak, Rochester, NY) and allowed to dry under red light conditions. Slides were placed in a light-tight black slide box with dessicant and stored in a dark refrigerator for 7 days. The slides were then developed, fixed, stained with methylene blue and photographed.

Results

Day 1

Autoradiography at day 1 revealed no labeling in either the medullary rays or preretinal blood vessels. Transmission electron microscopy showed relatively normal-appearing, wide-lumened blood vessels lined by thin endothelial cells and surrounded by an intact basal lamina (Fig. 1). Polymorphonuclear leukocytes, lymphocytes and fibroblasts were present in the vitreous at this and later times. The cells appeared suspended in the vitreous gel and were not in physical contact with the vessels or the retina.

Control eyes, injected with buffered saline, showed no labelling of cells at days 1, 2, 7 or 14. No changes in the vascular configuration or neovascularization were seen.[18]

Day 2

^{3}H-thymidine uptake was most apparent in the medullary rays (presumably glial cells) after 2 days (Fig. 2). Ten high-power fields of a representative eye revealed labelling of six medullary ray cells, one preretinal endothelial cell and one fibroblast in the vitreous. Electron microscopy revealed many small vessels with thickened endothelial cells with prominent Golgi apparati and extensive rough endoplasmic reticulum (Fig. 3). At this time, endothelial cells extended processes into the extracellular matrix (ECM) as well as into the lumen of the parent vessel. The ECM was multilaminar but fragmented, particularly in the areas of endothelial cell extensions into the ECM.

Days 3 to 7

Between 3 and 7 days, ^{3}H-thymidine uptake was heavy, particularly in the vasculature both adjacent and distant to the fibrous strand (Figs. 4, 5A). For

Fig. 1. Transmission electron micrograph of day 1 after fibroblast injection. The endothelium of a preretinal capillary is thin and had sparse rough endoplasmic reticulum. A possible fibroblast is seen above the capillary (×4650).
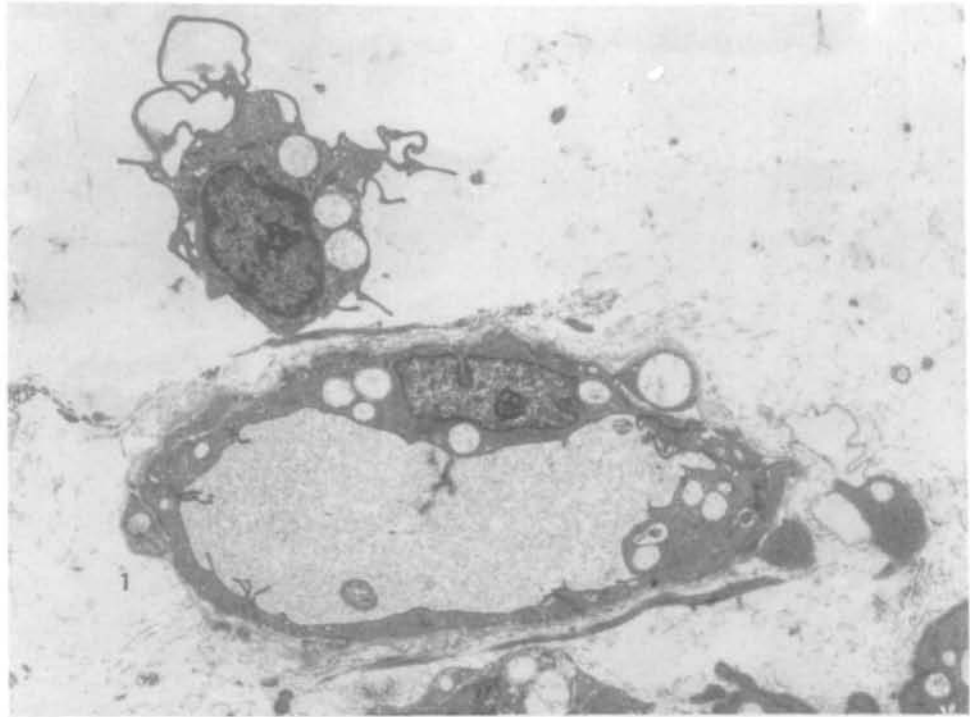
example, ten high-power fields of a representative eye from day 5 revealed labelling of 15 preretinal vascular and six medullary ray endothelial cells. Electron microscopy showed numerous endothelial cells migrating into the ECM (Fig. 5B). Many of these cells were polarized with luminal and abluminal surfaces. The lumens were partially obliterated and would have been difficult to recognize without the presence of intraluminal HRP. The migrating cells were surrounded by large amounts of multilayered basement membrane-like material. This material covered all of the cellular extensions but was more fragmented over the most distal tips of these extensions.

Serial light microscopic sections further documented the following morphological characteristics of the budding vessels: (1) ^{3}H-thymidine uptake was prominent in the parent venule but also in the most distal cells of the bud; (2) there were no free migrating endothelial cells (ie, no separations or gaps between endothelial cells); (3) there was no extravasation of intraluminal HRP; and (4) endothelial cells remained polarized retaining both a luminal and an abluminal surface.

Although budding areas were focal, they were clearly not restricted to one or two endothelial cells. Often vessels had a growing tip with seven to eight
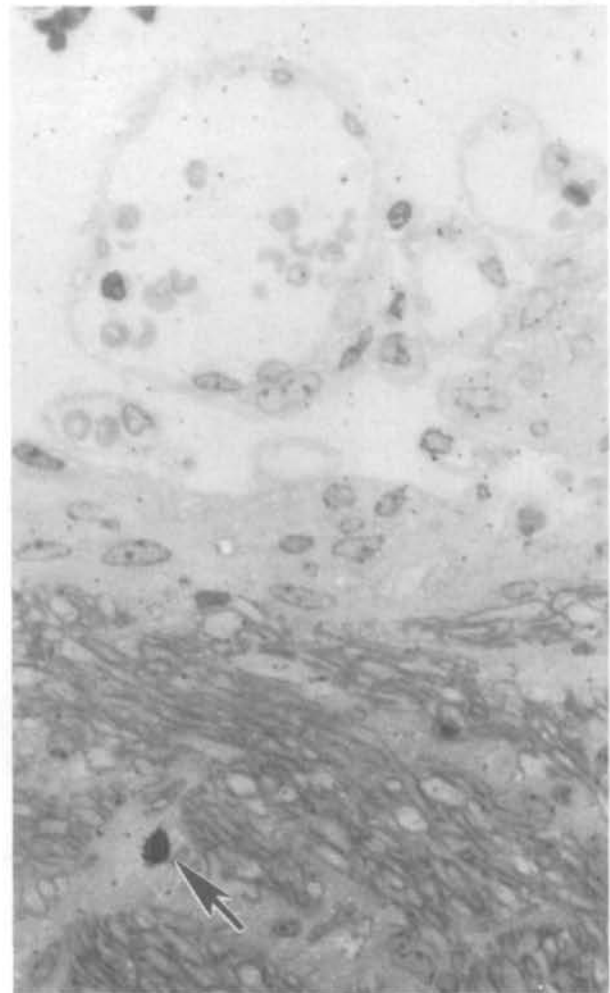
Fig. 2. Autoradiogram of day 2 after fibroblast injection. A single focus of ^{3}H-thymidine uptake is present in the medullary ray (arrow). None of the numerous thin preretinal capillary endothelial cells or any of the free floating cells in the vitreous are labelled with ^{3}H-thymidine (×420).
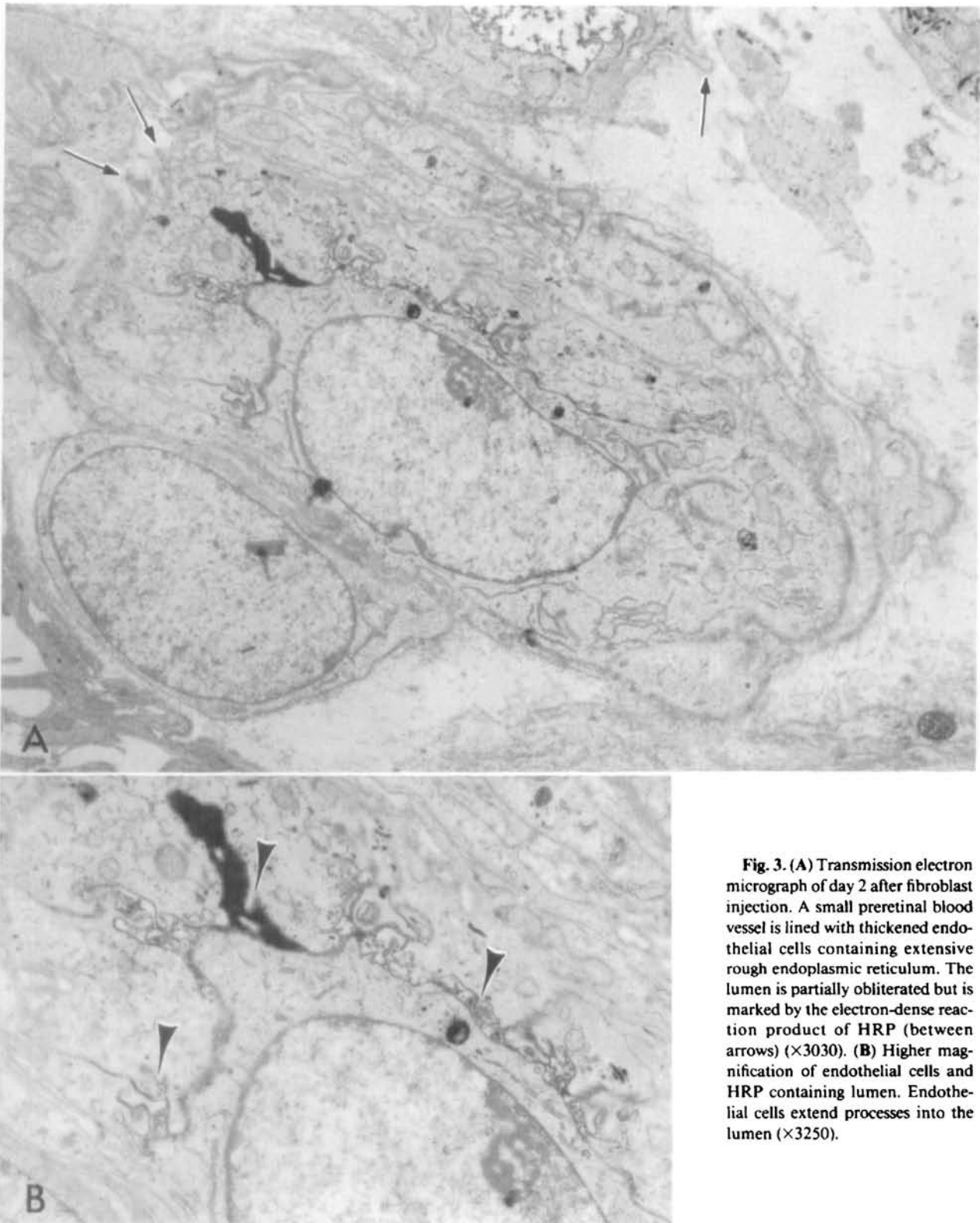
Fig. 3. (A) Transmission electron micrograph of day 2 after fibroblast injection. A small preretinal blood vessel is lined with thickened endothelial cells containing extensive rough endoplasmic reticulum. The lumen is partially obliterated but is marked by the electron-dense reaction product of HRP (between arrows) (×3030). (B) Higher magnification of endothelial cells and HRP containing lumen. Endothelial cells extend processes into the lumen (×3250).

thickened finger-like buds with tortuous lumens. Many cells that looked like pericytes at the light microscopic level were clearly seen to be endothelial cells with small HRP-containing lumens by electron microscopy (Fig. 5). However, even with serial thin sections, it was not possible to document in detail how the intricate maze of tunnels and extensions had formed.

Days 14 to 28

Few cells labelled with ³H-thymidine were present after 14 days. Ten high-power fields of a representa-
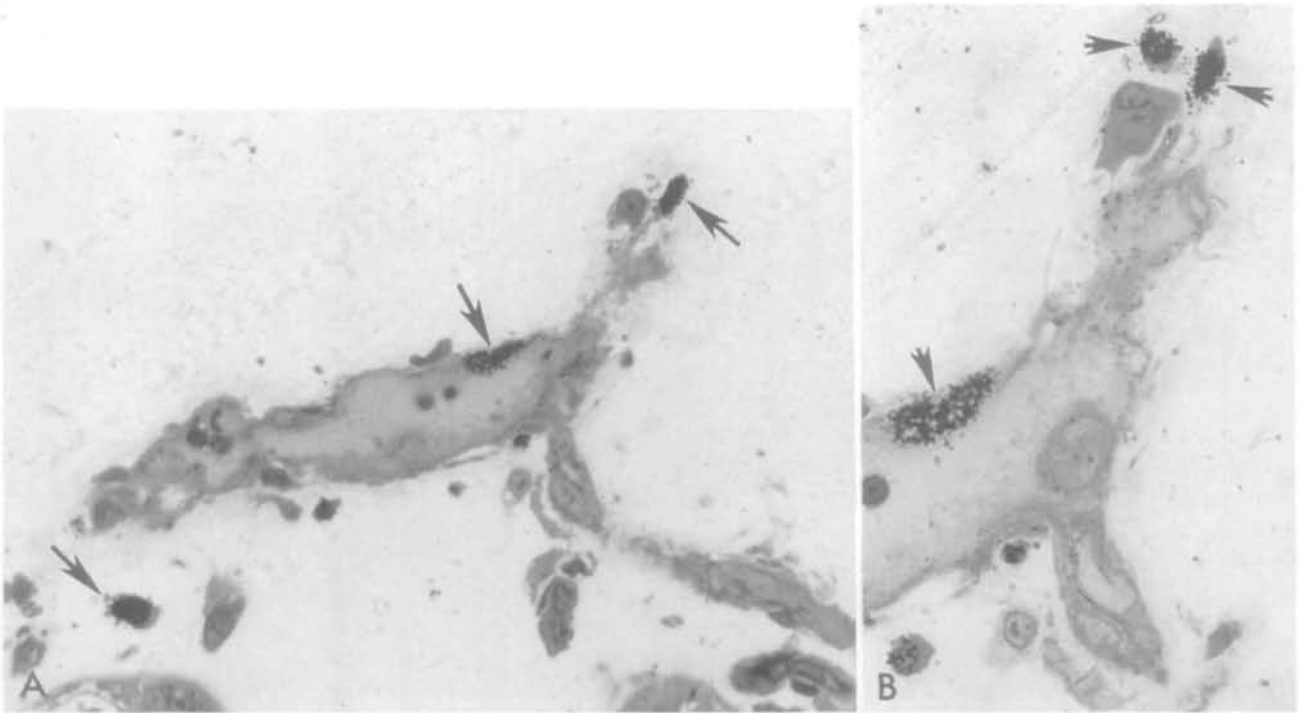
Fig. 4. (A) Autoradiogram of day 5 after fibroblast injection. ³H-thymidine uptake (arrows) is seen in the parent vessel, at the tip of the bud and in an extravascular cell (×420). (B) Higher magnification of the tip of the developing vessel in an adjacent section illustrates a continuity between the dividing cells at the tip and the parent vessel (×825).
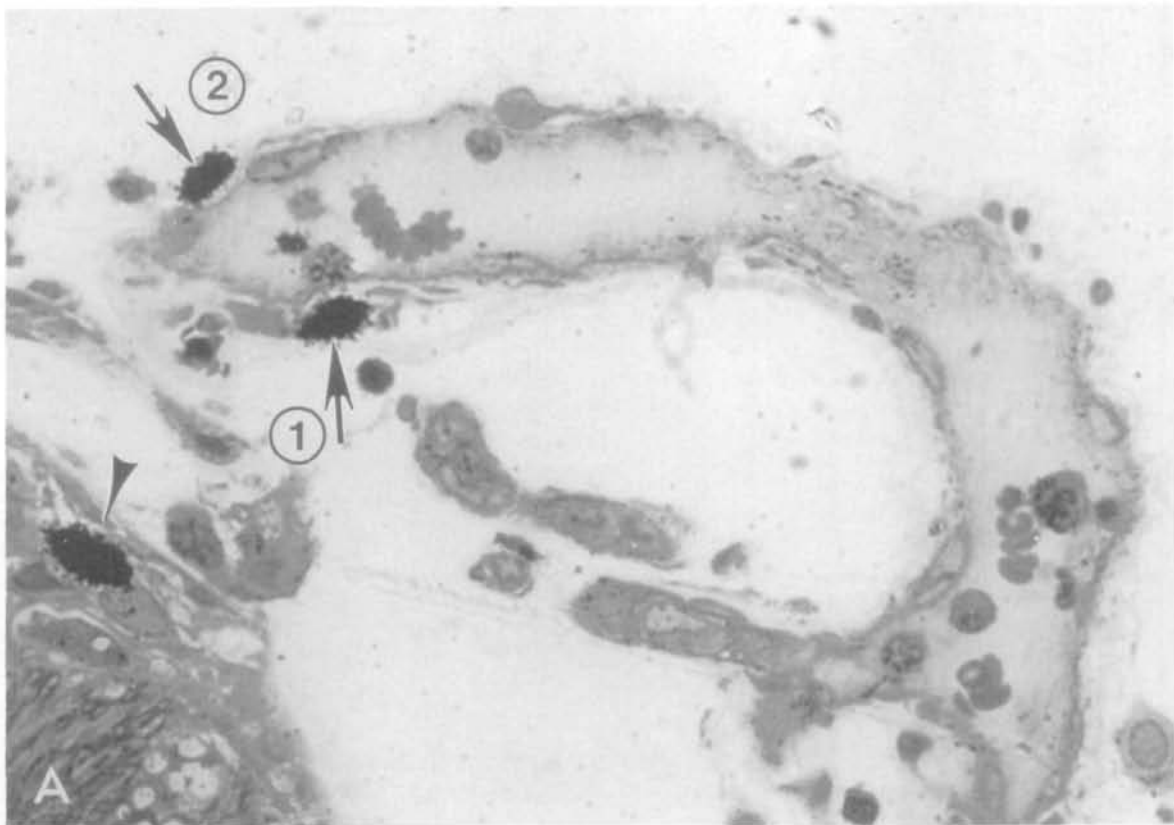

Fig. 5. (A) Autoradiogram of day 5 after fibroblast injection. Two vascular cells (arrows) and a cell near the surface of the medullary ray (arrowhead) are labelled with ³H-thymidine (×480). (B) Transmission electron micrograph of three developing tips (1, 2, 3) of the same blood vessel in an adjacent section. The luminal extensions are highly tortuous but are marked by the intraluminal HRP (arrows) (×800). (C) High magnification electron micrograph of area (1) in Figure 5B. Fragmentation of abundant multilayered extracellular matrix is apparent. The lumen of the vascular bud is marked with HRP. The endothelial cell marked (1) may correspond to the labelled vascular cell (1) seen in Figure 5A (×3000). (D) High magnification of another vascular bud seen in area (2) in Figure 5B. Again, the lumen is marked by HRP (between arrows) and the endothelial cells are plump and contain extensive rough endoplasmic reticulum indicating substantial protein synthesis (×3000).
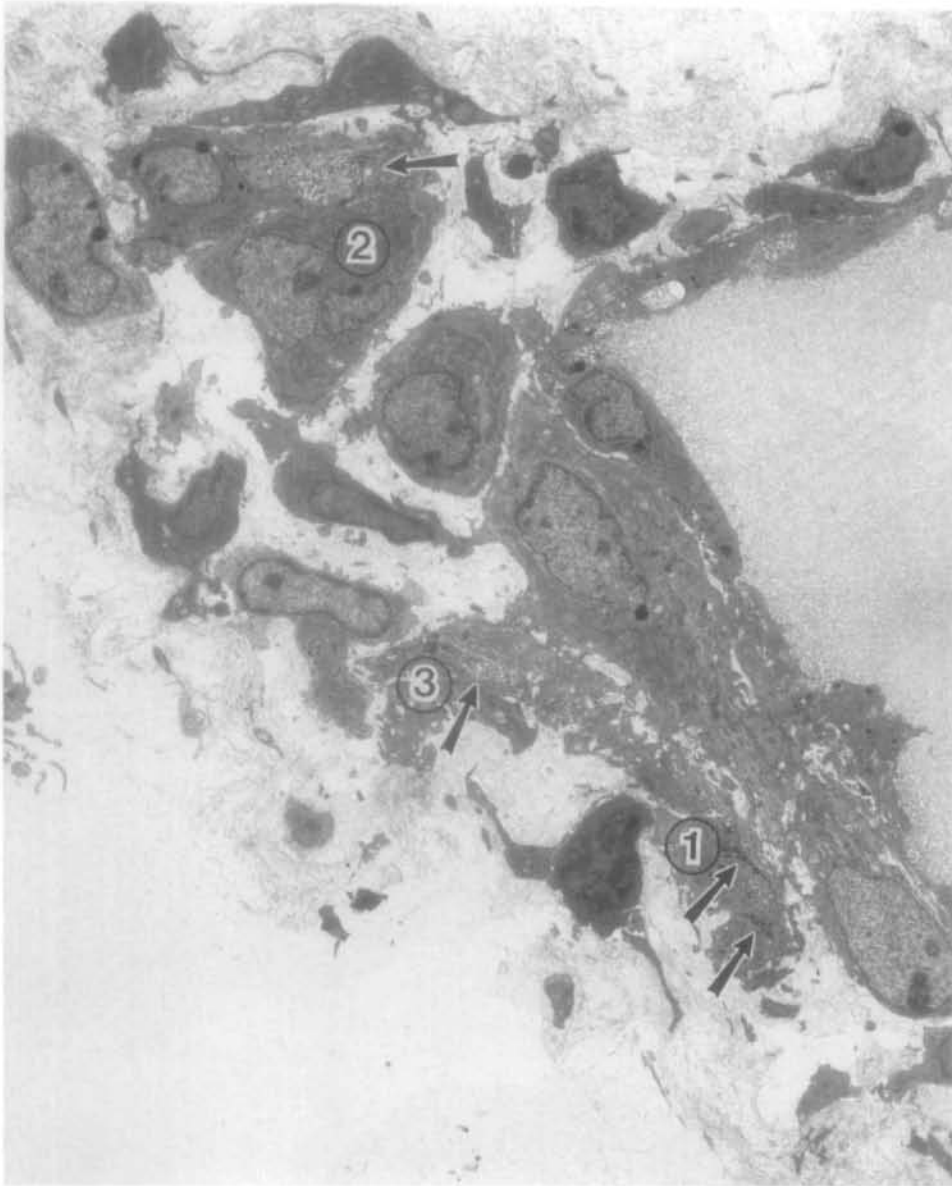
Fig. 5. (B) See legend under Figure 5A.

tive eye from day 14 revealed labelling of two medullary ray cells, one preretinal endothelial cell and one extraretinal fibroblast. Electron microscopy of the developing vessels revealed many mature thin-walled, wide-lumened vessels lined by thin endothelial cells (not illustrated). Few endothelial cell extensions were seen in the ECM and/or in the lumen. Well-defined basal lamina had now formed over the distal portions of the developing vessels. Rare polymorphonuclear leukocytes, lymphocytes and macrophages were scattered near the fibrous strand.

Discussion

Preretinal neovascularization induced by an injection of homologous fibroblasts into the vitreous cavity of rabbits was characterized by several sequential steps. Within 2 days, endothelial cells change in shape

from thin differentiated cells (which are proficient in nutrient exchange and waste removal) to thickened, synthetically active cells that have extensive endoplasmic reticulum and prominent Golgi apparati. Within 2–3 days, early capillary budding becomes evident. The process of new bud formation begins with endothelial cytoplasmic extensions which are usually surrounded by an exuberant multilayered ECM except at the very distal portions where it is fragmented. The cells also extend numerous projections into the lumen of the developing capillary. Concomitantly, numerous endothelial cells, both at the tips of new vessels and also in the parent vessel walls, begin DNA synthesis as evidenced by the uptake of ^{3}H-thymidine. Budding endothelial cells remain polarized, do not lose cell contact, or migrate individually into the ECM. A lumen is present throughout the
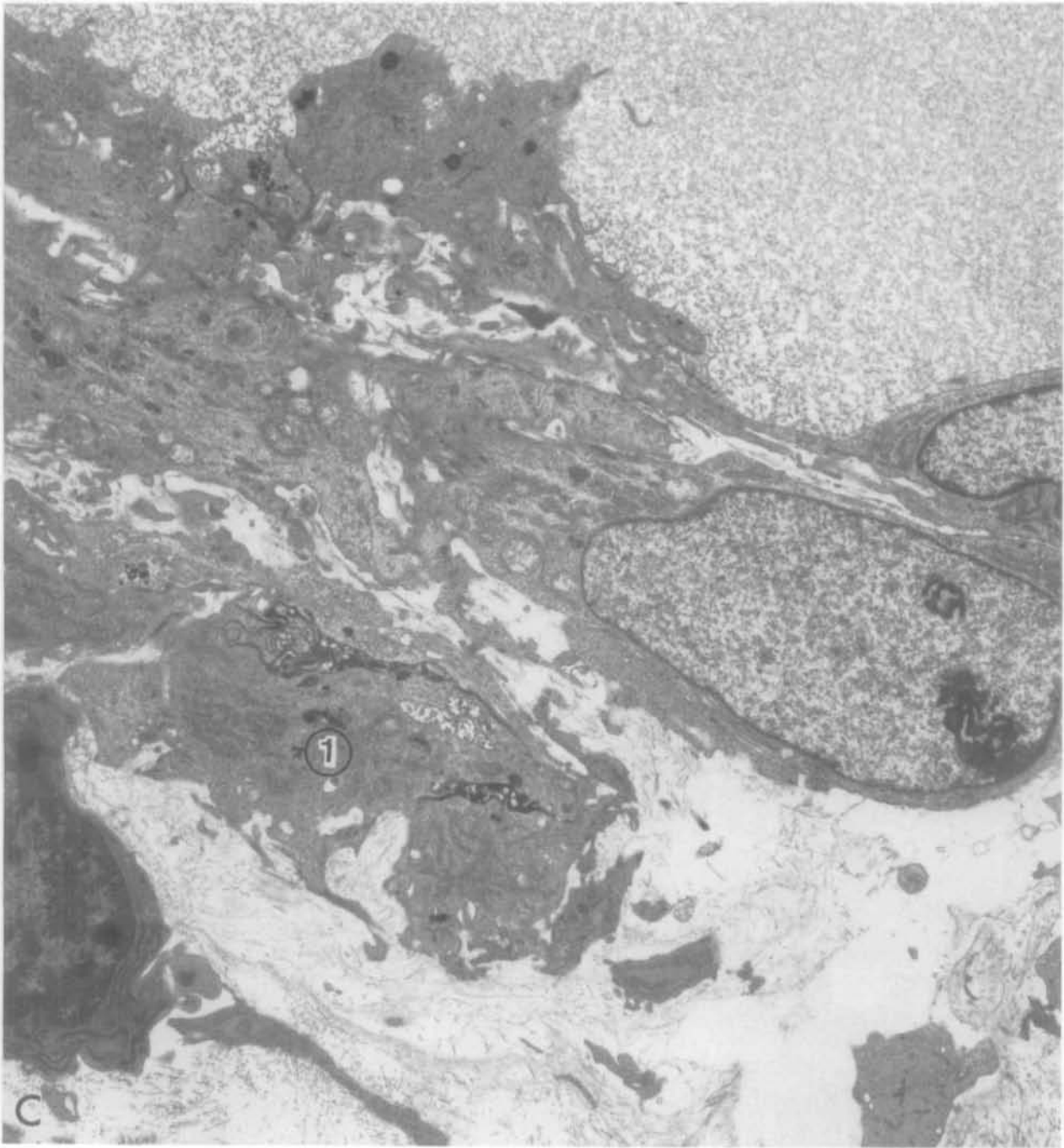
Fig. 5. (C) See legend under Figure 5A.

new bud, including the tip. Subsequently the vessel matures into a wide-lumened vessel lined with thin endothelial cells and surrounded by a well developed basal lamina.

In a previous study we showed that new blood vessels are permeable to fluorescein but not to HRP.[15] Ausprank and Folkman[19] described the histologic sequence of events in tumor-induced capillary proliferation in the rabbit corneal pocket assay and suggested that the following sequential events of neovascular-

ization are similar regardless of the source of the angiogenic stimulus.[16,19]

1) Within 1 day after the implantation of an angiogenic stimulus, resting luminal endothelial cells become thickened and contain extensive rough endoplasmic reticulum, free ribosomes, and prominent Golgi apparati.

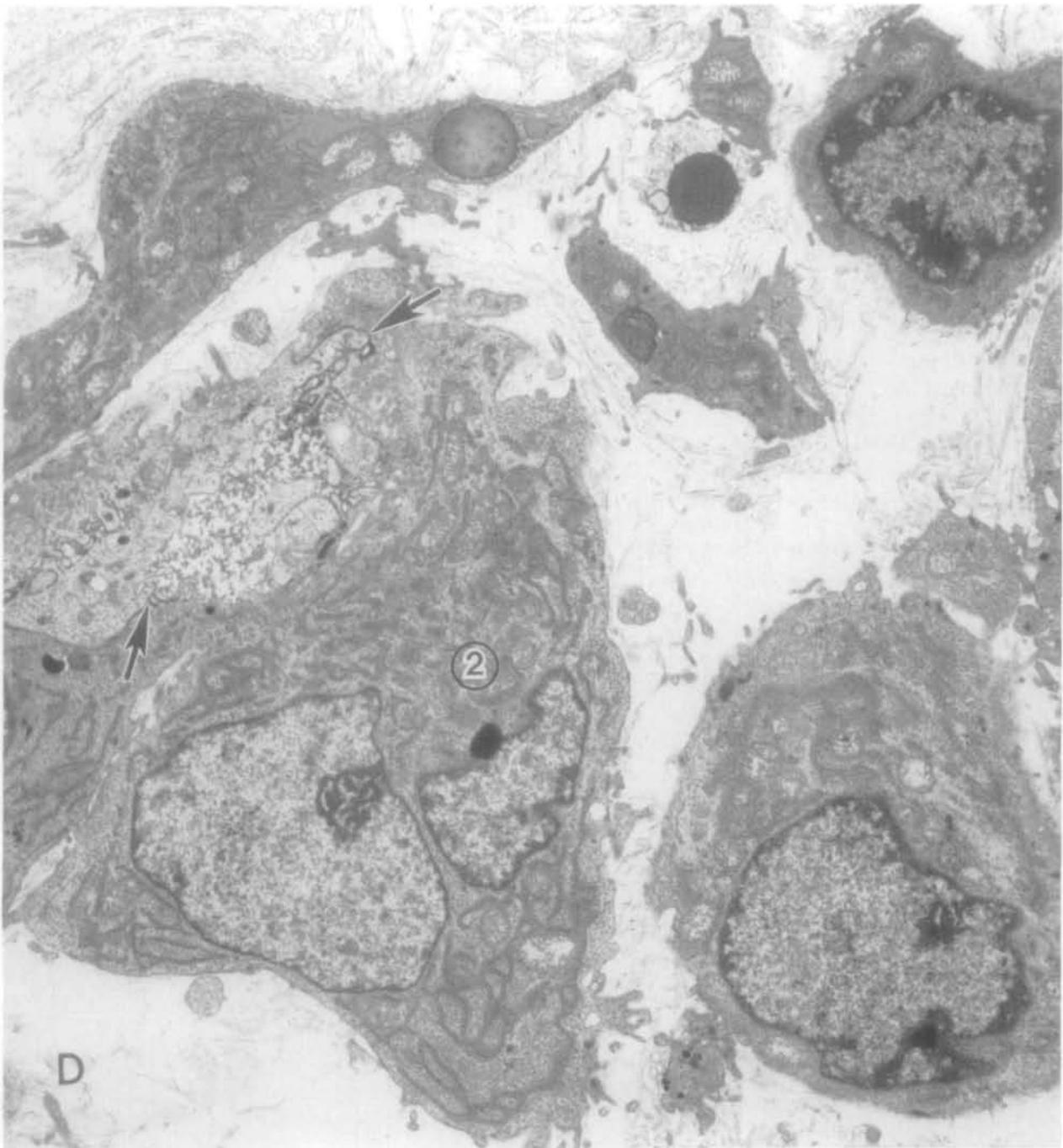2) On days 1–2, the basal lamina begins to fragment and small villous processes protrude through it.

Fig. 5. (D) See legend under Figure 5A.

3) On day 2, the endothelial cells migrate out into the perivascular space, with separations apparent between endothelial cells.

4) On days 2–3, increased DNA synthesis occurs primarily in the midcapillary region rather than at the tip of the growing vessel.

We demonstrate both similarities and important differences in the development of retinal compared to corneal neovascularization in the rabbit. Unlike corneal neovascularization, ECM production appears to

be prominent as evidence by extensive ECM surrounding the budding endothelial cells. Secondly, endothelial cells undergo DNA synthesis not only at the proximal regions but also at the tips of new vessels. In addition, serial sectioning shows that the apparent physical gaps (cell separations) seen between cells in some sections, are not present in other sections. This indicates that there is a continuity between cells. This is consistent with our previous results showing no leakage of HRP from the vessels.[15]

The process of retinal neovascularization in mam-

mals has been a subject of controversy for many years.[20-23] Studies of developing new vessels during embryogenesis using light microscopy suggested that solid cords of cells precede the development of vascular channels in normal retinal vascular development.[22,23] In our study solid cords of endothelial cells were not seen even though the lumens became extremely narrow (documented by HRP). The endothelial cells always maintained polarity (ie, retained a luminal and abluminal side).

The ultrastructure of new vessels in proliferative diabetic retinopathy[10,13,14,24-26] and retinopathy of prematurity[8,9] has been reported previously. Although inflammation does occur in the rabbit model when homologous fibroblasts are used and it may play a role important in new vessel formation,[27] the histopathology of experimental rabbit retinal neovascularization demonstrates numerous similarities to the process of neovascularization observed in retinopathy of prematurity[8] and proliferative diabetic retinopathy (ie, neovascularization associated with ischemia).[26] In these diseases, endothelial cells are thickened and have well developed, rough endoplasmic reticulum and Golgi apparatus. The endothelial cells, surrounded by fragmented but abundant basement membrane-like material, send projections into the surrounding ECM. No fenestrations or separations between endothelial cells are present.[8,26]

Experimental neovascularization in the rabbit has some significant differences when compared to rabbit corneal neovascularization. Moreover, this experimental model of retinal neovascularization parallels the steps of retinal neovascularization in both diabetic retinopathy and retinopathy of prematurity and may be useful for elucidating integral or sequential events in pathologic retinal neovascularization.

Key words: retina, neovascularization, histopathology, rabbit, autoradiography

Acknowledgments

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