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Demonstration of vasoproliferative activity from mammalian retina

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DEMONSTRATION OF VASOPROLIFERATIVE ACTIVITY
FROM MAMMALIAN RETINA

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ABSTRACT

Vasoproliferative activity has been demonstrated in extracts of retinas from human, bovine, and feline sources. These retinal extracts are capable of stimulating (a) proliferation and thymidine uptake of bovine vascular endothelial cells in culture and (b) neovascularization on the chick chorioallantoic membrane. Extracts of skeletal muscle, cardiac muscle, and liver lack similar stimulatory activity. The activity is nondialyzable, stable at 56°C, and inactivated at 100°C. Retinal extracts stimulate the proliferation of corneal fibroblasts but have no effect on the proliferation of vascular smooth muscle cells.

Indirect evidence suggests the liberation of a vasoproliferative factor from retina in several ocular disorders. The data in this report represent the first direct demonstration of vasoproliferative activity from mammalian retina.

KEY WORDS   endothelial cell   ·
neovascularization   ·   chorioallantoic
membrane   ·   growth factor   ·   retinas   ·
retinopathy

All adult tissues studied to date, with the exception of tumor (2, 3, 5, 6, 7, 13), corpus luteum (10), epidermis (25), and lymphatic tissue (2, 8, 23), are incapable of stimulating vasoproliferation. There is, however, considerable indirect evidence suggesting that adult retina, under certain conditions, is capable of stimulating vasoproliferation. This evidence stems from observations of several ocular diseases in which neovascularization closely parallels local or remote retinal nonperfusion (1, 14, 16, 21, 22). The treatment of the abnormal retinal tissue by laser or xenon arc photocoagulation results in the regression of these new vessels (15, 17, 19). In spite of this indirect evidence, a retina-derived substance capable of promoting vasopro-

liferation has yet to be demonstrated. The following experiments provide the first direct proof that mammalian retina can liberate a substance (or substances) with potent vasoproliferative activity.

MATERIALS AND METHODS

Tissue Extracts

RETINA: Eyes from recently slaughtered adult cows were obtained from a local abattoir. Retinas were removed from 14 eyes and suspended in 14 ml of balanced salt solution (BSS: 8 g/liter NaCl, 0.2 g/liter KCl, 0.2 g/liter $KH_2PO_4 \cdot H_2O$, 1.15 g/liter $Na_2HPO_4 \cdot 7H_2O$, 0.2 g/liter glucose, 0.01 g/liter phenol red) at room temperature and pH 7.2 for 2.5–3 h. The suspension was centrifuged at 850 g for 5 min at room temperature. The supernate was then decanted and filter-sterilized (0.2 μm, Millipore Corp., Bedford, Mass.). This retinal extract was used immediately or stored at −20°C. Thirty separate batches of bovine retinal extract were prepared and studied as described.

Human retinas from eye-bank eyes without known ocular disease were suspended in 0.5 ml of BSS per retina. The resulting extract was treated in the same manner as the bovine retinal

extract. These human eyes were obtained 2–4 d after death from donors ranging in age from 41 to 88 yr, with an average of 59 yr.

Ten adult cats were anesthetized intramuscularly with 25 mg/kg of ketamine hydrochloride (Ketaset, Bristol Laboratories Div., Bristol-Myers Co., Syracuse, N.Y.). One eye of each cat was enucleated, and the central retinal artery was cannulated with a small polyethylene tube (Intramedic, PE 50, Clay-Adams Div. Becton, Dickinson & Co., Parsippany, N.J.). The retinal circulation was then perfused for 5 min with BSS (without phenol red) at a pressure of 100 mm Hg. Each retina was examined with an operating microscope at 40 × to ensure that all vessels were cleared of blood. The retina was then removed and treated in the manner described for human retinas. The other eye of each cat was enucleated, and the retina was removed but was not perfused and treated in the same manner as the BSS-perfused retinas. Immediately after enucleation, all cats were sacrificed by intravenous injection of sodium pentobarbital in alcohol-propylene glycol base (Somlethal, Medtech, Inc., Elwood, Kans.).

OTHER TISSUES: Extracts of bovine skeletal muscle, cardiac muscle, and liver were prepared in the manner described for bovine retinas. The ratio of tissue weight to BSS volume was made equal to that used for bovine retinal extract.

Cell Proliferation Studies

REAGENTS: All reagents used were of tissue-culture grade. Minimal essential medium (MEM) and Medium-199 (M199) were purchased from Grand Island Biological Co., Grand Island, N.Y., collagenase (Type I) from Worthington Biochemical Corp., Freehold, N.J., and trypsin (Type III, bovine pancreas) from Sigma Chemical Co., St. Louis, Mo. Fetal bovine serum (FBS) was obtained from Sterile Systems Inc., Logan, Utah, and filter-sterilized before use. Tritiated thymidine (1 mCi/ml; 6.7 Ci/mM) was purchased from New England Nuclear, Boston, Mass. The scintillation fluid used consisted of 0.094 g of 2,5-diphenyloxazole (PPO), 4.15 g of 1,4-bis(2-[4-methyl-5-phenyl-oxazolyl])-benzene (dimethyl POPOP), 250 ml of Triton X-100, and 500 ml of toluene.

CELL CULTURES: Endothelial cells were enzymatically removed from fetal bovine aortas and cultured as previously reported (5). Cells were grown in MEM supplemented with glutamine (0.292 mg/ml), sodium bicarbonate (2.2 mg/ml), penicillin (200 U/ml), streptomycin (200 μg/ml) and 10% FBS at 37°C in 5% CO_2 with 85% relative humidity. These cells formed a characteristic mosaic of nonoverlapping, polygonal cells. The presence of Weibel-Palade bodies (20), which are associated with endothelial cells (24) and positive staining with anti-Factor VIII antibodies (12) showed them to be endothelial cells.

Smooth muscle cells were grown from explants of the medial layer of fetal bovine aorta and maintained in MEM with 10% FBS. Elaboration of an insoluble extracellular material and the peculiar pattern of cell growth as observed by phase-contrast microscopy (criteria suggested by Gimbrone and Cotran [9]) indicated their smooth-muscle-cell nature.

Corneal fibroblasts were grown from explants of corneal tissue according to the procedure of BenEzra (4) and were also maintained in MEM with 10% FBS.

TRITIATED THYMIDINE UPTAKE BY ENDOTHELIAL CELLS: Endothelial cells (passages 6–27) were plated in 24-well plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) in MEM with 10% FBS at a density of 20,000–25,000 cells/well (2.1-cm^2) for use that day or at 10,000–15,000 cells/well for use the following day. At this density, the cells

remained subconfluent for the entire experiment. The maximal final cell number was ~8.0 × 10^4 cells/well, about one-third of the cell number at confluence. After the cells attached, each well was rinsed with 1 ml of BSS before the addition of 1 ml of M199 with 1% FBS. 1–80-μl volumes of each retinal extract were added to triplicate wells, and control wells received equivalent volumes of BSS. The final dilution of retinal extract in each well was recorded. 42–48 h later, the cells were incubated for 1 h with 1 ml of tritiated thymidine (0.625 μCi/ml) in serum-free MEM. The incorporation was terminated by rinsing with cold BSS. The cells were then fixed by immersion for 3 min at room temperature in a 1:1 solution of BSS/ethanol-acetic acid fixative, for 20 min in ethanol-acetic acid fixative (three parts 70% ethanol to one part acetic acid), and for 10 min in 0.3 N perchloric acid followed by a thorough rinse with distilled water. The fixed cells were incubated for 20 min in 200 μl of 0.25 N NaOH before an equal volume of distilled water was added. The hydrolysate was then transferred to a glass scintillation vial with 3 ml of scintillation fluid, and the sample was counted for 1 min (20).

CELL COUNTING: Endothelial cells, smooth muscle cells, and corneal fibroblasts were plated in 24-well plates as described above. 1–80-μl volumes of the extract to be tested were added to triplicate wells, and the final dilutions were recorded. 42–48 h later, the cells in each well were rinsed with BSS, trypsinized (0.3 ml of 0.1% trypsin-0.05% EDTA), and the enzyme activity was quenched by the addition of 0.7 ml of MEM with 10% FBS. The entire 1.0 ml was then transferred to a plastic cuvette containing 9 ml of Isoton II, a balanced electrolyte solution (Coulter Diagnostics Inc., Hialeah, Fla.), and counted with a Coulter Counter Model 2-B (Coulter Electronics Inc., Hialeah, Fla.).

Chick Chorioallantoic Membrane (CAM) Assay

Extracts of retina, bovine skeletal muscle, cardiac muscle, and liver were lyophilized and incorporated into 1-mm^3 Elvax (an ethylene-vinyl acetate copolymer) pellets as described by Langer and Folkman (18). Each pellet contained 20–25 μl of crude extract. These pellets were assayed for their vasoproliferative activity on the CAM according to the technique described by Folkman et al. (3, 7, 13) with some modifications. Fertilized White Leghorn chicken eggs were used. When the eggs were 6 d old, a small hole in the egg shell overlying the air sac was made with a 20-gauge needle. A 1-cm^2 window was then made in the shell overlying the CAM, and the CAM was allowed to fall away from the window (11). The window was covered by clear tape, and the eggs were incubated at 38°C with 60% relative humidity. 2 d later, a 30-gauge hypodermic needle was used to puncture the membrane at the intended site of implantation. One of the Elvax pellets was placed over this puncture site, and the membrane was covered with a plastic coverslip (Thermanox, 15-mm round, Lux Scientific Corp., Newbury Park, Calif.) to limit pellet migration.[1] The CAM were examined daily at 16 × magnification and scored 4 d after implantation. The number of new vascular loops that had grown toward the pellet were counted. The membrane vasculature was then injected with India ink, excised, fixed in formalin, and photographed.

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[1] Folkman, J., Children's Hospital, Boston, Mass. As reported at the Juvenile Diabetes Foundation Workshop on Diabetic Retinopathy (Boston, Sept. 14–16, 1978).

RESULTS

Effects of Tissue Extracts on Vascular Endothelial Cells in Culture

The increases in cell number and tritiated thymidine uptake resulting from the addition of bovine retinal extract to cultured vascular endothelial cells are shown in Figs. 1 and 2. Increasing the concentration of retinal extract produces an increase in both cell number and tritiated thymidine uptake from control levels to a plateau of maximal stimulation. Comparison of Figs. 1 and 2 shows that the stimulation of tritiated thymidine uptake correlates well with the increase in cell number resulting from the addition of retinal extract. Mello (20) has demonstrated a similar correlation in response to tumor homogenates. Human retinal extracts, in an analogous study, have a comparable effect on tritiated thymidine uptake (Fig. 3). Comparison of Figs. 2 and 3 reveals that the effect of human retinal extract on thymidine incorporation is less than the effect of bovine retinal extract. As mentioned earlier, bovine eyes were obtained within 2–3 h after death of the animal, whereas human eyes were obtained 2–4 d after death. This prolonged time interval before removal of retinas from the human eye may have resulted in the loss or destruction of some of the angiogenic activity. We have found angiogenic activity in the vitreous
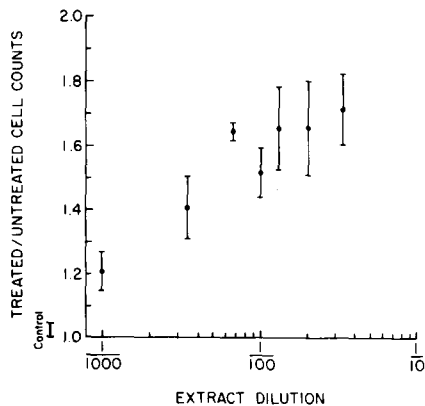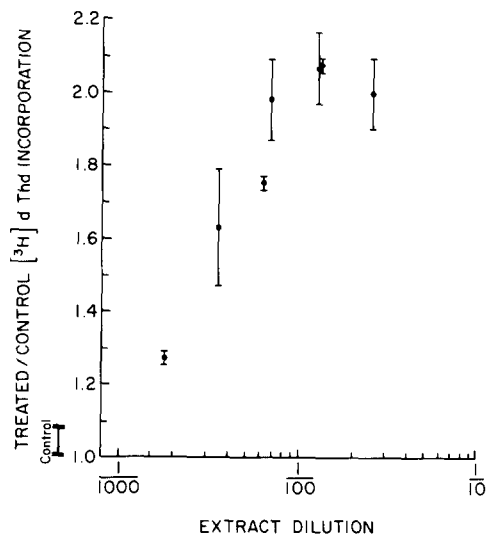

FIGURE 2 Effect of bovine retinal extract on tritiated thymidine uptake by bovine vascular endothelial cells in culture. All wells (2.1-cm^2) contained 20,000–25,000 cells in M199 with 1.0% FBS. 1–80-μl volumes of bovine retinal extract were added to triplicate wells. Control wells received equivalent volumes of BSS. 42–48 h later, the cells were incubated for 1 h with tritiated thymidine. The medium was removed, and the cells were washed, fixed, and lysed with 0.25 N NaOH. The hydrolysate was then counted. Control wells contained 13,500 ± 1,000 cpm. Each point represents the mean of triplicate wells. Standard deviation is represented by a vertical bar.

FIGURE 1 Effect of bovine retinal extract on the proliferation of bovine vascular endothelial cells in culture. All wells (2.1-cm^2) contained 20,000–25,000 cells in M199 with 1.0% FBS. 1–80-μl volumes of bovine retinal extract were added to triplicate wells. Control wells received equivalent volumes of BSS. 42–48 h later, the cells were trypsinized and counted. Control wells contained 40,000 ± 2,000 cells. Each point represents the mean of triplicate wells. Standard deviation is represented by a vertical bar.

of these human eyes, but we were unable to demonstrate such activity in bovine vitreous (data not shown). This finding suggests that some of the angiogenic activity of these human retinas may have been lost by diffusion into the vitreous during the prolonged interval before removal of the retina. In summary, bovine and human retinal extracts stimulate the proliferation and thymidine uptake of vascular endothelial cells in culture. This response is dose dependent.

Because the addition of serum to cultured vascular endothelial cells is known to stimulate tritiated thymidine incorporation (5, 20), it was necessary to demonstrate that the effect of retinal extracts was not the result of the small amount of serum retained in the retinal vasculature. To accomplish this, an extract from feline retinas whose vasculature was cleared of blood by perfusion with BSS was compared with an extract from untreated feline retinas. Equal dilutions of these two extracts stimulated equal levels of tritiated thymidine uptake by bovine vascular endothelial cells. There-

fore, the effect of retinal extract on endothelial cells is independent of any serum growth factors that may remain in the retinal vasculature.

Fig. 4 illustrates the effects of extracts of bovine cardiac muscle, skeletal muscle, and liver on vascular endothelial cell number. Addition of the same range of dilutions used for retinal extracts failed to stimulate the proliferation of endothelial cells. In fact, in contrast to retina, high concentrations of liver and cardiac muscle extracts appeared to have deleterious effects on cultured endothelial cells.

Effect of Tissue Extracts on the CAM

The vasoproliferative activities of various tissue extracts on the CAM are shown in Table I. 50% of the pellets containing retinal extract induced strong vasoproliferation, and 25% elicited a weak response. In comparison, none of the pellets containing extracts of other tissues induced strong vasoproliferation, and only 13% were capable of inducing a weak neovascular response. Representative photographs of the CAM neovascular response to tissue extracts in Elvax pellets are shown in Fig. 5. To investigate the statistical significance of the retinal extract-induced vasoproliferation,
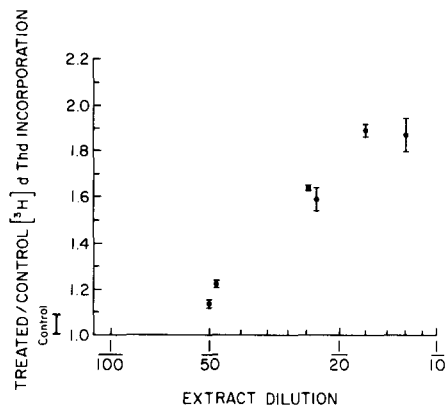
Fischer's exact test for 2 × 2 tables was used. The test was performed both with the weak responses grouped with the strong responses and with the weak responses grouped with negative responses. In both cases $P < 0.001$, indicating that extracts of adult retina are capable of inducing highly significant vasoproliferation in vivo. In comparison, extracts of the other adult tissues tested did not induce significant vasoproliferation.

Effect of Bovine Retinal Extract on Smooth Muscle Cells and Corneal Fibroblasts

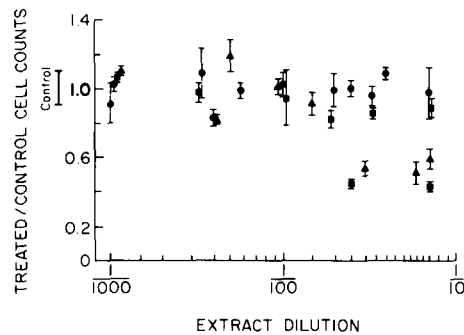The same range of dilutions of bovine retinal extract that stimulated vascular endothelial cells

FIGURE 4   Effect of extracts of bovine skeletal muscle (●), cardiac muscle (■), and liver (▲) on the proliferation of bovine vascular endothelial cells in culture. All wells (2.1-cm^2) contained 20,000–25,000 cells in M199 with 1.0% FBS. 1–80-μl volumes of each extract were added to triplicate wells. Control wells received equivalent volumes of BSS. 42–48 h later, the cells were trypsinized and counted. Control wells contained 40,000 ± 2,000 cells. Each point represents the mean of triplicate wells. Standard deviation is represented by a vertical bar.

FIGURE 3   Effect of human retinal extract on tritiated thymidine uptake by bovine vascular endothelial cells in culture. All wells (2.1-cm^2) contained 20,000–25,000 cells in M199 with 1.0% FBS. 1–80-μl volumes of human retinal extract were added to triplicate wells. Control wells received equivalent volumes of BSS. 42–48 h later, the cells were incubated for 1 h with tritiated thymidine. The medium was removed, and the cells were washed, fixed, and lysed with 0.25 N NaOH. The hydrolysate was then counted. Control wells contained 13,500 ± 1,000 cpm. Each point represents the mean of triplicate wells. Standard deviation is represented by a vertical bar.

TABLE I

Vasoproliferative Response on the Chorioallantoic Membrane

Tissue extract	Strong response*	Weak response‡	Negative response§
	No. of eggs		
Retina	19	9	11
Liver	0	1	7
Cardiac muscle	0	2	14
Skeletal muscle	0	1	6

* 4 or more new vessel loops directed toward the test pellet.
‡ 1–3 new vessel loops directed toward the test pellet.
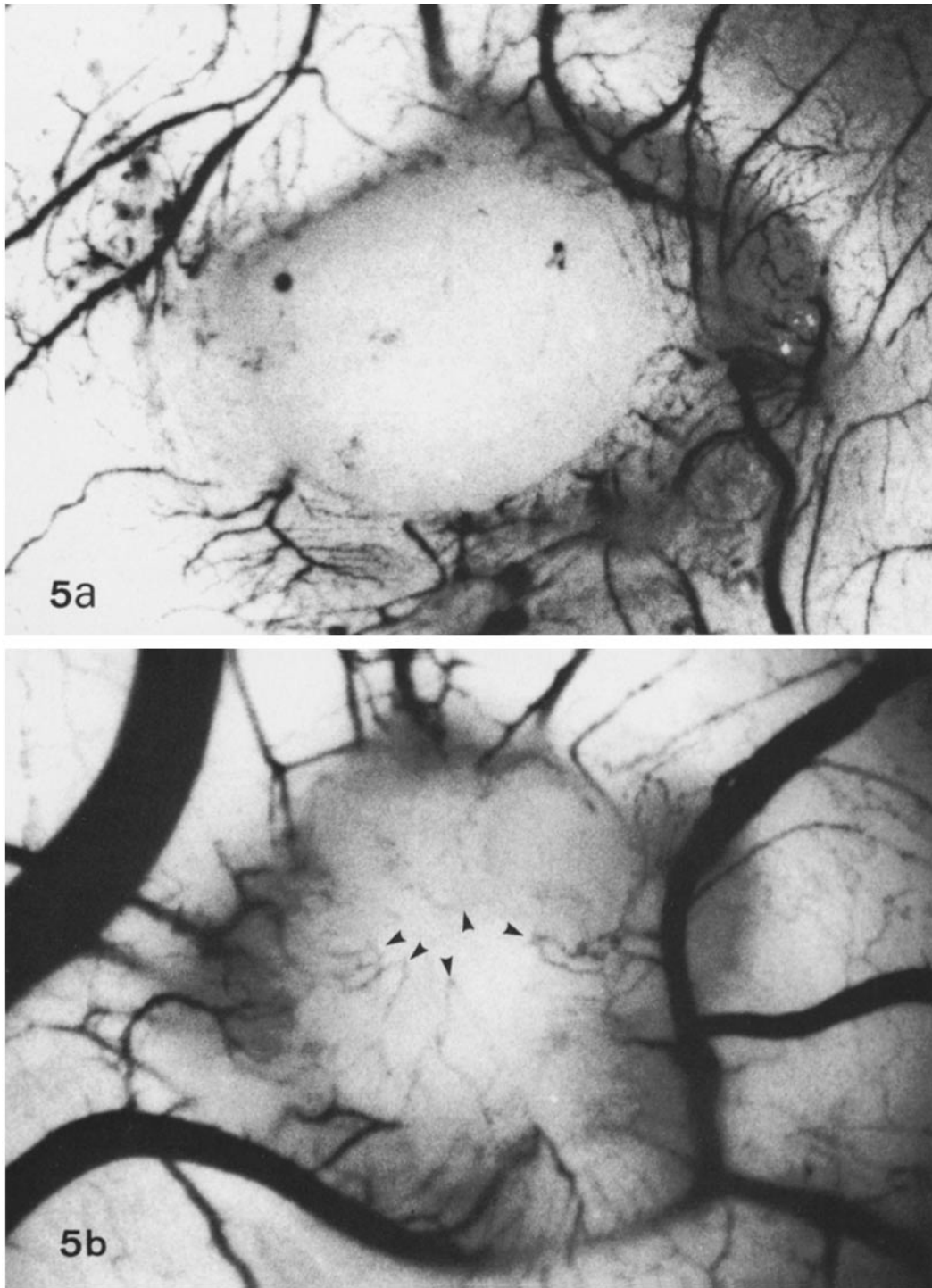§ No new vessel loops seen.

FIGURE 5   CAM vasoproliferative response. (a) Negative vasoproliferative response of CAM to extract of control tissue (skeletal muscle) in Elvax pellet. No new vessel loops are present. (b) Strong vasoproliferative response of CAM to retinal extract in Elvax pellet. Arrows show new vessel loops growing onto the test pellet. Formalin-fixed, India-ink injections. × 40.

did not stimulate the proliferation of vascular smooth muscle cells. However, a significant increase in cell number resulted from the addition of retinal extract to cultures of corneal fibroblasts (Fig. 6).

Physical Characteristics

Several physical properties of the active substance(s) contained in bovine retinal extracts were studied. The capacity of retinal extracts to stimulate endothelial cell proliferation and CAM neovascularization is unaffected by treatment at 56°C for 30 min. Heating of retinal extracts to 100°C for 2 min resulted, however, in a loss of this stimulatory activity. This activity is also nondialyzable (molecular weight cut-off of 10,000–12,000) and stable to lyophilization.

DISCUSSION

We have demonstrated vasoproliferative activity liberated by retina from several mammalian species. These retinal extracts induce a plateauing, dose-dependent stimulation of bovine aortic endothelial cells in culture. Studies using retinas cleared of blood prove that this stimulatory activity is not the result of serum growth factors that may remain in the retinal vasculature. The in vivo vasoproliferative activity of retinal extract is demonstrated by its effect on the CAM.

The vasoproliferative activity of adult retina contrasts with the observation that most other adult tissues are incapable of stimulating neovascularization. Fenselau and Mello (5) found that homogenates of liver and kidney from the adult cow and rat failed to stimulate proliferation of cultured bovine vascular endothelial cells. Ausprunk et al. (3) found that adult rat skeletal muscle, heart, liver, and kidney were incapable of inducing neovascularization on the CAM. In our study, extracts of bovine skeletal muscle, cardiac muscle, and liver were incapable of stimulating proliferation of cultured vascular endothelial cells or CAM neovascularization. The question that still remains is whether the tissues cited above can liberate a vasoproliferative substance under conditions so far not studied. The purification and characterization of a retinal vasoproliferative substance and the study of its production and release may aid in addressing this question.

The ability of a retina-derived substance to induce neovascularization correlates with the important role of vasoproliferation in several ocular
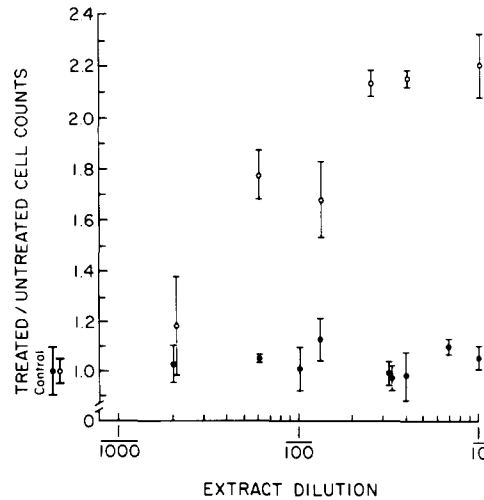

FIGURE 6   Effect of bovine retinal extract on bovine vascular smooth muscle cells (●) and corneal fibroblasts (○) in culture. All wells (2.1-cm^2) contained 20,000–25,000 cells in M199 with 1.0% FBS. 1–80-μl volumes of bovine retinal extract were added to triplicate wells. Control wells received equivalent volumes of BSS. 42–48 h later, the cells were trypsinized and counted. Each point represents the mean of triplicate wells. Standard deviation is represented by a vertical bar.

diseases and with the clinical evidence suggesting the liberation of a vasoproliferative factor from retina in these disorders. On the basis of clinical and laboratory research, Patz (21, 22) and Ashton et al. (1) independently demonstrated that areas of peripheral retinal capillary closure precede local retinal neovascularization in retrolental fibroplasia. Studies of patients with venous obstructive disease reveal a direct correlation between retinal capillary closure and neovascularization of the optic nerve and iris (14, 16). Clinical studies show that the destruction of retinal elements by laser or xenon arc photocoagulative treatment results in the regression of untreated disk, retinal, and iris neovascularization (15, 17, 19). These studies suggest that, under certain conditions, retina can liberate a substance (or substances) capable of inducing intraocular neovascularization. Despite the considerable indirect evidence previously reported for the existence of such a substance, it has never been directly demonstrated.

The capacity of retinal extract to stimulate cell proliferation appears to have some target-cell specificity because it acts as a potent stimulator of vascular endothelial cell and corneal fibroblast proliferation and has no effect on the proliferation

of vascular smooth muscle cells. The ability of retinal extracts to stimulate fibroblast proliferation may be important in view of the fact that intra-ocular neovascularization is often accompanied by fibrous tissue proliferation (26).

In conclusion, we have demonstrated, for the first time, a retina-derived substance (or substances) capable of promoting vasoproliferation. The mechanisms of production and release of this vasoproliferative substance and the conditions that promote its liberation by retina are being investigated in our laboratory. Characterization and purification of this substance are also proceeding. This vasoproliferative substance will play an important role in the investigation of intraocular neovascularization and angiogenesis, in general, and will aid in identifying substances capable of inhibiting the neovascular response.

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