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Capillary endothelial-cell mitogenic activity in experimental branch vein occlusion*

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Abstract. Since the 1940s, the major hypothesis regarding the stimulus for neovascularization occurring in diabetic retinopathy and other "ischemic" retinopathies has involved the release of a soluble angiogenic factor from the ischemic retina. We sought to test this by measuring the endothelial mitogenic activity that could be extracted from an ischemic retina caused by branch vein occlusion in the cat. We found that the extracable endothelial-cell mitogenic activity from normal retinal areas was similar to that in ischemic areas at 1, 2, and 7 days after the occlusion. In the area of occluded veins, the oxygen partial pressure was low (7 ± 7 mm Hg) compared with the normal value (23 ± 8 mm Hg). The data did not show any increase in soluble mitogenic factor release from the ischemic retina versus normal retinal areas under the conditions of this experiment.

Introduction

Retinal neovascularization is a common cause of blindness in this country, with diabetic retinopathy representing the major cause of new blindness in people under 64 years of age [10]. The association between capillary nonperfusion and retinal neovascularization is well established [11]. Forty years ago, it was suggested that vasoproliferation in the retina was stimulated by a soluble growth factor ("factor X" released as a result of hypoxia) [9, 15]. This hypothesis was supported by the discovery of angiogenic substances released by tumors and macrophages and by the discovery of endothelial cell growth factors obtained from mammalian retina [6].

However, there was no explanation as to how substances that stimulate endothelial cell mitosis in minute quantities could be extracted from "normal" adult retina that was not undergoing cell proliferation. Engerman and co-workers [3] demonstrated that a very low rate of endothelial cell turnover occurs in the retina (on the order of 0.10%). Under normal conditions, the brain contains as much as 1 mg fibroblast growth factor per kilogram of tissue, and it has been estimated that the retina contains a similar amount. This concentration is much higher (approx. 1000 times) than that at which fibroblast growth factor is maximally active in tissue culture, which is on the order of 1 ng/ml [4]. This presents a dilemma. What is the role of high concentrations of angiogenic growth factor in the adult retina, and how are these very potent angiogenic growth factors regulated?

Most current theories postulate either the release of growth factor as a consequence of endothelial cell death or its enzymatic release from the extracellular matrix [4]. It is presumed that retinal ischemia could activate these processes, resulting in the release of growth factor. Once the activity is released (by whatever mechanism), it can act on endothelial cells, stimulating their proliferation, migration, and tubule formation (i.e., angiogenesis). Central to these hypotheses, as they relate to Michaelson and Wise's original hypothesis, is an increase in angiogenic activity due to retinal ischemia. Little is known about the changes in either content or activity of extractable growth factors in ischemic retina. The purpose of this investigation was to test directly the hypothesis that retina made ischemic by experimental branch vein occlusion in the cat shows an increased release of extractable endothelial-cell mitogenic activity.

Materials and methods

We produced branch retinal vein occlusion in one each in 12 cats using the method of Chen et al. [1], which can be summarized as follows: cats weighing 2–5 kg were anaesthesized with intramuscular ketamine hydrochlorate (35 mg/kg) and acepromazine maleate (0.3 mg/kg). A bipolar coaxial diathermy was placed through a pars plana sclerostomy while it was observed by indirect ophthalmoscopy. The diathermy probe was guided to the exit of the retinal venules from the optic nerve head. Sufficient current was applied to the diathermy probe to cause white coagulation of the retinal vein. The adjacent retinal artery was avoided. A small amount of whitening occurred in the retina adjacent to the occluded vein and there was an immediate dilation in the distal vein, with a mottled appearance to the retina. The entrance wound was self-sealing. Subconjunctival gentamycin (5 mg) was injected.

The left eye was used as a control. After 1 ($n=1$), 2 ($n=3$), or 7 days ($n=8$), the cats were examined ophthalmoscopically. Anesthesia was induced with ketamine hydrochloride, after which the cats were killed with an overdose of intracardiac sodium pentabarbitol and the eyes, enucleated.

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Dissection and extraction

Under sterile conditions, the anterior segments of the eyes were removed and the posterior globe was everted. The retina was then removed by gently peeling it away with a sterile scalpel blade. The retina was transferred to the extraction fluid as rapidly as possible (3–6 min). The retina was then placed in 0.5 ml sterile phosphate-buffered saline (PBS) and extracted for 3 h at room temperature [6]. The vitreous of each eye was also collected and extracted in 0.5 ml PBS for 3 h.

Cell culture

Cloned bovine capillary endothelial cells (BCEC) were kindly donated by Dr. Judah Folkman. The cells were grown in gelatin-coated tissue-culture flasks (Corning, Fisher Biologicals) in Delbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and streptomycin/penicillin (Gibco Laboratories, Grand Island, NY) and 75 µg/ml endothelial cell growth supplement (ECGS) (Collaborative Research, Bedford, Mass). Cells were passaged every 7–10 days at a ratio of 1:3.

Proliferation assay

BCEC (between passages 12 and 19) were plated in gelatin-coated 24-well tissue-culture plates (Falcon, Fisher Biologicals, Raleigh, NC) at a density of 10000 cells/well and allowed to grow overnight in DMEM supplemented with 10% FBS and 75 µg/ml ECGS. Prior to the assay, the wells were washed with 1 ml PBS, and then 0.9 ml DMEM/10% FBS plus 0.1 ml retinal extract was added to each well. Each extract was assayed in triplicate or quadruplicate. The positive control wells received 1 ng basic fibroblast growth factor (FGF) (Amgene Biologicals, Thousands Oaks, Calif) and the negative ones, PBS (0.1 ml). The cells were incubated for 72 h at 39° C in an atmosphere containing 10% CO_2, after which the wells were trypsinized and the cells in each well, counted on a Coulter particle counter (Coulter Electronics, Hialeah, Fla).

Oxygen tension measurements

In three of the cats, the preretinal oxygen tension was measured 7 days after the vein occlusion using transvitreal polarographic oxygen electrodes, a technique described previously [12]. Briefly, the cats were placed under general anaesthesia with 20–30 mg/kg intramuscular ketamine hydrochloride, and anaesthesia was maintained with 80 mg/kg alpha chloralose given intravenously and repeated as necessary. The pupils were dilated with 0.25% tropicamide and 5% phenylephrine was applied to the cornea. The cats were placed in a stereotactic apparatus and the polarographic oxygen electrodes were advanced through the pars plana and transvitreally to the preretinal vitreous. The oxygen tension was simultaneously measured in the eye with retinal vein occlusion and in the normal fellow eye, except in one cat that had only one eye.

Fluorescein angiography

In selected cats, fluorescein angiography was carried out by intravenously injecting 0.3–0.5 ml 10% fluorescein sodium and taking fundus photographs through the appropriate excitation and barrier filters.

Results

On day 1 after branch vein occlusion with the diathermy, we found intraretinal hemorrhage and cystic oedema in the retina, which was sometimes followed by an exudative retinal detachment. By 1 week after occlusion, we observed continued retinal edema and hemorrhage as well as vascular remodelling, often with extensive collateral formation. Fluorescein angiography showed branch vein occlusion and collateral formation (Fig. 1).

In the endothelial cell mitogenesis experiments, we used 1 ng/ml basic FGF (Amgen) as positive controls, which gave cell counts of 1.6 ± 0.1 times the baseline value. Cell counts with retinal extracts gave values between the positive (FGF) and negative (PBS alone) control counts. Each retina with branch vein occlusion was compared with its control to ensure that the size of eyes and variation in individual animal responses would be controlled. A ratio was calculated for the endothelial cell count between the experimental and control eye. Both normal and ischemic retinae stimulated endothelial cell growth, but no significant difference was found between the cell counts. On day 1, the ratio (branch retinal vein occlusion over normal fellow eye) was 1 ($n=1$); on day 2, 0.98 ± 0.07 (mean $\pm$ SEM, $n=3$),; and on day 7 1.09 ± 0.08 ($n=8$, P value not significant) (Table 1). The average ratio for the cell cultures with vitreous samples was 0.94. Time-course experiments showed that the maximal extracted activity was usually obtained by incubation of retina for 1–3 h. There was no difference in when mitogenic activity was measured after extractions of 15 min and 1 h.

The preretinal oxygen tension in three eyes with vein occlusions was 7 ± 7 mm Hg; the normal fellow eyes in the same cats had preretinal oxygen tension of 23 ± 8 mm Hg. The preretinal oxygen tension in areas with branch retinal vein occlusion was compared with the oxygen tension in the preretinal vitreous in 13 normal cat eyes. The average value in normal cats was 21 ± 7 mm Hg, which is statistically significantly higher than that in areas with branch retinal vein occlusion (Student's t-test, $P=0.007$) (Table 2).

Discussion

Normal and ischemic retinae with branch vein occlusion contain extractable growth factors that stimulate endothelial cell mitosis equally. Our data clearly fail to support the hypothesis that retinal ischemia and/or hypoxia causes the production or release of an increased amount of soluble mitogens, as has previously been suggested.

Hayreh and Lata [7] have shown increased [^{3}H]-thymidine incorporation in endothelial cells exposed to intraocular fluids of primates with experimentally induced retinal vein occlusion. Taylor et al. [14] have shown that kitten retina has a greater procollagenase activity (a proposed marker for angiogenic activity) after oxygen-induced vaso-occlusion. Our data agree with an earlier study of Garner [5] who also failed to find increased endothelial-cell mitotic activity in extracts of ischemic retina as compared with normal retina. Our preretinal oxygen-tension measurements show that retinal hypoxia results from retinal vein occlusion. This agrees with studies on the hypoxic effect of branch retinal vein occlusion in the cat [13].

There are several arguments that urge caution in the interpretation of our data. For example, the results could

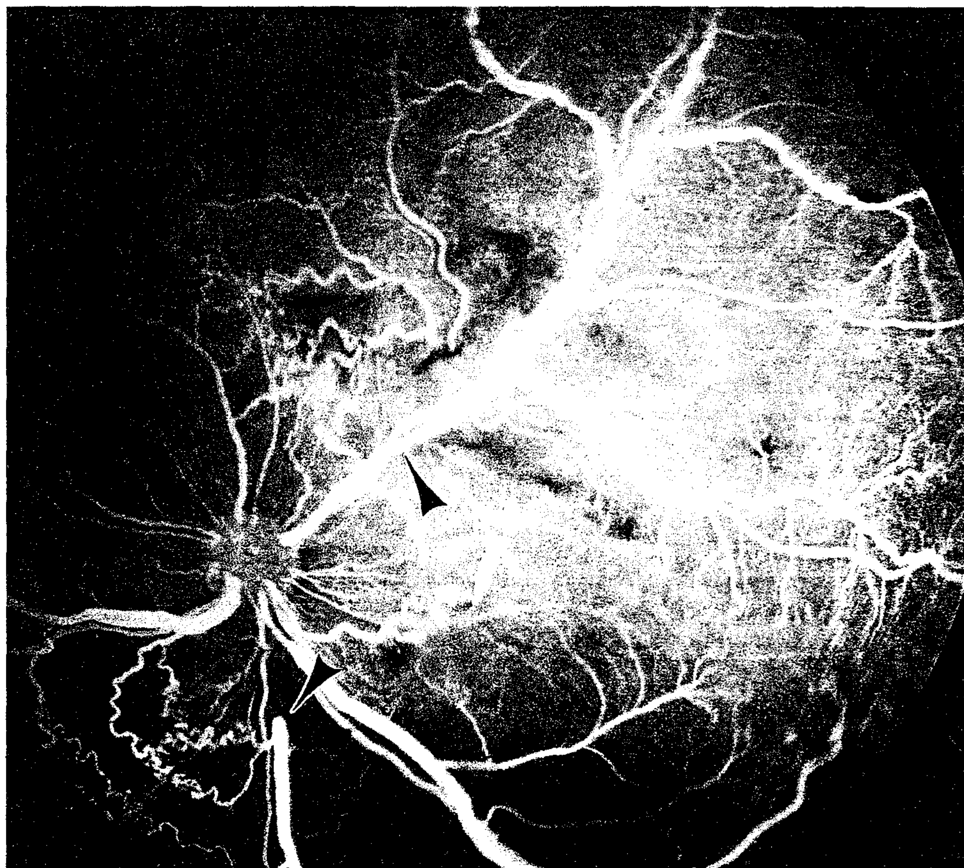

Fig. 1. Arteriovenous-phase fluorescein angiogram of a cat retina 7 days after vein occlusion with diathermy. A blocked vein (*arrow*) is seen, with extensive collateralization and intraretinal haemorrhage

Table 1. Extractable endothelial-cell mitogenic activity, showing the ratio of activity in retinal branch vein occlusion over that in normal retina

Days after branch vein occlusion	Branch vein occlusion/control retina (mean ± SE)
Day 1	1.0 ($n=1$)
Day 2	0.98 ± 0.07 ($n=3$)
Day 7	1.096 ± 0.08 ($n=8$)

Table 2. Preretinal oxygen pressure over tha in branch retinal vein occlusion 1 week after the onset of vein occlusion

	Normal retina ($n=13$)	Branch vein occlusion (day 7) ($n=3$)
PO_2	21 ± 7 mm Hg	7 ± 7 mm Hg ($P=0.01$)

be explained as a peculiarity of the cat, although we have evidence that a similar response occurs in the rat after acutely induced panretinal ischemia. Another argument could be that the cat retina was not ischemic, but dead. This possibility was minimized by the measurements of low oxygen pressure over the vein-occluded retina, indicating ischemia rather than death (i.e., ongoing oxygen consumption). It could be argued that our extraction times were so long that ischemic conditions were created in all samples during the extraction period. We mimicked the techniques previously reported for obtaining angiogenic growth factor from the retina [6], as well as checking shorter extraction periods (as early as 15 min), and observed similarly increased mitotic activity in both normal and ischemic retinae but no difference between them. It is also possible that the development of angiogenic activity release from retina takes longer than 7 days of ischemia. Another possibility is that proteases in our mixture could have reduced the extracted activity, although this would still not explain why we found no difference between the experimental and control groups. Finally, although branch retinal vein occlusion primarily affects the inner retina, the angiogenic activity may reside in the outer retina. This seems improbable, as ocular neovascularization is associated with branch retinal vein occlusion and other neovascular retinopathies primarily affecting the inner retina.

The hypothesis that the concentration of a single factor is responsible for the angiogenic response appears to us too simple. For example, after vitrectomy, neovascularization frequently appears at the iris but ceases at the retinal surface. Although it has been proposed that a retinal growth factor is responsible for iritic neovascularization, this hypothesis fails to explain why the growth factor acts on the iris, at a distant site where the concentration of the growth factor must be lower than at the retina itself, the presumed source of the angiogenic growth factor.

We suggest that although neovascularization in the eye probably involves soluble growth factors, the phenomenon cannot be explained by a "one-step" hypothesis; rather,

the growth factors are in part permissive and their concentration is to some degree immaterial. A second condition must be present in the tissue for the growth factor to exert its effects. We do not know what this condition is but have considered the following possibilities. Endothelial cell growth factors in vitro primarily stimulate the growth of nonconfluent tissue cultures. Once the endothelial cell monolayer becomes confluent, the mitogenic stimulation of the growth factor lessens dramatically. In normal vasculature, the endothelial cells are confluent and may therefore be insensitive to endothelial cell growth factors. It is possible that injury, hypoxia, endothelial cell death, or severe vasodilation might induce an effective nonconfluence, making the cells within the vessels sensitive to the growth factors. This hypothesis has an analogy in blood coagulation. In blood there is a high concentration of clotting factors. However, clotting does not occur unless tissue injury occurs, despite the concentration of the clotting factors. The latter are facilitative and necessary for coagulation but do not initiate it.

We conclude from our data that the cat retina that was hypoxic as a result of branch vein occlusion did not show an increased extractable endothelial-cell mitogenic activity and that the hypothesis that the concentration of soluble growth factors alone controls endothelial cell proliferation and neovascularization in vivo is invalid. We suggest that although soluble growth factors are a necessary component of neovascularization, other cellular events are necessary to initiate the neovascular response.

References

1. Chen CC, Rice T, Green W (1986) Experimental occlusion of the retinal vein. Graefe's Arch Clin Exp Ophthalmol 224:507–512
2. Cogan D, Troussaint R, Kuwabara T (1961) Retinal vascular patterns. IV diabetic retinopathy. Arch Ophthalmol 66:366–378
3. Engerman R, Pfaffenbach D, Davis MD (1967) Cell turnover of capillaries. Lab Invest 17:738–743
4. Folkman J, Klagsburn M (1987) Angiogenic factors. Science 235:442–447
5. Garner A (1976) The pathogenesis of ocular neovascularization. Eur Conf Microcirc 16:14–18
6. Glaser BM, D'Amore PA, Michels RG (1980) Demonstration of vasoproliferative activity from mammalian retina. J Cell Biol 84:298–304
7. Hayreh SS, Lata G (1986) Ocular neovascularization. Experimental animal mode and studies in angiogenic (factor)s. Int Ophthalmol 9:109–120
8. Kini MM, Heibowitz HM, Colton T (1978) Prevalence of senile cataract, diabetic retinopathy, senile macular degeneration, and open eye glaucoma in a Framingham eye study. Am J Ophthalmol 85:28–34
9. Michaelson IC (1948) The mode of development of the vascular system with some observations on its significance for certain retinal diseases. Trans Ophthalmol Soc UK 68:137–180
10. Patz A (1982) Clinical and experimental studies on retinal neovascularization (XXXIX Edward Jackson Memorial Lecture). Am J Ophthalmol 94:715–743
11. Shimizu K, Kobayashi Y, Muraoka K (1981) Midperipheral fundus involvement in diabetic retinopathy. Ophthalmology 88:601–612
12. Stefánsson E, Landers MB III, Wolbarsht ML (1981) Increased retinal oxygen supply following panretinal photocoagulation and vitrectomy and lensectomy. Trans Am Ophthalmol Soc 79:307–334
13. Stefánsson E, Novack R, Hatchell DL (1988) Hypoxia in branch retinal vein occlusion is prevented by vitrectomy. Invest Ophthalmol Vis Sci [Suppl] 29:220
14. Taylor C, Weiss J, Kissen IR, Garner A (1986) Effect of oxygen tension on the quantities of procollagenase-activity angiogenic factor present in the developing kitten retina. Br J Ophthalmol 70:162–165
15. Wise GN (1956) Retinal neovascularization. Trans Am Ophthalmol Soc 54:17–29

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