International Ophthalmology 9: 109–120 (1986)

Ocular neovascularization

Experimental animal model and studies on angiogenic factor(s)

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Key words: angiogenesis, angiogenic factor, blood vessels, iris, neovascularization, ocular, ocular hypotony, primate model, retinal vein occlusion, vasoproliferative factor

Abstract

We have been investigating experimentally the factors which initiate and perpetuate ocular neovascularization (NV). Our studies can be divided into four categories: (1) production of a primate experimental model of ocular NV, (2) morphological studies, (3) tissue culture studies, and (4) biochemical studies. We have been successful in producing a reliable primate model of ocular NV following retinal vein occlusion (RVO). The morphological and fluorescein angiographic studies have established that the new vessels seen in the animal model represent true NV and not simple dilatation of the pre-existing vessels. The tissue culture studies have shown the presence of vasoproliferative activity in the intraocular fluids in eyes with ocular NV in our animal model. The various biochemical studies conducted so far have provided preliminary results on various aspects, including the protein electrophoretic pattern and lysosomal enzymes in the intraocular fluids from eyes with and without ocular NV and effects of anoxia and of corticosteroids. The results of our various studies are very briefly reviewed and the importance of angiogenesis is discussed.

Introduction

In recent years there has been widespread interest in the phenomenon of angiogenesis, among scientists working in different fields of medicine. For example, the stimulation of growth of capillaries is important in the continued growth of malignant tumors, inflammation, wound healing, regeneration of tissues, cardiac hypertrophy, myocardial infarction, and during development, to mention only a few examples. In the field of ophthalmology, ocular neovascularization (NV) is a serious complication of a number of ocular diseases, and a major cause of permanent blindness or serious loss of vision. In spite of extensive studies in the field of angiogenesis, its pathogenesis still remains an enigma.

The primary objective of this paper is not to review the voluminous literature on angiogenesis or ocular NV, but to review very briefly our ongoing experimental studies, the primary objectives of which are:

1. To establish a reproducible and reliable experimental animal model of ocular NV simulating closely the clinical situation seen in man.
2. To investigate the factors which initiate and perpetuate ocular NV.

Present experimental studies

Our experimental studies on ocular NV can be divided into the following four categories:

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I. Production of an experimental animal model with ocular NV
II. Morphological studies
III. Tissue culture studies
IV. Biochemical studies

I. Experimental animal model with ocular NV

An important step in experimental investigation of a disease process is to have available an animal model simulating the clinical situation seen in man. The biggest problem in the past in the investigation of pathogenesis and management of ocular NV has been the absence of a suitable animal model. The animal model of retrolental fibroplasia has essentially formed the basis of various hypotheses on ocular NV; however, in retrolental fibroplasia there are immature retinal vessels which respond differently from the normal, mature retinal vessels. It is well established in man that retinal vein occlusion (RVO) is a common cause of ocular NV (22). About five years ago we successfully produced iris NV and optic disc NV with RVO in monkeys, and the changes closely simulated the clinical situation seen in man (18, 38).

In 32 eyes of cynomolgus monkeys, three of the four major retinal branch veins were occluded simultaneously by argon laser photocoagulation as close to the optic disc as possible, without occluding the accompanying retinal arteries (38) (Fig. 1, 2). All the eyes had the following studies performed before RVO and serially thereafter: (a) a detailed slit lamp examination for changes in the anterior chamber, angle and iris, (b) ophthalmoscopic examination, (c) stereoscopic color fundus photography and fluorescein fundus angiography,

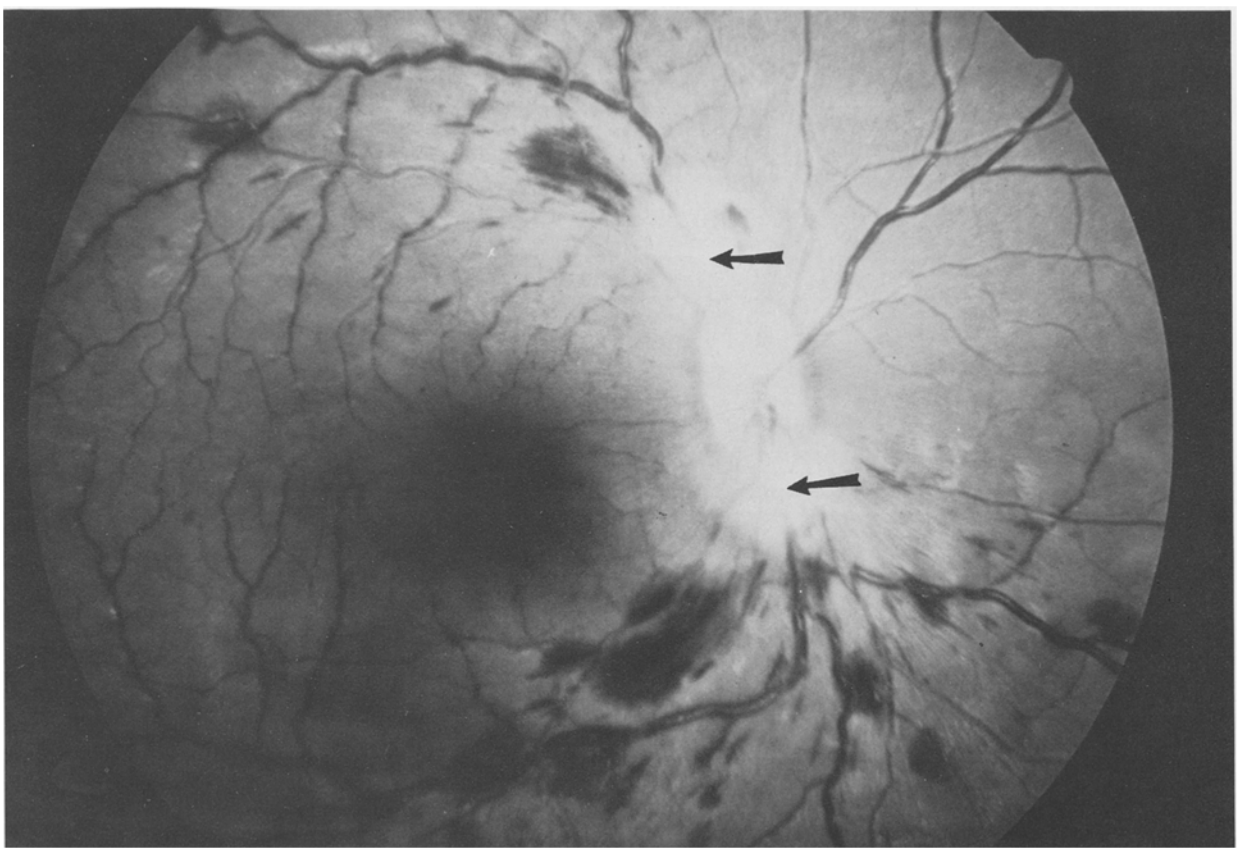

Fig. 1. Fundus photograph of the right eye, soon after the production of occlusion of all the major retinal veins except for the superonasal retinal vein, by laser photocoagulation (arrows).

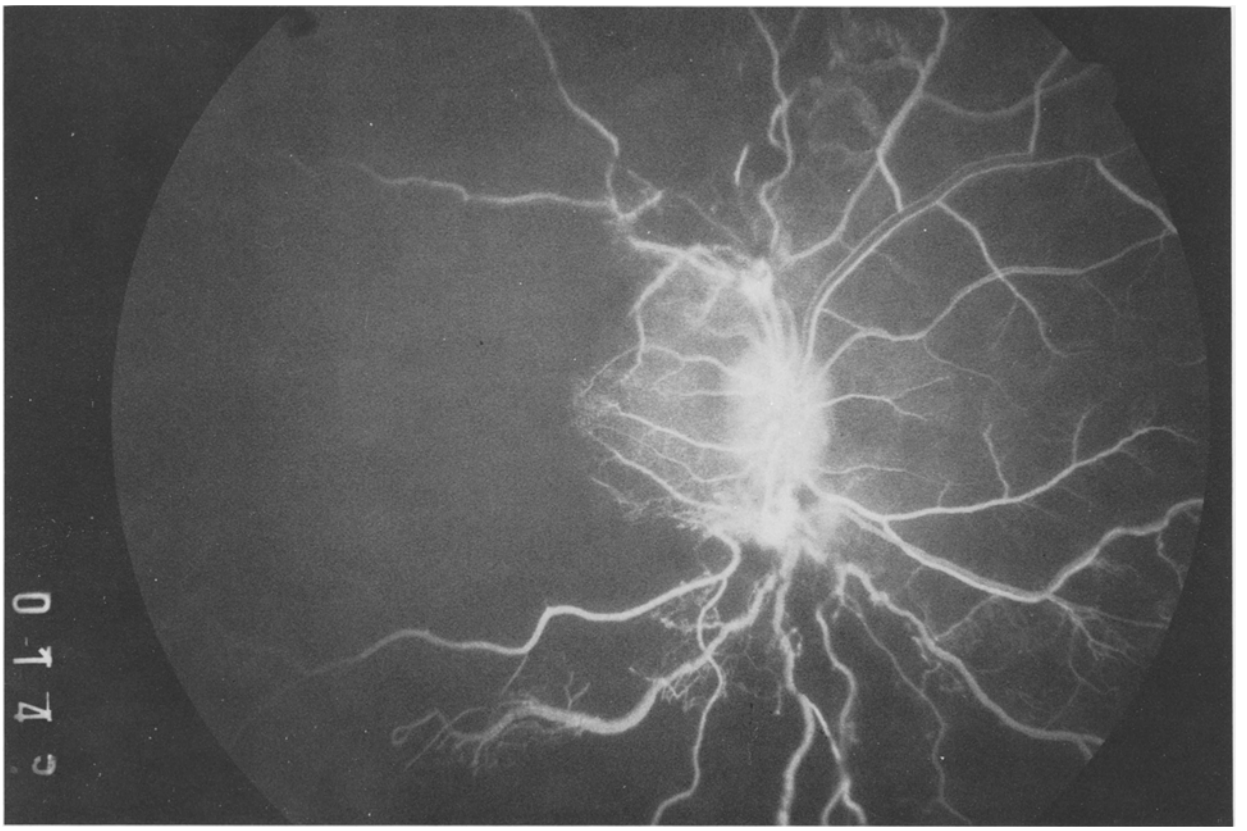

Fig. 2. Fluorescein fundus angiogram of the right eye after occlusion of superotemporal and inferior retinal veins but patent superonasal retinal branch vein. Note extensive retinal capillary non-perfusion in the distribution of the occluded veins but normal retinal capillary filling in the superior nasal quadrant and the area between the fovea and optic disc.

(d) iris color photography and fluorescein iris angiography (performed on a day when fluorescein fundus angiography was not done), and (e) intraocular pressure measurement with a Goldmann applanation tonometer mounted on a slit lamp. In 20 of the eyes lensectomy and vitrectomy were performed several months before the RVO (18) to evaluate the effects of this procedure on the NV of the anterior segment. Detailed ocular findings after RVO are given elsewhere (18, 38), and the following is a very brief summary of those. These eyes developed the following two types of changes: A. Anterior segment NV; and B. Ocular hypotony.

A. Anterior segment NV

Almost all the eyes developed iris NV (Fig. 3, 4), and 4 of the eyes (2 with lensectomy and vitrectomy and two without) also developed neovascular glaucoma (18, 31, 32). The mean time-interval between RVO and onset of iris NV was 3.1 ± 2.2 (S.D.) days and 5.8 ± 1.6 (S.D.) days with and without lensectomy and vitrectomy respectively (18), and the difference between the two groups was statistically significant ($p<0.01$); thus, lensectomy and vitrectomy accelerated the development of iris NV, which confirms the clinical experience. Fluorescein leakage from the iris on angiography appeared before the clinically apparent NV (38).

B. Ocular hypotony

This was a universal finding in these eyes. It usually developed on the day after the RVO (38). The eye with the RVO had a 4–10 mm/Hg fall in the intraocular pressure as compared to the fellow normal

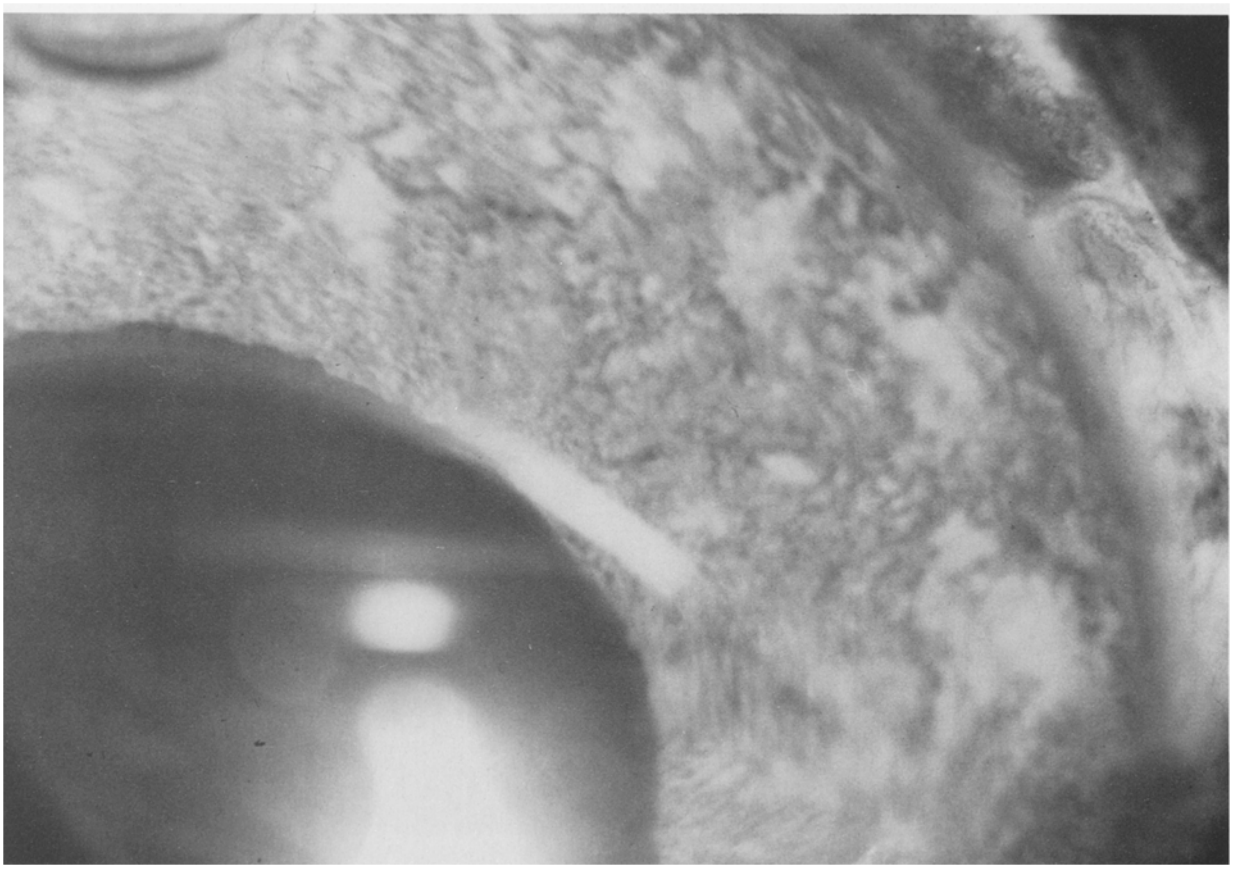

Fig. 3. Iris photograph showing marked NV on the iris surface in the eye shown in Fig. 2.

eye. The ocular hypotony lasted for a much longer time than the iris NV (38). Our clinical studies in patients also showed a significant ($p<0.0005$) fall of intraocular pressure in eyes with central and major branch RVO (20, 21). Thus, both the clinical and experimental studies showed that RVO produces a long lasting ocular hypotony.

Critics may argue that the vessels seen on the iris in our study simply represented the dilatation of the pre-existing normal iris vessels and not true NV. The following evidence indicates that this is not so. Firstly, iris fluorescein angiography revealed that the iris vessels seen after RVO had not existed prior to it (in Fig. 4 compare B with C and D); the new vessels formed tufts or even a vascular membrane on the surface of the iris (Fig. 3, 4). Secondly, the new vessels leaked fluorescein diffusely in a pattern similar to that seen in iris NV seen in man (Fig. 4E). Thirdly, the 4 eyes in this series developed neovascular glaucoma and peripheral anterior synechiae (18, 31, 32) – this never happens with simple dilatation of the pre-existing iris vessels. Fourthly, histopathological studies showed them to be new vessels.

II. Morphological studies

Many of the eyes with iris NV were examined histopathologically, and showed the neovascular nature of the iris vessels (31, 32). The histopathological studies also showed the presence of peripheral anterior synechiae in eyes with neovascular glaucoma (31, 32). In addition to these histopathological studies, 5 eyes, enucleated 5 to 133 days after RVO, had intracameral injection of tritiated thymidine and systemic intravenous injec-

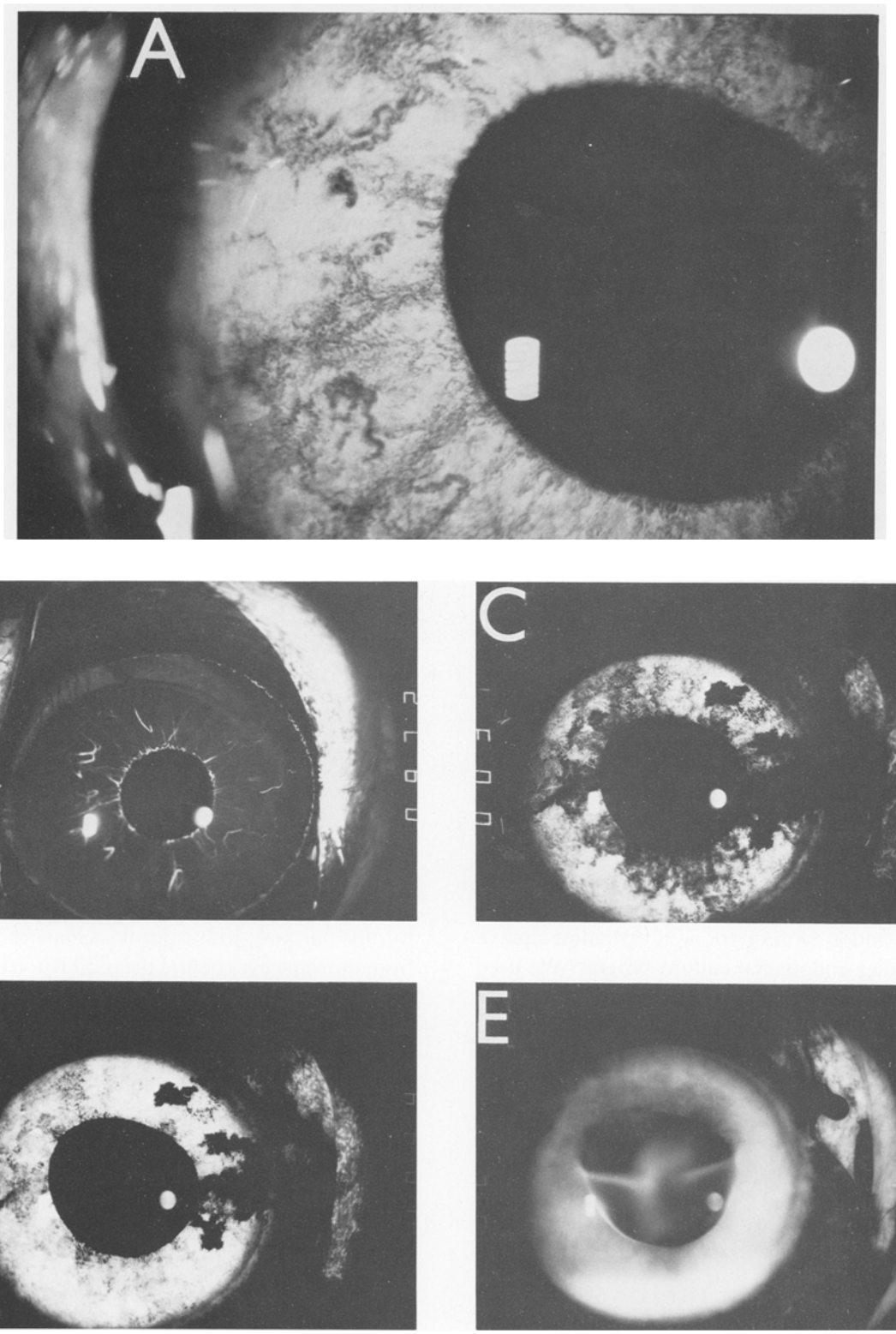

Fig. 4. Iris photograph and fluorescein angiograms of an eye. (A)–Iris photograph showing NV after RVO. (B)–(E) are fluorescein iris angiograms. (B) is before RVO, showing the normal iris vascular pattern. (C) to (E) are one week after (A), showing marked iris NV: (C) and (D) 3 and 4 seconds after the injection of fluorescein and (E) during the late phase. Compare the pattern of the normal iris vessels (in B) with that of the iris NV (in C and D). Normal iris vessels are radially arranged and do not leak fluorescein.

tion of horseradish peroxidase prior to enucleation, and were examined by light and electron microscopy and by autoradiography (31). These studies showed that during the early phases, there was an intense uptake of tritiated thymidine in the vascular endothelium (indicating marked proliferation of the endothelial cells), along with dilatation of the iris vessels, development of endothelial cell fenestrations with leakage of horseradish peroxidase, and deposition of fibrin outside of the iris vessel wall. Subsequently, there was a decrease in the tritiated thymidine uptake of the endothelial cells but a remarkable proliferation of stromal cells as documented by their increased thymidine uptake. The formation of a neovascular membrane was seen in association with the anterior migration of stromal cells and deposition of fine collagen fibrils. In the late phase, there was a decrease in the tritiated thymidine uptake in both endothelial and stromal cells, followed by regression of the neovascular membrane. These morphological studies firmly establish the iris NV as a true entity in these eyes.

III. Tissue culture studies

Vasoproliferative activity has been tested by different investigators using different assay systems, e.g., corneal pocket, chick chorioallantoic membranes, and growth or tritiated thymidine uptake by vascular endothelial culture systems. We used the tritiated thymidine incorporation by the cultured endothelial cells of human umbilical veins to test for the vasoproliferative activity in the following (18): (A) The various intraocular fluids in: (i) normal eyes, (ii) eyes with anterior segment NV in cynomolgus monkeys and human beings, and (iii) human eyes with proliferative diabetic retinopathy; (B) Bovine retinal extract; and (C) Normal serum from humans and cynomolgus monkeys.

The method used and the results obtained are discussed at length elsewhere (18). In summary, our studies showed that there was increase tritiated thymidine incorporation by (a) the aqueous, vitreous, and the intraocular fluid (which filled the eyeball after lensectomy and vitrectomy) removed from cynomolgus monkeys eyes with iris NV or with neovascular glaucoma that developed after experimental RVO, (b) by aqueous and vitreous removed from human eyes with neovascular glaucoma or proliferative diabetic retinopathy, (c) by serum, and (d) by the bovine retinal extract. There was no increased tritiated thymidine incorporation by the normal aqueous, vitreous, or the intraocular fluid filling the eyeball after lensectomy and vitrectomy. Thus, our tissue culture studies confirmed the findings of the previous workers about the presence of vasoproliferative activity in retinal extract, in serum, and in vitreous aspirated from eyes with proliferative diabetic retinopathy (18). In addition to that, we also demonstrated that the aqueous, vitreous, and the intraocular fluid (filling the eyes after lensectomy and vitrectomy) in monkey eyes with experimental RVO and associated iris NV or neovascular glaucoma show the presence of vasoproliferative activity, but not in the normal aqueous, vitreous, or the intraocular fluid (18).

IV. Biochemical studies

The main objective of our experimental studies was to investigate the biochemical nature of the angiogenic factor(s) responsible for initiation and perpetuation of ocular NV. We investigated the various intraocular fluids from eyes with ocular NV for this purpose, with control specimens obtained from the same eyes before the production of RVO. As discussed above, our tissue culture studies had shown that vasoproliferative activity was present in the former group but not in the latter group (18). In addition to the intraocular fluids, biochemical studies were also conducted on the bovine retinal extract. Before summarizing our findings on the biochemical studies, we want to stress that these are very preliminary observations and that we are still exploring the various avenues and methods to get more information about the biochemical nature of the angiogenic factor(s).

A. Bovine retinal extract

The following studies were performed on the bovine retinal extract:

(1). The supernatant from the incubated bovine

retinas was found to stimulate tritiated thymidine uptake in the human umbilical vein endothelial cell tissue culture (18). Partial purification of this extract by isoelectric precipitation, ion exchange chromatography and gel exclusion chromatography revealed an active preparation in the 15,000 to 25,000 molecular weight range. This preparation contained many proteins, as indicated by SDS-acrylamide gel electrophoresis. We have not carried out so far further purification of this extract but our preliminary results agree with the reported molecular weight range (i.e., about 18,000) (7).

(2). Incubation of the bovine retinas under nitrogen (anaerobic conditions) resulted in significantly greater ($p<0.01$) endothelial cell mitogenic activity by the supernatant as compared to the product from aerobic (air) incubation. We did not explore further the reasons for this difference but it may be consistent with retinal ischemia being an important factor in the development of ocular NV.

(3). We also investigated the effects of corticosteroids. Our preliminary studies indicated that in vitro addition of cortisol to incubating bovine retinas resulted in dose related decreases of up to 50% of controls in the mitogenic activity by the dialyzed incubated medium (using the human umbilical vein endothelial cell tissue culture); steroid concentrations were 100 picomolar to 1 μ molar. We can draw no definite conclusions from these experiments since several explanations are possible, related or unrelated to angiogenesis, but we are interested in further investigating the effects of steroid on angiogenesis.

B. Intraocular fluids

The following biochemical studies were conducted on the intraocular fluids obtained from: (i) normal eyes, (ii) eyes with anterior segment NV in cynomolgus monkeys and human beings, and (iii) human eyes with proliferative diabetic retinopathy:

(1). Prostaglandins' studies: Prostaglandins have been implicated as angiogenic agents in a number of studies (1, 14, 38, 40). We devised a protocol for measurement of PGE_2 and PGF_{2a} concentrations in the intraocular fluid of the experimental model monkeys before, during and following NV. Assays were performed by another laboratory for us, using a solid C_{18} cartridge (Sep-Pak from Waters Associates). Ninety monkey eye samples and 51 human eye samples were analyzed for PGE_2 and PGF_{2a}. Unfortunately a technical problem developed, invalidating most of the data; values obtained for samples with volumes less than 400 μl were artifically inflated. This was a highly disappointing experience, a waste of valuable samples and 3 years of study in this area.

(2). Protein electrophoretic studies: We investigated the nature of the angiogenesis factor(s) in the intraocular fluids, in which tissue culture studies (18) had shown a clear correlation between the mitogenecity and the degree of NV. With the assumption that the factor(s) might be a protein, we performed SDS-polyacrylamide gel electrophoresis on the intraocular fluid sample. At the same time serum of the monkey was also tested by the same method. Silver staining was used to visualize protein bands in these studies. The results to date indicate that there was a strong band corresponding to the serum albumin in all the samples of the intraocular fluid from eyes with and without iris NV; in addition to that, in the eyes without NV, there were also multiple bands of molecular weight higher than 40,000 daltons, with very few in lower molecular weight range (Fig. 5). When the NV was present, the total protein concentration increased markedly, and the gel display now more closely resembled that for blood serum, with some marked exceptions in the lower molecular weight range (<30,000 daltons). Our studies also revealed about a 100-fold increase in the protein concentration in the intraocular fluid in eyes with NV. So far we have found no appreciable change in any specific protein.

(3). Lysosomal Enzyme studies: These studies have been performed on the intraocular fluid obtained from some of the eyes of our monkeys with experimental RVO and ocular NV (29). The following lysosomal enzymes were measured:

(i) Beta-N-Acetyl-Glucosaminidase (NAGase);
(ii) Beta-Galactosidase;

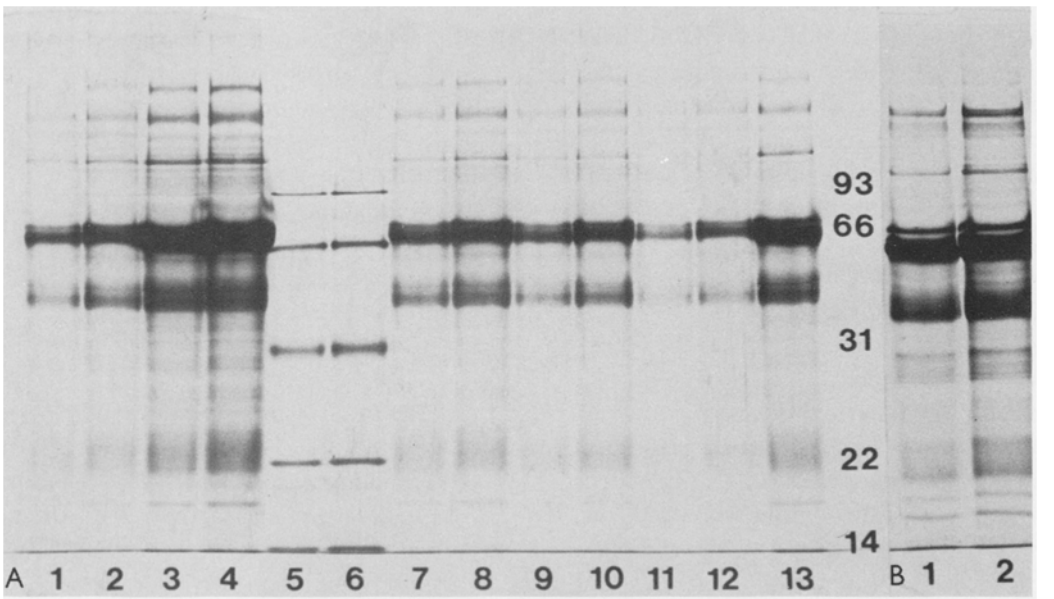

Fig. 5. Electrophoretic pattern: Silver stained SDS-Acrylamide Gels of monkey intraocular fluids (A) and serum (B). (A): Electrophoretic pattern of intraocular fluids from a monkey which developed iris NV after RVO – Odd numbered lanes bear 2.5 μl, and even numbered lanes 5 μl equivalent of intraocular fluid. Lanes 1 and 2 are one day post-RVO, lanes 3 and 4 are 7 days post-RVO, lanes 7 and 8 are 14 days post-RVO, lanes 9 and 10 are 19 days post-RVO, lanes 11 and 12 are 27 days post-RVO. Lanes 5 and 6 are of protein standards, with corresponding molecular weights ($\times 10^{-3}$) listed vertically after lane 13. (B): Electrophoretic pattern of the monkeys serum – lane 1 bears 0.22 μl and lane 2 bears 1.25 μl.

(iii) Alpha-Mannosidase;
(iv) Beta-Glucuronidase;
(v) Acid Phosphatase.

The NAGase level (μ moles/ml/hour) in the normal intraocular fluid was 0.036 to 0.088 (0.054 ± 0.018), but in eyes with iris NV it was significantly ($p = 0.001$) elevated and was 0.159 to 0.37 (0.22 ± 0.089). In contrast to that, there was no significant rise in Beta-Galactosidase and others. In other studies (29) NAGase activity was elevated in vitreous ($p<0.01$) and decrease in the retina ($p<0.01$) in experimental diabetic rats, suggesting that the NAGase might be leaking from the diabetic retina into the vitreous. These studies also showed that vitreous inhibited the NAGase activity by 34%. Purified NAGase, on ionic exchange chromatography and on chemical treatment, behaved similar to the retinal derived angiogenic proteins reported by other authors in the incubated bovine retina. Thus, these studies (29) indicate that NAGase, a lysosomal enzyme, has angiogenic activity; it plays a role in the breakdown of 5 of 7 proteoglycans. Morgan *et al.* (29) hypothesised that probably NAGase would carry out the initial basement membrane breakdown in the process of angiogenesis, but what releases the NAGase is still not known.

Discussion

The experimental production of ocular NV (particularly iris NV and neovascular glaucoma) following RVO, in cynomolgus monkeys, provides us with a very useful experimental animal model to study the various aspects of ocular NV. We found it to be a reliable model, mimicking very closely the naturally occurring disease and associated complications in man. These eyes with experimental RVO developed not only ocular NV but also invariably the associated ocular hypotony also seen in the human eyes with RVO (20, 21).

Thus, experimental RVO produced ocular NV (angiogenic effect) and ocular hypotony (hypotensive effect), as is also seen in patients, and these two effects must be humoral in origin, with the diseased retina liberating factor(s) that enter the intraocular fluids and circulate the ciliary epithelium, iris, and trabecular meshwork (20, 21, 38). The angiogenic effect is slow and short lasting, and for development of ocular NV the stimulus must be adequate and persist long enough (38). In contrast to that the hypotensive effect is very potent, rapid and long lasting (20, 21, 38). The various factors which influence them are discussed elsewhere (20, 21, 38). Whether the angiogenic and hypotensive factors are the same or different (20, 38) still remains a mystery.

The mechanism of the hypotensive effect of RVO is unknown, and the various possible mechanisms were discussed at length previously (20, 21, 38). In our previous publications (19, 38), we postulated that prostaglandins may be a possible factor in the production of both the ocular NV and ocular hypotony. Since then ample evidence has been presented in the literature about the hypotensive effect of prostaglandins, particularly PGE_2 and PGF_{2a} (2–5, 35) and also PGE_3 and PGD_3 (25). In the production of ocular hypotension with RVO, the other possible factor may be the liberation of the NAGase which has been shown to be significantly ($p = 0.001$) elevated in the intraocular fluid of eyes with experimental NV in the present studies. NAGase breaks down hyaluronic acid (27), and in the trabecular meshwork hyaluronic acid is responsible for outflow resistance (15, 17). NAGase could affect the glycosaminoglycans of trabecular meshwork, resulting in increased outflow facility, and consequently ocular hypotony. There is evidence indicating that prostaglandins (particularly PGE_1) may cause release of NAGase, thus suggesting a relationship between the two factors.

In the literature, there are a large number of reports giving evidence of the presence of angiogenic factors in a variety of tissues and agents, including human hepatoma (30), breast cancer (34), human adenocarcinoma (11), human myocardial infarction tissue (26, 34), synovial fluid from patients with joint disease (26, 34), non-luteal porcine ovary (28), activated macrophages (34), hypoxic macrophages (24), lymphocytes, fibroblasts, fibrin degeneration products (37), bovine retinal extract (7, 8, 18, 34), hypothalmus (7), lipids from omental fat extract (16), parathyroid tissue (33), placenta (30), brain (9), angiotensin II (10), lactic acid (23), degradation products of hyaluronic acid (39), adenine and adenine derivatives (36), and prostaglandins (1, 14, 40). However, angiogenic activity in all these studies were detected by using very different types of assays. The angiogenic factor isolated from the malignant tumors has been called 'tumor angiogenesis factor' (13). Recently the tumor angiogenesis factor isolated from human colon carcinoma cells has been christened 'angiogenin' (11) and its chemical composition has been determined by chromatography. It has been found to be a single-chain basic protein of molecular weight 14,400. Its complete aminoacid sequence has been determined and the angiogenin gene has been cloned. Whether all the angiogenic factors detected in the various tissues and agents mentioned above are identical in nature or totally different still remains to be determined. The biochemical nature of the angiogenic factor(s) responsible for various types of ocular NV in different conditions still remains a mystery. Most of the available evidence indicates that the angiogenic factor(s) is a protein. Our studies on the intraocular fluids revealed that the normal aqueous and the intraocular fluid that filled the eye after lensectomy and vitrectomy (many months after the surgical procedure when the eye was normal in all aspects) had proteins corresponding to the serum albumin and some proteins in the regions of molecular weight >40,000 daltons but fewer in the lower molecular weight range. When these eyes developed iris NV after RVO, there was about a 100-fold increase in the protein concentration in the intraocular fluid and the composition more closely resembled that for blood serum, except for the lower molecular weight (<30,000 daltons). This marked elevation in the protein contents of the intraocular fluid is most probably due to marked increase in the permeability of the new vessels in the iris so that the serum proteins are leaking into

the intraocular fluid. Thus, a marked leakage of the serum proteins from the iris NV adds to the difficulty of investigating the true biochemical composition and identification of the angiogenic factor.

A significant increase in the level of NAGase, without a corresponding rise in the level of other lysosomal enzymes, in the intraocular fluid of eyes with iris NV after RVO (29) suggests a possible role by it in the angiogenesis, particularly since NAGase has been found to have angiogenic properties. It is possible that in ischemic retinal vein occlusion, diabetic retinopathy and other proliferative retinopathies, the retinal damage following ischemic insult may result in damage to the lysosomes in the retinal tissues and production of NAGase; the latter would be then released from the retina and diffuse into the vitreous, and from there to other intraocular tissues to produce NV. This is simply a hypothesis at this stage and requires a good deal of work to confirm or refute.

Since the growth of the malignant tumors and of the new vessels in the eye depends upon the constant release of angiogenic factor(s), it is logical to think that agents inhibiting the activity of the angiogenic factor(s) would not only stop the growth but also cause regression of the malignant tumors and of the ocular NV. This has been suggested in the literature. For example, there is accumulating evidence that steroids may inhibit the growth of NV (6, 12, 40). In the published studies, the glucocorticoid-related steroids inhibited angiogenesis in the presence of heparin or heparin fragments of a specific size. According to Crum *et al.* (6) the anti-angiogenic effect was not coincident with glucocorticoid activity of the steroid. Folkman (12) found that injection of cortisol with hexasaccharide fragment into reticular cell sarcoma produced rapid regression of the tumor. Oral heparin with cortisone also produced regression of reticulum cell sarcoma, B16 melanoma, Lewis lung carcinoma and bladder carcinoma. He (12) also found that systemically administered protamine inhibited growth of lung metastases. Similarly Ziche *et al.* (40) found regression in growth of B16 melanoma, Lewis lung carcinoma and fibrosarcoma by cortisone alone which was slightly potentiated by heparin. In the eye, it has been reported that corticosteroids help to prevent or control the corneal NV. The mechanism of action of the steroid, however, is obscure. It might be speculated that synthesis of angiogenic factors in the cell is inhibited by steroids, or their destruction hastened. Thus, if a suitable inhibitor of angiogenic factor(s) is discovered, it would completely revolutionize our management of these dreaded conditions. The subject therefore assumes a tremendous clinical importance. For example, in the eye, at present the only known treatment effective in some conditions with ocular NV is panretinal photocoagulation, which is a destructive procedure; a drug therapy would be a highly desirable substitute.

Acknowledgments

We wish to acknowledge the help of many of our colleagues in this investigation. Drs. P.S. Virdi, X. Gu, G.E. Servais and Meena Beri helped in the clinical evaluation of the animals, Dr. A. Packer performed the lensectomy and vitrectomy, Dr. M.O.M. Tso, T.M. Nork, F.C. Blodi and L.E. Zimmerman helped with the morphological investigations, Dr. J. Hoak and Mrs. G. Fry for the tissue culture studies, and Dr. K.M. Morgan with the lysosomal enzyme studies. This research was supported by grants from the Iowa Heart Association, unrestricted grant from the Research to Prevent Blindness, EY-1576 from the National Eye Institute, and University of Iowa Committee on Ageing.

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