Components of vitreous-soluble proteins: Effect of hyperoxia and age. CHUNG-HO CHEN AND ARNALL PATZ.

In young puppies, the retina, which is incompletely vascularized at birth becomes fully vascularized at approximately four weeks of age. During this period of vessel growth the total content of vitreous-soluble protein was found closely associated with the rate of retinal vessel growth. As vascularization progressed toward completion, the protein originally present at birth decreased to a negligible or undetected amount. Intravitreal neovascularization was produced in young puppies by exposure to 85 per cent oxygen for four days, then removal to room air. This form of neovascularization resembles closely that observed in human proliferative diabetic retinopathy. The oxygen treatment, which initially produced retinal capillary closure, then neovascularization, was associated with a retention of vitreous-soluble protein at a high level for several days. The results raise the possibility that the vitreous protein(s) may be fundamentally involved in the process of normal vascularization of the retina and in retinal neovascularization.

Background diabetic retinopathy has been described in dogs with spontaneous diabetes and alloxan, or growth hormone-induced diabetes. Proliferative retinopathy with intravitreal neovascularization has not been reported in any of these models although Engerman and Bloodworth have described a form of "intraretinal" neovascularization in alloxan diabetic dogs. The latter was also observed by Kohner following experimental branch vein occlusion in monkeys. An experimental model of intravitreal neovascularization was developed by Ashton, Ward, and Serpell and Patz and co-workers. The pattern of neovascularization which represents the early proliferative stage of retrolental fibroplasia resembles that of human diabetic retinopathy. In the young kitten or puppy with an incompletely vascularized retina, continuous exposure to hyperoxia leads to destruction of the most anterior or immature retinal vascular complexes. Following removal to room air, intravitreal neovascularization develops from the vascular bed immediately adjacent to the zone of retinal capillary destruction or non-perfusion. A systematic study of this model has been instituted in our laboratory. This paper reports on the vitreous-soluble protein components in relation to normal vascularization and in the experimental oxygen model of neovascularization.

Methods.

Technique for induction of proliferative retinopathy with oxygen in puppies. Beagle puppies of both sexes at three to eight days of age were placed in a nursery incubator* and exposed to 85 per cent humidified oxygen continuously for 96 hours. The oxygen concentration in the incubator was monitored every two hours with a Beckman analyzer, Model D2, and the young animals were nursed twice daily by their mothers. Following oxygen treatment, the pups were removed to room air until sacrificed (from 0 to 29 days after return to air). Litter mates served as controls.

Fundus examination and histology. By the tenth day after removal from incubator to room air (average age 15 days), the media were sufficiently clear to resolve the retinal vascular details by indirect ophthalmoscopy. By three weeks after removal to air the neovascular tufts could be resolved. After ophthalmoscopic examination, 30 oxygen-treated animals were anesthetized with pentobarbital and a polyethylene catheter was placed in each carotid artery. Normal saline solution containing 2 per cent heparin was slowly infused until the blood vessels were free of visible blood (about 1,000 ml); a 50 per cent solution of India ink was then infused. The eyes were then enucleated and fixed in 10 per cent neutral formalin for 24 hours. On a random basis one eye selected for flat retinal mounts was opened at the pars plana and the cornea, iris, and lens dissected free. The retina and vitreous were removed with a scoop and mounted flat on a slide in Kaiser's glycerol-gel. The fellow eyes were then embedded in paraffin. Multiple 8 um thick sections were made and stained with hematoxylin and eosin and periodic acid–Schiff stain (PAS).

*Modified Isolette, Model C86, Air Shields Incorporated, Hatboro, Pa.
Preparation of vitreous homogenate. The following procedure was performed on 25 animals which did not receive India ink injection. Immediately upon sacrificing the animal by an overdose of pentobarbital, the eyes were enucleated, chilled in ice, and rinsed with ice-cold saline to remove all traces of blood. The cornea, iris, and lens were removed and the vitreous lifted from the posterior segment with a tweezer, then homogenized with a Potter-Elvehjem homogenizer without any medium added. The homogenate was then subjected to centrifugation at 100,000 x g for 20 minutes at 0 to 4° C. to remove the collagen.

Electrophoretic analysis of vitreous homogenate. Disc polyacrylamide gel electrophoresis was carried out according to the method of Brachneridge and Bachelard with a current of 2 mA per tube. After the termination of the electrophoresis (usually two to three hours for 10 cm. of gel), the gels were immediately removed and stained with 0.2 per cent amido black in 5 per cent acetic acid for 2 hours. Then they were destained in 7.5 per cent acetic acid by a diffusion device (Bio-Rad). In some experiments, the electrophoresis was also carried out by using a quartz tubing and the protein bands were detected directly by scanning the gel while still in the tubing with a densitometer, attached to a Gilford Spectrophotometer, without staining. PAS staining was then performed to identify glycoproteins.

Protein determination. The protein concentration was measured spectrophotometrically by the method of Murphy and Kies, with bovine serum albumin containing 200 μg per milliliter of hyaluronic acid as standard.

Results

Normal vascularization developmental pattern of puppy retinas. To interpret the histologic data, the vascularization developmental pattern of the normal puppy was analyzed and is illustrated in Fig. 1. At birth the newborn puppy retina is vascularized only to the equator temporally but to within one disc diameter of the ora nasally. At one week of age, vascularization reaches the ora nasally and by four weeks of age, retinal vascularization is complete temporally. Further examination of the growth pattern in the temporal retina revealed a rapid growth rate during the first two weeks of life and a progressively diminishing rate during the third and fourth week.

Correlation of vitreous-soluble proteins with retinal vascularization activity. The age-dependent normal vascularization rate of growth in the retinal temporal periphery was found closely as-
Fig. 2. Age-dependent vitreous-soluble protein concentration demonstrating the retention of the protein content at higher levels following hyperoxia when compared with controls. The insert in the upper right is the electrophoretic patterns (with anode at bottom) of the corresponding vitreous homogenates. Experimental details are as that described in Methods. A volume of 50 μl vitreous homogenate was used in each gel column in the electrophoresis. For the study on the effect of hyperoxia, eight-day-old puppies were incubated with 85 per cent oxygen for four days, then removed to room air until sacrificed. ••••, control; O—O, oxygen-treated.

associated with the total amount of vitreous-soluble proteins as shown in Fig. 2. The concentration of vitreous-soluble proteins was high when vascularization activity was greatest. As the vascularization became complete (28 to 30 days of age), the content of protein components, except the two fast migrating ones, decreased to undetectable levels based on the polyacrylamide gel electrophoresis. Two fast migrating components were also decreased significantly.

Effect of hyperoxia on vitreous-soluble protein components. The data in Fig. 2 demonstrate that, following oxygen treatment, the concentration of the vitreous-soluble protein was retained at appreciably higher levels than controls of the same age. It is significant that immediately at termination of oxygen, and prior to neovascularization, the concentration of vitreous-soluble proteins was retained at a higher level than in controls. Visible neovascularization began several days after removal to room air (Fig. 1) and the concentration of vitreous-soluble protein remained appreciably higher than in controls.

The composition of protein components of oxygen-treated animals was qualitatively similar to that of controls.

Comparison of the vitreous and serum protein patterns. The mobility of the vitreous-soluble protein components was different from that of serum, as demonstrated by the polyacrylamide gel electrophoresis (Fig. 3). The major band (the fastest moving band) of vitreous components has an electrophoretic mobility (Rf = 0.70 to 0.88) higher than that of serum albumin (Rf = 0.60 to 0.66). The difference between them was also indicated by the staining abilities with PAS reagent. Under the same staining condition, bands a, b, c, and d of vitreous proteins gave the intense characteristic pink color for glycoprotein (band e showed only slightly), while in serum only bands f and g showed intense PAS staining, and band h was negative. These observations are in general agreement with those of Balazs and Sundblad that the protein composition of vitreous body differs from that of serum.

Discussion. The vascularization pattern of the young puppy retina was found to be closely correlated with the content of vitreous-soluble proteins. As the vascularization progressed toward completion, the content of vitreous-soluble proteins decreased to a negligible or undetectable amount. Although closely correlated, this relationship of vitreous-soluble protein concentration and vascularization activity may only be coincidental. On the other hand, hyperoxygenated animals that were examined immediately after removal from oxygen and also during the period of greatest vaso-proliferation, showed significantly higher content of vitreous-soluble proteins. No further neovascularization developed five to six weeks after removal from the oxygen incubator, at which time the concentration of vitreous-soluble proteins had decreased to a very low level. Moreover, it is most unlikely that the higher vitreous-soluble protein concentration in hyperoxygenated animals resulted from the neovascularization as the protein concentration was found at a higher level immediately upon removal to air and long before neovascularization could have developed. Fluorescein angiography has revealed no dye leakage during the period of hyperoxia and vasoconstriction. In other words, if the neovascularization
leakage would account for the high vitreous protein concentration, the increase should not have occurred until neovascularization had developed. The correlation of greater vitreous protein(s) concentration during the peak of both neovascularization and normal retinal vascularization raises the possibility that the vitreous protein(s) may be fundamentally involved in the process of normal vascularization.

Although the findings are in agreement with those of Balazs and Sundblad⁹ that the vitreous protein patterns are different from that of serum, the question on the origin of vitreous proteins was not resolved. However, the data indicate that the vitreous proteins may be formed or deposited in the vitreous body during the embryonic development of the eye and that the proteins decrease rapidly during the first two weeks of life and the amount was negligible when the animal was mature. These findings also indicate that there is no significant synthesis or constant supply of these proteins from sources such as the retinal or ciliary body blood vessels after birth.

Studies are in progress to test the vitreous-soluble proteins identified in these experiments for possible vasoproliferative activity.

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REFERENCES


Essential fatty acid deficiency and renewal of rod outer segments in the albino rat.

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Biochemical studies in albino rats fed a lab chow diet (control) showed a 9 to 10 day turnover time for rhodopsin in rod photoreceptor membranes, whereas the turnover time in animals raised on a fat-free diet (experimental) was not easily measurable. The number of phagosomes in the pigment epithelium of the control group was three times that found in the experiments. These studies support earlier autoradiographic data which suggested that the renewal of new photoreceptor discs in the rat retina is controlled by the availability of polyunsaturated fatty acids.

†Dennis Landis, our friend and colleague died March 14, 1975.

Rod outer segments (ROS) from rat retinas contain large quantities of long-chain polyunsaturated fatty acids, the precursors of which must be obtained from the diet. Since rods are completely renewed every 9 to 10 days, we thought it possible to lower the levels of polyunsaturates in rat rod outer segments by raising them on diets deficient in the essential fatty acids. However, in albino rats raised for 10 weeks on a deficient diet, only a slight reduction (7 per cent) in the level of 22:6 

If membrane renewal by new disc formation is altered in animals raised on essential fatty acid-deficient diets, there should also be an alteration in the rate of rhodopsin turnover in the membranes as well as differences in the number of phagosomes in the pigment epithelium. These experiments are presented in this report.

Three-week-old weanling albino rats were raised under regulated light conditions (12 hours on and 12 hours off) on either an experimental (Fat-Free Test Diet, Nutritional Biochemicals, Cleveland, Ohio) or control (Lab Blox, Texas Laboratory Animal Feed Company, Houston, Texas) diet for 12 weeks, at which time they were injected via the jugular vein with 3.3 μCi per gram of body weight of a labeled amino acid mixture [equal activities of [side chain 2,3-3H]-L-phenylalanine (specific activity 50 Ci per millimole) and [4,5-3H]-L-leucine (specific activity 6 Ci per millimole), Amersham-Searle]. Groups of four to five animals each were sacrificed at various times following injection and, after dark adaptation, eyes were removed under dim red light and photoreceptor membranes prepared by a modification of the discontinuous sucrose gradient procedure of Papermaster and Dreyer. Two distinct bands could be seen, one at the 1.13 to 1.15 Gm. per cubic centimeter interface and one on top of the 76 per cent sucrose. Most of the rhodopsin was located in the upper band and always had a 278/498 absorbance ratio of less than 2.5. This material was washed twice in cold phosphate buffer, solubilized in 1 ml of 40 mM ethylhexadecyldimethyl ammonium bromide (EHDAB), and applied to a 0.9 by 130 cm. glass column filled with Agarose (Bio Rad Laboratories, A-0.5 M, 200-400 mesh). Fractions of 1 ml volume were