in this report, the investigators adhered to the Guide for Laboratory Animal Facilities and Care, as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.
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Angiogenic factor in vitreous from diabetic retinopathy

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Summary. Vitreous from patients with proliferative diabetic retinopathy contains an angiogenic substance which stimulates the proliferation of blood vessels on the chick chorioallantoic membrane, whereas vitreous from non-diabetics who do not have a proliferative retinopathy does not.

The development of retinal neovascularization in proliferative diabetic retinopathy poses a major clinical problem. The frequent bleeding from newly formed vessels into the vitreous cavity[3,4] results in impaired vision and loss of vitreous integrity, and frequently surgical removal becomes necessary. The underlying mechanism of retinal neovascularization is unknown. However, areas of ischaemia are commonly associated with neovascularization and it has been suggested that such areas generate diffusible angiogenic factors[5]. Recent work by Glaser and co-workers[6,7] has shown a relationship between the occurrence of proliferative diabetic retinopathy and the presence of angiogenic

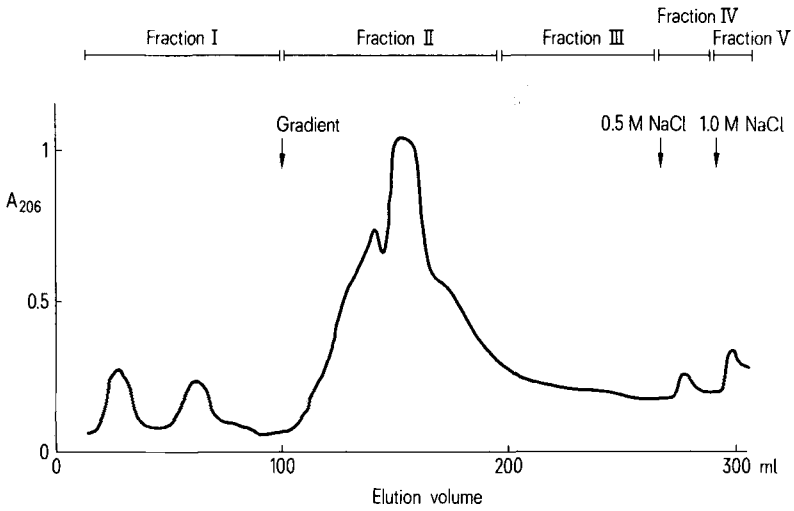

Figure 1. DEAE elution profile from a human diabetic vitreous.

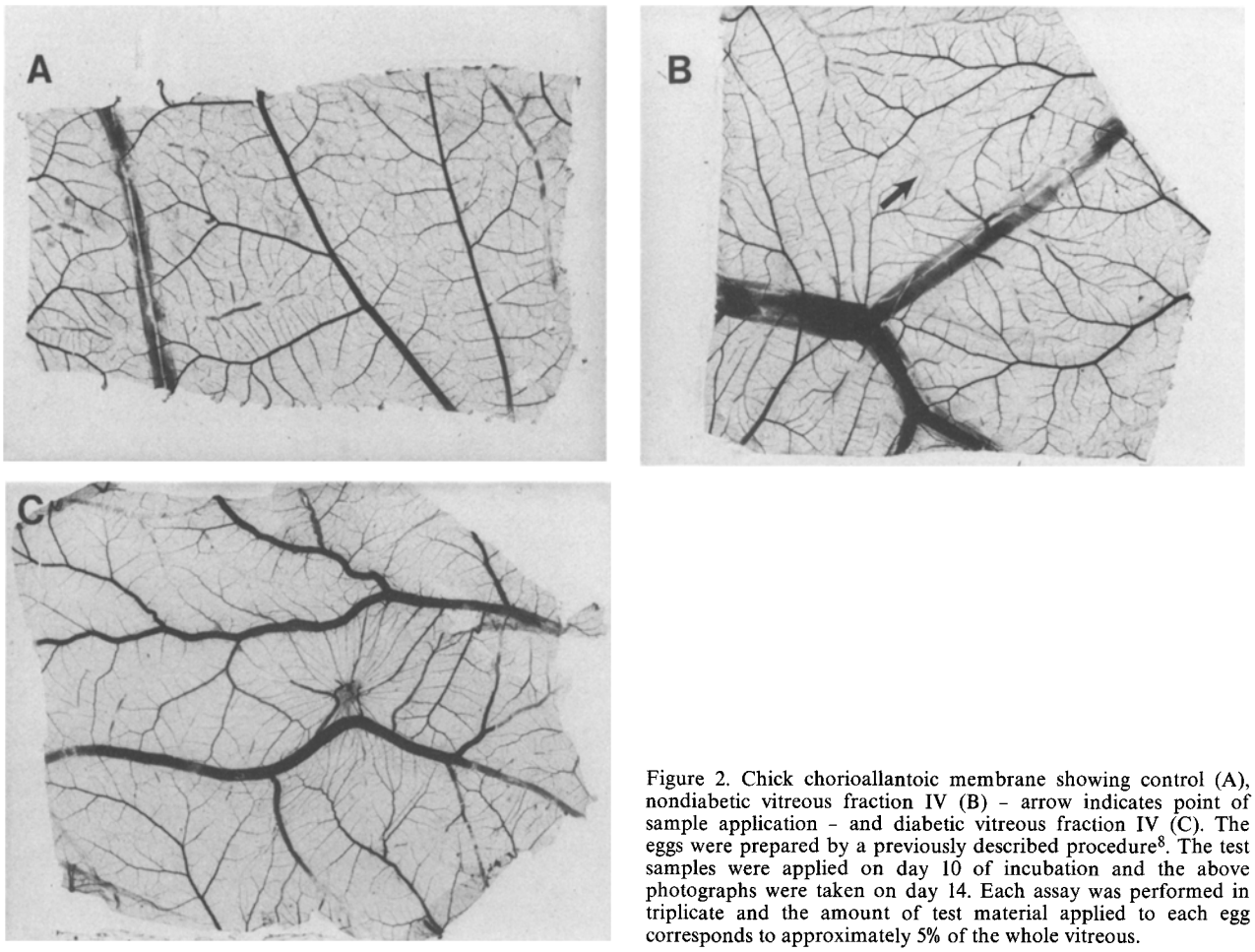

Figure 2. Chick chorioallantoic membrane showing control (A), nondiabetic vitreous fraction IV (B) - arrow indicates point of sample application - and diabetic vitreous fraction IV (C). The eggs were prepared by a previously described procedure[8]. The test samples were applied on day 10 of incubation and the above photographs were taken on day 14. Each assay was performed in triplicate and the amount of test material applied to each egg corresponds to approximately 5% of the whole vitreous.

substances in the vitreous itself. We report here the findings of a study in which we examined the levels of angiogenic activity in vitreous from diabetic and non-diabetic patients.

Materials and methods. Vitreous was obtained from 9 diabetic patients with proliferative retinopathy and from 8 nondiabetic who required vitrectomy but did not have a proliferative retinopathy. The vitreous was aspirated in Hartmann's or Cardiff solution and stored at $-20\,^{\circ}C$. After homogenisation for 30 sec at 4 °C in a glass Potter-Elvehjem homogenizer the vitreous was centrifuged at $100000\times g$ for 1 h at 4 °C. The supernatant was then dialyzed against 0.05 M ammonium bicarbonate (3×10 vol. at 4 °C) and fractionated by diethylaminoethyl (DEAE) cellulose column chromatography. The sample was applied to a column (10×1 cm) of DEAE-52 cellulose (Whatman) equilibrated with 0.05 M NH_4HCO_3 (pH 7.9) at 4 °C. Unbound material was eluted with the same buffer at a flow rate of 50 ml/h. The column effluent was monitored at 206 nm (Uvicord S, LKB, Sweden) and 14-ml fractions were collected. Bound material was eluted with a convex salt gradient (0–0.3 M NaCl) prepared in a constant level mixing device from 0.05 M NH_4HCO_3 (65 ml) and 0.05 M NH_4HCO_3 (100 ml) containing 0.3 M NaCl. The column was finally washed with 0.05 M NH_4HCO_3 containing 0.5 M NaCl and 1.0 M NaCl. A typical elution profile from a diabetic vitreous is shown in figure 1. Fractions I–IV were assayed for angiogenic activity by the chick chorioallantoic membrane (CAM) bioassay[8]. Samples were filtered through a 0.22-μm membrane and freeze-dried before application to the CAM.

Results and discussion. Vitreous from diabetic and nondiabetic patients was extracted by the method described above. Vitreous from both sources gave a similar DEAE elution profile (fig. 1). All fractions from both diabetic and nondiabetic vitreous were assayed for angiogenic activity using the chick chorioallantoic membrane bioassay. Fraction IV (fig. 1, 0.5 M NaCl) was positive for each diabetic vitreous extracted, whereas the remaining fractions were all negative. All fractions (I–V) were negative for vitreous from patients with no proliferative disease. A typical chorioallantoic membrane response to fraction IV from diabetic vitreous is shown in figure 2. The positive reaction in fig. 2C involves both the convergence of medium sized vessels on the point of sample application and the ingrowth of many small vessels resulting in the typical 'spoke-wheel' pattern. No such directional growth can be seen with either fraction IV from a non-diabetic patient (fig. 2B) or on a control membrane (fig. 2A).

The question remains as to the origin of the vitreous angiogenic substance. It has been recognized for a number of years that retinal neovascularization is virtually always preceeded by capillary non-perfusion[5] and it has been hypothesized that cells from such areas of ischemia may liberate a diffusable angiogenesis factor. Direct evidence for such a factor has only recently been forthcoming[9-11]. Furthermore, it has been shown that bovine vitreous contains inhibitors of neovascularization[12]. It would seem reasonable to suggest that the uncontrolled release of angiogenesis factor by diseased retina leads to its accumulation in the vitreous body resulting in the over-riding of the

natural ability of the vitreous to inhibit new blood vessel growth.

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Hepatic proline after bile duct ligation in rats

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Summary. Since biliary hyperplasia of fascioliasis correlated with hepatic proline level, we examined the occurrence of a similar chemical stimulus during bile obstruction. Uptake of tritiated proline and glycine rose in both hepatocytes and a bile duct enriched cell fraction, following duct ligation in rats. The increased hepatic content of proline but not glycine suggests that proline has a role in post-obstructive biliary proliferation.

Proline infused i.p. or into the common bile duct of rats induces hyperplasia of biliary ducts. Both models were suggested by experiments with *Fasciola hepatica* infestation[1,2]. The parasite secretes proline at a high rate into the bile fluid. When *F. hepatica* is placed within the bile duct, the rat liver shows early biliary hyperplasia prior to the development of inflammation and fibrosis. Similar duct excess appears after adult liver flukes, encased in mesh, are implanted into the abdominal cavity. The hyperplastic response may be inhibited by the concurrent administration of L-azetidine-2-carboxylic acid, a proline analog[3]. These observations indicate that a chemical stimulus induces biliary proliferation. We wondered whether a similar signal may also have a role in the biliary hyperplasia after obstruction of the common bile duct. Hence we undertook to measure the hepatic uptake and level of proline after ligating the duct in rats. For comparison glycine was also measured.

Methods. Male Sprague-Dawley rats, 250–300 g each, underwent under light ether anesthesia ligation of the distal common bile duct with double suture ligatures. Sham operated rats had their abdominal cavities opened and the bile duct gently touched.

1, 3 and 7 days after surgery the animals were sacrificed after perfusion and removal of their livers. 2 h prior to perfusion each rat received i.p. 250 μc of ^{3}H proline or ^{3}H glycine (each sp. act. 20 Ci/mmole). The liver was perfused in situ with 100 ml Krebs-Ringer buffer containing 0.1% collagenase and 0.05% hyaluronidase. The fluid was delivered by a constant infusion pump, retrograde into the hepatic vein, via the inferior vena cava after tying off the tributaries and the supradiaphragmatic segment. The excised liver was further digested with 1% collagenase for 10 min to prepare a suspension of single cells. The hepatocyte fraction was removed by centrifugation at 50 × g for 1 min. This fraction contained 95% hepatocytes. The bile duct enriched fraction in the supernatant was collected at 300 g for 5 min[4]. The hepatocytes, after 3 washes with Eagle's minimal essential medium (MEM, from GIBCO), were further purified by layering over lymphocyte separation medium (Histopaque, Sigma), and after centrifugation at 400 × g for 30 min collecting the pellet. The duct cell enriched fraction was suspended in 10 ml MEM and underlayered with 10 ml of 15% metrizamide (Winthrop Labs)[4]. Following spinning at 300 g for 8 min, the duct cells were removed from the interface. This fraction, observed under the light microscope, contained 40–60% duct cells, the remainder were Kupffer (20–30%), sinusoidal and other small oval cells (20–30%). Cells of the 2 fractions were solubilized in 1 N NaOH, and radioactivity counted in Aquasol II (New England Nuclear). Protein, proline and

Table 1. Hepatic proline content and uptake after duct ligation[a]

		Proline content (μmole/μg liver)	^{3}H Proline uptake[b] Hepatocyte	Duct cell enriched fraction
Control		0.22 ± 0.05	610 ± 60	610 ± 150
Sham operated day	1	0.31 ± 0.06	–	–
	3	0.43 ± 0.05	530 ± 150	590 ± 210
Post ligation day	1	0.71 ± 0.15*	1500 ± 470*	1100 ± 440*
	3	0.73 ± 0.16*	1500 ± 380*	2400 ± 550*
	7	0.63 ± 0.01*	2200 ± 450*	980 ± 380

Table 2. Hepatic glycine content and uptake after duct ligation[a]

		Glycine content (μg/mg liver)	^{3}H Glycine uptake[b] Hepatocyte	Duct cell enriched fraction
Control		1.7 ± 0.44	60 ± 10	80 ± 20
Sham operated day	1	2.2 ± 1.8	–	–
	3	1.5 ± 0.45	–	–
Post ligation day	1	1.7 ± 0.73	300 ± 80*	180 ± 10*
	3	2.6 ± 1.6	250 ± 10*	1200 ± 380*
	7	2.8 ± 1.8	230 ± 20	800 ± 100*

[a]Values in the table represent the mean ± SD, the result of 6 rats in each group. [b]cpm × 10^{-3}/μg liver protein. *$p < 0.05$ compared to controls.