

Normal Rabbit Aqueous Humour, Fibronectin, and Fibroblast Conditioned Medium are Chemoattractant to Tenon's Capsule Fibroblasts

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Summary

Some of the chemotactic and chemokinetic properties of rabbit Tenon's fibroblasts were examined in a 48-well micro-chemotaxis chamber. Normal rabbit aqueous humour, fibronectin, and fibroblast conditioned medium were used in the assay, and all were shown to be chemoattractant. In addition, aqueous humour was shown to be powerfully chemotactic. Since the failure of human trabeculectomies is associated with migration of fibroblasts to the operation site, the study of the chemoattractant influences acting on these cells may allow manipulation of their behaviour in order to influence favourably the outcome of surgery.

Of the trabeculectomies that fail the majority do so because of fibrosis at the operation site.¹ Factors leading to fibrosis and the role of aqueous humour in the success or failure of the procedure are poorly understood. Teng *et al.*² proposed that aqueous had a degenerative effect on collagen. Kornblueth *et al.*³ proposed that there is an inhibitory factor for fibroblast proliferation and iris tissue present in aqueous humour, while Herschler postulated the existence of an inhibitory factor for fibroblasts present in normal aqueous humour and absent in glaucomatous aqueous.^{4,5,6,7} In a model of failing fistulising surgery in the rabbit, Miller *et al.*⁸ have shown that fibroblast migration to the operation site, together with fibroblast proliferation, play a fundamental role in the obstruction of the fistula. In spite of work having been done on fibroblast anti-proliferative agents,⁹ little attention has been paid to the chemotaxis of these cells in this

context. Although fibroblast chemotaxis has been studied at other sites in the body, evidence suggests that fibroblasts in different locations behave differently.¹⁰ The present study was undertaken to elucidate some of the chemoattractant influences acting on ocular fibroblasts and to determine the chemotactic activity of normal rabbit aqueous humour.

Material and Methods

Fibroblasts Biopsies of Tenon's capsule and sclera were taken from 5 New Zealand White rabbits. These primary explants were placed in 25 cm² plastic tissue culture flasks (Sterilin) and incubated at 37 degrees C in 5 per cent CO₂/95 per cent air. The cultures were fed twice weekly with F10 medium with 10 per cent new born calf serum, containing 100 units per ml of streptomycin and penicillin, and 0.25 units per ml of amphotericin B. Cells grown from the primary explants reached confluence within 3 to 4 weeks, whereupon the cultures from the five different animals were bulked

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This paper is based on a poster displayed at the Ophthalmological Society of the United Kingdom Annual Congress in April 1987.

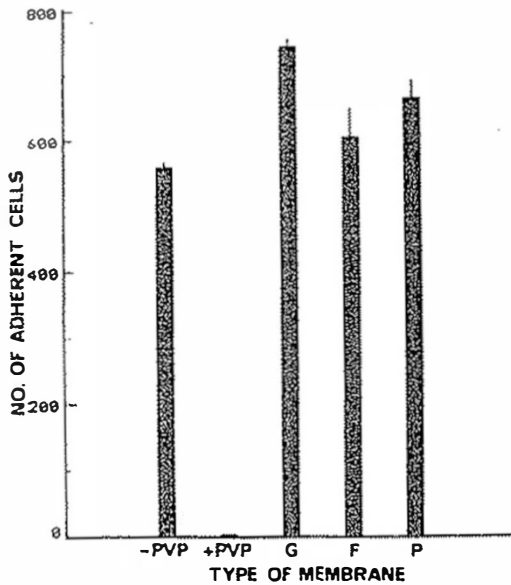


Fig. 1. Graph showing the adherence rate of rabbit Tenon's fibroblasts to various kinds of polycarbonate membranes: -PVP = Polyvinyl-Pyrrolidone Free; +PVP = With Polyvinyl-Pyrrolidone; G = Gelatin and PVP; F = Fibronectin and PVP; P = Poly-L-Lysine and PVP. The same number of cells was added to chambers containing the 5 types of membrane. After 4 hours incubation, the cells attached to the membranes in 20 1,000 \times fields were counted. Four individual chambers were counted for each membrane. Bars show the mean; lines the S.E.M.

together and passaged. Split ratios varied from 1-4 to 1-10. Tenon's fibroblasts were used between passages 2 and 4 in the chemotaxis experiments; scleral fibroblasts were used to prepare conditioned medium at 2nd passage.

Chemotaxis Chemotaxis was undertaken in a 48-well micro-chemotaxis chamber (Neuro Probe, Cabin John, Maryland), based on the original Boyden Chamber.¹¹ Polycarbonate membranes 25 \times 80 mm, 10 μ m thick and with pores 8 μ m in diameter were used (Nucleopore, Pleasanton, California). Membranes are available with or without the wetting agent polyvinyl-pyrrolidone (+PVP or -PVP). As obtained from the manufacturer, membranes with polyvinyl-pyrrolidone would not allow satisfactory adherence of fibroblasts and the morphology of cells adherent to the PVP free membranes was highly abnormal. Accordingly, membranes with PVP were treated in the following way to promote the adherence of the cells:

(a) Gelatin. Membranes were placed in a glass beaker containing 0.5 per cent acetic acid at 50

degrees C for 20 minutes. They were then washed twice in glass distilled water, following which they were placed in a solution containing 5 mg/l Porcine gelatin type 1, 300 bloom (Sigma) in distilled water at 100 degrees C for one hour. The membranes were then air dried, and subsequently placed in an oven at 100 degrees C for one hour.¹²

(b) Fibronectin. Membranes were placed in a solution of 100 μ g/ml of bovine fibronectin (Sigma) in distilled water for 30 minutes and then air dried.¹³

(c) Poly-L-Lysine. Membranes were soaked in 1 per cent Poly-L-Lysine (MW > 300,000 Sigma) in distilled water for two hours and then washed in distilled water for two hours.¹⁴

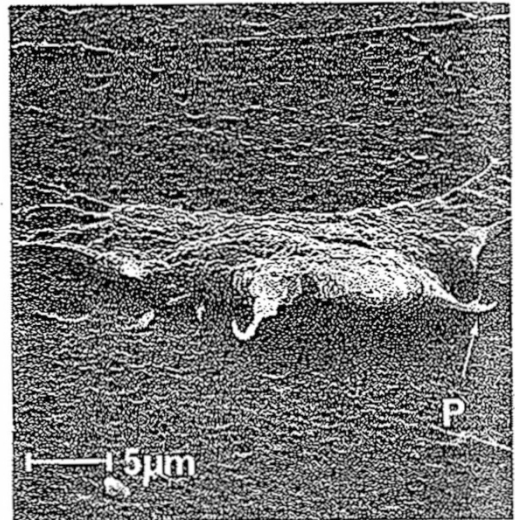


Fig. 2(a). Scanning electron micrograph of a Tenon's fibroblast with a process passing into an 8 μ m diameter pore (P) in the polycarbonate membrane.



Fig. 2(b). Light micrograph showing cells that have successfully migrated through the pores and are adherent to the membrane. Blurs in the background are cells adherent to the membrane that have not yet migrated.

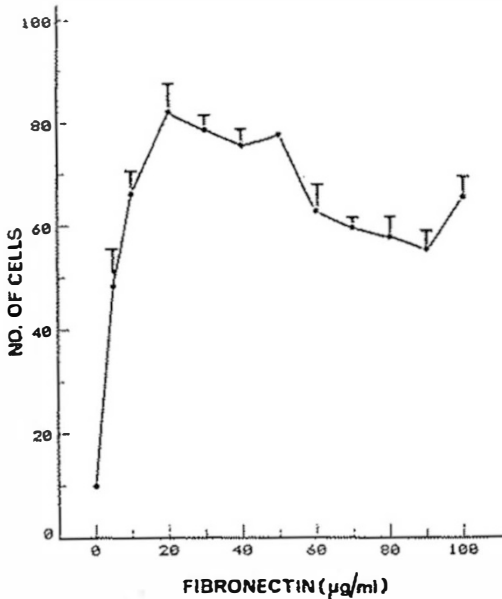


Fig. 3. Dose response curve of fibronectin. Cells that have successfully migrated through the pores have been counted in 20 1,000 \times fields (0.54 mm²). Each point is the mean of three chambers. Bars are standard errors of the mean.

Membranes with PVP, treated with gelatin, allowed the greatest fibroblast adherence rate (Fig. 1) as well as normal fibroblast morphology. Accordingly, gelatinised membranes were used in all the chemoattraction experiments. All membranes were used shiny side up in the chemotaxis chamber, according to the manufacturer's instructions. Chemoattractants were added to the lower chambers of the array and dilutions were made with serum free F10 medium.

The fibroblasts were used just on becoming confluent, approximately 4–7 days after passaging. The cells were removed from their culture flasks by brief exposure (3 minutes) to 0.25 per cent Trypsin and 0.02 per cent EDTA. Exposure to trypsin for longer than this leads to a reduction of chemotactic activity.¹² Cells were suspended in 10 ml F10 with 10 per cent new born calf serum, and centrifuged at 300 g for 10 minutes, following which they were washed once with serum free F10. Cell counts were made using a Coulter counter, and they were finally suspended at a concentration of 7×10^5 cells/ml. Fifty μ l of cell suspension was added to each of the top chambers of the array.

The micro-chemotaxis chamber was incubated at 37 degrees C in 5 per cent CO₂/95 per cent air for 4 hours. The polycarbonate membrane was removed

from the chamber, placed in 100 per cent ethanol for 30 seconds, air dried, and stained with haematoxylin.

Membranes were mounted on a glass slide and then examined with a light microscope at 1,000 \times magnification (Olympus, Tokyo). The number of cells that had migrated through the pores, towards the chemoattractant, was estimated by counting cell nuclei on the distal side of the membrane. Twenty fields were examined for each of the 48 chambers. This represented 0.54 mm² or 1/15th of the membrane corresponding to each chamber. A membrane was also prepared for scanning and transmission electron microscopy (Fig. 2).

Fibronectin Bovine fibronectin (Sigma) was stored at -20 degrees C until used. In all chemotaxis assays of fibroblast conditioned medium and aqueous humour, fibronectin was run as a positive

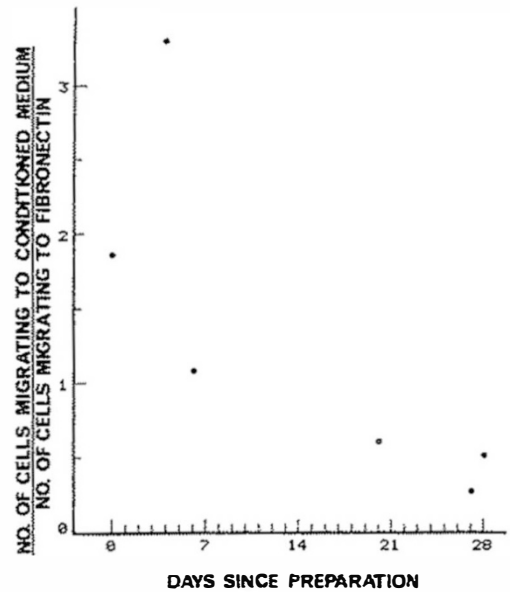


Fig. 4. Graph showing the decline in the relative chemoattractiveness of fibroblast conditioned medium over time. The medium was stored at -20 degrees C. The chemoattractive activity was measured as the ratio of the number of cells in 20 1,000 \times fields migrating to fibroblast conditioned medium versus the number of cells in 20 1,000 \times fields migrating to an optimal dose of fibronectin. Initially fibroblast conditioned medium was 2 to 3 times as active as fibronectin; after storage for 28 days this had reduced to about 0.5 times the activity. Each point is the mean of 3 observations. Standard errors of the mean for fibroblast conditioned medium were all less than 16 per cent.

control, with serum free F10 medium as a negative control.

Fibroblast Conditioned Medium A confluent culture of scleral fibroblasts was washed three times with phosphate buffered saline. It was then washed twice with 10 ml of serum free F10 medium, and a further 5 ml of this medium was incubated in the flask for 24 hours. This fibroblast conditioned medium was centrifuged at 300 g for ten minutes to remove floating cells. The supernatant was then aliquoted and frozen at -20 degrees C until used in the chemotaxis assay.¹⁵

Aqueous Humour Nine New Zealand White rabbits (18 eyes) weighing approximately 2 kg were used in the study. The rabbits were sacrificed by an overdose of barbiturates. Immediately post mortem, a paralimbal transcorneal paracentesis was made using a 25G needle and the aqueous humour was allowed to drip from the end of the needle. Approximately 100 μ l was obtained from each animal. The aqueous was either used immediately or was stored at -20 degrees C. Stored in this way the

aqueous retained its chemotactic activity for at least 6 weeks.

Results

Fibronectin is chemoattractant to rabbit Tenon's fibroblasts with an optimal dose of 20 μ g/ml (Fig. 3). At doses higher than this there is a slow decrease in effect. This continues to occur between 100 μ g/ml and 1,000 μ g/ml (data not shown). The optimal dose of 20 μ g/ml fibronectin has been used as a positive control in subsequent experiments. Boiling a solution containing this dose for 5 minutes is sufficient to deactivate the chemoattractive effect.

Scleral fibroblast conditioned medium is also strongly chemoattractant to Tenon's fibroblasts. When the conditioned medium was first prepared it was 2 to 3 times as potent in its chemoattractive effect as an optimal dose of fibronectin. After storage for 20 days at -20 degrees C, fibroblast conditioned medium was approximately half as potent as fibronectin (Fig. 4).

The response of the cells to the chemoattractants varies considerably depending on the confluency of the culture, the time since passaging, the time since the last feeding, and probably a number of other undefined factors. This makes comparison of results obtained on different days unreliable, in spite of using positive and negative controls in each run. However, all the specimens of normal rabbit aqueous humour were strongly chemoattractant, and the effect seen was always considerably greater than that of the optimal dose of fibronectin. An initial dose response curve was obtained using the aqueous from two eyes, as seen in Figure 5, where it is apparent that the optimal concentration is between 10 per cent and 20 per cent aqueous humour. This dose response curve has been confirmed four times. Using a smaller range of concentrations of aqueous humour it has been possible to compare 14 specimens (8 different animals) simultaneously (Fig. 6).

In a Zygmund-Hirsch¹⁷ chequer board analysis of a sample of aqueous humour (Fig. 7), it appears that the aqueous exerts a predominantly chemotactic effect, while at low concentrations there is a chemokinetic effect, which falls off at higher doses. Boiling rabbit

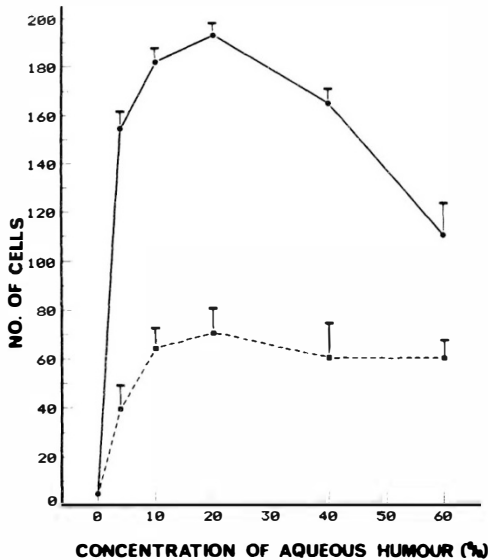


Fig. 5. Graph showing the chemoattractive activity of normal rabbit aqueous humour (—) and of similar aqueous humour that has been boiled for 5 minutes (---). The cell count was made in 20,000 \times fields. Each point is the mean of four chambers. The normal aqueous was taken from one eye of 2 rabbits, while the boiled aqueous was derived from their other eyes. Bars are 1 S.E.M. 20 μ g/ml fibronectin caused 90 cells to migrate. For 20 per cent aqueous, boiling reduces the chemoattractiveness activity by about 65 per cent ($p = 0.014$ Mann-Whitney U test).

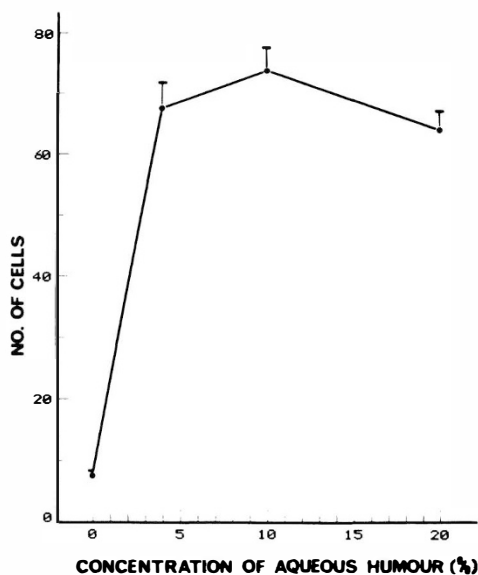


Fig. 6. Dose response curve of chemoattractive activity of normal rabbit aqueous humour. At 0 per cent aqueous (serum free F10 medium), point is the mean of 3 observations. Other points are the means of 14 aqueous specimens taken from 8 rabbits and run simultaneously in a single 48-well chemotaxis chamber. Cells have been counted in 20 1,000 \times fields for each sample. Bars are 1 S.E.M. 20 μ g/ml fibronectin caused 45 cells to migrate. 4 per cent aqueous causes a highly significant increase in the number of cells migrating across the membrane ($p < 0.001$ Student's *t* test $df = 15$). There is no significant difference between the effect of 10 per cent aqueous and 4 per cent aqueous ($0.1 < p < 0.5$) or between the effect of 20 per cent aqueous and 4 per cent aqueous ($P > 0.5$).

aqueous for 5 minutes still leaves it with a substantial chemoattractant effect (Fig. 5). Analysis of the chequer board for boiled aqueous, from the same animal as in Figure 7, shows that the predominant effect is still chemotactic, although this is reduced, and there is a similar chemokinetic effect (Fig. 8).

Discussion

Fibronectin is chemoattractive to rabbit Tenon's fibroblasts. This is relevant since fibronectin occurs in healing wounds¹⁸ and is responsible for the adherence of the cells to the connective tissue matrix.¹⁹ Fibronectin also occurs in aqueous humour,²⁰ and is probably one of numerous factors responsible for the migration of fibroblasts towards a healing trabeculectomy. Although we found that

20 μ g/ml was the optimal dose of fibronectin, the literature is confused on this and optimal doses of 1.2 μ g/ml,¹⁶ 20 μ g/ml²¹ and 100 μ g/ml^{15,22} have been mentioned. This may relate to differences in the measurement or preparation of fibronectin, as well as to the source of the fibroblasts used in the assay, since fibroblasts of diverse origins may respond differently to the same chemical mediator.¹⁰

Fibroblast conditioned medium is also a chemoattractant for rabbit Tenon's fibroblasts. This is interesting as it helps to explain the self-perpetuating nature of scarring. Once a few fibroblasts have been attracted to a healing trabeculectomy, they are able to call up reinforcements, and the process continues. Some of the factors known to be synthesised by fibroblasts are collagen, fibronectin, and proteoglycans. The former two are known to be chemotactic to further fibroblasts.^{15,16,21,23} However, Mensing *et al.* proposed that there is another, as yet unidentified, chemotactic

		UPPER CHAMBERS			
		% Aqueous			
		0	4	10	20
LOWER CHAMBERS	0	26	25	16	6
	4	87	63	40	20
	10	118	55	38	16
	20	152	88	54	15

Fig. 7. A chequer board analysis of normal rabbit aqueous humour. Aqueous humour is added to both the upper and lower chambers of the array, such that the Tenon's fibroblasts are exposed to (a) a positive gradient of the chemoattractant, (b) a negative gradient, and (c) no gradient but merely an increased concentration of the chemoattractant, equal in both the upper and lower chambers (between the diagonal lines). Normal aqueous humour is powerfully chemotactic (cf. optimal dose of fibronectin which caused 62 cells to migrate) but at low doses there is a moderate chemokinetic effect which diminishes at higher doses. A similar result has been seen in chequer boards done on 3 other aqueous specimens.

		UPPER CHAMBERS			
		% Aqueous			
		0	4	10	20
LOWER CHAMBERS	0	26	24	37	23
	4	60	36	47	29
	10	85	57	43	37
	20	79	51	55	37

Fig. 8. A chequer board analysis of aqueous humour derived from the other eye of the rabbit in Figure 7 and run simultaneously in the same experiment. This aqueous has been boiled for 5 minutes. The aqueous retains much of its chemotactic activity, with similar chemokinetic activity.

factor(s) in fibroblast conditioned medium besides collagen and fibronectin.¹⁵

Fibronectin and fibroblast conditioned medium are only two of numerous factors that have been demonstrated to be chemotactic, using non-ocular fibroblasts. These factors include elastin,²⁴ tropoelastin,¹⁴ collagen,²³ fibronectin peptides,²¹ platelet derived growth factor,²⁵ leukotriene B₄,²⁶ coagulation products,²⁷ lymphokines,²⁸ and activated complement.²⁹ Doubtless many of these substances also are chemotactic for ocular fibroblasts. It follows that any drug used to inhibit the process of fibroblast chemoattraction will have to be directed at the locomotory function of the cells, since a block against any one chemoattractant will be easily circumvented.

Normal rabbit aqueous humour is strongly chemotactic to rabbit Tenon's fibroblasts. This was confirmed in a chequer board analysis.¹⁷ Although the rabbit blood-aqueous barrier is very easily disrupted, the fact that the moderate chemokinetic effect seen at low concentrations of aqueous was reduced at 20 per cent aqueous, mitigates against a disruption of the barrier. Since protein can be chemokinetic one would anticipate a far greater chemokinetic effect which would increase with higher concentrations of aqueous, had

the blood aqueous-barrier been disrupted.³⁰ The aqueous was also removed immediately post mortem and was allowed to drip out of the eye under the influence of the intra-ocular pressure; this would also reduce the possibility of disrupting the blood-aqueous barrier.

We have found that boiling fibronectin containing solutions for 5 minutes is sufficient to deactivate the chemoattractant effect. Boiled aqueous humour retains a proportion of its chemoattractant activity which is confirmed by chequer board analysis, to be predominantly chemotactic. The reduction in the chemotactic activity caused by boiling is slightly more than can be accounted for by the effect of removing 20 µg/ml of fibronectin. The fibronectin concentration of bovine eyes has been measured at 2.46 µg/ml²⁰ or about 100th of the level in human blood. Fibronectin is also known to be produced by corneal endothelial cells³¹ and meshwork cells in tissue culture.³²

2.46 µg/ml is about a log unit lower than the optimal concentration of fibronectin that we found. However, as mentioned above, there are difficulties in estimating the true optimal dose of fibronectin. So, a component of the chemotactic activity of normal rabbit aqueous humour may be accounted for by fibronectin. Accurate measurement of the fibronectin content of rabbit aqueous humour will be needed to clarify this. However, what accounts for the rest of the chemotactic activity of rabbit aqueous humour is at present unknown. Some chemoattractants, e.g. platelet derived growth factor retain their chemotactic properties after boiling for 5 minutes,³³ and this substance could possibly contribute to the chemoattractiveness of normal rabbit aqueous humour.

Given that rabbit aqueous humour is powerfully chemotactic to rabbit Tenon's fibroblasts, it is surprising that it has not been found to be a chemoattractant for macrophages³⁴ and neutrophils.³⁰ In addition, Tapper *et al.*³⁵ examined the migration of capillary endothelial cells in response to normal rabbit aqueous humour, and found no stimulatory activity.

Rabbit eyes are known to exhibit a marked inflammatory response and to heal up a fistulising wound within 17 days.⁸ A major

part of this healing response may be attributable to the intrinsic chemotactic activity of normal aqueous humour, for fibroblasts, as demonstrated in this paper. The role of aqueous humour in the failure of human trabeculectomies remains to be clarified, and further studies are currently being undertaken to this end.

We gratefully acknowledge the generous financial support provided by the T. F. C. Frost Charitable Trust, the International Glaucoma Association and a Moorfields Endowment Grant.

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