

Effect of TNF, IL-1, and IL-6 on the proliferation of human Tenon's capsule fibroblasts in tissue culture

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Abstract

Trabeculectomy is a commonly performed procedure for primary open angle glaucoma and is successful in the majority of cases. However, certain factors including aphakia, previous surgery, secondary glaucomas, ethnic origin, and the long term use of topical antiglaucoma medications may be associated with a reduced success rate. The mechanism (or mechanisms) which influence clinical outcome following trabeculectomy remain elusive. Alterations in the composition of the conjunctiva or aqueous humour may be partly responsible for this effect, and this could be mediated by cytokines. In this study we found that tumour necrosis factor (TNF), and interleukin 1 (IL-1) were capable of stimulating the proliferation of Tenon's capsule fibroblasts in tissue culture. Interleukin 6 (IL-6) did not appear to have any effect. The relevance of this to wound healing following trabeculectomy is discussed.

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Trabeculectomy is commonly performed to control intraocular pressure (IOP) in glaucomatous eyes and is successful in the majority of cases.¹ Risk factors which may influence the outcome of filtering surgery include: aphakia,^{2–4} youth,^{5–7} ocular inflammation and neovascular glaucoma,^{8–12} ethnic origin,^{13–15} previous surgery,^{16–17} long term use of topical antiglaucoma medications,^{18–21} and possibly diabetes.²²

The mechanism or mechanisms by which these factors reduce success is not fully understood. Changes in the number of inflammatory cells and fibroblasts have been shown to occur in the conjunctiva of certain eyes at increased risk of failure,^{15 23–25} and it is possible that this may influence the wound healing process following surgery. It may be that cytokines released by these inflammatory cells are responsible for the effects seen.

Also, as a result of drainage surgery, aqueous humour flows into the subconjunctival space. The effect of this aqueous on the wound healing process should also be considered, as changes in the composition (that

may possibly occur in cases of neovascular or uveitic glaucoma) could also influence the scarring process.

In this paper, we examine the effects of three cytokines produced by inflammatory cells and possibly present in the aqueous under certain conditions on the proliferation of human Tenon's capsule fibroblasts in tissue culture.

Materials and methods

TISSUE CULTURE

Local ethics committee approval was granted for the work and patients gave their written informed consent. Cultures of human Tenon's capsule fibroblasts were established from biopsies taken from three different eyes (eyes 1–3) of patients (aged 57, 61, 77 years) undergoing ocular surgery. The biopsies were taken from Tenon's capsule on the superior aspect of the globe at the end of routine cataract surgery. The biopsies were placed in tissue culture medium and transported to the laboratory.

The biopsies were washed twice in phosphate buffered saline (PBS) containing penicillin 1% (Britannia Pharmaceuticals Ltd, Redhill, Surrey), streptomycin 1% (Sigma Chemical Company Ltd, Poole, Dorset), and fungizone 1% (Gibco Life Technologies, Paisley, Scotland). They were then dissected and placed in a tissue culture flask in Dulbecco's modified Eagles medium (DMEM) (Gibco Life Technologies) containing 10% fetal calf serum (FCS) (Advanced Protein Products Ltd, Briesly Hill, West Midlands) penicillin 0.1%, streptomycin 0.1%, and fungizone 1%. The biopsies were then incubated at 37°C in 5% carbon dioxide in a humidified air atmosphere. When the fibroblast cultures became confluent, they were detached from the flasks using trypsin and EDTA and replated into 75 cm² tissue culture flasks. The fibroblasts were characterised morphologically. Before each experiment the cell monolayer was washed twice with PBS, incubated at 37°C in trypsin and EDTA for 3–5 minutes until cells detached, washed by centrifugation at 230 g for 10 minutes in DMEM with 10% FCS, resuspended in serum free DMEM, and recentrifuged. The cells were then resuspended in serum free DMEM, and

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the number of viable cells determined using the trypan blue exclusion method. The cells were then diluted in DMEM with 10% FCS to a concentration of 2×10^4 live cells per ml.

CYTOKINES

Recombinant human tumour necrosis factor α (rhTNF- α) (specific activity 2.7×10^8 units/mg) was a generous gift from Strangeways Research Laboratories, Cambridge; recombinant human interleukin 1 β (rhIL-1 β) (specific activity 1×10^8 units/mg) was a generous gift from Immunex Research and Development Corporation, Washington, USA; and recombinant human interleukin 6 (rhIL-6) (specific activity 4.1×10^7 units/mg) was a generous gift from Imperial Chemical Industries Ltd, Cheshire. All cytokines were used in serial dilutions starting at 1000 units/ml.

PROLIFERATION STUDIES

Antibiotics were not used for the duration of proliferation studies. A total of 100 μ l of cells in DMEM with 10% FCS was plated into each well of a 96 well tissue culture plate. Cells were incubated at 37°C in 5% carbon dioxide in a humidified air atmosphere. After 24 hours, the medium was removed and the cells washed once with serum free DMEM.

The medium was replaced with 100 μ l of fresh serum free DMEM and the cells incubated for a further 24 hours at 37°C in 5% carbon dioxide in a humidified air atmosphere. Some 100 μ l of different cytokine concentrations in serum free DMEM were then added to the wells. Control wells received corresponding cytokine diluents in serum free medium. The cells were incubated for a further 48

hours. Volumes of 50 μ l of serum free medium containing 18.5 kBq of 3 H-thymidine was added to each well for the final 20 hours of incubation. The cells were then harvested onto filter paper and the amount of 3 H-thymidine incorporated into the DNA of the cells was determined by scintillation counting. Each cytokine dilution was performed in triplicate. The value obtained for each cytokine dilution was divided by the control mean to give percentage control values. Fibroblasts derived from three different eyes were tested twice using the three cytokines. Experiments were performed using cells from passages 5–8.

STATISTICAL ANALYSIS

Results from individual wells were compared with controls using analysis of variance (ANOVA) and the Dunnett multiple comparisons test.

Results

The results of the experiments are shown in Figures 1–3. The effect of each tested cytokine on the proliferation of fibroblasts derived from three different eyes (eyes 1–3) is shown as the mean (SEM) for each experiment, with the experiment being performed twice.

The effect of rhTNF- α on fibroblast proliferation was tested at concentrations ranging from 10^3 to 10^{-3} units/ml. A statistically significant 1.5–3.5-fold increase in proliferation on cells derived from eye 1 was produced by rhTNF- α at 100 units/ml ($p < 0.01$), 10 units/ml ($p < 0.01$), and 1 unit/ml ($p < 0.05$). A statistically significant inhibitory effect at 1000 units/ml ($p < 0.01$) was noted in one experiment. Scintillation counts for these cells

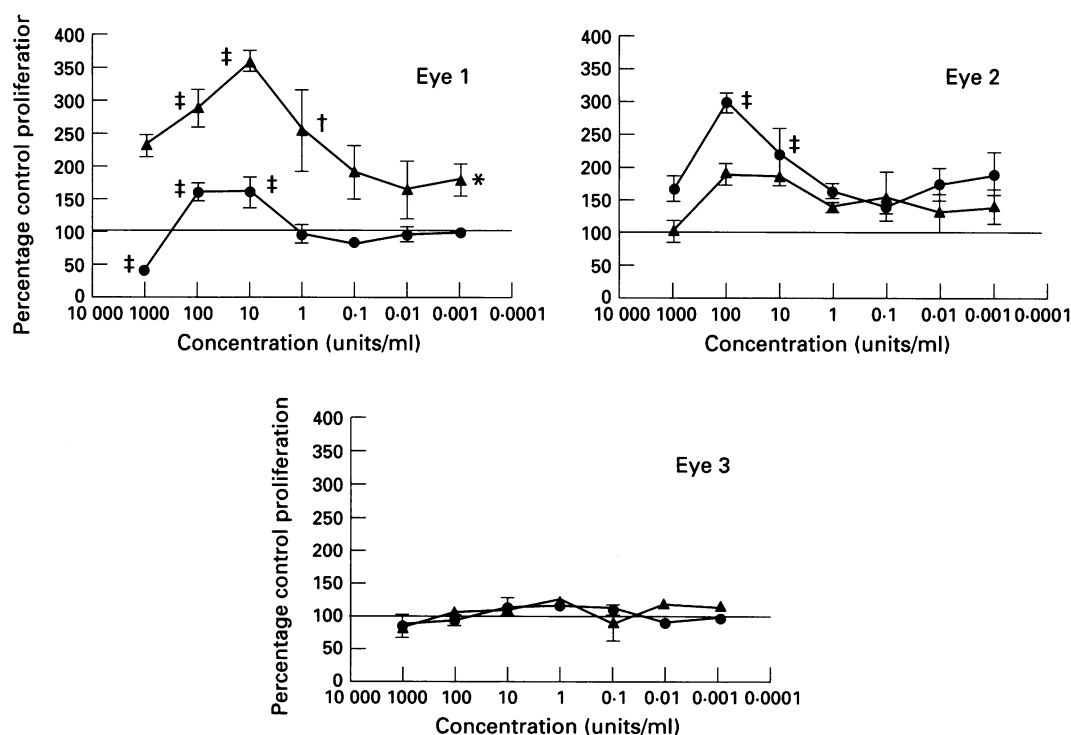


Figure 1 Effect of TNF- α on the proliferation of human Tenon's capsule fibroblasts in tissue culture. The results of two experiments on cells derived from three different eyes are shown. Each point shows the mean (SEM) percentage control proliferation for three wells. *Indicates result based on two wells. †= $p < 0.05$, ‡= $p < 0.01$.

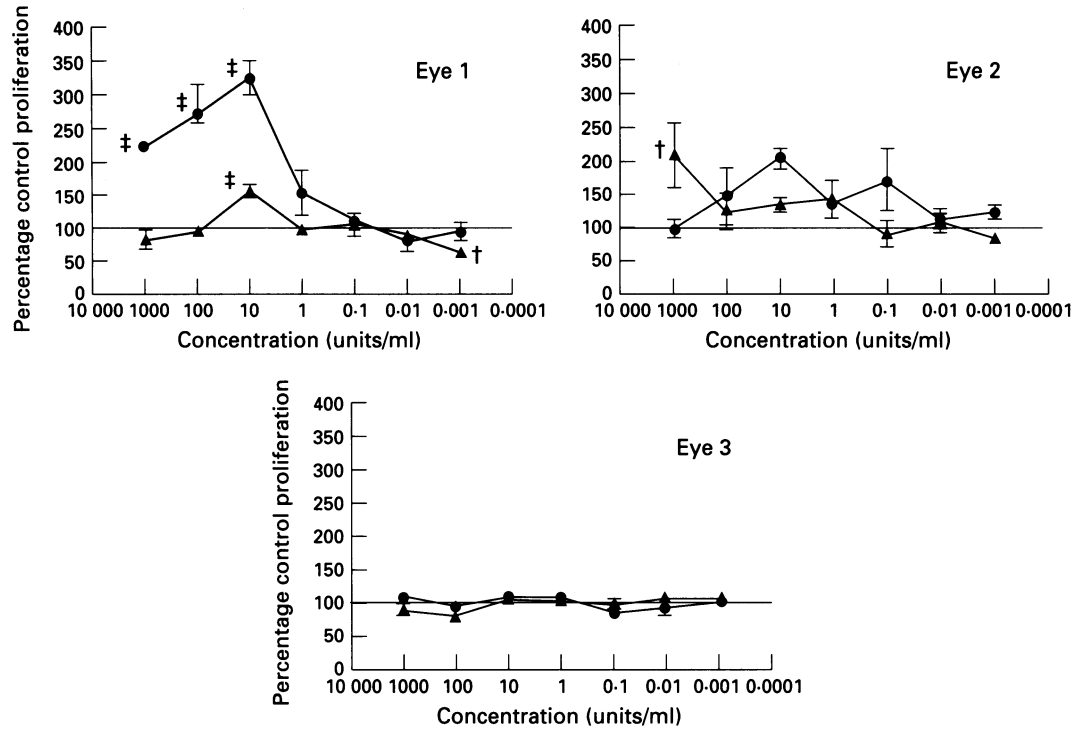


Figure 2 Effect of IL-1 β on the proliferation of human Tenon's capsule fibroblasts in tissue culture. The results of two experiments on cells derived from three different eyes are shown. Each point shows the mean (SEM) percentage control proliferation for three wells. †= $p < 0.05$, ‡= $p < 0.01$.

ranged from 316–4861 counts per minute (cpm). A statistically significant 2.0–3.0-fold increase in proliferation compared with controls was seen in cells from eye 2 at 100 units/ml ($p < 0.01$) and 10 units/ml ($p < 0.01$) and cpm ranged from 396–3440. rhTNF- α had no effect on cells from eye 3 (cpm ranged from 2171–12 646) (Fig 1).

rhIL-1 β was tested at the same concentrations as rhTNF- α and cells from eye 1 produced a statistically significant 1.5–3.0-

fold increase in proliferation compared with controls at 1000 units/ml ($p < 0.01$), 100 units/ml ($p < 0.01$), and 10 units/ml ($p < 0.01$). Scintillation counts ranged from 647–4740 cpm. Cells from eye 2 produced variable results that were only significant ($p < 0.05$) in one experiment at 1000 units/ml (cpm ranged from 493–4302). Cells from eye 3 did not give any statistically significant changes in proliferation compared with the controls (cpm ranged from 6143–12 139) (Fig 2).

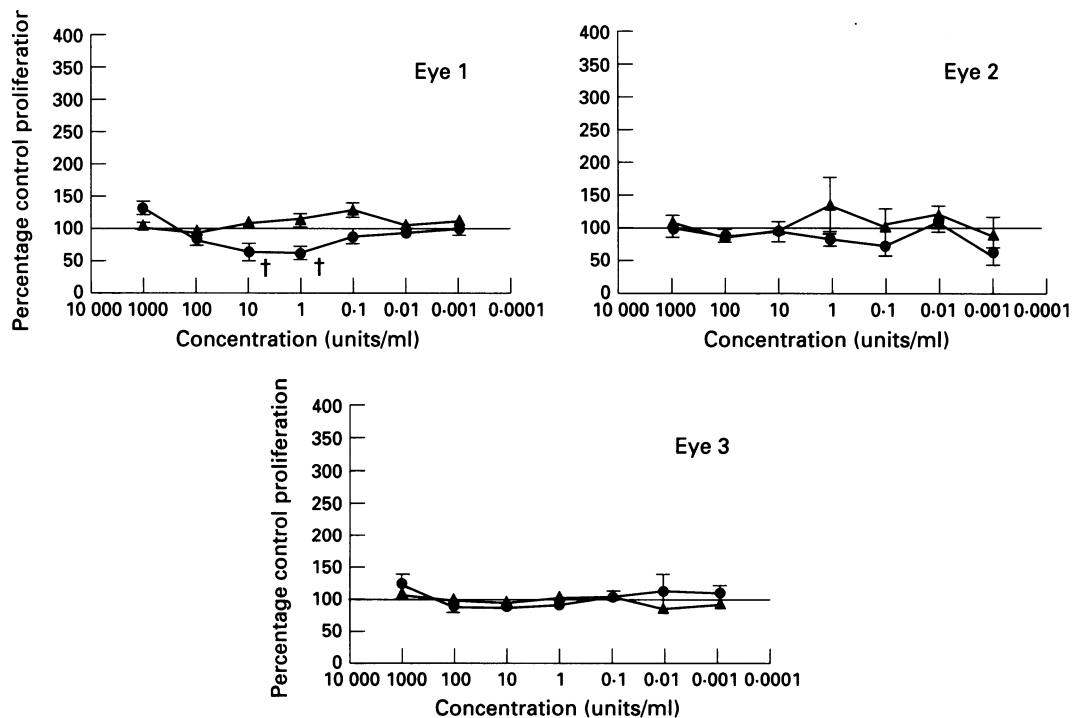


Figure 3 Effect of IL-6 on the proliferation of human Tenon's capsule fibroblasts in tissue culture. The results of two experiments on cells derived from three different eyes are shown. Each point shows the mean (SEM) percentage control proliferation for three wells. †= $p < 0.05$.

rhIL-6 did not appear to stimulate proliferation compared with controls in any of the experiments. A significant ($p < 0.05$) inhibition of proliferation was seen in one experiment in cells from eye 1 at 10 units/ml and 1 unit/ml. Scintillation counts ranged from 330–1980 for cells from eye 1; 227–3417 for cells from eye 2; and 5894–12 264 for cells from eye 3 (Fig 3).

Discussion

Several conditions may influence the outcome of trabeculectomy, including aphakia,²⁻⁴ young age,⁵⁻⁷ ethnic origin,¹³⁻¹⁵ secondary glaucomas (including uveitic),⁸⁻¹² previous drainage surgery,^{16 17} and the long term use of topical antiglaucoma medications.¹⁸⁻²¹

During the wound healing process, the proliferation, migration, and production of extracellular matrix and collagen by fibroblasts is an intricately regulated process. Inflammatory cells produce a multitude of cytokines, some of which are growth promoting and capable of regulating the function of fibroblasts. The effect of individual cytokines on any one cell type is dependent on many factors including the presence of other cytokines, the degree of cellular activation and the ability of the cell to produce biologically active products.²⁶ It may be that if the number or degree of activation of inflammatory cells is increased in the conjunctiva of certain eyes, they may exhibit an exaggerated wound healing response, which may be mediated by the cytokines released from the inflammatory cells. Alternatively, an alteration in the cytokine composition of the aqueous humour in certain eyes (such as eyes with uveitic or neovascular glaucoma) may influence the healing process once this is exposed to the subconjunctival fibroblasts following trabeculectomy.

In some of these cases the number of inflammatory cells in the conjunctiva has been shown to be increased. Broadway *et al*²⁵ have shown that in the conjunctiva of patients with uveitic glaucoma, there is an increase in the number of fibroblasts, lymphocytes, and macrophages. They proposed that the wound healing response may be upregulated by an increase in the number and degree of activation of these inflammatory cells, which in turn increases the number and activity of fibroblasts. Broadway *et al*²⁴ and Sherwood *et al*²³ have both shown an increase in the number of inflammatory cells and fibroblasts in the conjunctiva of patients on long term topical antiglaucoma medications, and suggest that this may have a role to play in the failure of filtration surgery. Broadway *et al*¹⁵ have shown an increase in the number of macrophages in the conjunctiva of black patients undergoing trabeculectomy, but this finding was not confirmed in a study by McMillan *et al*.²⁷ Stewart *et al*²⁸ looked at the effect of age on the conjunctival cell composition of patients with chronic open angle glaucoma, and did not find an increase in inflammatory cells between the age groups examined. However, this study did not examine specimens from eyes under 40 years of age.

The aqueous humour is known to contain several cytokines capable of affecting fibroblast activity.²⁹⁻³³ It is also known to contain substances which are cytotoxic to Tenon's fibroblasts.³⁴ In eyes with uveitis IL-6, IL-1, and IL-2 have been detected^{35 36} in the aqueous and in experimental endotoxin induced uveitis raised levels of TNF and IL-6 have been detected.³⁷ It may be that in eyes with uveitic glaucoma these cytokines are released onto the subconjunctival fibroblasts following trabeculectomy, and so affect the healing process. The aqueous of eyes with neovascular glaucoma may also contain cytokines not normally present in the aqueous.

Several cytokines including TGF- β , FGF, and IGF-1 are known to have effects on ocular fibroblasts^{29 33} and are probably involved in the ocular wound healing process. In this study we examined the effect of three cytokines (that are known to have effects on non-ocular fibroblasts) to see if they may be involved in the ocular wound healing process, via a stimulatory effect on the proliferation of human Tenon's capsule fibroblasts. TGF- β 1 has been shown to produce a two to three times greater stimulation of human Tenon's capsule fibroblast proliferation compared with media alone,²⁹ which is similar to some of the results seen in this study with rhTNF- α and rhIL1- β .

Macrophages and lymphocytes produce TNF- α ,³⁸⁻⁴⁰ which can stimulate the proliferation of certain types of fibroblasts,^{26 38-42} and also inhibit the proliferation of certain other cell types.⁴⁰ TNF- α has also been shown to affect fibroblast collagen production, collagenase activity, and extracellular matrix production.^{26 38 42 43} Induction of platelet derived growth factor production may be one mechanism by which TNF- α stimulates fibroblast proliferation⁴² via an autocrine mechanism. In one study,⁴¹ higher doses of TNF- α (greater than 500 ng/ml) caused cell death, and this may have been mediated in part by the production of prostaglandins as the cells appeared viable (although non-proliferative) when the experiment was performed in the presence of indomethacin, an inhibitor of PGE₂ synthesis. In this study we were able to demonstrate a proliferative effect of rhTNF- α on human Tenon's capsule fibroblasts derived from two different eyes in tissue culture. Cells from a third eye (eye 3) did not show a response and this may have been due to a lack of TNF receptors in these cells; an inability by the cells to process a response following TNF receptor stimulation; or that the cells were already proliferating at a maximum rate. Differences between cells from the same eye on repeat experiments are probably due to the biological variation of cells in culture. Monocytes/macrophages and lymphocytes are capable of producing IL-1 β ,^{40 44-46} which is able to stimulate or enhance fibroblast proliferation^{26 40 41 44 46 47} and can have different effects on different types of fibroblasts.⁴⁰ For example, it can stimulate dermal fibroblasts to proliferate, but has little effect on synovial fibroblasts. However, in the presence of indomethacin synovial fibroblasts will proliferate.

This may be related to PGE₂ production by the cells, and the fact that dermal fibroblasts can proliferate in the absence of indomethacin may be due to a limited ability to produce PGE₂.⁴⁰ IL-1 β, like TNF-α, may stimulate fibroblasts to proliferate by inducing the synthesis and secretion of platelet derived growth factor.⁴²⁻⁴⁶ It has also been shown to have effects on collagen production, collagenase activity, and extracellular matrix production.^{26 40 42 43 46 48 49} In this study, rhIL-1 β was shown to stimulate proliferation of human ocular fibroblasts from eye 1, but cells from eye 2 gave variable responses on repeat experiments and cells from eye 3 did not respond at all. The effect of PGE₂ was not examined in this study. The reasons for differences seen in the responses to rhIL-1 may again be related to a lack of receptors, an inability to process a response by some of the cells, or the fact that cells from eye 3 may have already been proliferating at a maximum rate. IL-6 is produced by monocytes/macrophages, lymphocytes, and certain other cell types.⁵⁰ IL-1 β and TNF-α can also stimulate fibroblast cultures to produce IL-6.⁵¹ IL-6 has not previously been shown to stimulate proliferation of fibroblasts^{40 41} and in this study we did not observe a stimulatory effect on the proliferation of human ocular fibroblasts at the concentrations tested. In one experiment we did observe some inhibition of proliferation, but this was not seen in the other experiments. IL-6 may, however, have effects on the production and metabolism of extracellular matrix.^{51 52}

It is always difficult to extrapolate from the tissue culture environment to the in vivo situation. In tissue culture, cells are in an isolated environment and not subject to the influences of other cell types as occurs in vivo. The effect of single cytokines on proliferation should be viewed cautiously as the effect of one cytokine may modulate the response to other cytokines. To show that the response to rhTNF-α and rhIL-1 β seen in these experiments is specific, further experiments using blocking monoclonal antibodies would be necessary. Despite the limitations of tissue culture, it is an accepted model for the investigation of cell responses, and does have the advantage that individual effects can be examined.

The function of fibroblasts in producing scar tissue is regulated in part by a complex network of cytokine effects. Unravelling this network may enable us to influence postoperative scar tissue formation by modulation of specific events. In certain eyes with glaucoma, that are thought to have a poorer outcome to trabeculectomy, alteration in the cellular composition of the conjunctiva or composition of the aqueous may be responsible for the enhanced wound healing seen postoperatively. This may be mediated in part by cytokines present in the aqueous or released from the inflammatory cells in the conjunctiva. The cytokines examined here may have a role to play in this process. Future work will hopefully elucidate events more clearly, and possible future modulation of these processes may provide mechanisms for control of postoperative scarring.

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