

SCIENTIFIC CORRESPONDENCE

In vivo production of interferon β by human Tenon's fibroblasts; a possible mediator for the development of chronic conjunctival inflammation

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Background: Chronic inflammation may develop from failure of the immune system to deactivate itself during resolution of the wound healing response, and is recognised as a major risk factor for trabeculectomy failure. Fibroblast/T cell interactions may contribute to aggressive scarring. Our previous research showed that in vitro human Tenon's fibroblast produced interferon β was responsible for preventing T cell apoptosis, suggesting that this interaction could contribute to the development of chronic inflammation.

Methods: Immunohistological techniques were used to investigate the in vivo components of this particular fibroblast/T cell interaction in conjunctival biopsies from glaucoma patients undergoing filtration surgery.

Results: Fibroblast produced interferon β and T lymphocytes were identified in human conjunctiva.

Conclusion: The components of fibroblast mediated prevention of T cell apoptosis were identified in vivo, suggesting that the development of this interaction is possible and that it may contribute to the development of chronic inflammation and excessive scarring.

Chronic inflammation is a clinically recognised risk factor for the failure of glaucoma filtration surgery owing to excessive conjunctival scarring.¹ However, the cellular mechanisms contributing to the pathogenesis of chronic inflammation in the conjunctiva are not fully understood. The immune system plays an essential part in the wound healing response after glaucoma surgery, especially during the inflammatory phase when there is an influx of neutrophils, macrophages, and lymphocytes into the injury site.^{2–4} During resolution of the wound healing reaction, the immune response must deactivate itself so that inflammatory cell numbers are reduced to avoid the potential damaging effects of a persistent population of activated inflammatory cells.^{5–6} Failure of the immune system to deactivate itself could play an important role in the development of chronic inflammation, where persistent inflammatory cells would continue to secrete cytokines and growth factors stimulating fibroblasts to produce excessive scar tissue.

The failure rate of filtration surgery in patients with uveitic glaucoma compared to patients with primary open angle glaucoma is generally higher, and this may be due to a difference in the conjunctival cell profile of these patients. Broadway *et al* showed that the preoperative conjunctiva of patients with uveitis contained significantly more fibroblasts, lymphocytes, and macrophages compared to patients with primary open angle glaucoma.⁷ The authors suggested that the presence of a greater number of inflammatory cells and fibroblasts predisposed uveitic patients to generating a more aggressive wound healing reaction following surgery.

Fibroblast/T cell interactions have been proposed to contribute to the development of excessive scarring—for instance, keloid scars may contain persistent fibroblasts and lymphocytes for several years.^{2–8} In addition, increased inflammation has been implicated in disease progression in the fibrosing conjunctival disease ocular cicatricial pemphigoid.⁹

Although the initial components of an antigen generated inflammatory reaction are specific, the factors promoting the development of a chronic inflammatory reaction may be quite similar. This has been suggested by Pilling *et al* and Orteu *et al* who showed that fibroblast produced interferon β (IFN β) prevented T cell apoptosis in diverse chronic inflammatory diseases such as rheumatoid arthritis and eczema.^{10–11} Previously, we showed that in vitro human Tenon's fibroblast produced IFN β prevented cytokine deprivation mediated T cell apoptosis.¹² We hypothesised that this interaction could result in persistent inflammatory cells and contribute to the development of a chronic inflammatory environment in the conjunctiva. In an aggressive wound healing reaction, chronic inflammatory cells could continue to secrete the cytokines and growth factors which would stimulate fibroblasts to produce excessive scar tissue. The aim of this study was to establish whether the components of this particular interaction were present in vivo in samples of human conjunctiva from a group of glaucoma patients.

MATERIALS AND METHODS

Patient selection

Conjunctival tissue was biopsied from glaucoma patients at the time of their filtration surgery as described before.¹³ The tenets of the Declaration of Helsinki were adhered to, with ethical approval obtained from the Moorfields Eye Hospital ethics committee and informed consent obtained from the patients before surgery. The aetiologies of our glaucoma patients consisted of primary open angle glaucoma, angle closure glaucoma, or pigment dispersion glaucoma. One subgroup of patients was about to undergo filtration surgery for the first time, a second subgroup of patients was considered higher risk for conjunctival scarring since these patients had already failed a previous trabeculectomy because of aggressive conjunctival scarring. All our patients had previously received eyedrop treatment for their glaucoma.

Immunohistology

The indirect immunoperoxidase technique was used to detect T cells and fibroblasts in the conjunctival sections. Frozen sections of 6 μ m were air dried for 2 hours and then fixed in acetone:chloroform 1:1 for 5 minutes. The sections were incubated for 10 minutes with normal rabbit serum (NRS) to minimise background staining by blocking non-specific binding sites. They were then incubated with 50 μ l of the primary antibodies—a pan-anti-T IgG monoclonal antibody (mAb) mix (RFT mix) or the AS02 mAb (Dianova, Hamburg,

Table 1 The demographics and immunohistology results of all the glaucoma patients investigated

	High risk	Low risk	p Value
Drug Hx (range months)	201.5 (68–356)	100 (9–285)	0.17
Age (range years)	71 (50–87)	70 (45–81)	0.65
Sex (%)			
M	3 (50)	8 (72.7)	
F	3 (50)	3 (27.3)	
Dx (%)			
1	5 (83.3)	7 (63.6)	0.57
2	1 (16.7)	1 (9.1)	
3	0	3 (27.3)	
Ethnicity (%)			
1	4 (66.7)	7 (63.6)	1
2	2 (33.3)	3 (27.3)	
3	0	1 (9.1)	
Fib grade (range)	1 (1–3)	1 (0–2)	0.44
IFN β grade (range)	2 (1–4)	2 (1–3)	0.3
RFT/UA (range)	1.93 (1.33–17.40)	4.28 (1.09–14.75)	0.6

Diagnosis (Dx): primary open angle glaucoma (1), chronic angle closure glaucoma (2), or pigment dispersion glaucoma (3).

Ethnicity: white (1), Afro-Caribbean (2), or Asian (3).

Patients were divided into 2 groups: low risk (no previous filtration surgery) and high risk (patients undergoing repeat filtration surgery).

Germany) previously used by our group as a specific fibroblast mAb at pretitrated concentrations for 45 minutes at room temperature.¹⁰ After a wash in phosphate buffered saline (PBS), 50 μ l of the secondary peroxidase conjugated goat antimouse IgG Ab (P161, Dako, Bucks) diluted 1/100 in PBS supplemented with 4% normal human serum (NHS) was applied for 45 minutes at room temperature. The reaction was developed using diaminobenzidine (D-5905, Sigma, Dorset, 10 mg of DAB dissolved in 16.6 ml of PBS, followed by 55.3 μ l of 3% hydrogen peroxide) and then counterstained with haematoxylin.

The positive controls used were sections of human tonsil for T cells and skin for fibroblasts. Two negative controls were included, omitting the first layer to detect background staining and using an isotype mAb (a mouse IgG2a, M9144, Sigma, Dorset) as an isotype control for the RFT mix. The isotype control for the fibroblast staining was the RFT mix.

The biotin/streptavidin/alkaline phosphatase technique is a more sensitive technique and was selected to identify IFN β in conjunctival tissue. Sections were incubated for 18 hours with 50 μ l of the mAb against IFN β (MAS291, Harlan Sera-Lab, Loughborough) at a pretitrated concentration diluted in PBS+0.1% BSA. After a wash in TRIS buffered saline (TBS), the sections were incubated with 50 μ l of the horse anti-mouse biotinylated second layer (BA-2000, Vector Laboratories, Peterborough) diluted 1/100 in PBS+0.1%BSA for 1 hour at room temperature. The sections were then incubated with 50 μ l of the streptavidin-alkaline phosphatase third layer (SA-5100, Vector Laboratories) diluted 1/100 in PBS+0.1% BSA for 1 hour at room temperature. The colour reaction was developed by the addition of 50 μ l of the substrate for 15 minutes (a combination of 0.005 g naphthol ASBI phosphate, 10 ml TRIS-HCl (pH 8.2), 200 μ l dimethylformamide, 0.01g fast red and 10 drops levamisole). The sections were counterstained with Mayer's haematoxylin (Sigma, Dorset), before being finally mounted in PBS:glycerol (9:1). The positive control used was sections of eczematous skin. The negative controls were omitting the first layer to detect background staining and an isotype mAb (IgG2a, M9144, Sigma, Dorset).

Quantification of immunohistology

An image analysis system (Seescan Imaging, Cambridge, magnification \times 320) was used to count the number of T cells per defined frame, in three random areas per section. This allowed the number of cells per unit area (UA) to be

calculated. A semiquantitative grading system was used to measure the fibroblast and IFN β staining. This was based on a grading system ranging from 1+ to 4+. An experienced masked observer performed the grading to reduce bias. Fibroblast grading 1+ represented scattered cells, 2+ represented more fibroblasts located especially beneath the epithelium, 3+ represented extensive cells staining positive. IFN β grading 1+ represented interepithelial staining, 2+ greater interepithelial staining, 3+ represented fibroblast-like cells staining in the stroma, 4+ represented more cells staining in the stroma. The number of IFN β and ASO2 positive fibroblasts was counted using a light microscope with a counting grid (\times 40 magnification) in three separate fields of view. The Mann-Whitney U test was used to analyse the numeric data and Fisher's exact test was used to analyse the quantitative data.

RESULTS

A total of 17 glaucoma patients were recruited of whom six underwent repeat filtration surgery. All had a diagnosis of primary open angle glaucoma, except for two with chronic angle closure glaucoma and three with pigment dispersion glaucoma. All were white except for five who were of Afro-Caribbean ethnicity and one of Asian ethnicity. The mean age was 68.3 years (range 45–87). The mean cumulative number of months of glaucoma eyedrop use was 154.5 months (range 9–356). Table 1 summarises the demographics of our patients.

Fibroblast and T cell staining

The conjunctiva of all glaucoma patients, except for two, contained spindle-shaped cells staining positive for the fibroblast marker ASO2 (Fig 1A and B). These cells were identified as Tenon's fibroblasts on the basis of their cell shape and positive ASO2 staining. It was noted that many of them were located beneath the conjunctival epithelium in Tenon's layer. Again, the conjunctiva of all of our glaucoma patients, except for one, showed RFT+ T cell staining (Fig 1C and D). Positive staining cells were located immediately beneath the conjunctival epithelium and in Tenon's layer. The biopsy sample of one patient was crushed and therefore was discounted from the analysis.

Of the two patients described as negative for fibroblast staining, one patient showed diffuse ASO2 positive staining, which was not fibroblast-like in distribution and therefore, was not included in the analysis. Other cells that stained positive but were not spindle-shaped were also not included in the quantitative analysis because these cells could not be

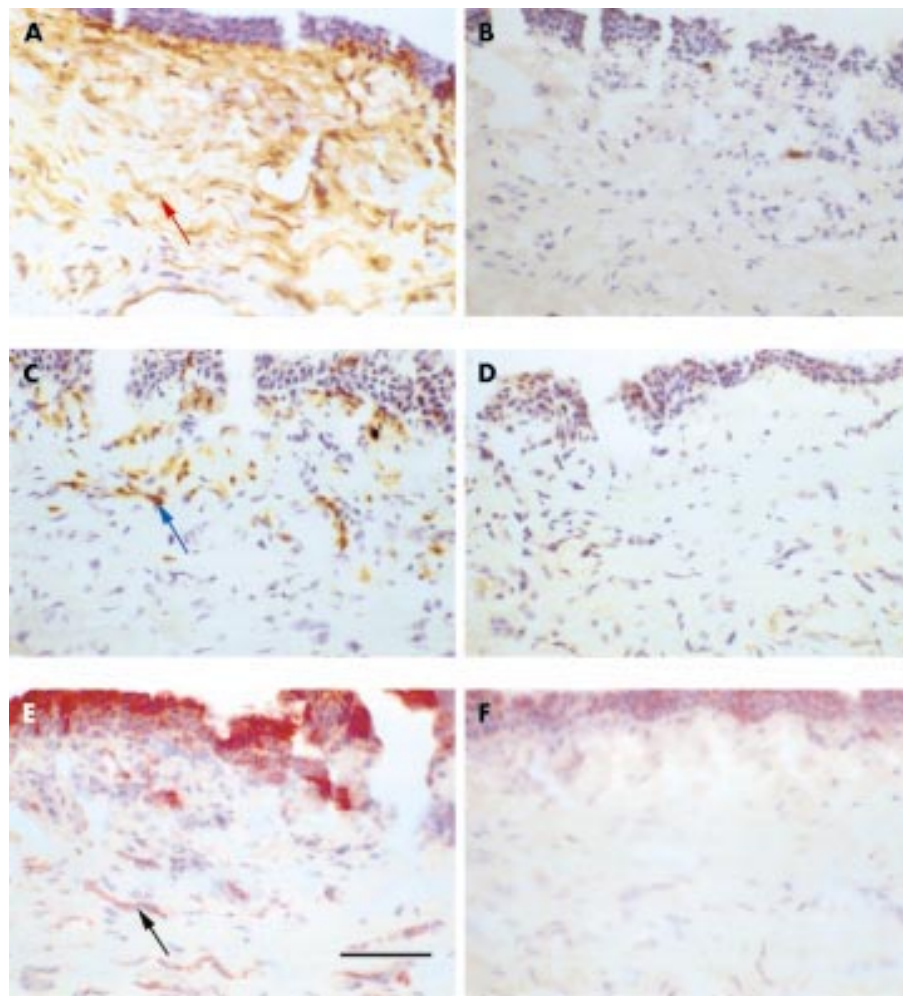


Figure 1 Human conjunctiva of glaucoma patients contains IFN β producing fibroblasts and T lymphocytes. The indirect immunoperoxidase technique was used to stain human conjunctiva for fibroblasts and T cells using the fibroblast specific mAb ASO2 and a pan-anti-T IgG mAb mix. Numerous spindle-shaped cells staining positive for ASO2 were identified in Tenon's layer (A, red arrow). The same conjunctival biopsy also showed RFT+ T cell staining (C, blue arrow). The negative isotype mAb controls did not stain positive (B and D). The biotin/streptavidin/alkaline phosphatase technique was used to stain human conjunctiva for IFN β using the mAb MAS291. Spindle-shaped fibroblast-like cells staining positive for IFN β were identified in Tenon's layer, although not all ASO2 positive fibroblasts stained positive for IFN β (E, black arrow). The negative isotype mAb control did not stain for IFN β (F). Scale bar = 150 μ m.

definitely identified as fibroblasts. In addition, ASO2 staining was noted in the endothelial cells of blood vessels in the conjunctiva. The positive controls tonsil and skin stained for T cells and fibroblasts (data not shown).

IFN β staining

Spindle-shaped fibroblast cells located in Tenon's layer beneath the epithelium stained positive for IFN β in the conjunctiva of three of our patients (two high risk and one low risk) (Fig 1E and F). It was noted that not all the ASO2 positive fibroblasts stained positive for IFN β . In addition, IFN β staining was found in the conjunctival epithelium of all of our glaucoma patients. This staining was especially strong in the outer layers and less strong in the inner layers of the epithelium. There were large mononuclear cells, which stained positive for IFN β . However, these cells were not included in the grading assessment because they were not fibroblast-like in shape. There were two technical failures in the low risk group that were not included in the analysis. Also, the blood vessel endothelial cells that had stained positive for the ASO2 fibroblast marker did not stain positive for IFN β .

Quantitative assessment of immunohistology

An image analysis system was used to count the number of T cells per defined frame. Three random areas per section were

selected to enable calculation of the number of cells per unit area (UA). The median RFT cell number per unit area (RFT/UA) was 3.15. Our grading system indicated that the median fibroblast grade was 1 and the median IFN β staining grade was 2. The mean IFN β positive cell count was 1.97 (SEM 0.20) compared to the mean ASO2 positive cell count of 30.53 (SEM 9.22). Therefore, 6.45% of the fibroblast cell population in these samples appeared to be producing IFN β . Table 1 summarises the results of the RFT staining and the grading for fibroblast and IFN β staining.

We divided our patients into those patients who underwent filtration for the first time (low risk group) and those who underwent repeat surgery (high risk group). The median RFT cell number per unit area (RFT/UA) for the high risk for scarring group was 1.93. The median RFT cell number per unit area for the low risk for scarring group was 4.28. After taking into account differences between the individual patients, there was no significant difference between the RFT cell number per unit area between the two groups ($p=0.60$). Again, there were no statistical differences in fibroblast and IFN β grading between the two groups ($p = 0.44$ and $p = 0.3$ respectively).

DISCUSSION

The aim of this study was to investigate whether the components for fibroblast mediated prevention of T cell apoptosis

were present in vivo in human conjunctiva. This is the first report of the in vivo production of IFN β by HTF, staining positive in the conjunctiva of three of our glaucoma patients. In addition, the conjunctiva of our glaucoma patients contained T lymphocytes. Therefore, our study has that all the components for this particular fibroblast/T cell interaction were present in human conjunctiva.

T cell/fibroblast interactions appear to have an important role in the development of abnormal scarring.² Hitchings and Grierson showed that early trabeculectomy failures were associated with excessive inflammation and increased conjunctival fibroblast numbers.¹⁴ Nuzzi *et al* demonstrated that the preoperative conjunctiva of repeat filtration surgery patients contained an increased number of activated T lymphocytes and fibroblasts.¹⁵ In our study, T lymphocytes and IFN β producing fibroblasts were present in the conjunctiva of two of our repeat trabeculectomy patients, who were a mean of 51.6 months (range 4–90) following their first failed trabeculectomy. This could suggest that conjunctival fibroblast mediated prevention of T cell apoptosis could continue for a prolonged period of time and thereby, contribute to the development of late as well as early failures.

Broadway *et al* showed that topical treatment altered the conjunctival cellular profile of glaucoma patients, with multiple therapy or treatment for more than 3 years resulting in an increase in lymphocytes, macrophages, and fibroblasts.¹⁶ Baudouin *et al* demonstrated that the preservative used in glaucoma drops was enough to stimulate conjunctival inflammation.¹⁷ All the patients in our group had received glaucoma eye drops as part of their medical treatment before undergoing filtration surgery. At present, the factors influencing IFN β production are not fully known, although our previous research did indicate that mitomycin C treatment (using a concentration of 0.4 mg/ml) of in vitro HTF increased HTF IFN β production.¹² Therefore, the medical treatment of our patients may have had some effect on HTF IFN β production. In our study, another control group would have been to investigate for the presence of IFN β producing fibroblasts in untreated conjunctiva; however, with current medical practice, we were unable to obtain ethical approval for biopsies of this type of tissue.

The interferons are a heterogenous group of cytokines consisting of the type I interferons (interferon α and β) and type II interferon (interferon γ).^{18, 19} The fibroblast is a common source of IFN β , and high levels are produced when fibroblasts are stimulated by viruses or agents such as polyinosinic:polycytidylic acid that mimic viral infections.²⁰ Our previous research showed that several types of fibroblasts including in vitro HTF produced IFN β constitutively.¹² IFN β is structurally dissimilar to IFN α but signals through the same receptor.

The interferons have long been recognised for their antiproliferative effects during viral infections.²¹ More recently, they have been shown to be able to reduce the wound healing response; Latina *et al* and Nguyen *et al* showed that IFN γ inhibited HTF collagen synthesis in vitro.^{22, 23} Gillies *et al* showed that intraoperative and postoperative subconjunctival injections of IFN γ reduced HTF proliferation after glaucoma filtration surgery, producing a success rate of 79% after 2 years follow up, similar to the success rate of 89% when the antimetabolite 5-fluorouracil was used.^{24, 25}

Our study has demonstrated the presence of the components of the interaction fibroblast-mediated prevention of T cell apoptosis in human conjunctiva; however, we did not demonstrate that this interaction was actually occurring. Our study demonstrated the presence of IFN β producing HTF in only three of our glaucoma patients. In addition, only 7.4% of the fibroblast population appeared to be producing IFN β . This was not unexpected because IFN β would not necessarily be produced by all fibroblasts at the same time or at the time of collection. Also, we were not able to demonstrate differences in the staining between glaucoma patients of different risk for

scarring. This may have been because the number of patients studied was quite small. Furthermore, the presence of different T cell subgroups was not investigated, although our in vitro research showed that fibroblast-mediated prevention of T cell apoptosis could be produced in both CD4+ and CD8+ T lymphocytes.²⁶

In conclusion, this study has identified the in vivo presence of the components of the interaction fibroblast mediated prevention of T cell apoptosis, indicating the possibility of a new potential role for HTF produced IFN β in the development of an aggressive wound healing reaction. The environmental milieu is crucial in directing the progression of a wound healing reaction and given the appropriate conditions, this particular interaction could promote the development of chronic conjunctival inflammation. This could subsequently promote greater fibroblast activity and ultimately excessive scar tissue formation. HTF IFN β mediated prevention of T cell apoptosis could have an important role in the pathogenesis of late surgical failures. Therefore, it will be important to further our understanding of this particular interaction with the aim of developing further strategies to control the wound healing reaction and improve our surgical success rate.

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