Human Tenon’s Fibroblast-Produced IFNβ and the Prevention of T-Cell Apoptosis

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PURPOSE. Fibroblast–T-cell interactions may contribute to the development of chronic inflammation, a risk factor for trabeculectomy failure. This study was undertaken to determine whether normal and growth-arrested human Tenon’s fibroblasts (HTF) can prevent cytokine deprivation–mediated T-cell apoptosis through the secretion of interferon (IFN)β.

METHODS. HTF were used either untreated or pretreated with mitomycin-C (MMC; 0.1 or 0.4 mg/ml) or 5-fluorouracil (5FU; 25 or 50 mg/ml). IL2-deprived T cells were cocultured with HTF. T-cell viability was measured at specific time points. Human Tenon’s fibroblast–conditioned medium was used either untreated or treated with a neutralizing antibody against IFNβ to block its action, after which IL2-deprived T cells were added and T-cell viability was measured. An image analysis system was used to determine the production of IFNβ by either untreated or MMC-treated HTF.

RESULTS. T-cell viability was significantly greater when T cells were cocultured with both untreated and growth-arrested HTF than when T cells were cultured alone (day 7, P = 0.0001). Neutralizing the action of IFNβ blocked HTF-mediated T-cell rescue from apoptosis. Both untreated and growth-arrested HTF secrete IFNβ, and MMC at 0.4 mg/ml appeared to increase IFNβ production.

CONCLUSIONS. Cytokine deprivation–mediated T-cell apoptosis can be prevented by the action of IFNβ secreted by both normal and growth-arrested HTF, which suggests that growth-arrested HTF can still participate in an aggressive wound-healing reaction by mediating a persistent inflammatory phase. This may partly explain why some trabeculectomies fail in high-risk patients, despite the use of antimetabolites. (Invest Ophthalmol Vis Sci. 2001;42:1531–1538)

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The major cause of failure after trabeculectomy is excessive conjunctival scarring.1 When patients undergo filtration surgery, they can be classified as low- or high-risk candidates for scarring. The surgical success rate for low-risk patients with glaucoma can be as high as 98%; however, it is usually considerably reduced in the high-risk groups.2,3 Success rates have been significantly improved in high-risk patients with the use of antimetabolites such as mitomycin-C (MMC) and 5-fluorouracil (5FU).3,4 However, complete success is still not guaranteed, and it is unknown why some patients have aggressive scarring, despite the use of these drugs.

The immune system is intimately involved in the wound-healing reaction, which can be arbitrarily divided into three phases: inflammatory, proliferative, and remodeling.6 The immune system appears to be most evident during the inflammatory phase, when there is an influx of neutrophils, followed by macrophages and T lymphocytes.7

Wound-healing models have indicated that inflammatory and fibroblast cell numbers decrease during resolution of the wound-healing reaction. Desmouliere et al.8 have shown that the number of fibroblasts is decreased by apoptosis. After an antigen-specific immune response, the associated increased T-cell population must be reduced to re-establish immune homeostasis to avoid the potentially damaging effects of persistent inflammatory cells.9 At the beginning of the immune response, this occurs by Fas-mediated apoptosis, and toward the end, it occurs by cytokine deprivation–mediated apoptosis.10–13 However, the clearance of inflammatory cells during the resolution of wound healing has not been examined, although it is possible that inflammatory cells are reduced similarly, as in an immune response.

Most clinicians recognize a persistent inflammatory response as a poor prognostic sign in glaucoma filtration surgery, but the mechanisms underlying this risk factor are poorly understood. It has been suggested that interactions between fibroblasts and T cells may contribute to the pathogenesis of aggressive wound healing.6,14 For instance, keloid scars have been shown to contain a persistent inflammatory infiltrate with increased fibroblasts for up to several years.15 Hitchings and Grierson16 showed that early glaucoma filtration failures were associated with an increase in inflammatory infiltrate and fibroblasts.

Therefore, we postulated that abnormal fibroblast–T-cell interactions might play a role in the development of chronic inflammation and the promotion of persistent conjunctival scarring. This problem may develop during the resolution of the wound-healing response, when apoptosis should decrease the number of T-cells. Our group and others have shown that human embryonic lung and skin fibroblasts, by production of the soluble factor interferon-(IFN)β, could prevent both types of T-cell apoptosis.17–19

The interferons are a group of cytokines that have been investigated for their wound-healing effects. The type I interferon family consists of IFNα and a group of related polypeptides collectively called IFNβ. IFNβ and IFNα are structurally dissimilar, but they signal through the same receptor. IFNβ is...
structurally distinct from IFNα and IFNβ, signals through a different receptor, and is called a type II interferon.

The interferons can exert quite different effects, depending on the environmental milieu. IFNβ and IFNα are best known for their antiviral effects, but IFNβ also has antiproliferative and antiapoptotic effects. Gillies et al. showed that IFNαb inhibits human Tenon’s fibroblast (HTF) proliferation; however, in a randomized phase II trial, they could not show that it was better than 5FU in improving the success rate of glaucoma filtration surgery. Nguyen et al. and Latina et al. showed that IFNγ inhibits HTF collagen synthesis in vitro.

We investigated the hypothesis that excessive HTF-mediated T-cell rescue from apoptosis might contribute to the development of chronic conjunctival inflammation and the promotion of excessive scarring. The purposes of this study were to investigate whether HTF prevents cytokine deprivation–mediated T-cell apoptosis and whether IFNβ is the soluble factor responsible for mediating this. We also investigated the effect of antimetabolites on this interaction and on IFNβ production by HTF.

METHODS

Fibroblast Cell Culture

HTF primary cell lines were established from subconjunctival Tenon’s biopsy specimens obtained from patients during glaucoma filtration surgery, as previously described. HTF were used in their exponential phase of growth between passages 3 and 7. Skin fibroblasts (SF) and human embryonic lung fibroblasts (HEL) were kind gifts from Cate Orteu and Jean Fletcher, respectively (Royal Free Hospital School of Medicine, London, UK). SF between passages 3 and 7 and HELF between passages 3 and 10 were used for our experiments. The study protocol adhered to the tenets of the Declaration of Helsinki.

All types of fibroblasts were cultured at 37°C in 5% humidified CO2 in RPMI (Life Technologies, Paisley, UK) containing 10% fetal calf serum (FCS; Life Technologies), 2 mM l-glutamine (Sigma, Dorset, UK), and 100,000 U/l penicillin (Sigma).

Preparation of T-Cell Lines

T-cell lines were established from peripheral blood mononuclear cells (PBMCs) that had been Ficoll isolated from healthy volunteers, as previously described. The PBMCs were activated with phytahemagglutinin (PHA; Sigma) and cultured at 37°C in 5% humidified CO2 in RPMI supplemented with 10% FCS, 2 mM l-glutamine, and 100,000 U/l penicillin.

Fibroblast–Lymphocyte Coculture

The HTF were cocultured with PHA-activated IL2-deprived PBMCs using a double-chamber system, as previously described. Briefly, HTF were grown in 24-well plates (Marathon, London, UK) in RPMI supplemented with 10% FCS, 2 mM l-glutamine, and 100,000 U/l penicillin at 37°C in 5% humidified CO2. The fibroblasts were used when confluent, and fresh complete culture medium was used at the start of each experiment.

Inserts (Marathon) were then placed inside the wells. The bottoms of the inserts were made from a semipermeable membrane containing pores (size, 0.4 μm) that allowed the free passage of soluble factors but prevented cellular migration. IL2-deprived T cells were then placed in the inserts. The negative control was IL2-deprived T cells in wells containing culture medium only. At specified time points, the number of viable T cells was measured using a trypan blue viability test, taking into account the change in volume. All experiments were performed at least three times.

The following coculture conditions were investigated: (1) HTF, HEL, or SF in coculture with T cells, and (2) antimetabolite-treated HTF in coculture with T cells.

HTF Antimetabolite Treatment

HTF seeded in 24-well plates were treated with a 5-minute application of MMC (Kyowa, Ealing, UK) at concentrations of 0.1 or 0.4 mg/ml or with a 5-minute application of 5FU (David Bull Laboratories, Warwick, UK) at concentrations 25 or 50 mg/ml. The dosage regimen used to treat the fibroblasts in vitro was selected to reflect clinical practice in glaucoma filtration surgery. After three washes with RPMI, the HTF were incubated in fresh complete culture medium for 4 hours. Activated IL2-deprived T cells were then cocultured with the treated HTF, as described above.

Two negative controls were used: untreated HTF cocultured with T cells and T cells cocultured in wells containing culture medium only. Viable T cells were subsequently counted at specified time points. All experiments were performed at least three times.

Modified Lactate Dehydrogenase Assay

A modified lactate dehydrogenase (LDH) release assay (Boehringer–Mannheim, Roche Diagnostics, E. Sussex, UK) was used to measure the number of viable fibroblasts. Lactate dehydrogenase is a stable cytoplasmic enzyme present in all cells. It catalyzes the reduction of a colorless tetrazolium salt to colored formazan, which absorbs light optimally at 492 nm. This commercially available assay normally measures the amount of LDH released by dead cells. However, if viable cells are lysed, then the assay can be used to measure the amount of LDH released from viable cells. At the end of each experiment, supernatant containing the apoptotic cells was discarded, leaving viable fibroblasts adherent to the culture wells. The viable cells were washed once with phosphate-buffered saline (PBS) and then permeabilized with 0.1% Triton X-100 (Sigma). To ensure total cell lysis, the fibroblasts were left for 5 minutes at room temperature. Catalyst solution (100 μl) was added to 100 μl of supernatant in a 96-well plate, which was read at 5 and 10 minutes using a microplate reader (Titertek Plus; ICN Pharmaceuticals, Hampshire, UK) and a 492-nm filter. We used this assay to establish that a linear relationship existed between fibroblast cell number and absorbance (data not shown).

IFNβ Immunostaining

The constitutive production of IFNβ by fibroblasts was investigated with a biotin/streptavidin/alkaline phosphatase immunostaining technique. Fibroblasts were seeded onto cell culture chamber slides (Life Technologies) and grown in complete medium, as described earlier. The fibroblasts were left for 1 to 3 days to allow them to reach the exponential phase of growth. During the last 4 hours of culture, old medium was replaced with fresh complete medium supplemented with monensin (Sigma) at a concentration of 3 mM, to arrest extracellular cytokine secretion. The cells were then washed twice with PBS and air dried overnight. Subsequently, the cells were fixed in acetone-chloroform (1:1) for 10 minutes, washed in PBS for 10 minutes, and stored at –20°C until used.

The fibroblasts were incubated overnight at 4°C in a moist chamber with the primary antibody diluted in PBS+0.1% bovine serum albumin (PBSA). IFNβ was labeled with the monoclonal antibody MAS291 (Harlan Sera Laboratory, Loughborough, UK) at a concentration of 50 μg/ml. An isotype-matched antibody (M9144; Sigma) or no primary antibody was used as a negative control. After an 18-hour incubation, the cells were washed in Tris-buffered saline (TBS; pH7.6) for 10 minutes and then incubated with the second layer, a biotinylated horse anti-mouse IgG (BA-2000; Vector, Peterborough, UK) diluted 1:100 in PBSA. IFNβ labeling was visualized using an ABC complex (Vector) and 5-bromo-4-chloro-3-indolyl phosphate. The stained slides were counterstained with hematoxylin and mounted in Pertex (Ted Pella, Inc., Redding, CA).
PBSA for 60 minutes at room temperature. After a wash in TBS, the third layer was added using streptavidin-alkaline phosphatase (SA-5100; Vector) diluted 1:100 in PBSA for 60 minutes at room temperature. The addition of the substrate (a combination of 0.005 g naphthol ASBi phosphate, 10 ml Tris-HCl [pH 8.2], 200 μl dimethyl formamide, 0.01 g fast red, and 10 drops levamisole) for 15 minutes at room temperature allowed the development of the color reaction. Finally, the cells were counterstained with Mayer’s hematoxylin (Sigma).

**IFNβ Immunostaining by Flow Cytometry**

The biotin/streptavidin/alkaline phosphatase immunostaining technique was modified to stain for IFNβ by flow cytometry. One million HTF were seeded in 25-cm² flasks (Falcon; Marathon) on t −2 days and cultured in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% FCS, penicillin, and L-glutamine. For the last 16 hours of culture, fresh medium was used supplemented with brefeldin A (concentration, 2.5 μg/ml; Epicentre Technologies, Cambridge, UK) and monensin (concentration, 1 μM), to arrest protein secretion.20

After the HTF were resuspended with trypsin-EDTA (Sigma) and two washes in PBS, they were fixed using 5% paraformaldehyde and 2% sucrose for 10 minutes and permeabilized using 0.5% Nonidet P-40, 10% sucrose, and 1% FCS in PBS for 5 minutes. The cells were washed once in 1% FCS in PBS and once in PBS. The primary layer was added using the monoclonal antibody (mAb) MAS291 (concentration, 25 μg/ml), to label for IFNβ, and incubated for 30 minutes at room temperature. The negative control was the IgG2a isotype control, M9144, or no primary layer. After a wash with 0.2% PBSA, the secondary biotinylated layer was added (BA-2000; Vector) at 10 μg/ml and incubated for 15 minutes at room temperature. After one wash in 0.2% PBSA, the fluorescent third layer was added, using fluorescein avidin-D (A-2001; Vector) at a concentration of 20 μg/ml and incubated for 10 minutes in the dark at room temperature. After two final washes with 0.2% PBSA, the cells were fixed in 2% paraformaldehyde and counted were acquired on a flow cytometer (FACSCalibur with CellQuest software; Becton Dickinson, Oxford, UK).

**Blocking Experiment**

To investigate whether IFNβ produced by HTF is the soluble agent responsible for rescuing T cells from apoptosis, fibroblast-conditioned medium (FCM) was collected from HTF and HELF cultures over a period of at least 1 week and then stored at 4°C until used. FCM was either untreated or incubated for at least 1 hour with a polyclonal neutralizing antibody against IFNβ (MCA1117; Serotec, Oxford, UK) using two final dilutions of 1:25 and 1:50. The T cells used were PHA-activated IL2-dependent T cells, cultured in IL2 for more than 10 days but less than 14 days. After the incubation period, T cells in fresh complete medium but without IL2 were added to the treated or untreated FCM in a ratio of FCM to fresh medium of 50:50. FCM collected from HELF was used as a positive control. Complete medium without IL2 was used as a negative control for the FCM. As a negative control for the neutralizing antibody, FCM was incubated with an irrelevant antibody, anti-human hepatocyte growth factor (AHP279; Serotec) at the same dilutions. The number of viable T cells was counted at 24 and 48 hours by using a trypan blue test.

**The Regulation of IFNβ Production**

To investigate whether the production of IFNβ by fibroblasts could be modified by treatment with antimetabolites, HTF grown in culture chamber slides were treated with the antimetabolites as described earlier. At specified time points, the slides were fixed and stored for staining at a later date, so that all the slides from one experiment were stained together. IFNβ was stained for by the biotin/streptavidin/alkaline phosphatase immunostaining technique, except that the counterstain step was omitted.

Quantification of the IFNβ staining was made using an image analysis system (Seescan Imaging, Cambridge, UK). Optical density measurements of fibroblast cytoplasmic IFNβ staining were made in 10 or 20 cells chosen at random and representative of the range of intensity of staining obtained. All experiments were performed at least three times.

**Statistics**

All in vitro experiments were performed at least three times, allowing mean and SEMs to be calculated. Two-way analysis of variance (ANOVA) was used to detect statistically significant differences between the factors of interest, after controlling for differences between patients and/or repeated experiments. After performing a global comparison, pair-wise comparisons between different treatment groups were performed using the Turkey test, ensuring that the overall type I error rate remained at 5%.

**RESULTS**

**Fibroblast–T-Cell Coculture and T-Cell Apoptosis**

PHA-activated IL2-deprived T cells were cocultured with HTF, and the number of viable T cells was measured on days 1, 4, and 7. When HTF were cocultured with IL2-deprived T cells, more T cells were prevented from undergoing cytokine deprivation–mediated T-cell apoptosis than when T cells were cultured alone (Fig. 1A). This experiment was repeated using four different donors, with T-cell viability measured on days 3 and 7 (data not shown). T-cell viability was significantly greater on day 7 when T cells were cocultured with HTF compared with when T cells were cultured alone, after controlling for the differences between donors using two-way ANOVA (P = 0.0001). Because a semipermeable membrane that allowed only the diffusion of soluble factors separated the HTF and T cells, it was also inferred that this rescue must be mediated by a soluble factor secreted by the HTF.

To investigate whether different types of fibroblasts mediated this rescue to different extents, HTF, HELF, and SF were cocultured with PHA-activated IL2-deprived T cells, and the number of viable T cells was counted on days 1, 4, and 7. All three types of fibroblasts appeared to rescue activated T cells from cytokine deprivation–mediated apoptosis to a similar degree (Figs. 1A, 1B, 1C).

**HTF-Produced IFNβ and the Prevention of T-Cell Apoptosis**

We wanted to verify that HTF-produced IFNβ was responsible for the rescue found in our experiments. Two different methods were used to investigate whether HTF produced IFNβ constitutively: a biotin/streptavidin/alkaline phosphatase immunostaining technique and flow cytometry. Both methods confirmed that HTF produced IFNβ constitutively (Figs. 2A, 3).

After 48 hours of culture, there was a greater number of viable T cells when IL2-deprived T cells were combined with FCM from HTF than in the control culture medium only (Fig. 4). When IL2-deprived T cells were combined with FCM from HTF treated with the neutralizing antibody against IFNβ, the number of viable T cells after 48 hours in culture was greatly reduced compared with when IL2-deprived T cells were combined with untreated FCM. Therefore, the addition of anti-IFNβ neutralizing antibody to FCM from HTF abrogated the ability of HTF to rescue IL2-deprived T cells from apoptosis. This indicated that IFNβ was the main soluble factor produced by HTF that was responsible for mediating the rescue of IL2-deprived T cells from apoptosis.

**The Effect of Antimetabolite-Treated HTF on T-Cell Apoptosis**

To investigate whether these growth-arresting agents affected the ability of HTF to prevent T-cell apoptosis, MMC, 5FU-
FIGURE 1. HTF, HELF, and SF were cocultured with PHA-activated IL2-deprived T cells. There was a significant difference in the number of viable T cells when T cells were cultured with HTF (A), HELF (B), or SF (C), compared with when T cells were cultured alone (P = 0.0001, day 7). HTF, HELF, and SF prevented T-cell cytokine-deprivation-mediated apoptosis to a similar extent.

FIGURE 2. A biotin/streptavidin/alkaline phosphatase immunostaining technique was used to detect the production of IFNβ. HTF produced IFNβ constitutively with the cytoplasm staining positively (arrow, A). HTF were treated with a 5-minute application of 0.4 mg/ml MMC and then were stained for IFNβ on day 3. MMC-treated HTF still produced IFNβ with positive cytoplasm staining (arrow, B). An isotype-matched antibody was used as the negative control (C).
treated or untreated HTF were cocultured with PHA-activated IL2-deprived T cells. T-cell viability was again measured to use the final number of fibroblasts at the end of each experiment. The modified LDH assay was used to measure the final number of fibroblasts at the end of each experiment.

Both growth-arrested MMC-treated or 5FU-treated fibroblasts rescued T cells from cytokine deprivation–mediated apoptosis, irrespective of the dosage used. In addition, the rescue compared similarly with the rescue produced by untreated fibroblasts (Figs. 5A, 5B). Multivariate analysis on day 7 showed that T-cell viability was not significantly different between these experimental groups (untreated, 0.1 mg/ml MMC, and 0.4 mg/ml MMC or untreated, 25 mg/ml 5FU, and 50 mg/ml 5FU). However, T-cell viability was significantly greater in all these experimental groups than in the control group (T cells only; \( P = 0.0001 \)).

The modified LDH assay showed that by day 7, there was an approximately 65% reduction in cell number when fibroblasts were treated with MMC at 0.4 mg/ml compared with the number of untreated HTF. When fibroblasts were treated with MMC at 0.1 mg/ml, there was an approximate 16% decrease in cell numbers (Fig. 6A). 5FU at 25 mg/ml and 50 mg/ml produced a 42% and 53% reduction in cell numbers, respectively. Therefore, although antimetabolite treatment decreased the number of fibroblasts, the rescue mediated by the remaining viable HTF was still comparable to that mediated by the untreated HTF.

HTF produced IFN\( \beta \) despite antimetabolite treatment (Fig. 2B). In fact, image analysis optical density measurements for IFN\( \beta \) production suggested that MMC (0.4 mg/ml) treatment may have increased IFN\( \beta \) production by HTF by day 7 (Fig. 7). The analysis of three repeats of this experiment measured the mean of IFN\( \beta \) staining for HTF treated with MMC (0.4 mg/ml) as 0.1441 ± 0.0075 (SEM), which was significantly greater than the mean of IFN\( \beta \) staining for untreated HTF, which was 0.0852 ± 0.0075 (\( P = 0.0001 \)).

**DISCUSSION**

We found that HTF can prevent cytokine deprivation–mediated T-cell apoptosis in vitro through the production of the cytokine IFN\( \beta \). This rescue is comparable to that mediated by other types of fibroblasts, such as human skin and embryonic lung fibroblasts, suggesting that fibroblast-mediated rescue of T cells from apoptosis may be a common feature of fibroblasts. By blocking the action of IFN\( \beta \) in FCM collected from HTF, we were able to confirm that HTF-produced IFN\( \beta \) is the main soluble factor responsible for mediating this rescue. A small amount of T-cell rescue (22%) was still measured after the action of IFN\( \beta \) was neutralized, suggesting that although IFN\( \beta \) is the main soluble factor responsible for this rescue, other as yet unidentified factor(s) may also play a role.

There is considerable evidence suggesting that aberrant interactions between T cells and fibroblasts contribute to the development of excessive scarring. Steroid eye drops are used after surgery to reduce the inflammatory phase of the wound-healing response and have been shown to significantly improve the success rate of trabeculectomies.\(^30\) Bernauer et al. showed that there are increased numbers of CD4\(^+\) and CD8\(^+\) T cells and fibroblasts in tissue specimens from patients with the fibrosing disease cicatrizing conjunctivitis.\(^30\) Greater disease activity and progression seems to be associated with increased conjunctival inflammation.\(^31\) The conjunctiva of pa-

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933588/) Prevention of T-Cell Apoptosis by IFN\( \beta \) in Tenon’s Fibroblasts

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933588/)
patients with glaucoma who had received multiple topical treatment for more than 3 years showed a significantly increased number of macrophages, lymphocytes, and fibroblasts, and these patients were more likely to have aggressive scarring after filtration surgery.32,33 The conjunctiva of early trabeculectomy failures show an increased number of activated CD4+ and CD8+ T cells and fibroblasts.34 However, the functional phenotype of these inflammatory cells has not been fully defined. Information of this sort would be important in indicating how inflammatory cells and fibroblasts interact to result in aggressive scarring and trabeculectomy failure.

Our group has suggested that fibroblast-mediated T-cell rescue from apoptosis may play a role in the persistence of chronic inflammatory conditions such as eczema and rheumatoid arthritis.18,35,36 In affected areas, there was a persistent T-cell infiltrate associated with an increased number of fibroblast-like cells producing IFN-β. Because we have shown that HTF are also capable of mediating this rescue, this mechanism may play a role in promoting a persistent inflammatory response in the conjunctiva after wounding and, subsequently, an aggressive wound-healing reaction.

Most antiscarring agents used in glaucoma filtration surgery have concentrated on reducing the ability of fibroblasts to proliferate.37 Antimetabolites such as MMC and 5FU are now used routinely in operations on patients who are considered higher risks for scarring, and these drugs have certainly improved the success rate of surgery in these patients.4,5 However, some patients still have aggressive scarring, despite the use of these drugs, and we do not fully understand what mechanisms are responsible.

Of particular interest, we found that antimetabolite-treated HTF were still able to prevent cytokine deprivation-mediated T-cell apoptosis. Even when MMC treatment (at a concentration of 0.4 mg/ml) reduced fibroblast numbers to 30% compared with untreated HTF, enough IFN-β appeared to be produced by the remaining viable HTF to prevent T-cell apoptosis. In fact, MMC treatment may actually increase IFN-β production, as suggested by the increased IFN-β staining measured in HTF treated with MMC (0.4 mg/ml). Because we investigated only one time point (day 3 after treatment), it is important to investigate whether this increased IFN-β production persisted.

The ability of growth-arrested HTF to mediate the rescue of cytokine-deprived T cells from apoptosis may partly explain why failures still occur despite the use of antimetabolites. Excessive continuation of this interaction may result in the persistence of inflammatory cells, which would continue to

**FIGURE 5.** HTF were treated with a 5-minute application of MMC at concentrations of 0.1 or 0.4 mg/ml (A) or 5FU at concentrations of 25 or 50 mg/ml (B). PHA-activated IL2-deprived T cells were then cocultured with untreated or treated HTF. Viable T cells were counted on days 3 and 7 of the experiment. Results are expressed as a percentage of T-cell viability ± SEM. By day 7, T-cell viability was significantly greater when T cells were cocultured with untreated and both types of growth-arrested HTF than T cells cultured alone (P = 0.0001). These results represent one of three experiments.

**FIGURE 6.** HTF were either untreated (unRx) or treated with a 5-minute application of MMC at 0.1 or 0.4 mg/ml (MMC0.1 and MMC0.4, respectively) or 5FU at 25 or 50 mg/ml (5FU25 and 5FU50). Cytokine-deprived T cells were then cocultured with HTF. A modified lactate dehydrogenase assay was used to measure the number of viable fibroblasts at the end of each coculture experiment, expressed as absorbance. At the end of the experiment, the assay indicated that there was a 16% and 65% reduction in HTF after 0.1- and 0.4-mg/ml treatments with MMC, respectively, compared with untreated HTF (A). There were 42% and 53% reductions after 25- and 50-mg/ml 5FU treatments, respectively, compared with untreated HTF (B).
produce the important growth factors responsible for continued fibroblast activity and wound healing. Occleston et al. have suggested that fibroblasts may perform other wound-healing functions despite being growth arrested. They demonstrated that growth-arrested fibroblasts still secrete certain growth factors such as TGFβ, still express growth factor receptors, and are still able to migrate. This may facilitate the activation of fibroblasts at the periphery of the treated area. Our findings suggest that the remaining fibroblasts in the anti-metabolite-treated area may also stimulate inflammatory cells in the aqueous that is present in the bleb and the surrounding area. This further emphasizes the importance of a wider surface area of treatment.

In summary, we have shown that both normal and growth-arrested HTF produce IFNβ, which is responsible for mediating the rescue of cytokine-deprived T cells from apoptosis. We suggest that if this fibroblast-T-cell interaction were to occur excessively, it might contribute to the excessive persistence of inflammatory cells, thus continuing the wound-healing reaction and promoting excessive scarring. By improving our understanding of the mechanisms that regulate IFNβ production, we may be able to develop more specific strategies to modulate chronic inflammation and the wound-healing response and ultimately may improve our glaucoma surgical success rate, even in patients in whom high-dose antimetabolite regimens presently fail.

References


FIGURE 7. IFNβ production was detected in cultured human Tenon's fibroblasts using a quantified biotin/streptavidin/alkaline phosphatase immunostaining technique. The amount of IFNβ production was compared in untreated HTF (control) versus MMC-treated HTF at concentrations of 0.1 and 0.4 mg/ml (MMC 0.1 and MMC 0.4, respectively). An image analysis system measuring the optical density of IFNβ staining was used to quantify IFNβ production. MMC treatment at 0.4 mg/ml compared with untreated HTF appeared to increase IFNβ staining.