

ACTIVATION AND SUPPRESSION OF FIBROBLAST FUNCTION

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SUMMARY

The fibroblast is the central player in the wound repair and scarring processes that occur in the anterior segment of the eye. Glaucoma filtration surgery is the ultimate example of the importance of the wound healing process, as this process is the major determinant of the success of this procedure. We highlight the role of the fibroblast, and discuss some of the growth factors stimulating fibroblast proliferation, migration and extracellular matrix production in the wound environment. We also review current methods of suppressing fibroblast proliferation, the new concepts that have arisen from laboratory studies, and future directions of investigation and treatment.

The processes involved in tissue repair play a critical role in either the pathogenesis or failure of treatment of most of the major blinding or visually disabling conditions in the world today. The biological processes that result in scarring account for a great deal of visual morbidity. In this review we use glaucoma filtration surgery as an example of the importance of scarring, as a model of the scarring process after ocular surgery, and also highlight the role of the fibroblast, the factors that may activate the fibroblast, and the effects of methods used to suppress fibroblast function.

GLAUCOMA FILTRATION SURGERY: AN EXAMPLE OF THE IMPORTANCE OF THE SCARRING PROCESS

The glaucomas are a group of disorders characterised by optic nerve head damage, visual field loss and an intraocular pressure sufficiently raised to affect function of the

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optic nerve head. Glaucoma is not a single disease process but rather a large group of diseases, which have in common damage to the optic nerve head. This group of diseases will soon be the second commonest cause of blindness in the world behind cataracts, affecting people in both developed and developing countries.^{1,2} Unlike cataracts, the blindness from glaucoma is essentially irreversible. Although many theories for the pathogenesis of optic nerve damage have been postulated, the major risk factor for glaucomatous optic nerve damage is the level of intraocular pressure. The higher the intraocular pressure, the greater the risk of optic nerve damage.^{3,4} Most treatment is aimed at reducing the intraocular pressure.

There are three main categories of treatment: medicine, laser or surgery. In recent years evidence has been accumulating that surgery is superior to either laser or medical treatment in lowering the intraocular pressure⁵ and preserving vision.⁶⁻⁹ In addition, successful surgery results in a better quality of life for patients compared with medical treatment,¹⁰ and the use of topical medication (eyedrops) increases the chance of subsequent surgical failure.¹¹ Furthermore, in many developing countries of the world, surgery is the only practical treatment to prevent blindness from glaucoma.

Glaucoma filtration surgery involves the creation of a new drainage channel for the aqueous humour to flow out of the eye and thus lower intraocular pressure. The commonest form of the procedure¹² involves making an incision in the conjunctiva, dissecting open the subconjunctival space and making a trapdoor incision in the sclera up to the junction between the cornea and the sclera. The eye is entered and a block of corneoscleral tissue removed to create a new channel for aqueous outflow, and the trapdoor sutured down to create a valve mechanism. The conjunctival incision is then closed and the aqueous allowed to flow out under the conjunctiva. The aqueous may then flow away subconjunctivally, through aqueous veins, through lymphatics channels or transconjunctivally.¹³

The successful lowering of intraocular pressure after fil-

tering surgery relies heavily on the nature of the healing response following the surgery. It is variations in this healing response that result in the majority of failed filtering procedures. Several risk factors may increase the chance of filtration surgery failing and these are shown in Table I. The reasons for the increased scarring response in patients with these risk factors are still unclear. Furthermore, just as important as absolute failure is suboptimal lowering of the intraocular pressure. There is evidence that the greater pressure lowering associated with surgery is associated with a better visual prognosis.⁹ The healing response is the major determinant of the final intraocular pressure. In terms of the entire glaucoma population undergoing surgery, this is probably a much more important problem epidemiologically than the far smaller but highly visible group that completely fail surgery. Thus the healing response is the single most important modifiable factor determining outcome in every patient undergoing glaucoma filtration surgery.

CELLULAR EVENTS AFTER GLAUCOMA FILTRATION SURGERY AND THE ROLE OF THE FIBROBLAST

The sequence of events following glaucoma filtering surgery can be illustrated in a simplified diagrammatic form (Table II), although some of the events obviously occur concurrently. When incisions are made in the conjunctiva and sclera, connective tissue and blood vessels are damaged. This results in the release of blood cells and plasma proteins into the damaged site. The clotting system is activated and clot forms around the damaged areas. This is similar to the events that occur after cutaneous or other wounding.

However, the glaucoma filtration surgical site differs in one fundamental aspect: it is bathed by aqueous humour emerging from the eye through the newly created fistula. This aqueous humour and its contents can have very significant effects on the healing response after glaucoma surgery. There have been suggestions that the aqueous contains an inhibitory factor or factors,^{27,28} and aqueous clearly modulates the wound as dissected conjunctiva normally scars down rapidly to the episclera without aqueous. However, aqueous also has stimulatory effects on fibroblasts *in vitro*^{29,30} and *in vivo*. A particularly good example is the aggressive scarring seen after filtration surgery in patients with new vessel formation within the eye. In this situation the aqueous humour, which is presumably full of stimulating factors, invariably induces rapid scarring and subsequent surgical failure. Even in a relatively undiseased eye (e.g. cataract extraction), when the eye is entered some

degree of blood–aqueous barrier breakdown occurs,^{31–33} bringing in serum factors to the wound site.

Polymorphonuclear cells move into the wound area followed by lymphocytes and macrophages. Fibroblasts then begin to migrate towards sites of damage. As they migrate they probably cause wound contraction. The tractional forces exerted by moving fibroblasts have been elegantly demonstrated by Harris *et al.*³⁴ who seeded fibroblasts onto ultrathin polymerised silicone. We have been using this model to investigate fibroblast-mediated wound contraction; the forces generated by the moving fibroblasts are clearly seen in Fig. 1. The fibroblasts also begin to proliferate, which is necessary to generate sufficient numbers of cells to carry out the various wound-healing processes in a relatively short time. This process peaks within the first 2 weeks after experimental glaucoma filtration surgery,^{35–37} which is similar to cutaneous wounding.

Fibroblasts secrete fibronectin, glycosaminoglycans and

Table II. Sequence of events in wound healing and potential areas of modification after glaucoma filtering surgery (simplified: events and agents have overlapping time duration and action)

Event	Possible areas of modulation
Conjunctival/episcleral/scleral incisions	Minimal trauma Less invasive surgical techniques
↓	
Damage to connective tissue Release of plasma proteins and blood Activation of clotting and complement Fibrin/fibronectin/blood cell clot Release of growth factors from blood	Haemostasis Agents preventing/removing fibrin (e.g. heparin, tissue plasminogen activator) Antagonists to growth factor production, growth factor activation (e.g. antisense oligonucleotides), antibodies to growth factors or receptors
↓	
Aqueous released from eye Breakdown of blood–aqueous barrier Release of growth factors into aqueous Aqueous begins to flow through wound	Blood–aqueous barrier stabilising agents (e.g. non-steroidal anti-inflammatory agents) Antagonists to growth factors
↓	
Migration and proliferation of polymorphonuclear neutrophil cells, macrophages and lymphocytes	Anti-inflammatory agents (e.g. steroids) Antiproliferative agents (e.g. 5-fluorouracil)
↓	
Migration and proliferation of fibroblasts Wound contraction	Antiproliferative agents Anticontraction agents (e.g. taxol)
↓	
Fibroblast synthesis of tropocollagen, glycosaminoglycans and fibronectin Collagen cross-linking and modification	Anticross-linking agents (e.g. beta-aminopropionitrile)
↓	
Blood vessel endothelial migration and proliferation	Inhibitors of angiogenesis
↓	
Continued collagen synthesis Capillary resorption and disappearance of fibroblasts	
↓	
Fibrous subconjunctival scar	

Table I. Risk factors for failure of glaucoma filtration surgery

- Topical medications >1 year^{11,14,15}
- Age <40 years^{16–20}
- Afro-Caribbean^{21–24}
- Previous failed filter²⁵
- Previous cataract surgery²⁵
- Uveitis²⁶
- Anterior segment neovascularisation²⁶
- Aphakia/pseudophakia²⁵

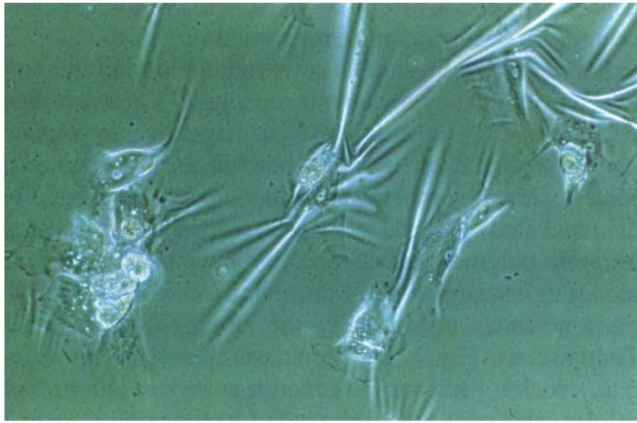


Fig. 1. Human Tenon's capsule fibroblasts seeded onto polymerised silicone sheets. As the fibroblasts move and migrate they create marked surface tractional forces.

tropocollagen which is then enzymatically cross-linked to form collagen. Collagen is a major component of the wound matrix and is a prominent feature of experimental filtering surgery failure in the rabbit and monkey.^{36,38} Many fibroblasts in the failing blebs of rabbits have cytoplasm rich in endoplasmic reticulum, with some distended cisternae. This appearance is consistent with an actively metabolising cell undergoing transcription.³⁶ Collagen accumulation is also a prominent feature of failing blebs in man.³⁹

This connective tissue is then remodelled continuously for several months⁴⁰ with continued synthesis and breakdown of the extracellular matrix. Eventually most of the active cellular processes cease and the final result is subconjunctival scar. Successful filtering surgery is usually associated with a subconjunctival accumulation of aqueous humour, or a 'bleb' (Fig. 2). Failed filtration surgery is associated with a dense scar in the subconjunctival space which closes this potential space and prevents aqueous flow. It is obstruction by scarring at the episcleral levels rather than the fistula itself which is the main reason for failure. The process of healing and the scarring response can be modulated at various points in the process, and these are represented in Table II. However, most of the efforts to suppress scarring have concentrated on the fibroblast cell,

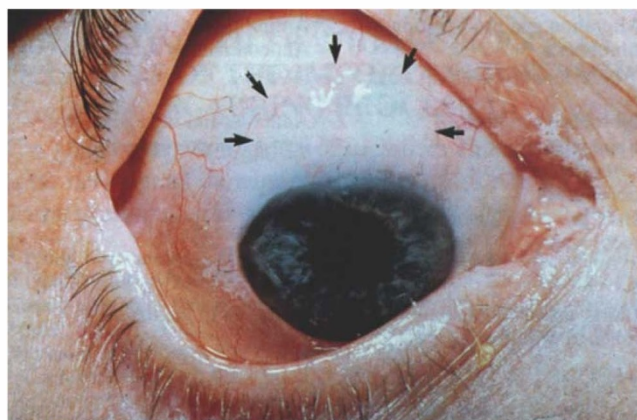


Fig. 2. A drainage bleb after glaucoma filtration surgery: collection of aqueous humour draining out of the eye under the conjunctiva. The edges of the bleb are marked with arrows.

which is the central cell of the scarring process. Filtration surgery most commonly fails because of the build up of extracellular matrix, in particular collagen which obstructs the flow of aqueous. Without the fibroblast cell this failure would not occur.

ACTIVATION AND STIMULATION OF THE FIBROBLAST

Normally, the fibroblast exists within the subconjunctival connective tissue as a quiescent undifferentiated mesenchymal cell, the fibrocyte. This cell is found in low numbers throughout connective tissue. The fibrocyte converts to the active fibroblast capable of carrying out the various cellular functions responsible for wound healing when stimulated by various factors in the environment. For instance, factors which are known to stimulate fibroblast migration are the complement component C5a,⁴¹ growth factors such as platelet-derived growth factor, transforming growth factor and fibroblast growth factor,⁴² elastin and collagen fragments,^{43,44} leukotriene, and fibronectin.⁴⁵ These factors are derived from damaged tissue, blood, inflammatory cells such as macrophages, lymphocytes and macrophages, and from the fibroblasts themselves. In the aqueous itself there are factors known to stimulate fibroblasts, and these are listed in Table III. The relative roles of these growth factors in healing after wounding of the anterior segment of the eye are unclear.

We previously investigated the role of several growth factors in the migration of corneal fibroblasts⁴⁶ and have more recently studied the effects of different concentrations (10^{-7} to 10^{-14} mM) of various growth factors including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor beta-1 (TGF- β 1) and insulin-like growth factor 1 (IGF-1) on the migration, collagen synthesis and proliferation of human Tenon's capsule fibroblasts. We found that all four growth factors stimulated proliferation, migration and collagen production to some degree, but that TGF- β 1 stimulated proliferation at much lower concentrations than the other growth factors. In addition, TGF- β 1 also stimulated much more collagen production than EGF and bFGF; it is of interest, though, that IGF-1 also stimulated the same amount of collagen production as TGF- β 1, albeit at a higher concentration. These results suggest that targeting specific growth factors, in particular the TGF- β family, may be a reasonable approach to preventing post-operative scarring in the eye. There is already evidence that modulating the profile of the TGF- β family after cutaneous wounding may reduce scarring.⁴⁷

Table III. Some stimulatory factors in the aqueous humour

Fibroblast growth factor
Epidermal growth factor ^a
Transforming growth factor beta-1 and beta-2
Insulin-like growth factor 1
Fibronectin
Transferrin
Interleukin 6

^aControversial.

Table IV. Examples of some agents used to modulate ocular fibroblast function (some have more than one action)

<i>Anticytoskeletal</i>
Colchicine
Cytochalasin b
Taxol
Nocodazole
Vincristine
Vinblastine
<i>Antiproliferatives</i>
Radiation
Doxorubicin
Bleomycin
Chlorambucil
Cytosine arabinoside
5-Fluorodeoxyuridine
5-Fluorouracil
Methotrexate
Mitomycin C
Gamma interferon
Anti-transferrin receptor monoclonal antibody conjugated to a cellular poison, ricin
<i>Immunosuppressives/anti-inflammatories</i>
Prednisolone
Dexamethasone
Triamcinolone
Indomethacin
Meclofenamate sodium
Cyclosporin
Diphenhydramine

MODULATION/SUPPRESSION OF FIBROBLAST FUNCTION

A great deal of research has been carried out on agents which directly or indirectly modulate various aspects of fibroblast function (Table IV). Attempts have been made to target specific cell functions such as migration. Examples are colchicine, cytochalasin B and particularly taxol (which all affect the cytoskeleton of the cell), which were all shown to inhibit the migration of fibroblasts to rabbit aqueous humour.⁴⁸ Taxol has been shown to prolong survival of filtration surgery in an aggressive model of filtration surgery.⁴⁹ Another example is the use of lathyrogenic agents such as beta-aminopropionitrile, which prevents collagen cross-linking by inhibiting the enzyme lysyl oxidase.⁵⁰ There is experimental and clinical evidence that this may work.⁵¹⁻⁵³ However, many of these 'specific' agents have effects on other fibroblast functions: for instance taxol also inhibits cell proliferation and BAPN may also have anti-inflammatory effects.⁵⁴ Most of the work aimed at inhibiting scarring after glaucoma filtration surgery has concentrated on the inhibition of proliferation of ocular fibroblasts,

Table V. Theoretical implications of the concept of long-term, titratable, focal tissue suppression of fibroblast function

Simultaneous corneal surgery without inhibition of healing
Titration of the final intraocular-pressure by changing agents, concentration and area treated
Creation of more diffuse thicker blebs (theoretically safer) by changing agents, concentration and area treated
Positioning of thin drainage areas in the fornix away from vulnerable areas such as limbus
Preoperative use of these agents may be possible
Appropriate treatment to maximise anti-scarring effect and minimise complications if individual healing responses are better understood

mainly on the premise that if there are inadequate numbers of cells, significant scarring cannot occur. However, it must be borne in mind that these agents clearly have effects on other aspects of fibroblast function.

Blumenkranz and colleagues^{55,56} established that various antiproliferative drugs, in particular 5-fluorouracil, inhibited the proliferation of fibroblasts during a period of continuous drug exposure in cell culture and prevented epiretinal scarring in the eye in an animal model. Stimulated by this initial research, a regimen involving subconjunctival injections of 5-fluorouracil was developed at Miami culminating in the multicentre 5-fluorouracil filtering surgery trial.²⁵ Many studies have also been carried out investigating the effects of 5-fluorouracil and many other antiproliferative agents on ocular fibroblasts in culture in an attempt to find an optimal agent,⁵⁷⁻⁶⁴ and also to develop an optimal delivery system that does not require multiple injections.^{49,65-70} However, Chen and colleagues^{71,72} had been using single applications of another anti-proliferative agent, mitomycin-C, for more than a decade, initially largely unnoticed. The use of this regimen has seen an exponential increase in popularity over the last few years because of the ease of application and effectiveness.

One of the reasons for the initial reluctance to use Chen's regimen was the lack of knowledge as to how a single application of these agents could suppress fibroblast proliferation adequately over the period of several weeks, during the period of maximal fibroblast proliferation. Our group has shown that several antiproliferative agents have long-term effects on the proliferation of ocular fibroblasts, even when the time of exposure is as short as 5 minutes.^{73,74} Given the appropriate concentration and agent, effective suppression of proliferation can be achieved for periods of up to 36 days without significant cell death.^{73,75} To our surprise, this long-term suppression also occurred with 5-fluorouracil, as the drug's inhibitory action on DNA synthesis through thymidylate synthetase should have been rapidly reversible. However, 5-fluorouracil, has, like all other drugs, secondary actions including interference with RNA synthesis, and these may explain the longer-term actions.

We have subsequently shown that this long-term suppression of proliferation occurs in an *in vivo* experimental model of glaucoma filtration surgery and appears to be titratable in terms of length of action.^{76,77} It is also focal in that only the fibroblasts in the treated areas are affected.^{78,79} The fact that this suppression of fibroblast proliferation is focal is very important clinically, and the theoretical implications are shown in Table V. Fibroblasts are not derived from the blood as was previously thought, but are derived from a local population of fibrocytes. Therefore recovery of local tissue healing due to fibroblasts can only occur if the local tissue fibroblasts recover from the focal treatment or if there is migration of fibroblasts into the area.

However, just as important as the effect on the cells themselves is the length and degree of stimulation present in the aqueous and the wound area. This stimulation can modify the overall profile of healing despite fibroblast modulation with the same treatment. A simplified model of

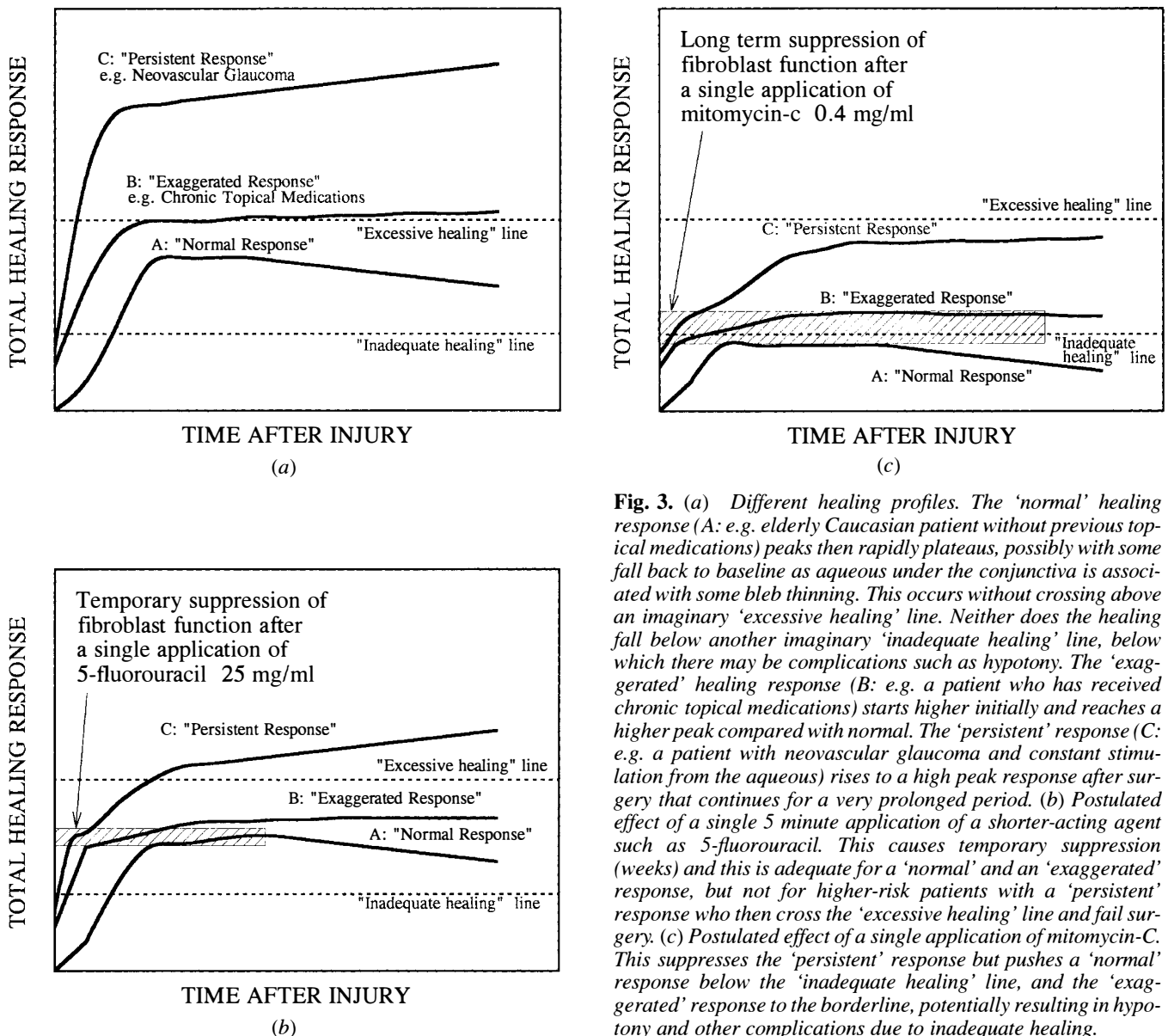


Fig. 3. (a) Different healing profiles. The 'normal' healing response (A: e.g. elderly Caucasian patient without previous topical medications) peaks then rapidly plateaus, possibly with some fall back to baseline as aqueous under the conjunctiva is associated with some bleb thinning. This occurs without crossing above an imaginary 'excessive healing' line. Neither does the healing fall below another imaginary 'inadequate healing' line, below which there may be complications such as hypotony. The 'exaggerated' healing response (B: e.g. a patient who has received chronic topical medications) starts higher initially and reaches a higher peak compared with normal. The 'persistent' response (C: e.g. a patient with neovascular glaucoma and constant stimulation from the aqueous) rises to a high peak response after surgery that continues for a very prolonged period. (b) Postulated effect of a single 5 minute application of a shorter-acting agent such as 5-fluorouracil. This causes temporary suppression (weeks) and this is adequate for a 'normal' and an 'exaggerated' response, but not for higher-risk patients with a 'persistent' response who then cross the 'excessive healing' line and fail surgery. (c) Postulated effect of a single application of mitomycin-C. This suppresses the 'persistent' response but pushes a 'normal' response below the 'inadequate healing' line, and the 'exaggerated' response to the borderline, potentially resulting in hypotony and other complications due to inadequate healing.

this concept is shown in Fig. 3. Essentially, this model represents three different profiles of healing, combined with a theoretical 'excessive healing' threshold line, which if crossed results in surgical failure. If excessive modulation is achieved, a theoretical 'inadequate healing' line is crossed and complications due to poor healing occur. The theoretical effects of a shorter-term inhibition (5-fluorouracil) and a longer-term inhibition (mitomycin-C) on these different healing profiles is shown. The inhibitory effect of mitomycin-C has been placed below the 'inadequate healing' line as we now have good *in vivo*⁷³ and *in vitro*⁸⁰ evidence that part of the effect of mitomycin-C at this concentration is due to actual cytotoxicity, while the remaining cells are growth arrested. Therefore, although we know we can adequately suppress fibroblast proliferation in a relatively titratable manner with practical single-application treatments, we still need to know more about the individual healing responses, and what functions the suppressed fibroblast is able to carry out under these conditions.

We have been looking at other aspects of fibroblast function following suppression of proliferation. Another vital function of the fibroblast is its ability to contract extracellular matrix. We have been using type I collagen gels populated with ocular fibroblasts. The fibroblasts rapidly contract these collagen gels when stimulated with serum. Five-minute exposures to 5-fluorouracil and mitomycin-C also inhibit the contraction of these gels. It is of particular interest that the effects of treatment are very different depending on the state of cellular activation of the fibroblast in the gel before treatment. A 'primed' cell may respond to treatment very differently from a 'quiescent' cell, further emphasising the importance of the environment of the tissues in individual patients before and after wounding. Topical medications, the commonest form of treatment for glaucoma, have a significant effect on conjunctival cellularity,⁸¹ amount of shrinkage⁸² and the response to surgery.¹¹

On a more molecular level, we have found that the 'suppressed' growth-arrested fibroblasts are still able to respond to stimulatory factors found in the wound environ-

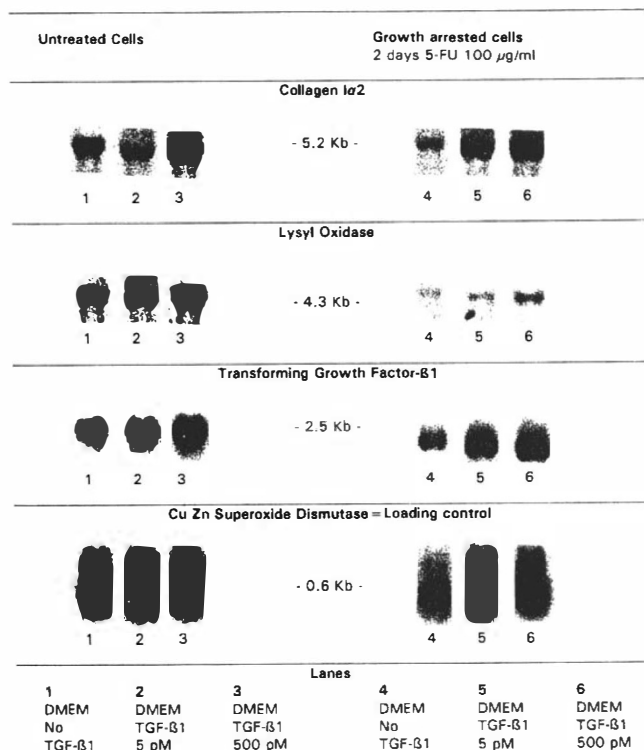


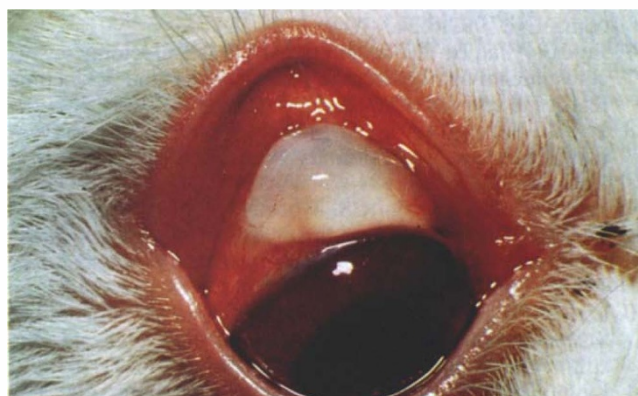
Fig. 4. Effects of transforming growth factor beta-1 (TGF-β1) stimulation on RNA levels of collagen, lysyl oxidase and TGF-β1 in normal and 'growth-arrested' ocular fibroblasts. RNA levels of collagen Iα2, lysyl oxidase and TGF-β1 all showed an increase when treated with 500 pmol TGF-β1. The cells growth arrested with 5-fluorouracil also showed relatively normal RNA levels of collagen and TGF-β1, with an increase when stimulated with TGF-β1. However, the levels of lysyl oxidase were markedly reduced, although there was still a response to stimulation with TGF-β1. NB. lane 4 underloaded.

ment such as TGF-β1. This is illustrated in Fig. 4. We found that Tenon's capsule fibroblasts show an increase in RNA levels of collagen Iα2 and TGF-β1 when exposed to increasing concentrations of TGF-β1. This has been well described in other fibroblasts. It is of interest too that the levels of lysyl oxidase are also upregulated by TGF-β1. After the cells have been growth arrested with 5-fluorouracil, they are still able to respond to TGF-β1 by upregulating levels of collagen Iα2 and TGF-β1 with slightly attenuated but nonetheless similar responses to untreated cells. It is interesting that there is a differential effect on the RNA levels of lysyl oxidase which were much lower, although there was still an increase in RNA levels with higher concentrations of TGF-β1. So suppressed cells, although growth arrested, are still able to respond to exogenous stimulation at the level of transcription.

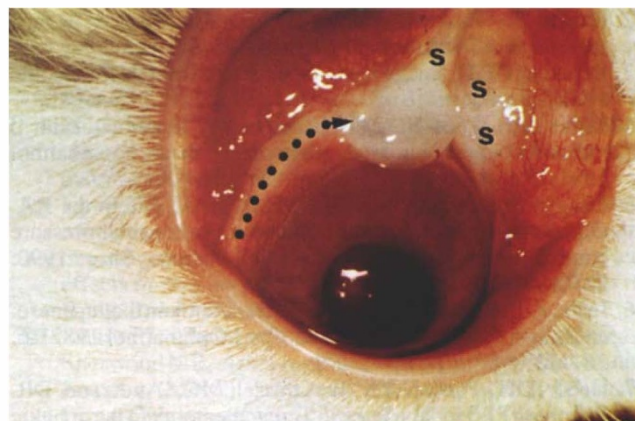
On the basis of these studies we have performed pilot studies *in vitro* on drainage blebs induced by mitomycin-C. When the blebs are injected with growth factors including TGF-β1 a healing response is generated which begins to reverse the effects of the mitomycin-C (Fig. 5). Thick scar tissue develops surrounding the bleb and the bleb begins to opacify and shrink compared with the effect of a control injection of carrier only. We are currently investigating the cellular and molecular mechanism of this scarring. How-

ever, what this pilot experiment confirms is that the effects of local suppression of fibroblasts can be significantly altered by changes in the wound environment.

In summary, we now have a variety of effective methods to suppress fibroblast function. In particular, the single exposures to antiproliferative agents are at present the most practical method of achieving fibroblast suppression after glaucoma filtration surgery. From basic research we now understand a great deal more about how these treatments work *in vitro* and *in vivo*. However, we have also shown that the state of activation of the cell, and the stimulatory factors activating the cell, can have profound effects on the healing response mediated by the 'suppressed' cells and tissues as a whole. A deeper understanding of the basic cellular and molecular biology of the healing process, variations in different individuals and circumstances and the effects of modulating agents on the fibroblast in particular are essential. With this knowledge, we may then be able to achieve totally safe but effective control of the scarring process, not just in the eye but in the whole human body.



(a)



(b)

Fig. 5. Effect of exogenous growth factor (TGF-β1) on the area growth arrested with a single 5 minute application of mitomycin-C. (a) Rabbit eye 40 days after treatment with a 5 minute exposure to mitomycin-C during experimental filtration surgery. Injection of control carrier at day 30. The bleb is still enlarged and draining. (b) Rabbit eye 40 days after treatment with a 5 minute exposure to mitomycin-C before glaucoma filtration surgery. Injected with TGF-β1/carrier into bleb. The bleb has contracted from the periphery inwards. Thick subconjunctival scar tissue is present in surrounding tissue(s) including the injection track (dotted line with arrow).

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Key words: Antiproliferative agents, Fibroblast, Growth factors, RNA, Scarring, Wound healing.

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