Corneal Repair following Keratectomy

A Comparison between Conventional Surgery and Laser Photoablation

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We have used the fluorescent dye dichlorotriazinyl aminofluorescein (DTAF) to demonstrate corneal remodeling following keratectomy in the rabbit. The dye was applied to the surface of 3.5 mm diameter anterior keratectomy wounds produced by either lamellar dissection or photoablation with an excimer laser (193 nm) to a depth of 15, 50 or 75 μm. Stromal wounds that had been ablated in 12 concentric steps to produce a graded profile with a central depth of 15 or 30 μm were also studied. The repair process was followed for periods of up to 6 months. These results were compared to wounds of similar dimensions in which an intrastromal keratectomy was performed and the anterior stromal surface replaced. Sections examined by fluorescence microscopy showed that connective tissue was deposited beneath the epithelium of all anterior keratectomy wounds irrespective of their mode of induction or depth. The deposition of this new tissue, and an associated thickening of the epithelium over the wound surface, appeared to be complete by 1 month and tended to restore the original surface contour. The synthesis of connective tissue, but not the hyperplasia of the epithelium, was reduced by local steroid treatment. In contrast, an intrastromal keratectomy only stimulated the deposition of small amounts of new connective tissue at the wound junction without as marked a thickening of the overlying epithelium. These observations emphasize the importance of the epithelium in moderating repair after stromal loss, and suggest that remodeling may result in corneal haze and a change in the desired refraction if refractive surgery is attempted by anterior keratectomy. Invest Ophthalmol Vis Sci 30:1769-1777, 1989

Argon fluoride excimer lasers (193 nm) can accurately etch plastic and biological materials by a mechanism termed ablative photodecomposition.1,2 The proposed clinical applications of excimer lasers for corneal surgery include the fashioning of radial or arcuate incisions for the correction of myopia or astigmatism3,4 and the direct rep.profiling of the anterior surface of the cornea—photorefractive keratectomy (PRK).5,6

PRK differs in concept from other refractive surgical operations in that it would alter the power of the eye by removing tissue from the anterior stromal surface of the optical zone. For the results of such an operation to be clinically acceptable the cornea must remain transparent after the keratectomy and the induced refractive correction must be stable and permanent. It is therefore necessary that the repair processes that follow surgery are predictable and that they do not adversely effect the final visual acuity.

After a linear incision has been made in the cornea of a rabbit, the epithelium that covers the wound becomes thicker than normal to plug the defect and collagenous scar tissue is deposited beneath the epithelial layer.7 A similar repair process follows anterior keratectomy, when the combined response may entirely fill a shallow stromal defect.8,9 However, after a deep keratectomy, repair may be incomplete and a residual depression will persist. Over a period of several months a gradual maturation of scar tissue occurs which, in the rabbit cornea, may become completely transparent.10,11

When corneal repair is complete it is difficult to identify the original surface of a wound at histology, and thus to analyze the extent of new connective tissue synthesis. A simple technique to study corneal remodeling has been described in which a fluorescent dye is used to label connective tissue adjacent to a freshly wounded surface.12,13 The dye forms an irreversible covalent bond with the exposed collagen and, because remodeled collagen is unlabeled, the original wound surface can be identified by fluorescence microscopy for as long as 1 year after surgery. We have used this technique to examine stromal repair in rabbit corneas after either surgical lamellar keratectomy.
or photoablation with an excimer laser. We have also studied the effect of wound depth and steroid treatment on scar formation. Finally, we have studied repair after intrastromal keratectomy to define the role of the anterior corneal surface in moderating stromal regeneration.

Materials and Methods

Thirty-eight New Zealand white rabbits and six Dutch Belted rabbits were used in this study. The majority were approximately 6 months old at the time of surgery, but selected procedures were performed in parallel on 2-year-old animals to determine the influence of increased age upon the repair process. Prior to surgery, anaesthesia was induced by an intramuscular injection of Hypnorm 0.75 ml/kg (Janssen Pharmaceuticals Ltd., Oxford, UK) and topical proparacaine hydrochloride. Only one eye of each animal was operated upon and all the animals used in these studies received adequate care and humane treatment in keeping with the ARVO Resolution on the Use of Animals in Research.

Laser Excisions

Circular discs were ablated into the center of the cornea using a series 2000 Questek excimer laser emitting at 193 nm (Questek Inc., Bedford, MA). The gas mixture of argon, fluorine and helium buffer in the cavity was that recommended by the manufacturer. The most uniform area of the beam was selected with a 5 mm mask which was then imaged onto the cornea with a plano-convex quartz lens f = 35 cm (Spectrall II, UCG., Cambridge, UK) to produce a 3.5 mm diameter anterior keratectomy. All ablations with this laser were performed at a pulse repetition rate of 10 Hz and the diameter of the beam was checked by ablating a piece of exposed photographic paper placed in the plane of the cornea. The energy per pulse at the irradiated surface was measured with a Joulemeter (Gen-tec ED 200, Ste-Foy, Canada) via a storage oscilloscope (Tektronix 7633, Beaverton, OR) and adjusted to 100 mJ/cm². We noted that during ablation a reduction of fluorescence occurred which seemed to coincide with the interface between the epithelium and the stroma, and this was confirmed histologically. Using the change in fluorescence as a marker, and assuming that the stroma ablated at approximately the same rate as the epithelium, we produced stromal cuts to depths of 50 μm or 75 μm after ablation of the epithelium. Stromal healing was also studied following ablation to a depth of 15 μm after the central epithelium had first been debrided with a blunt spatula.

All of the discs produced with the Questek laser had a single step when viewed in section. The effect of wound profile on healing was evaluated by ablating discs with a terraced lenticular configuration. These were produced with a Summit UV 200 excimer laser (193 nm, 200 mJ/cm², 5 Hz) (Summit Technologies Inc., Watertown, MA). This instrument produces flattening of the anterior corneal surface by reducing the area of tissue that is ablated during the period of laser exposure. Using the approach of Munnerlyn and associates, 3.5 mm discs were cut with 12 concentric steps of equal depth, to produce stromal cuts with a central depth of either 15 or 30 μm. These discs had an aspect ratio over the whole wound that was appropriate to either a 5 diopter or a 10 diopter myopic correction.

Lamellar Keratectomy

Anterior lamellar keratectomy wounds were studied to permit a comparison between healing after excimer laser photoablation and mechanical wounds. The eye was propsected and a partial thickness incision was centered on the apex of the cornea with a 3.5 mm trephine. A superficial keratectomy of approximately 75 μm was then completed by blunt dissection.

An intrastromal keratectomy was devised to investigate remodeling after recession of the anterior surface of the cornea and to define the role of the corneal epithelium and its basement membrane. A 3.5 mm lamellar keratectomy was fashioned as above and a second keratectomy specimen was then removed from the wound bed. The superficial keratectomy button was then replaced and secured using eight interrupted 10.0 monofilament nylon sutures (Ethicon Ltd., Edinburgh, Scotland). This operation was repeated in other animals in whom the epithelium had first been debrided.

Fluorescent Labeling

The stromal surfaces exposed at surgery were irrigated with a freshly prepared 0.5% solution of DTAF (Sigma Chemical Co., St. Louis, MO) dissolved in 0.2 M sodium bicarbonate. After 30 sec the excess dye was removed by flushing the surface with phosphate-buffered saline and a drop of 0.5% chloramphenicol instilled.

In a parallel experiment we attempted to prevent the development of subepithelial haze after laser keratectomy by using local steroid treatment. A 10 diopter disc was ablated into one eye of six rabbits. In three of the six animals a subconjunctival injection of betamethasone (4 mg/ml) was placed in four quad-
Clinical photographs taken 3 months after laser keratectomy (10 diopter discs). (A) No postoperative steroid treatment. (B) With local steroid treatment. Note that a slight subepithelial haze has developed despite the use of steroids (bar marker is 1 mm).

Results

Clinical Examination

The regenerative processes that followed both laser and lamellar anterior keratectomy were similar and they will therefore be considered together. On macroscopic examination all of the discs had reepithelialized by the fifth postoperative day, but one instance of secondary epithelial loss was observed 2 weeks after laser keratectomy. In all of the rabbits, regardless of the wound depth or the profile of the wound edge, the site of the disc was evident with tangential broad beam illumination as a circular area of white subepithelial haze (Fig. 1A). This haze could be easily distinguished from the green fluorescence of bound DTAF. The intensity of the haze gradually reduced over the course of the experiment (6 months) but it
Table 1. Summary of data from rabbits without steroid treatment 3 months after keratectomy

<table>
<thead>
<tr>
<th>Wound profile</th>
<th>Laser keratectomy</th>
<th>Mechanical keratectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Noncontoured (1 step)</td>
<td>Contoured (12 steps)</td>
</tr>
<tr>
<td>Maximal depth of stromal cut (µm)</td>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td>Collagen synthesis (%)*</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Stromal haze†</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Change in contour‡</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

* Estimated as percentage of total depth of wound remodeling.† Estimated with oblique broad-beam slit-lamp viewing (± = just detectable, +++ = severe).
‡ As detected by photokeratoscopy, arbitrary units (0 = none, +++ = easily detected).

...did not entirely disappear in any animal. Postoperative treatment with steroid markedly reduced the intensity of this haze (Fig. 1B). A facet of thickened but clear epithelium could be seen over some wounds. A change in the surface contour at the site of all keratectomies was evident by photokeratoscopy at 1 month, but the site of 15 µm stromal cuts could not be identified at 3 or 6 months.

After intrastromal keratectomy a well defined depression in the contour of the anterior corneal surface was easily detected. A faint haze persisted at the site of the wound interface, but this was less marked than the subepithelial haze observed after anterior keratectomy (Table 1).

Histological Examination

On histological examination there was a thickening of the epithelium over the bed of all anterior keratectomy specimens (Fig. 2A). This was due to both a vertical elongation of the basal cells and an increase in the number of the superficial cell layers. By the seventh day after surgery a hypercellular zone in which the regular array of the stromal lamellae was lost had developed beneath the epithelium. The thickness of this zone increased over a period of 1 month. After this time its depth remained constant, although there was a reduction in cellularity.

Fluorescence Microscopy

On fluorescence microscopy the subepithelial zone was noted to correspond to a layer that was not labelled with DTAF, and which was therefore interpreted to be connective tissue that had been synthesized since the time of surgery (Fig. 2B, C). The non-fluorescent connective tissue was never observed on the surface of a wound that had not been covered by epithelium. It first appeared in the periphery of wounds after reepithelialization, and had usually extended over the entire wound bed between the 7th and 14th postoperative days (Fig. 3). The final thickness of the new tissue was greatest in the periphery of the wounds, especially of the 75 µm single stepped cuts, and it had achieved its maximum thickness by one month. An interleaving between the fibrils of the labeled and the unlabeled collagen was evident, particularly in the deeper portions of the wound (Fig. 2). After anterior keratectomy the relative contribution of either epithelial thickening or new connective tissue deposition to the restoration of the final surface contour was not constant between specimens. Thus in animals in which only a thin layer of new collagen was deposited, the amount of epithelial thickening was correspondingly greater.

Unlabelled connective tissue was evident over the shallowest wounds studied—the 15 µm disc cut after epithelial debridement and the 5 diopter terraced cut (Fig. 4A, B). Thus, once the anterior stromal surface had been destroyed, the depth of a laser keratectomy had no demonstrable influence on the stimulus to synthesize new connective tissue. In animals that received postoperative treatment with steroid there was only a thin subepithelial zone of newly synthesized collagen (Fig. 4C). However, of particular interest was the observation that the reactive thickening of the epithelium over the site of a keratectomy was still present after the stromal response had been suppressed. New collagen was deposited after anterior keratectomy in 2-year-old rabbits. Although it was our impression that the synthesis was less than in 6-month-old animals, further studies are needed to permit a quantitative comparison between the growing and the mature eye.

After intrastromal keratectomy the deposition of connective tissue was observed at the vertical edges of the wound. In contrast, only a relatively small amount of unlabeled collagen was observed at the interface of the intralamellar wound, and none was...
Fig. 2. Three months after a 3.5 mm diameter anterior keratectomy. (A) Light micrograph of a 75 \( \mu \text{m} \) depth laser keratectomy. (B) Fluorescence photograph of a section adjacent to (A). (C) Fluorescence photograph after a lamellar keratectomy. Note the altered orientation of the regenerated collagen fibers within the scar tissue (asterisk) in the light micrograph, and the demonstration by fluorescence microscopy of scar tissue (S) as unlabeled connective tissue beneath the epithelium (bar marker is 100 \( \mu \text{m} \)).

detected immediately beneath the epithelial layer (Fig. 5). There was a slightly greater deposition of connective tissue at the intralamellar surface if the epithelium was debrided at the time of surgery, with small areas of synthesis within the button. The epithelium that covered the surface of an intrastromal keratectomy was of a more normal appearance than was seen after deep anterior keratectomy, but there was a hypertrophic response at the disc margins.

**Discussion**

We have demonstrated in the rabbit a mechanism to restore the original corneal contour after loss of the anterior stromal surface. This response involves both hyperplasia of the corneal epithelium that covers the wound and the deposition of new stromal collagen. These two processes are complementary and restore the original surface contour of shallow wounds (15
Fluorescence photographs of early stromal regeneration (10 diopter discs) (A) 7 days post-surgery, (B) 14 days post-surgery. Note that the deposition of connective tissue is mainly in the subepithelial zone. The collagen fibers within the scar tissue (S) appear to be only loosely aggregated in this early phase of regeneration (bar marker is 50 μm).

μm, but remodeling of deeper wounds (50 and 75 μm) is incomplete and a residual depression in the surface persists at 6 months. We observed that the synthesis of connective tissue only began after reepithelialization of the wound surface, that it was subsequently produced for a period of approximately 1 month, but we could not detect subsequent remodeling by histological examination. We were unable to demonstrate any quantitative difference in the synthesis of new connective tissue over a rough wound made by lamellar keratectomy or a wound produced by excimer laser photoablation. When we compared these results with contoured wounds we were unable to demonstrate an influence of the depth of the steps comprising the wound upon the stimulus to initiate stromal regeneration.

There are presumably a number of factors that control the regeneration of connective tissue after the loss of the anterior stromal surface. It is interesting that a depression in the surface contour produced by intralamellar keratectomy did not initiate this response when the original anterior corneal surface of epithelium and anterior stroma was preserved. If the epithelium was debrided at the time of surgery then there was only a limited deposition of connective tissue within the cornea. This would indicate that the ante-
rior corneal surface can prevent stromal remodeling, an effect that is only partly dependent upon the presence of intact epithelial cells. In contrast, once the anterior surface has been disrupted and the stroma exposed, the regrowth of epithelial cells over the wound is then essential before the synthesis of new connective tissue can occur. Therefore, our data agree with the observation previously reported by Gasset and Dohlman that the corneal epithelium must be present before stromal wound healing will occur.\(^\text{15}\) The nature of this cellular cooperation is uncertain, but a stimulatory influence of epithelial cells upon keratocytes during wound healing has been proposed,\(^\text{16}\) and epidermal growth factor is a potent accelerator of experimental stromal wound healing in rabbits.\(^\text{17}\)

The origin of the subepithelial haze that develops after anterior keratectomy may be the result of light scattered by newly synthesized collagen fibers, which have larger diameters and greater interfiber spacing than normal.\(^\text{18,19}\) Alternatively, it has been proposed that collagen fibers cut at the wound surface can themselves scatter light, but the persistence of disorganized collagen fibers after anterior keratectomy and keratomileusis, despite improving transparency, argues against this theory.\(^\text{20,21}\)
Fig. 5. Repair after intrastromal keratectomy. (A) Epithelium intact at the time of surgery. (B) Epithelium debrided at the time of surgery. No significant synthesis of new connective tissue is evident at the wound interface (W) if the epithelium is preserved. Note that there is a narrow zone of connective tissue synthesis at the wound interface if the epithelium is debrided, but no connective tissue is deposited beneath the epithelium (E) (bar marker is 100 μm).

We observed that the stromal haze and the thickness of the subepithelial layer of new collagen could be reduced by local steroid treatment. Intense local or systemic steroid treatment is known to reduce the numbers of fibroblasts around corneal wounds, to reduce the deposition of connective tissue and to delay wound healing. This may be mediated by an anti-inflammatory effect or more probably by direct inhibition of corneal fibroblast recruitment and proliferation. This latter theory is supported by the observation that dexamethasone has a suppressive effect upon fibroblast proliferation in tissue culture. However, we still observed epithelial hyperplasia over wounds that had received steroid treatment and the site of discs in steroid-treated animals were not more distinct at photokeratoscopy.

It has been established that the cornea of the rabbit has considerable regenerative potential. We are not aware of DTAF being used to document stromal repair in primates, but an alternative approach has been to demonstrate immunohistochemically the macromolecules involved in the repair process. Results using fibronectin and type III collagen as markers for stromal repair show that a similar process of collagen deposition occurs in the rabbit and the monkey cornea after anterior keratectomy. Whether the primate cornea will not mount a fibroblastic response if excisions are confined to the acellular Bowman’s layer requires verification. However, restricting a procedure to Bowman’s layer limits the degree of correction that can be achieved for a given diameter of optical zone.
The attractive feature of the excimer laser for use in refractive surgery is its ability to precisely remove graded amounts of tissue from the anterior corneal surface. It has been proposed that this facility could be used to recontour the optical zone and thus correct refractive error. The most finely graded discs that we studied consisted of 12 concentric steps ablated to a central depth of 15 μm. Although it is possible to produce a more gradual contour, with this configuration we found no evidence that any properties inherent in the mechanism of ArF excimer laser ablation would prevent subsequent stromal regeneration or the development of subepithelial haze after anterior keratectomy in the rabbit. We consider that stromal repair contributes to subepithelial haze and that remodeling will reduce the effect of a refractive correction. Although our preliminary experience indicates that the subepithelial haze and the fibroblastic response may be reduced with steroid therapy, epithelial hyperplasia was not prevented and the optical significance of thickened epithelium over a contoured wound awaits further study.

Key words: cornea; fluorescent dyes, lasers, wound healing

Acknowledgments

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