Stromal remodelling following photorefractive keratectomy

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Abstract

An excimer laser (193 nm) was used to ablate discs 3.5 mm in diameter into the anterior surface of rabbit corneas. The bed of each disc was then labelled with dichlorotriazinyl aminofluorescein and animals sacrificed at intervals of one or two months. This technique demonstrates the deposition of new collagen beneath the epithelium during the process of corneal remodelling after deep keratectomy.

Introduction

A surgical technique has recently been proposed that would alter the refractive power of the eye by using laser energy to reprofile the anterior corneal surface. Most studies of this procedure, termed photorefractive keratectomy (PRK), have used an argon fluoride (ArF) excimer laser (193 nm) because of the clean wound surface that is produced. The properties of tissue photoablation that are fundamental to this type of surgery can be summarized as follows: Firstly, the limited penetration of the cornea by ultraviolet radiation at 193 nm and the nature of the photoablative process restricts the effect of each laser pulse to a superficial layer with only a narrow zone of conductive damage to adjacent tissue. Secondly, the amount of tissue removed per laser pulse is energy dependent once the ablation threshold has been attained. Tissue can thus be removed from the target surface in predictable increments. Thirdly, the ablated surface is exceptionally smooth and sealed by a pseudo-membrane, leaving a suitable substrate to preserve the new optical properties of the eye.

Mathematical models predict that significant changes in refractive power can be achieved by volume ablation confined to Bowman’s layer. Experiments in animals have demonstrated that photoablation can effectively alter the refractive properties of the eye and suitable delivery optics have been designed to flatten or steepen the curvature of the anterior surface of the cornea. For PRK to be a clinically acceptable procedure, a number of requirements must be satisfied. Epithelial migration across the wound must proceed without significant delay or loss of adhesion to the underlying stroma. Secondly, the ablative process should not result in visually significant stromal opacification from either acute thermal effects or sub-epithelial scarring from tissue repair. Finally, the contour of the reprofiled surface must remain constant within narrow limits; a tendency for either the forward movement of thinned cornea or for epithelium and new stromal collagen to fill ablated areas would defeat the long-term objective of a predictable and stable refractive change.

Studies of deep (150 μm) PRK wounds have not demonstrated a significant latency of migration or
Fig. 1. (a) Photograph of 3.5 mm disc ablated into the cornea of a rabbit. (b) With ultraviolet light the site of the disc is accentuated by fluorescence from bound DTAF. (c) Light micrograph of a section at the edge of a disc from a rabbit one month post surgery. Hyperplasia of the epithelium is marked over the edge and base of the disc, and the stroma beneath the disc has a disordered orientation (toluidine blue). (d) Photograph taken by fluorescence microscopy of a section adjacent to (c) demonstrates a band of collagen that has a lower level of fluorescence than the epithelium or the labelled stroma.
abnormal epithelial adhesion, although epithelial hyperplasia overlying the disc has been reported. However, there is some debate in relation to stromal changes. A percentage of the ablation sites develop a sub-epithelial haze, and a degree of disorganisation of the underlying collagen has been evident at histology. When care has been taken to limit the maximum ablation depth to 50 μm or less these problems are less apparent. The aetiology and nature of this opacity need to be defined.

Clinical experience indicates that the cornea has a considerable ability to synthesise new tissue and in so doing to regain contour lost after injury or inflammation. An alteration of the topography of the optical zone following PRK would provide evidence of remodelling, but there is no suitable technique to monitor changes in the shape of this small corneal area in vivo. Keratoscopic evaluation following deep ablation of the monkey eye suggests that there is a tendency for the induced correction to regress, but similar studies following shallow PRK procedures are not available. Although corneal scarring may be evident clinically it is difficult to distinguish between normal stroma and scar tissue on conventional histopathological preparations. Clearly some remodelling must occur and a technique to discern the original wound surface in a healed cornea would be of value for following this reaction. Davison and Galbavy have described a technique whereby freshly exposed stromal tissue is covalently labelled with the fluorescent dye dichlorotriazinyl aminofluorescein (DTAF). DTAF is retained in the stroma for at least a year and is not mobilised during remodelling. This paper reports our preliminary results with this technique in the study of collagen repair in the rabbit model for PRK.

Material and methods

New Zealand white rabbits of approximately 2.5 kg were used throughout this study. Anesthesia was induced by intramuscular injection of Fentanyl 0.75 ml/kg (Hyn norm) and local application of proparacaine hydrochloride. A circular disc of 3.5 mm diameter was ablated into the centre of the cornea of the right eye of each animal using a Questec series 2000 excimer laser filled with ArF to the manufacturers' specifications. The beam of the laser was cropped by a 5 mm mask before it was passed through a plano-convex lens (focal length 35 mm) of UV grade quartz (Spectracil II), and the mask imaged on the cornea. The beam energy was calibrated at the image plane on the cornea with a Joulemeter (GenTec ED 200) and was adjusted to 100 mJ/cm² with a repetition rate of 10 Hz. It was found empirically that 1000 pulses were required to produce a disc of approximately 75 μm in the stroma after ablation of the corneal epithelium. At the completion of irradiation the wound was irrigated with a 0.5% solution of DTAF dissolved in 0.2 M sodium bicarbonate. After 30 sec the excess dye was washed from the wound with phosphate buffered saline and a drop of 0.5% chloramphenicol instilled. As re-epithelialisation progressed, the epithelial cells masked the fluorescence from the underlying stroma which permitted the process of re-epithelialisation to be followed by illuminating the cornea with an ultraviolet source (UVS-11). Animals were examined twice daily until re-epithelialisation was complete and twice weekly thereafter.

Animals were killed at an interval of one or two months after the procedure by intravenous Pentobarbitone (Euthetal). Following enucleation the epithelium was fixed for 30 min in a solution of 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, a sclerotomy was then performed and the globe returned to the fixative. After 24 h the cornea was dissected from the globe and divided into two segments. Tissue for light microscopy was dehydrated through an alcohol series and embedded in JB4 resin. Sample sections of each disc were cut with a glass knife and stained with toluidine blue for histological examination and adjacent 1 μm sections photographed using an epi-fluorescent microscope.

Samples for TEM were first fixed for one hour in 0.1% osmium tetroxide before being dehydrated and embedded in Araldite. Sections were cut with a diamond knife on a Reichart OUM4 microtome, stained with lead citrate and uranyl acetate before being viewed in an JEOL 100S microscope.
Results

Following the ablation of a 3.5 mm disc in the rabbit cornea all wounds had re-epithelialised by three days with no evidence of subsequent wound breakdown during the period of follow-up. Sub-epithelial haze beneath the area of the disc was noted at both the one and two month observation periods in all animals, and this could be easily distinguished using a slit lamp microscope from the fluorescence due to bound DTAF.

Histological examination of stained sections confirmed the presence of an ablated disc in the stroma of the cornea (Fig. 1). The edge of the wound appeared wider at the lip than at the base. The epithelium that covered the wound was hyperplastic with an accentuated columnar appearance to the basal cells. The thickened epithelium gradually merged into the normal epithelium at the edge of the disc. Immediately beneath the epithelium the orientation of the lamellae was irregular. Keratocytes appeared to be present in normal numbers and no inflammatory cells were noted.

Fluorescence microscopy demonstrated a background fluorescence throughout the specimen but fluorescence due to DTAF bound to the stroma of the cornea was clearly demonstrated over the wound bed. A zone of collagen with a reduced level of fluorescence was observed beneath the epithelium and this gradually reduced in thickness as it ascended the edge of the wound. At the interface between the two zones of labelled and unlabelled collagen there was some undulation of the fluorescent border. We interpret the junction between the fluorescent and the non-fluorescent collagen as representing the site of the original wound surface.

On examination of transmission electron micrographs of the interface of normal basal epithelium and stroma a number of features are apparent (Fig. 2). The epithelial cells are separated from the superficial stroma by their basement membrane (50 to 70 nm). There is a slight undulation of the membrane, with an excursion of up to 100 nm and a periodicity of 300 nm. The basal cells adhere to the basement membrane at hemidesmosomes distributed every 300 to 400 nm. The superficial 2 μm of stroma does not have the organized collagen arrays of the deeper lamellae but is formed of a random array of fibers 30 to 35 nm in diameter with an interfibrillar matrix of a similar density to the basement membrane of the epithelium. Keratocytes populate the stroma immediately beneath the basement membrane.

In the base of the photoablated discs the basement membrane was less regular in thickness (50 to 150 nm) with less marked undulations. There were normal numbers of basal hemidesmosomes of normal morphology. The underlying stroma did not show the 2 μm layer of disorganised collagen, but a much deeper layer of poorly organised material 25 μm thick. The collagen fibers in this area averaged 25 to 30 μm in diameter. There was electron dense interfibrillar material throughout the depth of the remodelled zone. In this study we have not correlated TEM of the ablated region with the interface defined by fluorescence.

Discussion

The potential benefit of a method to precisely manipulate the curvature of the corneal optical zone would be considerable. The feasibility of PRK has been demonstrated in animals and the acute histological changes have been documented. This paper addresses the nature of corneal repair in a rabbit model following 193 nm excimer laser PRK.

Collagen fibers deposited during embryogenesis are arranged in a regular pattern of lamellae and it is thought that both the restriction in fiber cross-sectional diameter and the regular interfibrillar
spacing is necessary for corneal transparency;\textsuperscript{4, 13} thus the stereo-spatial morphology of collagen in part determines corneal transparency and conversely an increased fiber thickness and interfibril spacing or a decreased density of fibers are associated with a reduction in transparency. The regularity of the collagen fibrils may reflect the pattern of their component collagen. To date, of the 11 types of collagen that have been characterized, types I, II, V and VI have been identified as major components of human corneal stroma.\textsuperscript{16, 18} Bowman’s layer differs from the rest of the stroma in that it is acellular and contains mainly type V collagen, while the epithelial basement membrane is formed mainly by type IV collagen.\textsuperscript{1}

Repair of corneal wounds does not follow the sequence of normal embryonal fibrogenesis, with differences in both keratocyte organisation and collagen deposition.\textsuperscript{2, 3} The physical properties of corneal stromal scars also differ significantly from normal cornea but it is uncertain if this is the result of differences in the types of collagen fibers laid down by keratocytes, which change during development, or an altered cross-linkage between the collagen fibers.\textsuperscript{2, 3} In the rabbit there is an increased transparency of the wound after prolonged periods with a gradual process of fiber rearrangement to a regular morphology and a reduced cellularity.\textsuperscript{12} Tensile strength of the wound, however, remains permanently impaired.

It is usual to distinguish between wounds of different depth when considering corneal healing. A mechanical abrasion removes the epithelial layer, but the basement membrane and the attachment complexes remain intact and available for the migrating epithelium to use, therefore healing is usually rapid and complete: a keratectomy removes part of the anterior stroma in addition to the basement membrane, while deep lacerations may pass through Descemet’s membrane and enter the anterior chamber. Injuries in which corneal stroma is lost are usually associated with an attempt to reform the original contour. This may be achieved by an epithelial plug in superficial wounds, whereas more severe stromal loss is invariably followed by stromal scarring and permanent corneal thinning. The majority of stromal repair studies have used deep linear incisions. Following this type of injury, Matsuda and Smelser\textsuperscript{12} noted degeneration of damaged fibroblasts within a zone of 300 µm from the wound edge followed by fibroblast re-population within 24 h and the onset of collagen deposition. While keratocyte and epithelial cell interaction is important for scar formation in the superficial portion of the wound, the authors suggested that the origin of collagen in the deep part of the wound was predominantly of an endothelial cell origin.\textsuperscript{12}

Although the photoablated discs in our experiments were square edged in profile and passed deeper into the stroma than is anticipated for clinical PRK,\textsuperscript{15} the epithelium was able to migrate rapidly over the wound edge and to cover the base. However, the regenerated epithelial layer was thicker than normal producing an apparent levelling effect, a phenomenon that has previously been noted to occur following lamellar keratectomy in the rabbit.\textsuperscript{7} The observed deposition of new collagen beneath the thickened epithelium would tend to enhance this filling effect and produce sub-epithelial haze on clinical examination. It has been noted from clinical specimens that collagen deposition is less marked after superficial than deep keratectomies, and whereas the rabbit does not have a keratocyte-free Bowman’s layer, it is possible that wounds limited to this layer of the primate cornea will not demonstrate collagen deposition. Clearly the influence of edge profile, wound depth and other variables upon this reparative response needs to be further investigated.

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References


