Intact corneal epithelium is essential for the prevention of stromal haze after laser assisted in situ keratomileusis

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Abstract

Aims—To determine the effect of intact corneal epithelium on stromal haze and myofibroblast cell formation after excimer laser surgery.

Methods—Denuded epithelium alone, photorefractive keratectomy (PRK), laser in situ keratomileusis (LASIK), or LASIK with denuded epithelium was performed in rabbit eyes. Postoperative anterior stromal haze was assessed employing a standard scale. Immunohistochemical methods were used to detect alpha smooth muscle actin (α -SMA), a marker for myofibroblastic cells, and type III collagen in subepithelial corneal tissue.

Results—Three weeks after surgery, the presence of α -SMA positive long extended and spindle-shaped stromal cells, and synthesis of type III collagen were observed in the subepithelial stromal layer corresponding to corneal haze in PRK and LASIK with denuded epithelium, but not in denuded epithelium alone and LASIK.

Conclusion—The intact corneal epithelium may play an important part curbing subepithelial haze and differentiation of myofibroblasts in corneal wound healing.

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The excimer laser has the potential of producing tissue ablation with a high degree of precision and with minimal damage to the adjacent structures.¹ Photorefractive keratectomy (PRK) has become a favourable surgical procedure to correct refractive error including myopia,² hyperopia,³ and astigmatism.⁴ Although the performance is effective and safe, some patients may develop a corneal subepithelial haze as a postoperative wound healing response especially in patients with allergic conjunctivitis,⁵ and those who complain of glare or disturbance of contrast visual functions.^{6–7} Histopathological studies revealed that subepithelial haze contains newly synthesised collagen such as type III collagen, type IV collagen, fibronectin, laminin, and proteoglycans.^{8–10} During the wound healing process, wound fibroblasts differentiate into contractile, smooth muscle-like cells, “myofi-

broblasts” that express smooth muscle specific alpha actin (α -SM actin) and synthesis type III collagen.¹¹ Jester *et al* reported that the expression of α -SM actin is co-localised to intracellular stress fibres within myofibroblasts contained solely within the corneal wound.¹²

Recently, laser in situ keratomileusis (LASIK) was introduced to the refractive surgery field. This technique combined the excimer laser’s accuracy in removing corneal tissue and the microkeratome’s ability to access the inner stroma, to preserve corneal epithelium and Bowman’s membrane, thereby reducing the effect of wound healing and problems associated with surface PRK.^{13–14} Thus, we have hypothesised that intact epithelium is the key to the prevention of stromal haze after photoablation.

In this article, to investigate this hypothesis, we have evaluated the subepithelial haze and the difference of the expression of α -SM actin during corneal wound healing after denuded epithelium alone, PRK, LASIK, and LASIK with denuded epithelium procedures in the rabbit, respectively.

Materials and methods

LASER SYSTEM

Technolas excimer laser (Bausch & Lomb, Claremont, MA, USA) was used as a source of a 193 nm argon fluoride excimer laser. To ablate cornea in constant thickness, the PTK mode excimer laser was used. The central cornea was ablated using a 400 mJ/pulse at a frequency of 50 Hz.

MICROKERATOME

The automated corneal shaper microkeratome (Bausch & Lomb, Claremont, MA, USA) was used as a cornea lamellar microkeratome. The corneal flap was made with a nasal hinge.

LASER TREATMENT

The animals used in this study were treated in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Thirty two pigmented chinchilla rabbits (2.0–2.5 kg) were divided into four groups: denuded epithelium alone (n=8 eyes), PRK (n=8 eyes), LASIK (n=8 eyes), and LASIK

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with denuded epithelium (n=8 eyes). In all groups, ketamine hydrochloride (4.7 mg 100 g body weight) (Sankyo, Tokyo, Japan) and 2% xylazine hydrochloride (0.46 mg 100 g body-weight) (Bayer, Leverkusen, Germany) were injected intramuscularly and a topical anaesthetic agent was applied to the eyes preoperatively.

In the denuded epithelium alone group, a 6.0 mm diameter of rabbit corneal epithelium was denuded with spatula manually.

In the PRK group, a 6.0 mm diameter of rabbit corneal epithelium was denuded with the spatula manually, then a 4.5 mm diameter and 90 µm deep stromal PTK was performed in the centre of denuded site.

In the LASIK group, an 8.0 mm diameter and 130 µm deep nasal hinged corneal flap was made with a microkeratome, and a 4.5 mm diameter and 90 µm deep ablation was performed in the exposed anterior corneal stroma.

In the LASIK with denuded epithelium group, a 6.0 mm diameter of rabbit corneal epithelium was denuded with spatula manually, then LASIK was performed as previously described.

After surgery, an antibiotic topical agent was applied to all treated eyes for 5 days. Anti-inflammatory drugs were not used during the study.

STROMAL HAZE

Postoperative anterior stromal haze was graded on a scale of 0 to 4 as follows: 0 = clear; 0.5 = faint corneal haze; 1 = mild corneal haze seen only by oblique indirect illumination; 2 = moderate corneal haze seen by direct illumination; 3 = easily visible opacity not affecting refraction; and 4 = dense opacity that impairs view of intraocular structures, possibly affecting refraction.

HISTOPATHOLOGICAL STUDY

The rabbits were killed with an overdose of intravenous sodium pentobarbitone (Dainihonsei-yaku, Osaka, Japan) 1, 3, 6, and 9 weeks after surgery, and the globes were fixed with 10% neutral formalin, embedded in paraffin after dehydration, and cut into 6 µm slices.

The labelled streptavidin-biotin method using a Histostein-SP kit (Zymed, South San Francisco, CA, USA) was used to detect α -SMA and type III collagen immunohistochemically. Sections were deparaffinised with xylene, rehydrated in a graded series of alcohol, and immunostained for α -SMA and type III collagen according to the manufacturer's instructions, with the following modifications. Sections for type III collagen underwent digestion by 2% proteinase (Dako Proteinase Enzyme Digestion, code no S3004, Dakopatts, Denmark) for 10 minutes at room temperature to unmask the antigens. Endogenous peroxidase activity was quenched by treating the sections with 3% hydrogen peroxide in methanol for 20 minutes at room temperature. Non-specific background staining was eliminated by incubating the sections with non-immune goat serum (reagent 1A; H-SP kit) at room

Table 1 The course of subepithelial haze in the different treatment groups

Weeks	Denuded epithelium alone	PRK	LASIK	LASIK with denuded epithelium
1	0	0	0	0
3	0	2	0	3
6	0	1	0	2
9	0	0.5	0	1

temperature. A primary mouse monoclonal antibody directed against α -SMA (IgG2a, clone 1A4, code no M851, Dakopatts, Denmark) or type III collagen (IgG1/k, clone 3-53, code no F-58, Fuji chemical Corp, Japan) was diluted 1:100 with primary antibody diluting buffer (Biomedica Corp, Foster City, CA, USA). Sections were incubated with this diluted antibody in a moist chamber at room temperature for 30 minutes. Sections were then rinsed three times with phosphate buffered saline (PBS), incubated with a biotinylated secondary antibody (goat anti-mouse IgG; reagent 1B; H-SP kit) at room temperature for 10 minutes, and washed three times with PBS. Streptavidin-peroxidase was added (reagent 2; H-SP kit), and the sections were incubated at room temperature for 15 minutes, then rinsed three times with PBS. Finally, peroxidase was visualised by the addition of a solution containing 3-3'-diaminobenzidine hydrochloride (0.3 mg/ml), 0.005% hydrogen peroxide, and 50 mM TRIS-HCl buffer at room temperature for 4 minutes. Sections were then counterstained with haematoxylin.

Mouse monoclonal IgG2a antibody (clone Dak-G05, code no X943, Dakopatts) was used as a negative control. No immunoreaction was detected in the negative control. As internal positive controls, the sphincter and dilator muscles of the iris were used for α -SMA, and the Tenon's capsule was used for type III collagen.

Results

In the denuded epithelium alone group, the PRK group and the LASIK with denuded epithelium groups, re-epithelialisation had completed within 1 week after surgery.

STROMAL HAZE

The course of stromal haze in the different treatment groups is shown in Table 1. In the PRK and LASIK with denuded epithelium groups, stromal haze increased significantly 3 weeks after operation, and then tended to resolve subsequently (Fig 1B, 1C). In the denuded epithelium alone and LASIK group, no stromal haze was observed during the entire period of examination after the operation (Fig 1C, 1D).

MICROSCOPY

In the denuded epithelium alone group, no significant change and expression of α -SMA and type III collagen was observed in the stroma during the entire period of examination after the operation (Fig 2A, 3A).

In the PRK and LASIK with denuded epithelium groups, at 1 week after surgery, one or two layers of migrated corneal epithelial

Figure 1 Rabbit eyes 3 weeks after operation. (A) Grade 0 subepithelial haze after denuded epithelium alone. (B) Grade 2 subepithelial haze after PRK. (C) Grade 3 subepithelial haze after LASIK with denuded epithelium. (D) Grade 0 subepithelial haze after LASIK.

Figure 2 Immunolocalisation of α -SMA in cornea of rabbit eyes 3 weeks after operation (magnification $\times 40$). (A) The central area after denuded epithelium alone. (B) The central area ablated with PRK. (C) The central area after LASIK with denuded epithelium. (D) The central area after LASIK. Long extended and spindle-shaped stromal cells stained with α -SMA appeared in the subepithelial stromal layer after PRK and LASIK with denuded epithelium (arrows), but not after denuded epithelium alone and LASIK.

Figure 3 Immunolocalisation of type III collagen in cornea of rabbit eyes 3 weeks after operation (magnification $\times 40$). (A) The central area after denuded epithelium alone. (B) The central area ablated with PRK. (C) The central area after LASIK with denuded epithelium. (D) The central area after LASIK. Staining of type III collagen appeared in the subepithelial stromal layer corresponding to corneal haze after PRK and LASIK with denuded epithelium (arrows), but not after denuded epithelium alone and LASIK.

cells were observed and no significant change was seen in the stroma. However, at 3 weeks epithelial hyperplasia was observed and the density of long extended and spindle-shaped stromal cells was increased in the subepithelial stromal layer corresponding to corneal haze. Staining of α -SMA and type III collagen also appeared in the subepithelial stromal layer (Figs 2B, C, 3B, C).

In the LASIK group, the stroma generally maintained its lamellar structure in the central surgical site during the entire period of examination after the operation. The number of long extended and spindle-shaped stromal cells increased in the microkeratotomy wound site, but not in the central site. Expression of α -SMA and type III collagen was not observed in the central site at all after operation (Figs 2D, 3D).

Discussion

The first stage of wound healing in the cornea after PRK is epithelial migration along the ablated stromal bed. After re-epithelialisation, compensatory epithelial hyperplasia occurs.^{15–17} In rabbits, this hyperplasia may return to normal in 3–12 weeks.^{10 18 19} Corneal epithelial regeneration is followed by stromal regeneration. During this phase, the number of stromal spindle-shaped keratocytes that are positive for α -SMA, increase in the subepithelial stromal

layer.²⁰ α -SMA is known as a marker of myofibroblasts,²¹ and these activated stromal keratocytes (myofibroblasts) lay down an extracellular matrix.²² From a month to a few months after PRK the number of keratocytes tends to gradually diminish. The degree of corneal stromal haze after PRK correlates with the number of active fibroblasts and the amount of new extracellular matrix.²³

Masur *et al* reported, with use of cultured corneal fibroblasts, that myofibroblasts differentiate from fibroblasts during corneal wound healing in which losses of cell-cell contact between fibroblast cells participate.²⁴ In the present study, expression of α -SMA was not observed in the denuded epithelium alone and the LASIK group, but in the PRK and the LASIK with denuded epithelium group. These findings suggest that loss of cell-cell contact between fibroblast cells may not contribute to myofibroblast differentiation *in vivo*, and myofibroblast differentiation from keratocyte is not induced by only stromal injury but by both epithelium and stromal injury. The basal corneal epithelium anchors itself to the underlying tissue by forming an adhesion complex and the superficial epithelial cells maintain this barrier function by their cell membranes and tight junctions. After PRK, the corneal epithelium had an abnormal barrier function to topically applied sodium fluorescein, and it

recovered to the baseline barrier function 2–4 weeks later.^{18,19} Many cytokines and growth factors are involved in corneal wound healing—epidermal growth factor, keratinocyte growth factor, hepatocyte growth factor, fibroblast growth factor 1 (FGF-1), FGF-2, vascular endothelial growth factor, transforming growth factor alpha (TGF α) and TGF β .^{25–30} Recent studies showed that TGF β 1 plays a central part in the differentiation of myofibroblasts.^{31,32} The abnormal barrier function of corneal epithelium is permeable to these cytokines and growth factors from tear fluid into the corneal stroma, and it may cause activation of keratocytes. On the other hand, Mishima *et al* reported that the contraction stimulating factor derived from corneal epithelial cells modulates the contraction in the corneal stroma,³³ and Wilson *et al* reported that apoptotic cytokines released from injured epithelium trigger keratocyte apoptosis and this results in activated keratocytes replenishing the anterior stroma.³⁴ This suggests that the interaction between epithelial cells and keratocytes is an important factor in corneal wound healing. The present findings in the LASIK with denuded epithelium group did not show the expression of α -SMA and type III collagen close to the irradiated inner stroma but in the subepithelial stroma. This suggests that interaction between earlier regenerated epithelial cells and keratocytes is an important factor in corneal wound healing.

Further investigations are needed to reveal the role of epithelium in corneal wound healing. Thus, intact corneal epithelium may have an important role in curbing subepithelial haze and myofibroblast differentiation. Furthermore, epithelial damage of the corneal flap due to severe dehydration or mechanical injury during LASIK procedures might evoke excessive wound healing.

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