
Wound healing in rabbit corneas after photorefractive keratectomy and laser in situ keratomileusis

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Purpose: To compare the wound-healing process in the rabbit cornea after photorefractive keratectomy (PRK) and laser in situ keratomileusis (LASIK) with the same refractive correction.

Setting: Department of Ophthalmology, Wakayama Medical University, Wakayama, Japan.

Methods: Adult albino rabbits (N = 24) were used. One eye of each animal had PRK or LASIK with the same refractive correction. Each animal was killed after an interval of up to 6 months. The expression pattern of corneal stromal injury-related molecules with the 2 treatments were compared. Paraffin sections of the cornea were processed immunohistochemically for α -smooth muscle actin (α -SMA), collagen type IV [α 1(IV)]₂, α 2(IV), and heat shock protein (HSP) 47 as well as other HSPs. Sections were also examined after hematoxylin and eosin or periodic acid-Schiff staining.

Results: Hematoxylin and eosin staining showed the central epithelium to be thick in PRK-treated corneas. The thick epithelium was restricted to the area around the corneal flap edge adhesion in LASIK-treated corneas at 3 months. Periodic acid-Schiff staining showed an absence of or interruption in the epithelial basement membrane in PRK-treated corneas for up to 6 months. Heat shock protein 47 was detected in keratocytes on day 3 but not after that in PRK-treated corneas. There was no difference in the expression of other HSPs. Alpha-smooth muscle actin was expressed in keratocytes repopulated in the central anterior cornea of PRK-treated corneas at 28 days. Keratocytes with immunoreactivity for these 2 proteins were not seen in LASIK-treated corneas. Collagen IV [α 1(IV)]₂, α 2(IV) was not detected in either group of corneas. The central epithelium became transiently thicker in PRK-treated corneas.

Conclusion: Keratocyte responses to laser stromal ablation were more marked in corneas treated with PRK than in those treated with LASIK.

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Excimer laser ablation of the corneal stroma is now popularly used to correct a refractive error by altering the anterior corneal curvature. Currently, 2 procedures are performed to treat myopia: photorefractive keratectomy (PRK) and laser in situ keratomileusis

(LASIK).¹⁻⁶ Although LASIK is becoming more popular than PRK, it can result in various complications associated with the creation of an anterior corneal flap.^{3,6-10}

Several molecules are known to be involved in corneal epithelial and stromal wound healing.¹¹ Among them, a variety of extracellular matrix metabolism-related proteins have important roles: collagen types,¹² adhesive glycoproteins,¹³ proteoglycans,¹⁴ and matrix metalloproteinases and their endogenous

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inhibitors.^{15,16} Aspects of corneal wound healing are reported by Jain and Azar,¹⁷ Kao and Ishizaki,¹⁸ Zieske,¹⁹ and Saika et al.²⁰

Although Hutcheon and coauthors²¹ have compared the proliferating activity of keratocytes in corneas treated by PRK and LASIK, the corneal wound-healing process (expression of matrix molecules and keratocyte phenotype modulation) after PRK and LASIK has not been studied. We compared the wound-healing process in the 2 procedures but did not investigate the basic biological mechanism of corneal wound healing. We performed PRK and LASIK in the corneas of adult albino rabbits and then examined the tissue immunohistochemically, focusing on the expression patterns of 3 wound-healing-related molecules—heat shock protein (HSP) 47, α -smooth muscle actin (α -SMA), and collagen type IV [α 1(IV)]₂, α 2(IV).

Collagen type IV [α 1(IV)]₂, α 2(IV) is not observed in the normal corneal epithelial basement membrane but is immunohistochemically detected in the epithelial basement membrane of severely damaged corneas such as alkali-burned corneas.^{22–26} Heat shock protein 47 works as a molecular chaperone of collagen precursors in the cytoplasm and is known to be an up-regulator of collagen type I production in keratocytes.²⁷ It serves as a marker of accelerated collagen biosynthesis because keratocytes up-regulate collagen expression during healing. Alpha-smooth muscle actin is known to be expressed in myofibroblasts but not in keratocytes in the normal/quiescent condition.²⁸

Materials and Methods

Experiments were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Adult Japanese albino rabbits (N = 24) were generally anesthetized by intramuscular injection of ketamine hydrochloride and xylazine as reported.²⁹ The right eye of each animal received PRK or LASIK using a Nidek EC-5000 excimer laser at a frequency of 30 Hz. The mean preoperative anterior corneal curvature in the rabbit eyes was 44.0 diopters (D). The optical zone in all eyes was 6.0 mm and the refractive correction, -5.0 D. The ablation area was adjusted to be the same in PRK and LASIK. The corneal flap in LASIK was cut using an Automated Corneal Shaper[®] (Bausch & Lomb) with a 160 μ m plate. Ofloxacin ointment was applied to reduce the risk of posttreatment infection.

Each animal was killed immediately after the treatment, at 3 or 28 days, or at 3 or 6 months by an intravenous overdose

of pentobarbital. Each eye was enucleated and well fixed in paraformaldehyde 2.0% in 0.1 M phosphate buffer. Paraffin sections of 5.0 μ m were processed for hematoxylin and eosin (H&E) staining, periodic acid-Schiff (PAS) staining, and immunohistochemical analysis.

Immunohistochemistry

Indirect immunostaining was used as previously reported.²² Briefly, a deparaffinized section was allowed to react with each antibody adequately diluted in phosphate-buffered saline (PBS) following a blocking procedure. The antibodies used were mouse monoclonal anti- α SMA antibody ($\times 200$ in PBS, Sigma), goat polyclonal anticollagen type IV [α 1(IV)]₂, α 2(IV) antibody ($\times 200$ in PBS, Southern Biotechnology), and goat polyclonal anti-HSP47 antibody ($\times 200$ in PBS, Santa Cruz Biotechnology). Antibodies against HSP27, 60, 70, and 90 were also used ($\times 200$ in PBS, Santa Cruz Biotechnology). After the specimens were washed in PBS, they were allowed to react with a peroxidase-conjugated secondary antibody ($\times 100$ in PBS, Cappel, Organon-Technika). Peroxidase reaction was done with 3,3'-diaminobenzidine, and counterstaining was performed with methyl green as previously reported.²²

Results

Light Microscopic Histology

In PRK-treated corneas, H&E-stained specimens showed an epithelial defect and thinned central stroma immediately after treatment. At 3 days, the central ablated stroma was covered with a regenerated thick epithelial sheet, and the peripheral corneal epithelium was thinner than normal epithelium (Figure 1, *A1* and *A2*). Periodic acid-Schiff staining showed that at this time, the central cornea lacked an epithelial basement membrane (Figure 1, *A1* and *A2*). At 3 months, the regenerated central epithelium was thicker than the peripheral corneal epithelium outside the ablated area (Figure 2). The irregular PAS staining pattern of the epithelial basement membrane of the cornea was observed until 6 months posttreatment (Figure 1, *A1*, *A2*, *C*, *E*, and *G*).

In LASIK-treated corneas, hyperplastic epithelium and an accumulation of keratocytes around the stromal incision were seen at 3 days. At 28 days, keratocyte distribution in the central stroma appeared normal. The PAS-stained scar tissue of the corneal stroma was observed in the area of the flap stromal bed at 6 months (Figure 1, *B*, *D*, *F*, and *H*).

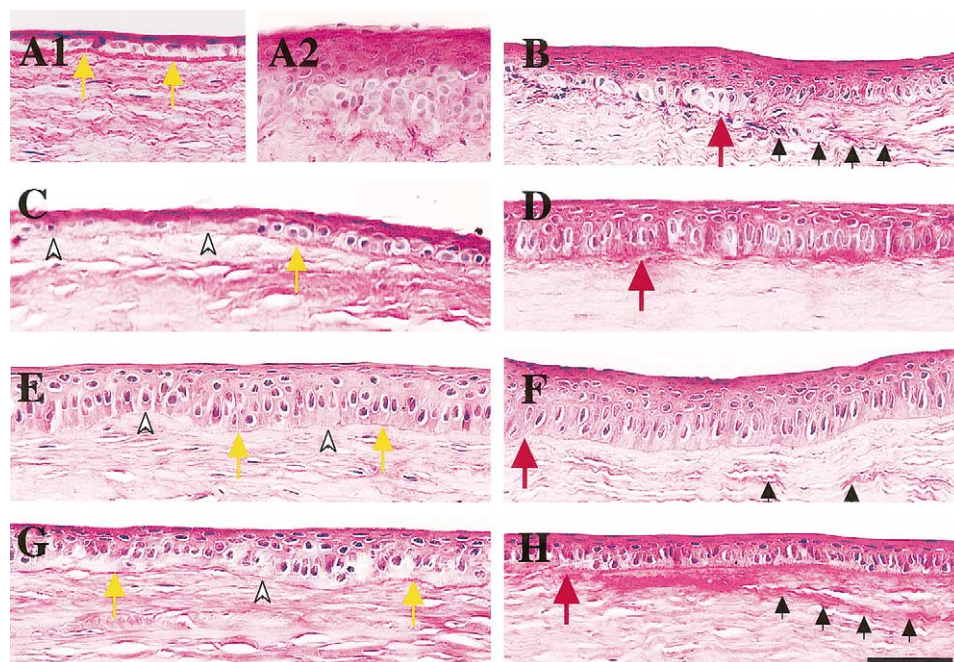


Figure 1. (Miyamoto) Histological findings in rabbit corneas after PRK or LASIK shown by PAS staining. A1: Peripheral area of corneal epithelium 3 days after PRK. A2: Central area of corneal epithelium 3 days after PRK. B: Area of adhesion between the corneal flap and stromal bed 3 days after LASIK. C: Central area of the corneal epithelium 1 month after PRK. D: Area of adhesion between the corneal flap and stromal bed 1 month after LASIK. E: Central area of the corneal epithelium 3 months after PRK. F: Area of adhesion between the corneal flap and stromal bed 3 months after LASIK. G: Central area of the corneal epithelium 6 months after PRK. H: Area of adhesion between the corneal flap and stromal bed 6 months after LASIK. The PAS-positive epithelial basement membrane (yellow arrows in A1, C, E, and G) is missing or interrupted beneath the regenerated epithelium in corneas after PRK (open arrowheads in C, E, and G). The epithelial basement membrane in the central cornea is intact but is interrupted at the site of the microkeratome incision in a LASIK-treated cornea (red arrows in B, D, F, and H). A PAS-positive matrix substance is also observed in the central stroma (black arrowheads in B, D, F, and H), the presumed adhesion site between the corneal flap and stromal bed even 6 months after LASIK (bar = 50 μm).

Immunohistochemistry

In PRK-treated corneas, HSP47 was immunohistochemically detected in keratocytes at 3 days but not thereafter; no such immunoreactivity was seen in LASIK-treated corneas (Figure 3). At this time, the expression of HSP47 seemed to be up-regulated in the regenerated central epithelium of PRK-treated corneas (Figure 3). Other HSP proteins (27, 60, 70, and 90) were detected in the corneal epithelium in both treatment groups, but no difference was observed between normal and regenerated epithelia. In PRK-treated corneas, α -SMA was expressed in keratocytes located in the central stroma beneath the regenerated epithelium at 28 days but not at 3 months or later (Figure 4). In LASIK-treated eyes, no α -SMA-positive keratocytes were detected during the healing intervals (Figure 4). Collagen type IV [α 1(IV)]₂ α 2(IV) was not detected at any time in either group.

Discussion

We immunohistochemically detected transient up-regulation of 2 wound-healing-related proteins, α SMA and HSP47, in the keratocytes in rabbit corneas after PRK but not after LASIK. This indicates that PRK stimulates keratocytes in the affected stroma more markedly than LASIK; ie, the presence of the covering tissue (corneal flap) on the ablated stroma may prevent keratocyte activation.

Repopulated keratocytes in the central anterior corneal stroma that had been resurfaced 3 days after PRK were found to express HSP47. This protein was not immunohistochemically detected in keratocytes of LASIK-treated corneas at the same interval. Up-regulation of HSP47 indicates acceleration of collagen production; ie, HSP47 works as a molecular chaperone for collagen precursor peptides in the cytoplasm. Accelerated collagen production in anterior keratocytes in

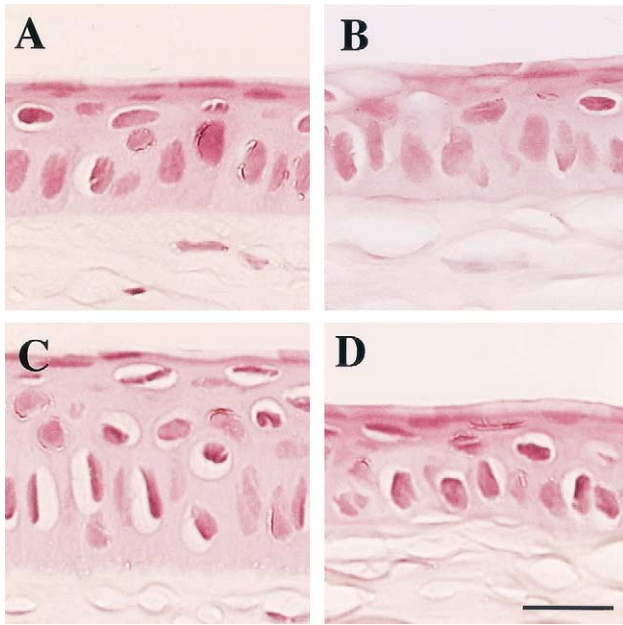


Figure 2. (Miyamoto) Epithelium in the central and peripheral areas of the cornea 3 months after PRK or LASIK. In PRK-treated corneas, the central regenerated epithelium (A) was thicker than the peripheral epithelium (C). This abnormality was not seen in LASIK-treated corneas (B: central area of the corneal epithelium; D: peripheral area) (H&E stain; bar = 20 μm).

PRK-treated corneas, indicated by HSP47 expression, may induce corneal stromal opacity. The regenerated epithelium of PRK-treated corneas also transiently up-regulated HSP47, suggesting the up-regulation of collagen biosynthesis in the healing corneal epithelium.

In contrast, in the early stage of healing after LASIK, the corneal flap may not completely heal histologically to adhere to the stromal bed; later, it may gradually attach to the bed by the PAS-positive matrix. Blunt trauma has been reported to dislocate the corneal flap even 2 months post-LASIK.³⁰ The regenerated epithelial basement membrane after PRK was not labeled with an antibody against collagen type IV [$\alpha 1(\text{IV})_2, \alpha 2(\text{IV})$] during the follow-up, suggesting that underlying stroma had been minimally damaged by PRK; this subtype of collagen IV is reportedly expressed and deposited primarily by keratocytes in severely damaged corneas, such as alkali-burned corneas.

We then examined the expression pattern of α -SMA, the myofibroblastic marker, to compare it with the expression pattern of HSP47. Alpha-smooth muscle actin is known to be expressed in keratocytes following myofibroblastic conversion in severely damaged corneal

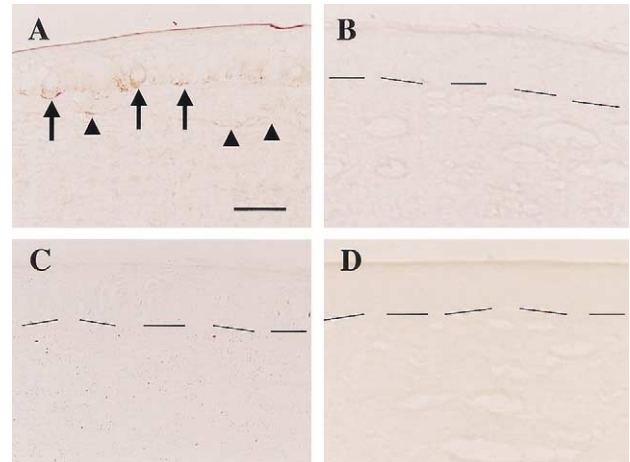


Figure 3. (Miyamoto) The expression pattern of HSP47 in rabbit corneas 3 days after PRK or LASIK. Keratocytes (arrowheads) positive for HSP47 immunoreactivity were observed in the central anterior stroma under the regenerating epithelium of the PRK-treated rabbit corneas (A) but not in the peripheral anterior stroma (B). Corneal epithelial cells (arrows) also expressed HSP47 in a PRK-treated central cornea. These cells were not seen in the stroma of the LASIK-treated corneas (C: central cornea; D: peripheral cornea). Dotted lines (in B, C, and D) indicate the location of the epithelial basement membrane (indirect immunohistochemistry; bar = 20 μm).

stroma such as alkali-burned corneas and not to be expressed in keratocytes in injured corneal stroma following epithelial debridement only.³¹ Myofibroblasts

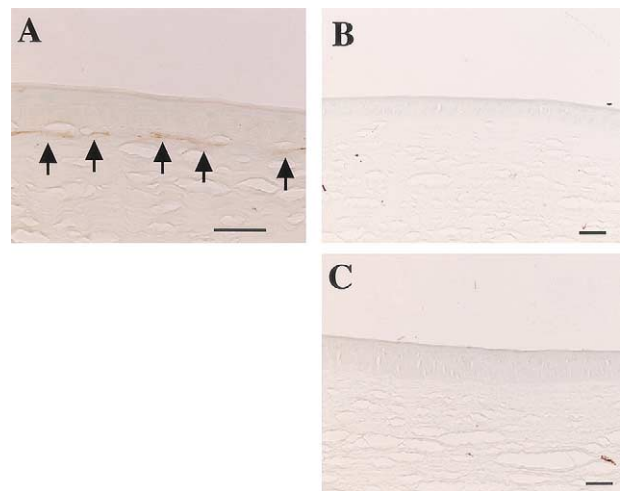


Figure 4. (Miyamoto) Expression pattern of α -SMA in rabbit corneas 4 weeks after PRK or LASIK. Keratocytes expressing α -SMA (arrows) are detected in the central anterior stroma under the regenerating epithelium of the PRK-treated cornea (A). These cells are not seen in the stroma of the central (B) and peripheral (C) cornea after LASIK (indirect immunohistochemistry; bar = 20 μm).

expressing α -SMA are known to have a characteristic to contract tissue. The results showed that up-regulation of α -SMA was detected in the keratocytes in the central anterior stroma 4 weeks posttreatment. A similar finding of the up-regulation of α -SMA is reported in rabbit corneas after PRK.³² However, the peak of α -SMA up-regulation did not coincide with that of HSP47, suggesting the regulation of the up-regulation of these 2 molecules may differ. Nevertheless, this indicates that PRK damages corneal stroma more severely than LASIK and that α -SMA-positive corneal cells may contract tissue, causing a refractive error after PRK. A similar expression pattern of α -SMA has been observed in human eyes after phototherapeutic keratectomy.³³

Regenerated epithelium was thickened in PRK-treated corneas 3 months posttreatment. It is well known that regenerated corneal epithelium becomes thicker on bared stroma without a basement membrane barrier.³⁴ The thickened epithelium may reduce the refractive correction by compensating the bare stroma ablated by PRK. Snibson and coauthors³⁵ report that the mean refractive correction by PRK becomes less than predicted 1 month postoperatively. In healing corneas after LASIK, no such abnormal hyperplasia of the central epithelium was detected at any interval; however, local hyperplasia of the repaired epithelium around the adhesion between the corneal flap and stromal bed was seen.

In conclusion, the cellular responses of keratocytes and epithelial cells were more marked in corneas treated by PRK than in those treated by LASIK. Undesirable cellular reaction by PRK may cause postoperative complications such as stromal opacification and power errors in the refractive correction. Recently, a new procedure, laser-assisted subepithelial keratectomy (LASEK), was introduced. An epithelial flap covers the ablated stroma to reduce the risks associated with PRK. The question now is whether preserving the epithelium during LASEK reduces the keratocyte reaction seen in PRK-treated corneas.

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