Baseline Membrane and Collagen Deposition After Laser Subepithelial Keratomeileusis and Photorefractive Keratectomy in the Leghorn Chick Eye

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Objective: To evaluate corneal scar formation and new collagen deposition after laser subepithelial keratomileusis (LASEK) compared with photorefractive keratectomy (PRK) in the leghorn chick corneal model.

Methods: Leghorn chick corneas treated with LASEK surgery (using 20% ethanol for 30 seconds) or PRK were evaluated by indirect confocal immunofluorescence and transmission electron microscopy at 1, 2, 7, 14, and 28 days after surgery. New collagen deposition was determined by dichlorotriazinylaminofluorescein staining 2 and 4 weeks after surgery.

Results: Laminin was detected around the basal layers during the immediate postoperative period and 4 weeks after LASEK surgery, and from day 2 onwards after PRK. Collagen III deposition in the cornea was about 3 times greater with PRK than with LASEK. The thickness of new collagen deposition at 4 weeks was 34 µm ± 2.5 µm in the PRK group, which was significantly greater than that of the LASEK group (11 µm ± 1 µm; \( P < .001 \)).

Conclusions: Reduced subepithelial stromal tissue deposition was observed in LASEK-treated eyes compared with PRK-treated eyes. Postoperative preservation of the epithelial basement membrane and survival of epithelial cells in LASEK and possibly in epithelial laser in situ keratomileusis may contribute to this phenomenon.

Clinical Relevance: An advantage of LASEK over PRK is the reduction of postoperative haze.

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ANIMALS

White leghorn chicks, purchased from Truslow Farms (Chester-town, Md), were cared for using Purina Laboratory Mill Start & Grow (St Louis, Mo) feed in a well-ventilated, lightly- and climate-controlled environment. The chicks were anesthetized with an intramuscular mixture of ketamine hydrochloride (20 mg/kg), xylazine hydrochloride (5 mg/kg), and an adjunctive topical opthalmic anesthetic solution (proparacaine hydrochloride). All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and a protocol approved by the Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary, Boston.

The chick corneas were treated with mechanical epithelial debridement or 20% ethanol for 30 seconds for ethanol-assisted flap creation. The chick eyes were propsected anteriorly after medial and lateral canthotomy. For eyes treated with PRK, a 3-mm trephine was used to demarcate the central cornea followed by epithelial debridement using a No. 15 Bard-Parker blade (Becton Dickinson, Franklin Lakes, NJ).

In creation of an epithelial flap for LASEK, a 3-mm trephine was applied to the cornea and several drops of 20% ethanol, enough to cover the entire epithelial surface, were instilled. After 30 seconds, the trephine was removed and the eye irrigated with balanced salt solution (BSS Plus; Alcon Laboratories, Houston, Tex). One arm of a jeweler’s forceps was then inserted under the epithelium and traced around its delineated margin leaving 2 to 3 clock hours intact. The loosened epithelium was peeled and pushed as a single sheet using a dry Merocel sponge and jeweler’s forceps leaving a flap of epithelium to be repositioned and allowed to dry for 1 minute. The lower lid was pulled and repositioned to prevent excess dehydration from the exposure. Photothermal keratome was done using a 2.3-mm-diameter, 24-µm-deep ablation with an excimer laser (Apex SVS; Summit, Waltham, Mass) in both PRK and LASEK procedures. The pulse energy density was 160 mJ/cm² and the repetition rate was 10 Hz. Slitlamp examination of the corneas was not performed; gross examination of the corneas did not reveal evidence of clinically visible haze. Corneoscleral rims were removed with corneal surgical scissors (Bausch & Lomb, Manchester, Mo) and processed for TEM and indirect immunofluorescence.

IMMUNOHISTOCHEMISTRY

The frozen sections (8 µm each) were warmed to room temperature for 30 minutes, immersed in chilled acetone for 5 minutes, and then air dried. After rehydrating in phosphate-buffered saline (PBS) (Invitrogen Corp, Carlsbad, Calif) for 10 minutes, the slides were incubated with blocking solution (1% bovine serum antigen (Sigma-Aldrich Corp) in PBS for 30 minutes) followed by incubation with a primary antibody for 1 hour at room temperature.

The monoclonal antibodies for laminin (1:1 dilution) and collagen III (1:1.5 dilution) were obtained from the Developmental Studies Hybridoma Bank. Slides were washed 3 times in PBS for 5 minutes and incubated at room temperature with fluorescein isothiocyanate–conjugated, affinity-purified, donkey anti-mouse IgG (Jackson Immunoresearch, West Grove, Pa) at a dilution of 1:400. Then the slides were washed again 3 times in PBS and mounted using the anti-fading medium Vectorashield with propidium iodide (Vector Laboratories, Burlingame, Calif). A scanning laser confocal microscope (Leica Lasertechnik, Heidelberg, Germany) was used to document all findings.

TRANSMISSION ELECTRON MICROSCOPY

The corneoscleral rims were fixed in half-strength Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde) and processed in 0.2M cacodylate buffer (pH 7.4) overnight. The samples were then postfixed in 1% osmium tetroxide, dehydrated in graded ethanol, and embedded in epoxy resin (Embed 812; Electron Microscopy Sciences, Fort Washington, Pa), and oven dried at 60°C for 48 hours. Sections 1-µm thick were stained with toluidine blue for orientation. Subsequent ultrathin sections were obtained using an ultramicrotome counterstained with 2% uranyl acetate and lead citrate and analyzed using a TEM (model 410; Philips, Eindhoven, The Netherlands).

DTAF STAINING

The frozen sections (8 µm each) were fixed in 10% forminal for 30 minutes. The sections were washed 3 times in PBS and mounted using Vectashield with propidium iodide. A scanning laser confocal microscope was used to document the images. The newly formed extracellular matrix and cellular components were quantified using the Image software (National Institutes of Health, Bethesda, Md). The images of each cornea were collected to calculate the area of new collagen.

RESULTS

Laminin was visualized in the center region of the cornea 4 hours after LASEK surgery (Figure 1A). However, no laminin staining was observed in the corresponding region of the cornea 4 hours after PRK treatment (Figure 1B). In the normal cornea, laminin was immunolocalized to the corneal epithelial basement membrane and Bowman membrane (Figure 1C). In the periphery of the cornea, laminin was visualized on the basement membrane in LASEK-treated eyes (Figure 1D). In PRK-treated corneas, laminin staining was shown to grow toward the laser-treated, central corneal area (Figure 1E). Epithelial cell growth lagged behind the laminin expression 4 hours after laser surgery.

Faint laminin staining was observed the first day after LASEK surgery (Figure 2A), and the staining remained constant between the day 2 and week 4 time points (Figure 2B-D). Hemidesmosomes were visualized by TEM at the epithelium-stroma interface in LASEK-treated eyes during days 1 and 2 (Figure 2E and F) and weeks 1 and 4 (Figure 2G and H). No laminin staining was observed the first day after PRK treatment (Figure 2I), but laminin was expressed uniformly on day 2, week 1, and week 4 (Figure 2J-L). Under TEM, no hemidesmosomes were seen on days 1 and 2 (Figure 2M and N) post-PRK treatment. However, hemidesmosomes were formed at weeks 1 and 4 (Figure 2O and P) post-laser treatment.
Type III collagen plays an important role in the healing of the corneal stroma. Normal untreated and treated corneas did not demonstrate type III collagen immunostaining immediately after creation of the epithelial flap. The level and pattern of type III collagen expression is very similar in PRK-treated and LASEK-treated corneas (Figure 3A and D). Enhanced type III collagen expression was observed at week 2 and continued through week 4 after LASEK (Figure 3B and C) and PRK (Figure 3E and F) treatment.

Dichlorotriazinylaminofluorescein is a vital dye that covalently binds to the stroma bed and delineates the boundaries of new collagen synthesis. Using DATF for labeling, little demonstrable difference was observed in the amount of subepithelial, newly formed tissue among the LASEK (Figure 4A) and PRK (Figure 4D) groups 2 weeks after surgery. There was, however, significantly less newly formed collagen over the treatment bed in the LASEK (Figure 4B) compared with the PRK (Figure 4E) group 4 weeks posttreatment. No new collagen was formed after ethanol-assisted flap creation (Figure 4C) or mechanical epithelial debridement (Figure 4F) without excimer laser application.

The thickness of newly synthesized collagen was calculated by dividing the total area of newly synthesized collagen by the base length. The percent (±SD) increase in thickness of new collagen deposition at 4 weeks was 34%±2.5% in the PRK group and 11%±1% in the LASEK group (Figure 4G). The difference in newly synthesized collagen was statistically significant.

**COMMENT**

Photorefractive keratectomy has become an accepted surgical procedure to correct refractive errors including myopia, hyperopia, and astigmatism. Although the procedure has been proven effective and safe, some patients may develop subepithelial haze as a postoperative, wound-healing response.6,7

In LASIK, the corneal epithelium and Bowman membrane are preserved, thereby reducing the effects of wound healing and problems associated with surface PRK.8,12 Laser subepithelial keratomileusis and Epi-LASIK may offer some of the advantages of both the LASIK and PRK procedures.13-15 These techniques have the potential to offer faster visual rehabilitation and reduced subepithelial corneal haze formation than PRK, and also avoid microkeratome-related and flap-related complications associated with LASIK.14,16,17
tact epithelium most likely represents the key issue in suppression of a wound reaction after LASEK. It is likely that the results that we observed in our chick LASEK model may be different than that of an Epi-LASIK model. Additional studies comparing our results with those after Epi-LASIK in the leghorn chick model may help determine the extent to which ethanol devitalization of some of the epithelial cells interferes with scar deposition after surgery.

We evaluated the effects of the epithelial flap on scar and neocollagen formation by demonstrating the presence or absence of the basement membrane, scar tissue components, and neocollagen in LASEK-treated and PRK-treated eyes. We used immunohistochemical methods to localize a basement membrane component, laminin, and the amount of stromal scarring and newly formed stromal tissue using collagen III and DTAF, respectively, in a temporal and spatial manner.

Dichlorotriazinylaminofluorescein binds irreversibly to the exposed collagen by forming covalent bonds to amino groups, and since the remodeled collagen and other extracellular matrix components are unlabeled, the original wound surface can be identified by fluorescent microscopy for as long as 1 year after surgery. We used this technique to demonstrate new stromal tissue formation after LASEK and PRK (Figure 4). There was hardly any noticeable difference in newly formed subepithelial tissue between LASEK and PRK groups 2 weeks post-treatment. However, 4 weeks after treatment, there was less neosubepithelial tissue formed in the LASEK-treated group compared with the PRK group. The difference between the 2 groups was statistically significant.

Cytokines released from an injured corneal epithelium may induce a cascade of events affecting the underlying keratocytes, which may result in scar formation and haze. The prevention or lessening of this cytokine release may be a way to avoid undesirable sequelae. Corneal epithelial vitality may be an important factor in this issue. Gabler et al stained the epithelial flap of human cadaver eyes with 0.1% trypan blue after ethanol exposure. They found that vital epithelial cells were still visible after a maximum of 45 seconds of exposure to 20% ethanol.
ethanol, and longer exposure times had a direct effect on epithelial cell survival. We have also shown the effect of ethanol concentration and duration of exposure to epithelial cell survival.

Both corneal epithelial cells and activated keratocytes have the ability to synthesize components of the basement membrane as well as adhesion structures. Basal epithelial cells play the more predominant role in this function.

The separation plane between the epithelium and underlying stroma is variable in the human eye after LASEK and Epi-LASIK. This phenomenon may in part be due to the variability of the shearing forces on the epithelium during surgery. In the chick eye, our LASEK studies demonstrated that the predominant separation plane is between the basal epithelial layers and the basement membrane. We used laminin immunolocalization to identify the basement membrane in LASEK and PRK because it is an ubiquitous basement membrane component important to adhesion complexes and is also a known participant in the attachment, migration, proliferation, and differentiation of various types of cells. We found irregular fragments of the basement membrane attached to the basal cells at the posterior surface of the epithelial flap, indicating that the plane of ethanol separation might be within the basement membrane or between the basement membrane and Bowman layer.

Complete regeneration of the basement membrane after keratectomy in rabbits has been shown within 6 to 8 weeks. The adherence of the basement membrane to the basal layer of the epithelium is significant because it is believed that the basement membrane provides the stability and support that keeps the epithelium intact even with manipulation, thereby preserving the integrity and viability of the entire corneal epithelium.

Our results show laminin visible beneath the corneal epithelial flap within 4 hours of the procedure and at day 1 after LASEK, whereas no laminin was demonstrable during the same time points after PRK (Figure 1A, B, D, and E). The laminin immunopositive basement membrane attached to the epithelial flap may also act as a matrix to facilitate epithelial migration and proliferation and affect the adhesion of cells sitting on it.

Subepithelial corneal haze is related to several substances such as glycosaminoglycans, fibronectin, type III collagen, keratin sulfate, and hyaluronic acid. Our results show a slightly increased immunohistochemical localization of type III collagen in the PRK group compared with the LASEK group (Figure 3A-F). There was no immunoreactivity for type III collagen in normal, untreated corneas. The greater reduction of haze in LASEK than in PRK is still anecdotal and has not been proven clinically. Our study shows that when no excimer treatment was rendered, no new collagen was seen either in LASEK or PRK. This is consistent with the concept that collagen deposition may be related to the depth of stromal ablation.
In LASEK, the presence of the epithelium over the bare stroma may serve as a mechanical barrier which confines the epithelium-stroma interaction to an area around the epithelial flap margin. Although certain cytokines such as Fas ligand and interleukin 1 are released after epithelial injury, this may not have been sufficient to result in exaggerated keratocyte loss and apoptosis after surgery in our LASEK chick model. Also, the epithelial flap reduces mechanical trauma to the migrating (and regenerating) epithelium, which may play a role in reducing scar formation. In addition to its barrier function, the epithelium and its basement membrane may also be a source of active molecules that protect the keratocytes, or it may serve as a neutralization site for fear factors that would trigger keratocyte death.

In summary, our findings of reduced subepithelial scar deposition after LASEK and possibly Epi-LASIK compared with PRK in the leghorn chick model is consistent with clinical reports suggesting this potential advantage. It is not clear whether the reduction in subepithelial scarring is related to the method of epithelial separation, the intactness of the epithelial flap, the viability of the epithelial cells, the presence of an intact epithelial basement membrane, or the alterations in epithelial-stromal interactions and tear/epithelial cytokine release. It is reasonable to assume that the amount of collagen deposition may be related to the degree of clinical haze, but it is difficult to postulate that this link is causal. Additional studies comparing the results of our study with those using a mechanical separator (Epi-LASIK) in the

Figure 4. Fluorescence micrograph labeled by dichlorotriazinylaminofluorescein in white leghorn chick corneas post–laser subepithelial keratomileusis (LASEK) during week 2 (A) and week 4 (B) and post–photorefractive keratectomy (PRK) during week 2 (D) and week 4 (E). Untreated controls are shown during week 2 (C) and week 4 (F). The graph (G) shows the significantly greater amount of new collagen in PRK-treated corneas compared with LASEK-treated corneas during week 4.
leghorn chick may help in furthering our understanding of the pathogenesis of subepithelial scar formation after PRK and other stromal surface ablation procedures.

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