Corneal Molecular and Cellular Biology Update for the Refractive Surgeon

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Abstract

PURPOSE—To review clinically relevant progress in understanding cellular and molecular interactions in the cornea that relate to refractive surgical outcomes in patients.

METHODS—Recent published literature focused on femtosecond LASIK and surface ablation procedures, such as photorefractive keratectomy, was reviewed and correlated with clinical results of surgery.

RESULTS—The femtosecond laser has a direct necrotic effect on stromal keratocytes, resulting in the release of cellular components that are chemotactic to bone marrow-derived inflammatory cells. Developments of the femtosecond laser led to lower energy delivery to the stroma and altered laser ablation profiles that decrease epithelial damage during the side-cut, and have markedly improved femtosecond LASIK to the point that the overall early postoperative healing response is indistinguishable from microkeratome LASIK. New studies have directly demonstrated the importance of surface irregularity and resulting structural and functional defects in the epithelial basement membrane, in the generation and persistence of anterior stromal myofibroblasts and haze following surface ablation procedures. These defects augment penetration of epithelium-derived TGF-β, which is a critical modulator of myofibroblast development in the stroma. Studies on the mechanism of action of mitomycin C treatment to prevent haze have confirmed that the most powerful effect is on stromal cell proliferation and, therefore, decreased population of the anterior stroma with myofibroblast progenitor cells. An undesirable long-term effect of mitomycin C is diminished anterior stromal keratocyte density due to diminished keratocyte re-population. This raises concerns regarding future corneal anomalies in treated corneas.

CONCLUSIONS—Basic research studies of refractive procedures provide important insights into the effects of wound healing on surgical outcomes.

Important progress in research over the past two decades has led to better understanding and control of molecular and cellular interactions involved in the response of the cornea to refractive surgical procedures such as photorefractive keratectomy (PRK) and LASIK. Many key advances have derived from work in animal models that closely parallel responses noted in humans during the postoperative period extending from hours to months.1,2 Our review published 10 years ago3 in the Journal of Refractive Surgery detailed fundamental processes such as keratocyte apoptosis, keratocyte repopulation, and change in keratocyte phenotype to a more metabolically active fibroblastic cell. Since that time, however, many new observations have been made that provide important insights into processes such as the wound healing differences between LASIK performed with the microkeratome and femtosecond laser, haze generation and resolution, and the effect of mitomycin C treatment on corneal morphology.
after surface ablation. In the current review, we highlight several recent advances and detail their effects on corneal refractive surgical procedures as well as some associated complications.

**FEMTOSECOND LASER AND MICROKERATOME FLAPS DURING LASIK**

The femtosecond laser has revolutionized flap formation in LASIK surgery. Results with earlier models of the laser, however, such as the IntraLase 6-kHz and 15-kHz models (IntraLase Corp, Irvine, Calif), were at times sub-optimal, with reports of marginal diffuse lamellar keratitis (DLK), increased frequency of central DLK, slower visual recovery in the first weeks after surgery, and, occasionally, a syndrome of good visual acuity with extreme sensitivity termed transient light sensitivity syndrome. Cellular biological studies performed in rabbits with the 15-kHz femtosecond laser demonstrated increased levels of keratocyte cell death along the interface in the hours following surgery (Fig 1). These dying cells were initially identified with the TUNEL assay that detects fragmented DNA ends characteristic of apoptosis. Although the TUNEL assay is relatively specific for apoptotic cell death—a non-inflammatory form of controlled cellular death—additional investigations with the transmission electron microscope demonstrated that the femtosecond laser actually triggers keratocyte necrosis. Necrosis is another form of cell death that promotes more inflammation (Fig 2). This is in contrast to LASIK performed with a microkeratome that has been shown to trigger keratocyte cell death mediated primarily by apoptosis in the same model system.

The level of cell death associated with femtosecond laser flap formation was markedly decreased with the development of the 30 kHz, and subsequently the 60 kHz, femtosecond lasers produced by IntraLase. Optical and other changes in these newer models allowed the per pulse side-cut and lamellar-cut energies to be reduced to ≤1.0 μJ and also decreased the total femtosecond laser energy delivered to the cornea during flap formation. This decrease markedly reduced the level of keratocyte necrosis to the point that the inflammatory response became indistinguishable from that noted with LASIK performed with a microkeratome.

Another beneficial change made in the later femtosecond models, based on earlier histological observations, was a decrease in the width of the side-cut ablation. The 15-kHz and early 30-kHz models created a furrow in the epithelium at the flap edge that was obvious to the surgeon (Fig 3) and facilitated lifting the flap. This, however, produced extensive epithelial damage that led to massive release of pro-inflammatory cytokines (eg, interleukin-1) and growth factors that promote myofibroblast cell differentiation, such as transforming growth factor β (TGF-β), from these cells. These modulators pass into the stroma, bind to keratocyte receptors, and trigger chemokine production and more inflammatory cell infiltration (see Fig 2), resulting in greater healing response at the flap edge (see Fig 3). The augmented wound healing response with early femtosecond laser models generates a highly opaque flap edge, which is at times visible as a double ring around the flap. This ringed opacity is due to enormous numbers of myofibroblasts developing at the epithelial-stromal penetration site and correlates with increased difficulty lifting the flap for enhancement. Thus, whereas the edge of the flap is typically easy to identify, it may be much more difficult to lift, even as early as 1 month after surgery. In later 30-kHz (model II) and 60-kHz models (models I and II), the diameter of the side-cut is markedly reduced so lifting the flap for retreatment is no more difficult than it is after LASIK performed with a microkeratome.

The changes incorporated into the 60-kHz IntraLase femtosecond laser have resulted in a LASIK procedure with no more inflammatory response than microkeratome LASIK. Standard four times per day of 1% prednisolone acetate, or equivalent corticosteroid treatment, is typically all that is required in the early postoperative period after LASIK performed with the modern femtosecond laser. In our experience, flap lifts after LASIK with the 60-kHz model II IntraLase femtosecond laser are no more difficult than those performed after LASIK with the microkeratome, even when retreatment is needed 1 year or more after the original surgery.

*J Refract Surg. Author manuscript; available in PMC 2010 May 1.*
STROMAL HAZE AFTER SURFACE ABLATION: ETIOLOGY, PREVENTION, AND TREATMENT

Anterior corneal opacity, or haze (Fig 4), occurs in 1% to 4% of eyes that undergo surface ablation procedures, such as PRK or laser epithelial keratomileusis (LASEK) without mitomycin C treatment. The condition tends to be rare after correction of <6.00 diopters (D) of myopia, but can occur. The incidence of haze increases as the level of attempted correction increases >6.00 D. Typically, the patient who develops haze does well for 2 to 3 months after surgery and then has sudden onset of decreased distance vision, along with glare and other visual symptoms in both eyes. When the surgeon examines the corneas at the slit lamp, a layer of opacity is noted immediately beneath the epithelium.

Several clinical factors have been correlated with haze formation. These include the depth of ablation and the smoothness of the stromal surface after the ablation. Clinicians such as Vinciguerra et al. and Daniel Epstein, MD, PhD (personal communication, 2000) have advocated smoothing procedures after PRK to reduce the incidence of haze. Typically, these smoothing procedures are performed with the excimer laser in phototherapeutic keratectomy (PTK) mode with a masking agent such as 1% Healon (Pharmacia, Uppsala, Sweden) in basic salt solution. Most refractive surgeons, however, have preferred treatment with mitomycin C, probably because the additional surgical step with this treatment is relatively simple and quick, and few, if any, long-term effects of mitomycin C treatment have been noted when using concentrations between 0.02% and 0.002% on the cornea. Many surgeons limit their use of mitomycin C to higher corrections (>5.00 to 6.00 D of myopia) and eyes that had previous refractive surgical procedures such as radial keratotomy (RK), astigmatic keratectomy, and LASIK. In addition to the clinical factors that are associated with haze generation, genetic, dietary, and environmental factors may contribute to haze formation in some patients.

The tendency to develop haze at times varies with the excimer laser used for surface ablation. Prior to 2003, the authors had performed several thousand PRK and LASEK procedures with the VISX models S2, S3, and S4 (Santa Clara, Calif) without mitomycin C treatment and did not have a single case of clinically significant haze. In 2005, the authors began using the LADARVision 6000 laser (Alcon Laboratories Inc, Ft Worth, Tex) and had two patients with corrections <3.00 D of myopia develop severe haze. This prompted us to begin using mitomycin C for all levels of PRK for myopia when this laser was used. In retrospect, haze in corrections for low myopia with the LADARVision 6000 laser was likely related to surface irregularities, such as central islands, that led to the recall of that laser model. Subsequently, we resumed using the VISX S4 laser and discontinued use of mitomycin C in PRK for <5.00 D of myopia without noting any cases of late haze in these lower corrections.

Recent studies in our laboratory have confirmed the clinical impression that postoperative smoothness of the stromal surface is an important factor contributing to subepithelial haze formation. In these experiments performed on rabbits, the degree of surface irregularity was carefully modulated with a fine screen and the excimer laser. A direct correlation was noted between the level of surface irregularity and the level of clinical haze or myofibroblast density in the anterior stroma. It was discovered that stromal surface irregularity correlated with structural and functional defects in the epithelial basement membrane that facilitated movement of TGF-β and possibly other epithelial-derived cytokines into the stroma. Persistence of these basement membrane abnormalities leads to exposure of stromal progenitor cells to sufficient concentrations of TGF-β that they differentiate into myofibroblasts localized immediately beneath the basement membrane (Fig 5). These myofibroblasts are not only opaque themselves due to down regulation of the corneal crystallins expressed in keratocytes but also produce large amounts of disorganized collagen and other matrix material that are also opaque. Thus, corneal transparency is altered overlying the area of the original stromal ablation. This
association between haze and defects in the epithelial basement membrane is likely also the mechanism for formation of haze in corneas with persistent epithelial defects—after PRK or otherwise—where the basement membrane is absent for extended periods of time.

Even after normal PRK, the native conformation of the extracellular matrix is altered deep into the stroma. Every refractive surgeon can see this by merely performing LASIK retreatment on a cornea that had previous PRK. When the LASIK flap is lifted, a ring of stroma with altered texture, corresponding exactly to the original ablation zone, will be observed in the bed many years after the original PRK procedure. We have noted this in all LASIK retreatments for previous PRK with flaps from 90-μm to 170-μm thick (unpublished data, 2007). Thus, the PRK procedure triggers persistent changes deep in the stroma. We believe these changes are due to differences in the stromal matrix in areas where keratocyte apoptosis and subsequent cellular repopulation occurred after the original PRK procedure.

Corneas that develop haze can be divided into those that are corticosteroid-sensitive and those that are corticosteroid-resistant. Currently, these groups can only be distinguished through a trial of intensive topical corticosteroid treatment such as with 1% prednisolone acetate eight times a day for 1 week. If the haze is steroid-responsive, the patient will note a marked improvement in vision and visual symptoms, such as glare, and the clinician will note a marked decrease in corneal haze at the slit lamp. In responders, the corticosteroids must be tapered slowly, typically over a period of months or years, or the haze and symptoms will recur. An alternative treatment for steroid-sensitive corneas is to begin topical 0.05% cyclosporine (Restasis; Allergan, Irvine, Calif) twice a day as the corticosteroids are tapered over a 2- to 3-week period. Topical cyclosporine treatment is safer and usually prevents recurrence of haze after the corticosteroids have been stopped and can be maintained for 1 to 2 years until the cornea has had sufficient time to repair the basement membrane abnormalities that generated myofibroblasts and haze (see prior discussion). If the haze is steroid-resistant, no improvement in vision will be noted by the patient and no change in haze at the slit lamp will be noted by the clinician within 1 to 2 weeks of beginning corticosteroid treatment. In these patients, topical corticosteroids are rapidly tapered and stopped over a period of a few days. We suggest all patients who develop haze have the corticosteroid trial as anti-inflammatory treatment is effective in the corticosteroid-sensitive group.

The steroid-responsive group is, unfortunately, the smaller of the two haze groups. In our experience, the steroid-responsive patients only comprise 10% to 15% of haze patients. The pathophysiological basis for the two haze groups remains unknown. Our working hypothesis is that the primary precursors for myofibroblasts in corneas that are corticosteroid-sensitive are bone marrow-derived cells, as has been noted for myofibroblasts in skin, lung, and liver, whereas in corneas that are corticosteroid-resistant the primary precursors are keratocyte cells. This hypothesis is being tested in animal models.

Another interesting observation regarding haze is that even the most severe cases usually resolve spontaneously over a period of 1 to 3 years after the original surgery that triggered the haze response. In animal studies of haze,12,13 myofibroblasts that produce haze slowly undergo apoptosis over a period of months to years (Fig 6). Presumably, myofibroblasts begin to undergo apoptosis when epithelial basement membrane structure and function is restored and epithelial-derived stromal TGF-β falls to a level that is insufficient to maintain myofibroblast viability. Cellular apoptosis after growth factor withdrawal is a common mechanism to rid organs of unwanted cells in many organ systems. Abnormal collagen and other matrix materials deposited by the myofibroblasts are then reabsorbed by keratocytes that repopulate the stroma. The natural history of haze resolution is dramatically altered in eyes that received mitomycin C treatment but developed “breakthrough haze.” In these eyes, there is little tendency for the haze to resolve spontaneously.
An attempt can be made to remove haze with a combination of epithelial scrape and PTK. However, more severe haze tends to recur. This tendency can be at least partially blocked with mitomycin C treatment but the refractive outcome of this therapy is unpredictable. Therefore, we typically favor observation until the haze spontaneously resolves, as most of these patients function surprisingly well with spectacle correction. Also, when the haze resolves spontaneously, the initial effect of the refractive surgery procedure on the refractive error is often unmasked so that the patient’s good uncorrected vision is restored. Thus, heroic surgical attempts to remove the haze may yield poorer visual outcomes than merely waiting for spontaneous resolution.

MITOMYCIN C TREATMENT AFTER PRK AND OTHER SURFACE ABLATION PROCEDURES

Mitomycin C treatment after PRK or other surface ablation procedures has been used empirically for years to block haze formation.14 Recently, animal studies have been performed to determine the mechanisms through which mitomycin C blocks the haze response. Although mitomycin C augments the normal apoptosis response that occurs after epithelial scrape performed during PRK,15,16 Netto et al16 demonstrated that the most notable effect is in the inhibition of mitosis of cells that function to repopulate the anterior stroma (Fig 7). Thus, progenitor cells to myofibroblasts, in addition to keratocytes, are blocked from proliferating. The end result of this treatment is that the anterior stroma has profoundly diminished cell density lasting for more than 6 months after mitomycin C treatment.16 It has not been determined when, if ever, more normal keratocyte density is restored in the anterior stroma after mitomycin C treatment. Because keratocytes function to maintain collagen, glycosaminoglycans, and other matrix materials in the stroma, there is concern regarding the long-term effects of mitomycin C, possibly measured in decades, on corneal morphology and function.16 Some patients have been followed for 10 years after PRK and mitomycin C treatment without significant side effects noted.14 However, follow-up measured in decades in large numbers of patients may be required to ensure there are no long-term effects from changes such as diminished anterior stromal keratocyte density. Thus, mitomycin C has long-term effects on many cellular processes in the stroma besides myofibroblast development. Efforts should be directed toward development of more focused treatments to block myofibroblast generation without affecting keratocyte repopulation. Cytokines, such as TGF-β, that modulate myofibroblast development are excellent potential targets for new treatment approaches, and investigators have demonstrated that treatment with antibodies blocking TGF-β binding to receptors effectively decreases haze.17

In an attempt to limit the effects of mitomycin C treatment on corneal morphology, animal and clinical studies have been performed to determine optimal concentrations and duration of treatment.16,18 These studies found that 0.002% mitomycin C was nearly as effective as traditional 0.02% mitomycin C14 and that prolonging exposure from 12 seconds to as long as 2 minutes produced only marginal additional effects. Based on these studies, we began using 0.002% mitomycin C for 30 seconds as our standard treatment.16 However, in using this treatment, we noted several patients had “breakthrough haze” despite administration of mitomycin C. One patient who was treated with 0.02% mitomycin C developed breakthrough haze, which was also noted by other clinicians,19 but the incidence appears to be greater with the lower concentration of mitomycin C. Regardless of the concentration of mitomycin C used, a disturbing observation was there is little tendency for spontaneous resolution of the haze, even after >3 years’ follow-up. Presumably, this is attributable to diminished keratocyte density in the anterior stroma, and therefore decreased cellular function in removing abnormal collagen and other matrix components deposited by myofibroblasts. Thus, because few keratocytes are present in the anterior stroma after mitomycin C treatment, there is diminished capacity to
remove the wound healing collagen and other matrix materials associated with haze. Based on these observations, we no longer use the 0.002% mitomycin C concentration. Rather, 0.02% mitomycin C for 30 seconds has become our standard treatment for eyes having primary PRK. In eyes that had previous surgical procedures, such as RK, LASIK, or LASIK with buttonhole complications, we use 0.02% mitomycin C for 1 to 2 minutes.

A better understanding of the cellular mechanisms involved in response to corneal refractive surgery and associated treatments can provide insight in the development of further treatments to improve outcomes and decrease the likelihood of complications from these treatments.

Acknowledgments

Supported in part by US Public Health Service grants EY10056 and EY15638 from the National Eye Institute, National Institutes of Health, Bethesda, Md; and Research to Prevent Blindness, New York, NY. Dr Wilson is the recipient of a Research to Prevent Blindness Physician Scientist Award.

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Figure 1.
Death of stromal cells detected with the TUNEL assay (red cells [arrows]) at 24 hours after LASIK flap formation with the A) Hansatome microkeratome, B) 15-kHz femtosecond laser, and C) 30-kHz femtosecond laser. Blue 4′,6-diamidino-2-phenylindole (DAPI) stains all intact cell nuclei. In A and B, DAPI staining of stromal nuclei anterior and posterior to the region of TUNEL-stained cells is less distinct due to tissue edema, but keratocytes are present in these zones outside the region of cell death caused by the microkeratome or femtosecond laser. (Original magnification ×200.) Reprinted with permission from SLACK Incorporated.4
Figure 2.
CD-11b-positive (green stain [arrows]) monocyte cell infiltration into the cornea at 24 hours after LASIK flap formation with the A) Hansatome microkeratome, B) 15-kHz femtosecond laser, and C) 30-kHz femtosecond laser. Blue 4',6-diamidino-2-phenylindole (DAPI) stains all intact cell nuclei. (Original magnification ×200.) Reprinted with permission from SLACK Incorporated.4
Figure 3.
Mitotic cells (red cells [arrows]) detected by immunohistochemical staining for mitosis-specific antigen Ki-67 24 hours after LASIK flap formation with A) the Hansatome microkeratome, B) the 15-kHz femtosecond laser, and C) the 30-kHz femtosecond laser. (Original magnification ×400.) Note the larger diameter gap (arrowheads) in the epithelium and stroma at the epithelio-stromal junction in eyes treated with the femtosecond lasers (B) and (C) compared with the small gap in eyes treated with a microkeratome (A). Blue 4′,6-diamidino-2-phenylindole (DAPI) stains all intact cell nuclei. Reprinted with permission from SLACK Incorporated.4
Figure 4.
Slit-lamp photograph of haze occurring in an eye that underwent treatment with 0.002% mitomycin C for 15 seconds after photorefractive keratectomy for -9.00 diopters of myopia. (Original magnification ×20.)
Figure 5.
Basement membrane defects and myofibroblast cells after photorefractive keratectomy (PRK).
A) Note the uniform basement membrane (green cells [arrows]) regeneration in a cornea without haze at 1 month after -4.50 diopter (D) PRK. B) At 1 month after -9.00-D PRK, the basement membrane has obvious disruptions (green cells [arrows]), and beneath this area, red-stained alpha-smooth muscle actin-positive myofibroblast cells (arrowheads) are noted. The basement membrane is highlighted by immunohistochemical staining for integrin beta4. Blue 4',6-diamidino-2-phenylindole (DAPI) stains all intact cell nuclei. (Original magnification ×400.) Reprinted with permission from Elsevier.13
Figure 6.
Myofibroblast apoptosis in a rabbit cornea with severe haze after -9.00 diopter photorefractive keratectomy. DAPI staining of cell nuclei appears blue in all panels. **A)** When the section is stained for the myofibroblast marker alpha-smooth muscle actin, myofibroblasts (arrows) are detected as green in the anterior stroma beneath the epithelium. **B)** When the section is imaged with a red TUNEL assay for apoptosis, TUNEL-positive cells (arrows) are detected in the same location as myofibroblasts in **A**. **C)** When both the red and green are imaged, some myofibroblast cells in the anterior stroma are noted to be undergoing apoptosis (arrows) and therefore appear yellow. (Original magnification ×500). Reprinted with permission from Elsevier.\textsuperscript{13}
Figure 7.
Mitomycin C blocks proliferation of cells in the anterior stroma after photorefractive keratectomy (PRK). A) At 24 hours after PRK and treatment with 0.02% mitomycin C for 2 minutes, there are very few cells undergoing mitosis (arrowheads) in the anterior stroma detected by immunohistochemical staining for the mitosis marker Ki-67. B) In contrast, at 24 hours after PRK and treatment with balanced salt solution vehicle for 2 minutes, numerous cells are proliferating (arrowheads) in the anterior stroma. 4’,6-diamidino-2-phenylindole (DAPI) staining of cell nuclei appears blue; green stain is Ki-67. (Original magnification ×400.) Reprinted with permission from SLACK Incorporated.16