The Wound Healing Response After Laser In Situ Keratomileusis and Photorefractive Keratectomy

Elusive Control of Biological Variability and Effect on Custom Laser Vision Correction

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Biological diversity in the wound healing response is thought to be a major factor limiting the predictability of the outcome of refractive surgical procedures such as laser in situ keratomileusis and photorefractive keratectomy. Corneal wound healing is critical to the success of topography-linked or wave front–linked excimer laser ablation to optimize visual performance. This is because of the importance of retaining subtle features of custom ablation and the tendency of epithelial hyperplasia and stromal remodeling to obscure these features following either procedure. The corneal wound healing response is exceedingly complex. Keratocyte apoptosis, which occurs in response to epithelial injury, is the earliest observable event in the wound healing cascades and is therefore an excellent target for pharmacological intervention. Alterations of surgical technique can be designed to limit keratocyte apoptosis and the subsequent events in corneal wound healing. Abnormalities of the cascades could contribute to the pathogenesis of corneal diseases. For example, recent data have suggested that perturbation of the keratocyte apoptosis/mitosis balance could underlie the development of keratoconus in a proportion of patients.

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Rapid progress is being made in improving the quality of excimer lasers, microkeratomes, and other technology needed for refining the results of laser in situ keratomileusis (LASIK) and photorefractive keratectomy (PRK). Similarly, advances in technique are also improving the safety of PRK and LASIK. Despite these improvements, patients and surgeons continue to be vexed with variations in outcomes. Factors such as corneal hydration, variability in the instruments, and efficiency of plume removal may be important factors. Biological variability in the wound healing response between different patients or even the 2 eyes of a single patient is also likely to be an important factor. Attempts at corneal topography-linked or wave front–linked excimer laser ablation will probably be confounded by the biological variability of wound healing and the tendency of the cornea to smooth out features of surface or interface stromal ablation through stromal remodeling or epithelial hyperplasia. Many features that will be incorporated into custom ablation to correct aberrations will be subtle and readily masked by wound healing–related alterations (**Figure 1**). Further progress in modulation of wound healing will be critical if these efforts to precisely alter corneal contour to eliminate refractive error and aberrations are to be effective.

In the recent past, the stoma of the adult cornea was thought of as being relatively quiescent. During the past 5 years, there have been tremendous advances in our understanding of the complex events that occur in the stoma and epithelium in response to injury or surgery. These contributions are bringing us closer to being able to manipulate corneal wound healing to clinical advantage.

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This review will highlight some of the important new insights that have been gained through basic science investigations of corneal cell biology and physiology. These observations have potential clinical implications and point to areas in which further work is needed to characterize cascades that contribute to wound healing in the cornea.

THE MYSTERY OF THE DISAPPEARING KERATOCYTE

Despite the dogma that has existed regarding the quiescence of keratocytes, reports contradicting this dogma
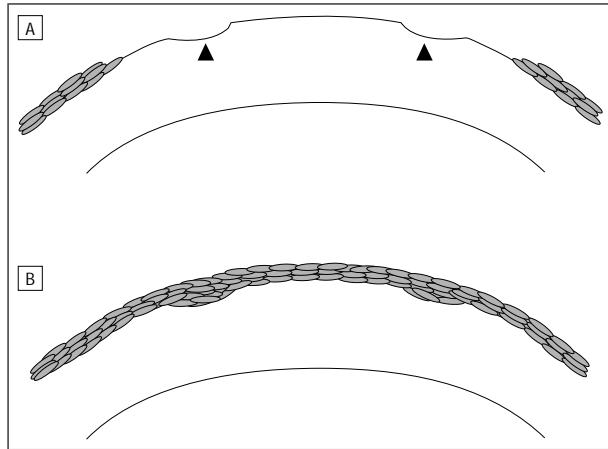
Figure 1. *Potential effect of corneal wound healing on custom cornea excimer laser ablation. A, Surface photorefractive keratectomy surface ablation was performed to eliminate myopia. This simulation includes hypothetical symmetrical alterations designed to limit aberrations (arrowheads). B, Following healing there is epithelial hyperplasia at the site of the custom alterations that tends to mask the custom effect. Custom features designed to alter aberrations may be subtler than in this schematic example. Similar epithelial hyperplasia has been noted with laser in situ keratomileusis.[27] Stromal remodeling may also influence these alterations.*

go back more than 25 years. One of the earliest observations was the disappearance of superficial keratocytes following corneal epithelial scrape injury (**Figure 2**). This observation was made first by Dohlman et al[1] in 1968, but was ignored and forgotten because its relevance was not clear. During the decades that followed, this same observation was made independently 3 times in a variety of species.[2-4]

Investigators speculated that the disappearance was owing to artifact, corneal hydration changes, exposure to the atmosphere, or other factors.[1-4] We reported in 1994[5] that the disappearance of the keratocytes was mediated by apoptosis. Apoptosis is a controlled, relatively gentle form of cell death in which cells are dismantled and eliminated with minimal release of intracellular components that could seriously damage surrounding cells and tissues. Since that time, there have been other observations of manipulations such as PRK and LASIK (**Figure 3**) triggering specific patterns of keratocyte apoptosis. This localization was observed to lead to localization of subsequent wound healing events that occurred in the stroma and epithelium.

WHY WOULD KERATOCYTES DISAPPEAR IN RESPONSE TO EPITHELIAL INJURY?

Studies were initiated to explore the physiologic and evolutionary significance of keratocyte apoptosis occurring in response to epithelial injury. Corneal epithelial scrape and refractive surgery were probably not the pressures that led to the evolution of this response. We hypothesized that perhaps this could represent some form of early response to viral infection of the corneal epithelium, a cellular "fire break" to retard posterior extension of viruses such as herpes simplex virus or smallpox virus that
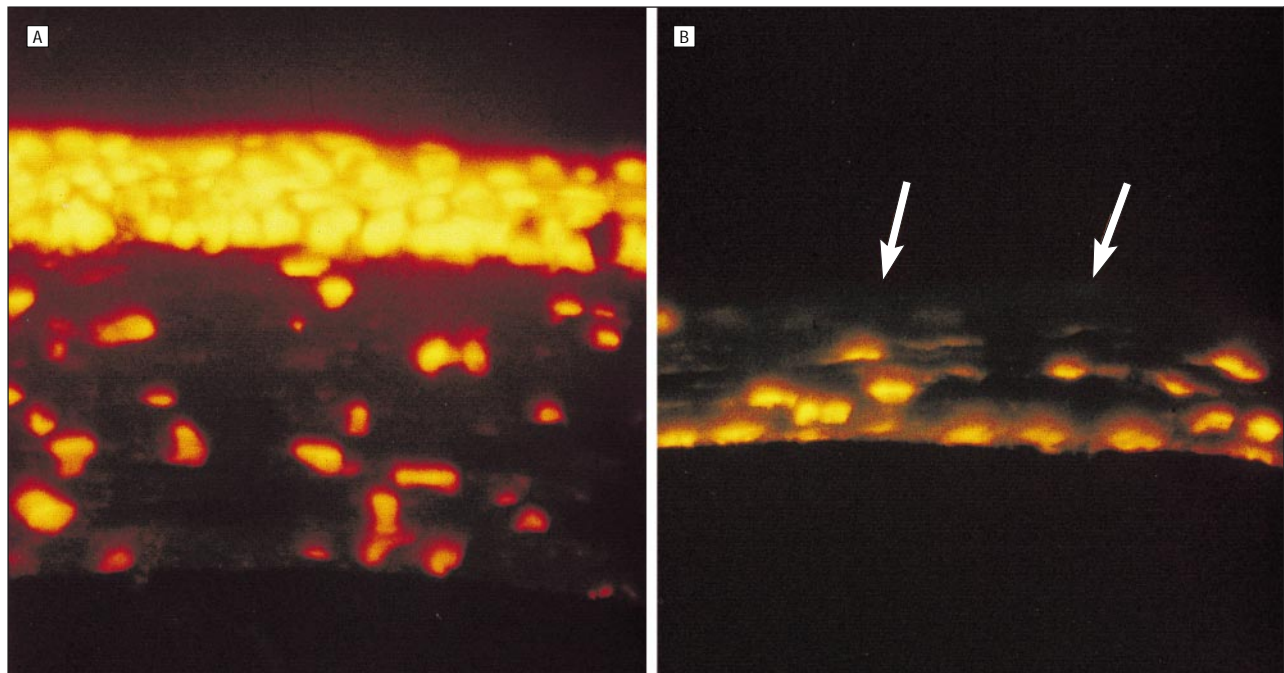

Figure 2. *A, Propidium iodide staining of mouse corneal sections. Propidium iodide stains nucleic acids and shows the position of cells. A, In an unwounded cornea, keratocytes are distributed throughout the stroma with many lying just beneath the overlying epithelium. B, Propidium iodide staining of a cornea at 4 hours after epithelial scrape injury. Arrows show the anterior stromal surface devoid of epithelium. Note the low density of keratocytes in the anterior stroma (original magnification ×200).*
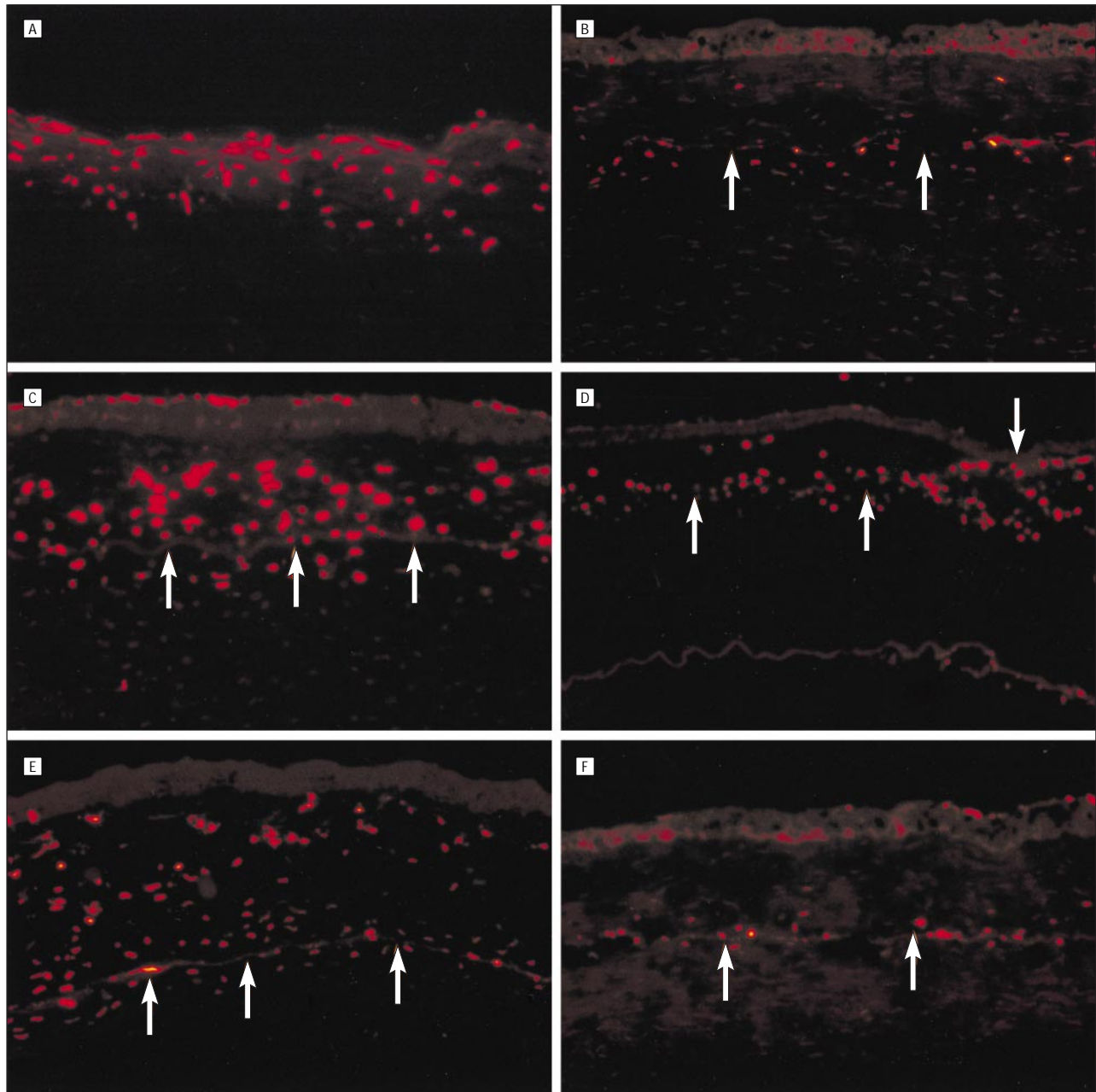
Figure 3. *Thymidine-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) assay to detect apoptosis. A, TUNEL assay in a rabbit cornea 4 hours after photorefractive keratectomy shows marked apoptosis of superficial keratocytes. B, TUNEL assay at 4 hours after laser in situ keratomileusis (LASIK) shows keratocytes anterior and posterior to the interface (arrows) undergoing apoptosis. Note most of these keratocytes are far away from the overlying intact epithelium. C, A cornea with a relatively high keratocyte apoptosis response 4 hours after LASIK. The keratocyte apoptosis response is much closer to the overlying epithelium than in B, and the subsequent events in the wound healing response are also likely to be nearer to the overlying epithelium. D, In the periphery of a cornea that underwent LASIK, the apoptosis response is marked near the point of epithelial entry by the microkeratome blade (downward pointing arrow). Upward pointing arrows denote the LASIK interface. E, A rabbit cornea that had epithelial debris from another cornea deliberately introduced beneath the flap 4 hours after surgery. Arrows indicate the interface. Note the high level of keratocyte apoptosis surrounding this epithelial debris. F, In a control cornea no epithelial debris was introduced and the level of keratocyte apoptosis is lower. Arrows indicate the LASIK interface in this cornea (original magnification ×400).*

require living cells to spread. These viruses typically infect the corneal epithelium, but may also infect the underlying keratocytes and endothelial cells and extend to other cells of the eye and central nervous system. Studies we performed[6] provided evidence to support this hypothesis. When rabbits were infected with herpes simplex virus, without initial scarification, thymidine-medicated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) assay and electron microscopic analysis revealed keratocytes beneath areas of epithelial in-

fection undergoing apoptosis. Stat-1 null mice have a defective keratocyte apoptosis response to epithelial scrape injury.[7] When Stat-1 null mice were infected via corneal surface inoculation with herpes simplex virus, they had overwhelming corneal infection that often led to central nervous system infection and death (James Hill, PhD, et al, unpublished data, 1999). It is difficult to verify whether diminished keratocyte apoptosis was the sole source of this fulminate infection since Stat-1 null mice also have defects in interferon production.
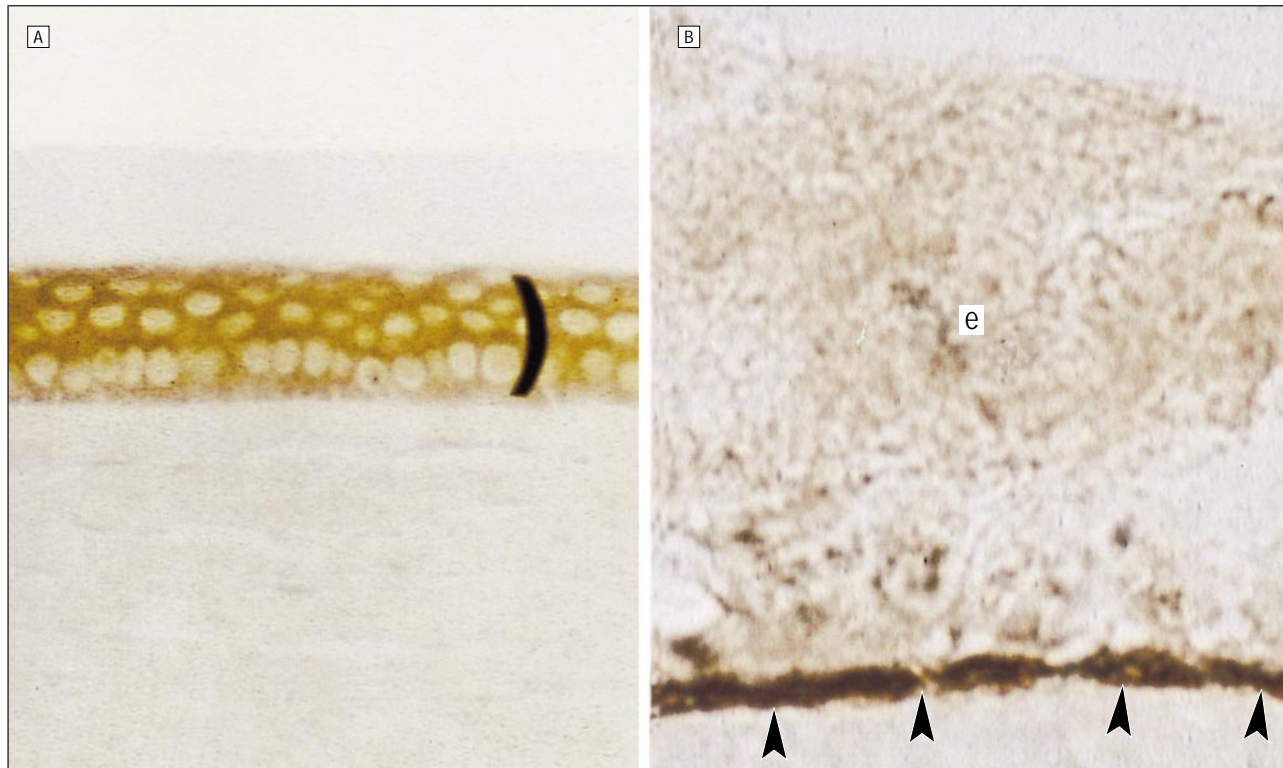
Figure 4. *Interleukin 1α (IL-1α) and platelet-derived growth factor (PDGF) detection in human corneal epithelium using immunocytochemistry. A, IL-1α is constitutively produced in the epithelium (bracket), but is not detected in the keratocytes in this unwounded cornea. Interleukin 1α is released primarily through injury or death of the epithelial cells and immediately binds IL-1α receptors expressed by the keratocyte cells (original magnification ×500). Reprinted with permission from Wilson et al. Exp Eye Res. 1994;59:63-72. Copyright 1994, London, England: Academic Press. B, PDGF is localized at high levels in the basement membrane (arrowheads) of the epithelium (e) and is likely released following damage to the epithelium and basement membrane (original magnification ×400).*
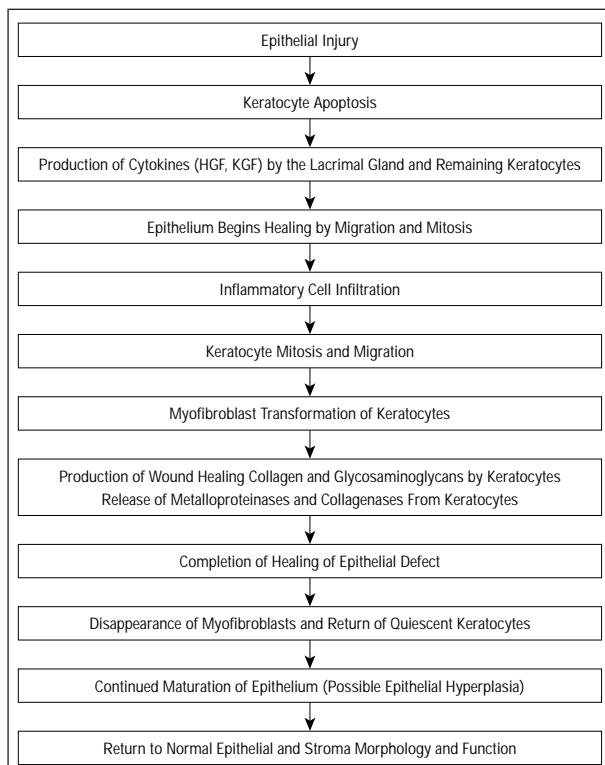

Figure 5. *This schematic diagram indicates some of the important aspects of the wound healing cascade in the cornea. Only major events are noted and the cascade is simplified. Cytokines and receptors likely regulate each of these components.*

The keratocyte apoptosis response occurs almost immediately after epithelial injury. Thus, if the corneal epithelium is injured and the eye immediately removed, the anterior keratocytes have already begun the apoptosis process (detected using electron microscopy). If this hypothesis is correct, the response that is seen following epithelial injury associated with LASIK or PRK likely represents mechanical triggering of this early defense system.

HOW IS KERATOCYTE APOPTOSIS FOLLOWING EPITHELIAL INJURY MEDIATED?

Several studies have suggested that injury-induced keratocyte apoptosis is mediated by the release of proapoptotic cytokines from the injured epithelium.[8] Cytokines that have been implicated in this response include interleukin (IL)-1, Fas ligand, bone morphogenic protein 2, bone morphogenic protein 4, and tumor necrosis factor α.[9-13] We believe redundancy may be designed to limit the capacity of viruses to evolve strategies to overcome this defense mechanism. For example, keratocyte apoptosis still occurs, albeit at significantly reduced levels, following epithelial scrape injury in Fas-null mice.[11] Many of these cytokines are constitutively produced by the epithelium (**Figure 4**), vigilantly awaiting epithelial injury. When epithelial injury occurs, the cytokines are immediately released and bind to receptors on keratocytes within moments. Thus, the apoptosis pathways are activated in the keratocytes almost immediately following epithelial injury.
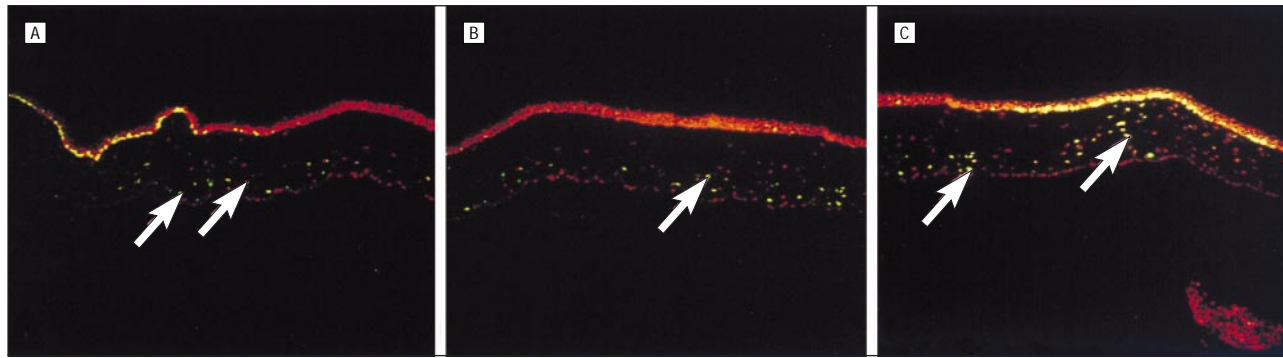
Figure 6. *Mitosis detected in keratocyte cells 24 hours after epithelial scrape injury in the rabbit. Cells undergoing mitosis stain green for the mitosis-associated antigen Ki-67 (arrows). A and B show the central cornea in 2 different specimens. C shows the peripheral cornea near the edge of the scrape injury. Note that keratocyte mitosis is localized in the peripheral and posterior cornea in a band surrounding the area where keratocyte apoptosis occurred immediately following the scrape injury. The red counterstain is propidium iodide. Figure provided by Sabino R. Guimaraes, MD, Audrey E. K. Hutcheon, BS, and James D. Zieske, from their studies presented at the Association for Research in Vision and Ophthalmology annual meeting, Ft Lauderdale, Fla, April 28, 1999.*

Virtually any source of epithelial injury can trigger the release of the cytokines. Epithelial scrape, microkeratome cuts through the peripheral epithelium during formation of a LASIK flap (Figure 3), diamond blade cuts through the epithelium in radial keratotomy, or even firm pressure on a contact lens or cloning cylinder placed on the intact corneal surface triggers apoptosis in the underlying keratocytes.

These cytokine systems may interact in the induction of keratocyte apoptosis. One of the more interesting interactions involves the IL-1/IL-1 receptor and Fas/Fas ligand systems. Experiments in our laboratory showed that microinjection of mouse IL-1α into the central stroma of the mouse cornea triggered apoptosis of the keratocytes at the site of injection.[9] Subsequent studies demonstrated that IL-1 stimulated keratocytes to produce Fas ligand messenger RNA and protein.[11] Keratocytes constantly produce the receptor Fas, but do not produce Fas ligand in vivo or in vitro in the absence of induction by IL-1.[11] Thus, when stimulated by IL-l, these cells produce both Fas ligand and Fas. Our data suggest that simultaneous production of the ligand and the receptor can result in apoptosis via "autocrine suicide."[11]

THE SUBSEQUENT EVENTS IN CORNEAL WOUND HEALING FOLLOWING KERATOCYTE APOPTOSIS

Keratocyte apoptosis is the first detectable event following epithelial injury. It occurs almost immediately and sets in motion a complex cascade of events in the stroma and epithelium (**Figure 5**). Hutcheon et al[14] have shown that within 12 to 24 hours of keratocyte apoptosis the remaining keratocytes surrounding the zone of dropout begin to undergo proliferation (**Figure 6**). These proliferating cells give rise to wound healing–type keratocyte-derived cells called *myofibroblasts*.[15,16] These myofibroblasts migrate in to repopulate the stroma and produce disorganized collagen, glycosaminoglycans, growth factors that stimulate healing of the overlying epithelium, and other components. Proliferation and migration of these myofibroblast cells are probably mediated by growth factors such as platelet-derived growth factor released from the epithelium (Figure 4).[17]

At this time, various inflammatory cells invade the cornea.[18] Little is known about what signals these cells to invade, although a recent study has shed some light on how signals that attract immune cells may be generated.[19] This study demonstrated that corneal fibroblasts stimulated with IL-1 markedly up-regulated monocyte chemotactic and activating factor. Thus, it could be that keratocytes stimulated by IL-1 at levels insufficient to induce apoptosis via autocrine suicide are competent to produce factors that attract inflammatory cells into the corneal stroma. Similarly, cytokines released from the epithelium by injury could directly attract inflammatory cells. Just how inflammatory cells interact in the wound healing response is poorly understood. Studies are underway to characterize these inflammatory cells and their involvement in corneal wound healing at the molecular and cellular levels.

Other cytokines such as transforming growth factor β (TGF-β) also serve important roles in modulating the wound healing response in the stroma.[20,21] One study demonstrated that TGF-β is likely involved in aggressive wound healing responses in which there are higher levels of opacity in the stroma.[20]

Cytokines produced by myofibroblasts regulate the proliferation, migration, and differentiation of the overlying healing epithelium.[22] Such interactions between the myofibroblasts and the epithelial cells are referred to as *stromal-epithelial interactions*. Key growth factors regulating this response are hepatocyte growth factor and keratinocyte growth factor.[22,23] The receptors for these cytokines are expressed by the epithelium and are up-regulated in response to injury.[24] Interleukin 1 and other cytokines also stimulate keratocyte/myofibroblast cells to produce metalloproteinases, collagenases, and other enzymes that are involved in the stromal wound healing response.[25] These regulatory interactions are referred to as *epithelial-stromal interactions*.

The cornea eventually returns to a more normal morphology and function. Myofibroblast cells disappear from the cornea during a period of weeks to months. At present it is not clear how these cells are eliminated from the stroma, but there is some evidence that late apoptosis may play a role.[26]

There are numerous complex interactions between epithelial cells and keratocytes/myofibroblasts that are

mediated by cytokines during the corneal wound healing cascade. Studies suggest that epithelium-derived cytokines trigger keratocyte apoptosis and stimulate mitosis and chemotaxis of myofibroblasts, and in turn, the myofibroblast-derived cytokines stimulate epithelial cell proliferation and migration. One can imagine the difficulty in regulating any single step in this complex interaction in an attempt to control the overall corneal wound healing response.

CLINICAL IMPLICATIONS IN UNDERSTANDING THE WOUND HEALING RESPONSE

This complex wound healing response has important implications for PRK and LASIK. The end result is variable stromal remodeling and epithelial hyperplasia associated with regression, haze, and other poorly controlled factors affecting the predictability of surgery.

The difference in the location of the keratocyte apoptosis response and the subsequent events in the wound healing cascade between PRK and LASIK may provide an explanation for the difference in clinical outcome between these 2 procedures, especially in eyes with higher levels of myopia. Similarly, differences in induced keratocyte apoptosis may explain some of the variation in achieved correction between different eyes having the same attempted correction.

The keratocyte apoptosis response occurs immediately beneath the healing epithelium in PRK (Figure 3). Subsequent events in wound healing are also localized immediately beneath the epithelium. When the myofibroblast cells produce hepatocyte growth factor and keratinocyte growth factor, the effects of these cytokines in stimulating proliferation and inhibiting terminal differentiation of epithelial cells (effects that together will tend to trigger epithelial hyperplasia) are in immediate proximity to the overlying epithelium. Studies have demonstrated that epithelial hyperplasia is an important factor in regression in PRK.[22,27]

In contrast, LASIK keratocyte apoptosis and the subsequent cascade occur at the level of the interface and are further removed from the overlying epithelium (Figure 3). Therefore, the epithelial hyperplasia response is likely to be attenuated in LASIK compared with PRK, although recent studies have demonstrated that epithelial hyperplasia can occur in LASIK.[27] This correlates with the clinical observation that eyes with identical levels of myopia require less laser ablation when LASIK is performed than when PRK is performed. Eyes that have LASIK are also less likely to have substantial regression. For example, with our own algorithm developed for the VISX Star S2 laser (VISX, Santa Clara, Calif), there are adjustments of −8% to −24% for LASIK treatment compared with PRK treatment. The downward adjustment of correction depends on the level of myopia (the higher the myopia to be treated the greater the adjustment) and patient age (the higher the patient age the greater the adjustment).

It follows that variability in flap thickness (Figure 3) could be an important factor in variation in wound healing and achieved correction between different eyes that have undergone LASIK. Thus, the closer the wound healing response is to the overlying epithelium, the more likely epithelial hyperplasia will be stimulated by growth factors released from keratocytes or myofibroblasts.

Clinical studies by Johnson et al[28] have indicated that more refractive correction is obtained per laser pulse with true transepithelial PRK than with traditional epithelial scrape followed by PRK or laser-scrape PRK. Laboratory studies have found that less anterior keratocyte apoptosis occurs following transepithelial PRK than laser-scrape PRK.[29] This study suggests that the difference is attributable to ablation of the proapoptotic cytokines in the epithelium with true transepithelial PRK. It is important that the surgeon know that new algorithms are needed to prevent overcorrection if true transepithelial PRK is adopted.

Since epithelial-derived cytokines seem to be the inducers of keratocyte apoptosis, the surgeon should do everything possible to limit the introduction of epithelial tissue into the interface (Figure 3). Thus, LASIK is best performed with sharp blades that perforate the epithelium with minimal trauma rather than dull blades that will tend to tear through the epithelium and release more cytokines. Even a sharp blade will track some epithelial debris into the interface as the flap is cut. Strategies such as limited irrigation with balanced salt solution and use of an aspirating eyelid speculum may be useful in controlling the LASIK-associated wound healing response. A laser with properties such as the fentosecond laser could be useful in producing a flap with uniform thickness with minimal introduction of epithelial debris and cytokines into the interface. However, at some point the epithelium must be breached to lift the flap. Cytokines will be released at this site and may diffuse into the interface following surgery even if a new technique eliminates transfer by a blade.

The ultimate goal is to develop pharmacological agents that could be applied to the cornea prior to surgery to limit or normalize the subsequent wound healing response. There is a tendency to think in terms of blocking the wound healing response, but that may not be attainable or necessary. If we could merely normalize the wound healing response between different eyes and different patients, so that it is consistent, we could easily adjust the laser algorithms to compensate. Our main target in this effort is the initial keratocyte apoptosis response. Our working hypothesis is that if the initial keratocyte apoptosis can be inhibited or blocked, the subsequent events in the wound healing cascade will also be attenuated. This is not to say that the subsequent events in wound healing are not important. However, once the "cow is out of the barn" and dozens of interacting cytokine-mediated cascades are activated, it seems unlikely that we will be able to obtain the fine control necessary to modulate the refractive effect of surgery and prevent wound healing from confounding attempts at customized ablation. Control of later events could be helpful in some situations. For example, patients who develop excessive haze following PRK might benefit from blockade of the TGF-β cytokine receptor system.[20]

How can keratocyte apoptosis be controlled? Apoptosis is mediated by a series of defined steps much like glycolysis or the Kreb cycle.[30] Enzymes called caspases

that mediate the apoptosis process are attractive targets for pharmacological control. We have recently completed studies in which a well-known caspase-inhibitor, zVAD-FMK, was applied prior to epithelial scrape in an attempt to block keratocyte apoptosis.[31] Early results were promising. There was apparent inhibition of apoptosis monitored by the TUNEL assay. However, transmission electron microscopy revealed that the superficial keratocytes were dying by an alternative form of cell death called *necrosis* that was not detected by the TUNEL assay. This study confirms the importance of verifying inhibition of apoptosis using methods other than the TUNEL assay, at least in a pilot study performed with each experimental model. We are still hopeful that other inhibitors will be developed that block apoptosis without triggering necrosis. We are concentrating our efforts on more apical participants in the apoptosis cascade.

Other strategies may also be possible. The cornea is uniquely accessible for gene transfer. Thus, it may be possible to block apoptosis through the introduction of inhibitory genes. It may only be necessary to transiently inhibit keratocyte apoptosis to control wound healing in the cornea. This is an important point and differentiates efforts to control apoptosis in the cornea during wound healing from those in other tissues. For example, if blockage of apoptosis were effective in treating some forms of retinal degeneration, the effect would need to be prolonged to be clinically useful. In contrast, inhibition of apoptosis in the cornea might only need to last hours, or days at the most, to effectively modulate the wound healing response. It seems likely that it is not a question of whether methods will be found to inhibit this response, but rather when the appropriate drug or combination of drugs will be identified. We predict that frustrating efforts to introduce stable custom corneal ablations will once and for all bring corneal wound healing research to the forefront.

COULD UNWANTED ACTIVATION OR UNCONTROLLED PROGRESSION OF THE WOUND HEALING RESPONSE BE ASSOCIATED WITH CORNEAL DISEASE?

An intricate and highly regulated process such as the corneal wound healing response could occasionally go awry and precipitate disease or trigger an abnormal outcome to surgery. Such abnormal activation or progression could be related to environmental and genetic factors.

Late corneal haze that occurs following PRK likely represents such an abnormality.[32,33] Almost all patients who have PRK have some level of stromal haze in the months following the procedure. Such haze is probably related to disorganized collagen deposition and opacity of the cells that repopulate the anterior stroma.[34] This mild haze is benign and typically self-limited, resolving during the first year or two after surgery. In some patients, however, a very severe bilateral haze develops in the anterior stoma underlying the epithelium. In these cases, the patient typically has an excellent outcome for a period of 3 to 6 months and then suffers regression and loss of best spectacle-corrected visual acuity associated with the dense superficial haze. Late haze after PRK is exceedingly rare in patients with attempted corrections of less

than 5 diopters, and increases in frequency as the level of attempted correction increases.[35-37] Our own results are similar. It has been suggested that this late haze response is associated with ultraviolet light exposure.[38] Whatever the exact mechanism, somehow the typical wound healing response of the cornea is abnormal in these patients. Retreatment with the excimer laser is associated with recurrence with increased severity. The abnormality typically resolves during a period of years, but the morbidity during the period of time required for resolution is troubling to the patient and physician. Recent reports have suggested that mitomycin C treatment may be beneficial,[39] but the mechanism is unknown.

Keratoconus is an ectatic dystrophy of the cornea associated with progressive thinning and structural changes. A variety of stromal abnormalities such as increased collagenases and other components have been noted in keratoconus cornea.[40] Clinical studies have associated chronic eye rubbing, poorly fit contact lenses, and atopic eye disease with keratoconus. These associated environmental factors all have chronic corneal epithelial injury in common. A recent study has suggested the unifying hypothesis that keratoconus is associated with increased keratocyte apoptosis within the stroma.[8,41] The working hypothesis is that chronic ongoing epithelial injury, perhaps associated with underlying genetic abnormality, leads to accelerated keratocyte apoptosis and compensatory mitosis. Chronic ongoing cell turnover may trigger the release of intracellular components such as degradative enzymes that damage the corneal stroma. Thus, during a period of years, the homeostatic balance between degradative and repair processes in the normal cornea may be tipped toward degradation. In chronic cell death, some of the intracellular components may escape into the tissue and cause damage to the Bowman layer and the stroma. Again, genetic factors could play a role in determining which individuals are susceptible.

These are just 2 examples in which abnormal activation or regulation of systems that are associated with the wound healing response may somehow lead to alterations in corneal morphology and function. Further investigation into the normal response to injury may provide insights into other corneal disorders where etiology remains enigmatic.

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From the Archives of the ARCHIVES

A look at the past . . .

My impression was deep that here, at the southern end of the Mississippi Valley, the majority of our patients with acute iritis are also victims of the specific disease. Is it possible, I have often asked myself, that in so vast a country as the United States the prevalent causes and the course of diseases may be somewhat different in widely separated areas? Or do variations arise solely from the different racial stocks preponderating, the densities of population and the unlike conditions such differences entail?

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