

# ANALYSIS OF THE KERATOCYTE APOPTOSIS, KERATOCYTE PROLIFERATION, AND MYOFIBROBLAST TRANSFORMATION RESPONSES AFTER PHOTOREFRACTIVE KERATECTOMY AND LASER IN SITU KERATOMILEUSIS

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## ABSTRACT

*Purpose:* To test the hypothesis that (1) there are quantitative differences in the cellular responses in the corneal stroma after photorefractive keratectomy (PRK) for low myopia compared to high myopia and (2) there are both qualitative and quantitative differences in the cellular responses in the corneal stroma after PRK for high myopia and laser in situ keratomileusis (LASIK) for high myopia.

*Methods:* PRK for low myopia (-4.5 diopters [D]), PRK for high myopia (-9.0 D), and LASIK for high myopia (-9.0 D) were performed in rabbit eyes, and corneas were obtained for examination at 4, 24, and 72 hours, 1 and 4 weeks, and 3 months after surgery. A total of 144 rabbits were included in the study. Stromal apoptosis, necrosis, mitosis, myofibroblast generation, and inflammatory cell infiltration were evaluated by immunohistochemical methods and electron microscopy.

*Results:* Keratocyte apoptosis/necrosis and the subsequent proliferation and density of myofibroblasts were qualitatively and quantitatively different in PRK for high myopia compared to either PRK for low myopia or LASIK for high myopia. Significant inflammatory cell infiltration was noted in both PRK and LASIK but appeared to be greater in PRK for high myopia.

*Conclusions:* The qualitative and quantitative differences in the cellular wound healing response after PRK for high and low myopia and LASIK for high myopia are likely determinants of the clinical differences in refractive outcome and some of the complications, such as regression and haze, seen after these procedures.

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## INTRODUCTION

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It is essential to consider the evolutionary context to understand the importance of the wound-healing response in the cornea. Adequate vision was certainly essential for the survival of most animals, and this resulted in selective pressure to develop the ability to recover from a variety of corneal injuries. Abrasions to the vertebrate cornea from branches, projectiles, and other sources were probably common. Infectious agents such as herpes simplex virus, smallpox virus, or related viruses may have posed an even greater threat because of the potential for extension into the eye and even the brain. The wound-healing response to these injuries would likely have evolved to restore the protective epithelial surface, maintain the integrity of the cornea, and maintain or restore

the corneal clarity necessary for vision. Systems designed to rapidly restore the integrity of the epithelium, reestablish the structure and clarity of the stroma, and impede the spread of pathogens until the immune response eradicated the invaders would probably have provided strong selective advantages to organisms, animal or human, dependent on sight for survival.<sup>1</sup>

Within the context of modern medicine and surgery, wound healing remains critical to the maintenance of corneal health and vision. The wound-healing response is a major factor in the outcome of all corneal surgical procedures and contributes to the pathophysiology of many corneal diseases. The healing response at the donor-recipient interface plays an important role in determining the efficacy of corneal transplantation and also contributes to the development of complications, including astigmatism, graft overdrive, and others.<sup>2</sup> The stromal wound-healing response that occurs in diseases such as recurrent herpes simplex keratitis is a major determinant of the amount of corneal scarring that develops in individual eyes.<sup>3,4</sup>

The corneal wound-healing response also contributes to the efficacy and safety of refractive surgical procedures

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such as photorefractive keratectomy (PRK) and laser in situ keratomileusis (LASIK). It is a major factor in the development of overcorrection, undercorrection, haze, and other complications that occur when these surgeries are performed for the correction of myopia and hyperopia,<sup>5,6</sup> as well as astigmatism.<sup>7,8</sup>

Experimentally, PRK and LASIK have been used to provide standardized animal models in which to study the corneal wound-healing response. Although many of the studies performed to date have contributed important insights into wound healing in the cornea, most have been limited to some extent in terms of the number of eyes (and hence statistical analysis), choice of time points, length of follow-up, and/or aspects of the cellular responses examined. It is important, however, to understand the knowledge derived from these previous investigations in order to allow interpretation of the results of the work performed for this thesis.

#### COMPONENTS OF THE CORNEAL WOUND-HEALING CASCADE

The corneal epithelium, stroma, and nerves participate in homeostasis of the ocular surface. The lacrimal glands and tear film also contribute to maintaining ocular surface smoothness and integrity. Following most injuries, these components are involved in an orchestrated wound-healing response that efficiently restores corneal structure and function. This process is modulated by a variety of cytokines and receptors that are produced locally in the cornea and lacrimal glands. Also, immune cells that function to eliminate debris and microbes are attracted to the site of injury.

Studies performed over the past decade have revealed many of the events that make up the wound-healing cascade in the cornea. Most of these studies, however, especially those examining the stromal response, have focused on the early phase of the response—from the time of injury to a few hours after wounding. It is important to understand the contributions of each of the components of this cascade and their interactions in order to appreciate the overall corneal healing response. Figure 1 provides a framework for considering the corneal wound-healing cascade. (It should be noted that many of these events actually occur simultaneously and, therefore, the “cascade” should be viewed as such only in rough terms. Also, some of the processes that are important components in the overall wound-healing response, but are not directly related to these studies, are omitted for the sake of clarity.)

#### *Keratocyte Apoptosis*

Early studies noted that keratocytes seemed to disappear from the anterior stroma after corneal epithelial scrape injury;<sup>9-12</sup> explanations offered for this phenomenon

included osmotic changes from the loss of epithelium, exposure to the atmosphere, and artifact resulting from tissue processing. In 1996, however, it was demonstrated for the first time that the early disappearance of keratocytes after epithelial injury is mediated by a process known as apoptosis.<sup>13,14</sup> Apoptosis is a gentle, involuntal form of cell death that occurs with little release of lysosomal enzymes or other intracellular components and the resulting damage to surrounding cells or tissues that are characteristic of necrotic cell death. Subsequent studies have suggested that apoptosis is mediated by cytokines released from the injured epithelium, including interleukin-1 and tumor necrosis factor alpha.<sup>15-17</sup>

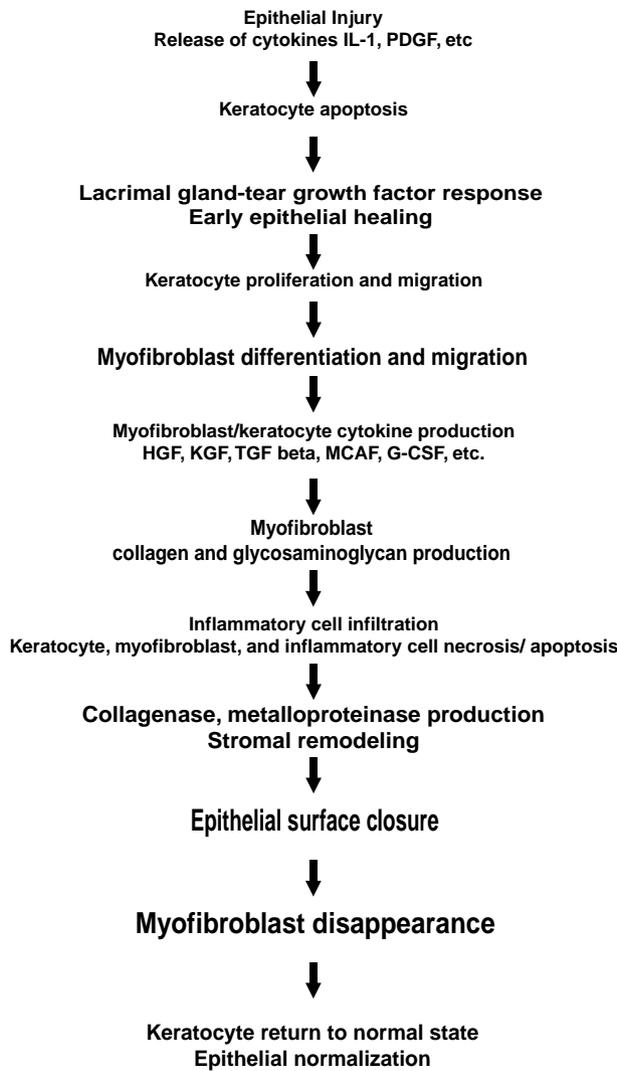
Virtually any epithelial injury induces keratocyte apoptosis. Among the causes demonstrated in animal models are mechanical scrape,<sup>13</sup> corneal surgical procedures such as PRK and LASIK,<sup>18</sup> herpes simplex keratitis,<sup>1</sup> incisions,<sup>18</sup> and the application of a plastic ring pressed firmly against the epithelial surface.<sup>14</sup> Recently, we have shown that apoptosis occurs in the keratocytes underlying Bowman's layer in the human eye after epithelial scrape injury (Figure 2) (unpublished data).

Keratocyte apoptosis near the surface of the cornea is the first observable stromal change after epithelial injury. The rapidity with which apoptosis occurs after injury can be detected by electron microscopy. If one euthanizes a mouse, enucleates the eye, performs a single scrape across the corneal epithelium, immediately places the eye into fixative, and processes the cornea for electron microscopy, one finds that the specimens already demonstrate chromatin condensation and other morphologic changes in the keratocytes consistent with apoptosis (Figure 3). Thus, the cornea is primed and ready to respond immediately to injury. Such an immediate response would be expected if one of the functions of this process is to retard dissemination of viral pathogens by removing accessible cells until the immune system can respond to the invader.<sup>1</sup>

Keratocyte apoptosis can also be demonstrated by means of the terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay, which detects fragmented ends of the DNA strands produced in the cell during the apoptotic process. DNA fragmentation, which takes longer to develop (10 to 30 minutes) compared with the electron microscopic evidence, has been shown to be most prominent approximately 4 hours after epithelial scrape injury in mice and rabbits.<sup>13,18</sup>

Keratocytes undergo apoptosis to a depth of one third to one half of the stromal thickness, depending on the species and the type of injury. In the unwounded cornea, cellular processes called gap junctions connect keratocytes to form a syncytium.<sup>19,20</sup> Thus, cytokines released from the injured epithelium could bind to receptors on

*Analysis of the Keratocyte Apoptosis, Keratocyte Proliferation, and Myofibroblast Transformation*

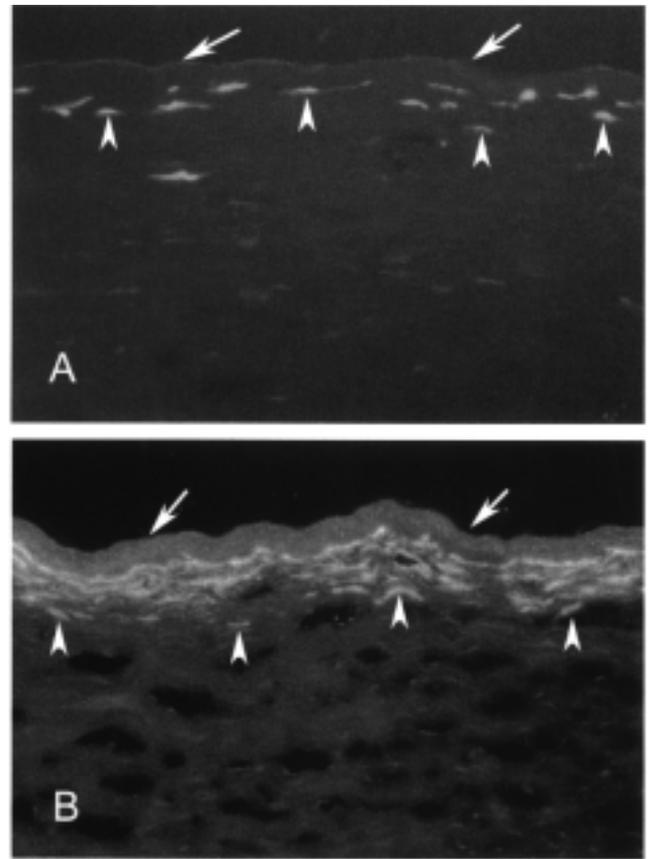


**FIGURE 1**

Schematic diagram showing some of the events that make up corneal wound-healing response that occurs after corneal epithelial injury or surgical procedures such as photorefractive keratectomy or laser in situ keratomileusis. Note that this scheme is simplified; not all events that may be important to the total wound-healing response are included. Additionally, events in the cornea do not necessarily follow the precise sequential nature of the diagram. For example, keratocyte apoptosis is the first observable phenomenon after injury, but many of the subsequent events occur in parallel.

the most superficial keratocytes and signal adjoining cells to undergo apoptosis via these intercellular communication channels. Alternatively, pro-apoptotic cytokines from the injured epithelium may penetrate into the stroma and stimulate the keratocytes directly. It has been suggested that keratocyte apoptosis continues for at least 1 week after PRK in the rabbit cornea.<sup>21</sup>

Localization of the keratocyte apoptosis response in the stroma varies with the type of corneal epithelial injury.<sup>18</sup> Injuries such as epithelial scrape and epithelial viral infection trigger keratocyte apoptosis in the superfi-

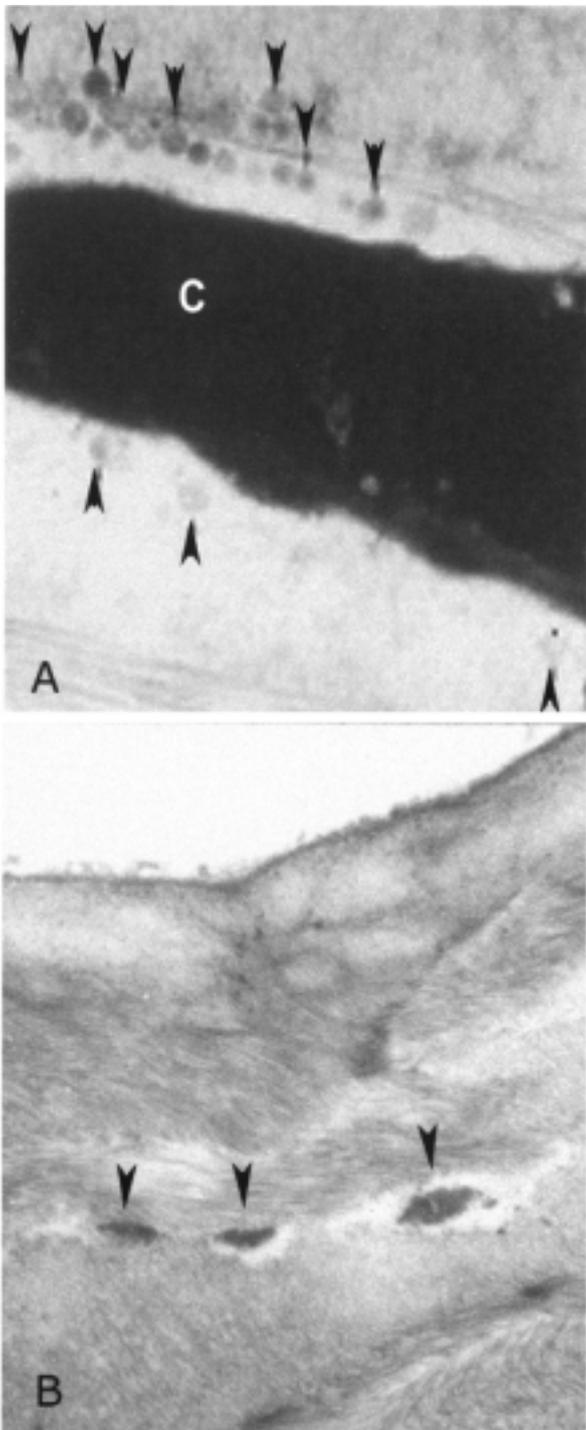


**FIGURE 2**

Keratocyte apoptosis detected at 4 hours (A) and 65 hours (B) after epithelial scrape injury in a human eye prior to enucleation for a choroidal melanoma. Terminal deoxynucleotidyl transferase-mediated dUTP nick end label (TUNEL)-positive keratocytes (arrowheads) are seen in anterior stroma beneath Bowman's layer (arrows) (magnification x200). (This experiment was approved by the institutional review board of the author's university.)

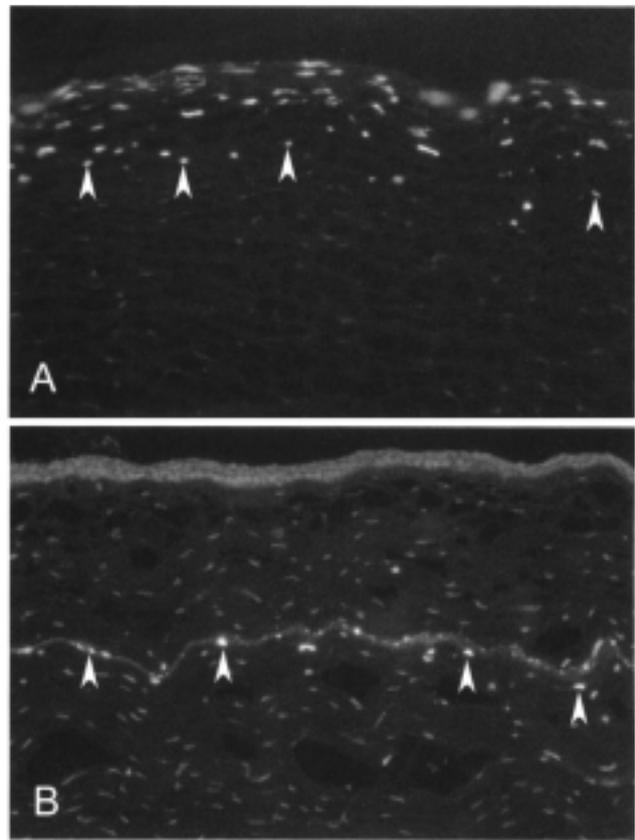
cial stroma (Figure 4A). Similarly, PRK, which is in effect an ablative epithelial scrape, results in anterior stromal keratocyte apoptosis. By contrast, a lamellar cut across the cornea produced by a microkeratome, as performed for LASIK, induces keratocyte apoptosis not only peripherally at the site of epithelial injury, as would be expected, but also along both sides of the lamellar interface created deeper in the stroma by the microkeratome cut (Figure 4B). This extension of the apoptosis-inducing effect from the site of epithelial injury is thought to be attributable to tracking of epithelial debris, including pro-apoptotic cytokines, into the interface by the microkeratome blade.<sup>18</sup> Alternatively, cytokines from the injured peripheral epithelium could diffuse along the lamellar interface and into the central stroma.<sup>18</sup>

In turn, the location of keratocyte apoptosis influences the location and effect of events that occur later in the wound-healing cascade. This may be important in determining clinical and biological differences between



**FIGURE 3**

A, Transmission electron micrograph of keratocyte apoptosis in the mouse eye. One hour after epithelial scrape injury, superficial keratocytes show shrinkage of cytoplasm, chromatin condensation (C), and formation of apoptotic bodies (arrowheads) that contain mitochondria, lysosomes, and other cellular components. These apoptotic bodies disperse into stroma and are absorbed by other cells that remain alive (magnification x6,000). B, At 4 hours after epithelial scrape injury, only condensed chromatin (arrowheads) remains from a keratocyte that underwent apoptosis (magnification x1,000). Both figures reprinted with permission from *Experimental Eye Research*.<sup>13</sup> Copyright 1994, Academic Press, London.



**FIGURE 4**

Localization of keratocyte apoptosis in rabbit cornea, as detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, depends on type of injury. A, Epithelial scrape injury, as occurs in photorefractive keratectomy, triggers superficial stromal keratocyte apoptosis (arrowheads). B, Lamellar microkeratome cut, such as would be made for laser in situ keratomileusis, triggers keratocyte apoptosis peripherally where blade penetrates epithelium (not shown) and along lamellar interface (arrowheads) (magnification x200).

PRK and LASIK for high myopia in terms of, for instance, epithelial hyperplasia, which characterizes later stages of corneal wound healing. Thus, superficial keratocyte apoptosis (such as that triggered by PRK) may be more likely to result in epithelial hyperplasia than deeper keratocyte apoptosis (such as that noted in LASIK) because of the localization of the epithelium-modulating growth factors produced by the proliferating keratocytes and myofibroblasts involved in repopulating the areas denuded of these cells during the early stages of wound healing.

#### *Keratocyte Proliferation and Migration: Myofibroblasts*

As noted above, the wave of keratocyte apoptosis that occurs within the first few hours of corneal epithelial injury produces an area of stroma that is devoid of keratocytes. Some of the remaining keratocytes in the posterior and peripheral cornea begin undergoing mitosis about 12 to 24 hours after injury,<sup>22</sup> as can be shown by bromodeoxyuridine incorporation or immunocytochemical stain-

ing for a mitosis-specific antigen, Ki-67.

The cytokines and other factors that regulate the proliferation and migration of the keratocytes are not well understood. Platelet-derived growth factor (PDGF) expressed by the epithelium and PDGF receptors expressed by the keratocytes may play a role, in that PDGF has a mitogenic and chemotactic effect on these cells.<sup>23-25</sup> Keratocyte proliferation after epithelial scrape injury may continue for several days.<sup>22</sup> To date, however, no studies have reported the long-term time course of keratocyte proliferation after PRK or LASIK.

Studies have suggested that keratocyte proliferation after injury results in the generation of a type of cell called the myofibroblast.<sup>26-29</sup> Most of these studies, however, have been in vitro tissue culture-based investigations. Little information is available regarding whether this transformation takes place in the keratocytes that undergo mitosis after PRK,<sup>29</sup> and nothing has been reported about the status of these cells after LASIK.

Myofibroblasts are characterized by expression of alpha smooth-muscle actin (SMA). This allows them to be detected in vitro and in situ by immunocytochemistry.<sup>26-29</sup> They are thought to have altered transparency in vivo related to corneal crystallin expression.<sup>30</sup> They are also thought to produce high levels of growth factors such as hepatocyte growth factor and keratinocyte growth factor, which have the specific functions of regulating cellular proliferation, migration, and differentiation associated with healing of the corneal epithelium.<sup>31</sup> There is also evidence that myofibroblasts express the types of collagen associated with wound healing rather than the normal collagen types produced by keratocytes.<sup>32,33</sup> They may also produce collagenases, gelatinases, and metalloproteinases associated with the remodeling of stromal collagen,<sup>34-38</sup> a process that is thought to play an important role in regression following PRK or LASIK.

The ultimate fate of the myofibroblasts that appear in the corneal stroma after PRK and LASIK is also uncertain. Two possibilities are that myofibroblasts transdifferentiate into keratocytes as the wound-healing process is completed or that myofibroblasts are terminally differentiated and are slowly eliminated by apoptosis. Greater knowledge of the role and fate of the myofibroblasts may be important in our understanding of the wound-healing events that occur after PRK and LASIK, as well as in the development of approaches to the control of these events to prevent unsatisfactory outcomes after these refractive surgical procedures.

### *Inflammatory Cells*

Very little is known about the role of inflammatory cells in the wound-healing response after PRK and LASIK. In the only published study to date,<sup>39</sup> inflammatory cells were

identified in the stroma by hematoxylin-eosin staining beginning about 24 hours after PRK. However, this staining technique was unable to distinguish specific types of inflammatory cells in these corneas. It is important to clarify which types of inflammatory cells are involved in the corneal wound-healing response after PRK and LASIK by using more sensitive methods, such as electron microscopy.

It seems likely that the inflammatory cells attracted to the cornea after PRK and LASIK are eventually eliminated by apoptosis once the response has fulfilled its function, in that apoptosis has been shown to be the cause of immune cell death in other organs. However, there is no concrete information concerning how these cells are eliminated in the cornea after refractive surgery.

### **RESOLUTION OF THE WOUND-HEALING RESPONSE**

In the months after injury, the wound-healing response is completed and there is a return to normal structure and function in the corneal stroma. This process is associated with elimination of some of the cells involved in wound healing, as well as remodeling of the disordered collagen produced by the myofibroblasts and/or keratocytes during the wound-healing process.<sup>40,41</sup> This remodeling process, which begins within a few weeks after injury, can continue for years, depending on the severity of the injury. Studies of later time points after laser refractive surgery are needed to understand these processes that return the cornea to its normal prewounding state.

Restoration of the epithelial cell layer after injury is also essential to the function and survival of the cornea. However, this response is often exaggerated. Eyes that have undergone identical laser refractive surgical corrections have been shown to develop widely variable amounts of epithelial hyperplasia. This phenomenon is thought to be an important mechanism in regression of the refractive effect of PRK and LASIK.<sup>42-45</sup> Additionally, remodeling of the epithelium may take place over a period of months to years, and this prolonged response may result in instability of the refractive effect of PRK and LASIK. The regulatory mechanisms that modulate the return to normal corneal epithelial morphology have not been characterized.

In the present study, we have examined several important aspects of the wound-healing response after PRK for low and high myopia and LASIK for high myopia in a large number of rabbit eyes over a substantial period of time, from the earliest observations at 4 hours to the latest at 3 months after surgery. Our goal was to test the hypotheses that (1) there are quantitative differences in the cellular responses in the corneal stroma after PRK for low and high myopia and (2) there are both qualitative and quantitative differences in the wound-healing

response after PRK for high myopia and LASIK for high myopia. We have also used the results of these experiments to draw some conclusions about possible relationships between wound healing and the complications associated with these refractive surgical procedures in the clinical setting.

## METHODS

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### ANIMALS

All animal studies described in this thesis were approved by the Animal Control Committee at the author's university. All animals were treated in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

A total of 144 12- to 15-week-old female New Zealand white rabbits weighing 2.5 kg to 3.0 kg each were included in the study. One eye of each rabbit, selected at random, was subjected to PRK or LASIK. Three corrections were performed: -4.5 D PRK (low myopia) (N=48 eyes); -9.0 D PRK (high myopia) (N=48 eyes); and -9.0 D LASIK (high myopia) (N=48 eyes). The contralateral eyes served as unoperated controls (N=144).

Anesthesia was obtained by intramuscular injection of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg). In addition, topical proparacaine hydrochloride 1% (Alcon, Fort Worth, Texas) was applied to each eye just before surgery. Euthanasia was performed with an intravenous injection of 100 mg/kg pentobarbital while the animal was under general anesthesia.

Six time points were examined: 4 hours, 24 hours, 72 hours, 1 week, 4 weeks, and 3 months. These time points were selected because they include all of the major early events that have been noted in previous studies of corneal scrape, PRK surgery, and LASIK surgery, and also provide later time points to follow the return to normalcy. Time points earlier than 4 hours were not used because previous studies found no relevant differences between earlier times and 4 hours, except that the TUNEL assay was more strongly positive at 4 hours than it was earlier.<sup>13</sup>

Eight corneas with each of the three refractive surgical procedures were examined at each of the six time points. Six of the eight corneas were preserved by cryofixation for the TUNEL assay and immunocytochemistry, and two corneas were processed for light microscopy and transmission electron microscopy (TEM).

### PRK TECHNIQUE

With the animal under general and local anesthesia, a wire lid speculum was positioned and a 7-mm-diameter area of epithelium overlying the pupil was removed by scraping with a No. 64 blade (Beaver; Becton, Dickinson & Co, Franklin Lakes, New Jersey). A laser ablation with the

corresponding sphere correction without astigmatism correction (Star S2 Excimer Laser System, VISX, Inc, Santa Clara, California) with a 6.0-mm-diameter optical zone was performed. The -4.5 D correction had a depth of 53 mm, and the -9.0 D correction had a depth of 106 mm. Two drops of ciprofloxacin hydrochloride 0.3% (Ciloxan, Alcon, Fort Worth, Texas) were instilled into the eye at the end of the procedure. A temporary tarsorrhaphy was placed with a double-armed 5.0 silk suture (Alcon) for the first 24 hours after surgery. Any eye that developed infiltration suggestive of infection or an epithelial defect persisting beyond 1 week after surgery was excluded from the study and an additional animal was included in its place.

### LASIK TECHNIQUE

With the animal under general and local anesthesia, the eye was proptosed anterior to the eyelids and temporarily retained in that position by clamping the temporal upper and lower eyelids together with a mosquito clamp. The base of the microkeratome (Hansatome, Bausch & Lomb, Rochester, New York), set to cut a flap 8.5 mm in diameter and 160  $\mu$ m in thickness, was placed on the corneal surface, and a clamp was placed on the suction tubing that runs from the microkeratome base to the power supply. This was necessary because of the difference in the curvature of the rabbit cornea, compared with that of the human, in order to allow the instrument to sense suction in the tubing. Otherwise, the safety features of the instrument would have prevented the automated microkeratome head from cutting the flap.

After the tubing was clamped, the microkeratome base was pressed firmly onto the cornea, and suction was activated with the microkeratome power supply. The microkeratome head was placed into position on the base and the forward pedal activating the motor was depressed. The base was pressed firmly against the cornea to simulate the suction obtained in the human eye while the head of the microkeratome coursed across the gear track to cut the flap. The head was returned to its original position by depressing the reverse pedal, and the base and head of the microkeratome were removed from the eye. A smooth round spatula was inserted into the stromal interface, and the flap was reflected on its hinge against the conjunctiva to expose the bed. A -9.0 D correction identical to that used in the PRK eyes was performed (Star S2 Excimer Laser System, VISX). The flap and stromal bed were irrigated with approximately 0.5 mL of 0.2  $\mu$ m filtered balanced salt solution (Alcon). The flap was returned to its original position with the spatula.

After 1 minute, two drops of ofloxacin ophthalmic solution 0.3% (Ocuflox, Allergan, Irvine, California) were instilled into the eye. Corticosteroid drops were not used to eliminate the potential confounding effect of this type

of drug on the corneal wound-healing response. The flap was protected with a contact lens (Soflens, 66 F/M base curve, Bausch & Lomb, Rochester, New York) for the first day after surgery. The eyelids were closed for 24 hours after surgery with a temporary tarsorrhaphy.

Rabbits with flaps that were displaced or that had visible striae on the first day after surgery were excluded, and replacement animals were added to complete the treatment groups.

#### **TISSUE FIXATION AND SECTIONING**

Rabbits were euthanized, and the corneoscleral rims (operated-on eyes and unoperated control eyes) were removed with 0.12 forceps and sharp Westcott scissors. For histological analyses (TUNEL assay, Ki-67 immunocytochemistry, and alpha-SMA immunocytochemistry), the corneas were embedded in liquid OCT compound (Sakura Finetek, Torrance, California) within a 24 mm x 24 mm x 5 mm mold (Fisher Scientific, Pittsburgh, Pennsylvania). The tissue specimens were centered within the mold so that the block could be bisected and transverse sections cut from the center of the cornea. The mold and tissue were rapidly frozen in 2-methyl butane within a stainless steel crucible suspended in liquid nitrogen. The frozen tissue blocks were stored at  $-85^{\circ}\text{C}$  until sectioning was performed.

Central corneal sections (7  $\mu\text{m}$  thick) were cut with a cryostat (HM 505M, Micron GmbH, Waldorf, Germany). Sections were placed on 25 mm x 75 mm x 1 mm microscope slides (Superfrost Plus, Fisher) and maintained frozen at  $-85^{\circ}\text{C}$  until staining was performed.

#### **TUNEL ASSAY AND IMMUNOCYTOCHEMISTRY ASSAYS**

To detect fragmentation of DNA associated with apoptosis,<sup>13</sup> tissue sections were fixed in acetone at  $-20^{\circ}\text{C}$  for 2 minutes, dried at room temperature for 5 minutes, and then placed in balanced salt solution. A fluorescence-based TUNEL assay was used according to the manufacturer's instructions (ApopTag, Cat No. S7165; Intergen, Purchase, New York). Positive (4-hour mechanical corneal scrape) and negative (unwounded) control slides were included in each assay. Photographs were obtained with a fluorescence microscope (Nikon E600, Melville, New York).

Immunocytochemistry for Ki-67 and alpha-SMA was performed as previously described.<sup>46</sup> The monoclonal antibody against Ki-67 (Zymed Laboratories, South San Francisco, California) was used at the stock concentration of 214.6 mg/L in 1X phosphate-buffered saline (PBS), pH 7.4, with 1% bovine serum albumin. The alpha-SMA antibody (DAKO Corporation, Carpinteria, California) was used at a concentration of 85 mg/L in PBS with 1% bovine serum albumin. In each case, the

sections were incubated with the diluted antibody for 1 to 2 hours at room temperature, followed by a 1-hour incubation with the secondary antibody (fluorescein isothiocyanate-conjugated donkey anti-mouse IgG; Jackson ImmunoResearch, West Grove, Pennsylvania). Cover slips were mounted with a protective mounting medium containing propidium iodide or 4',6-diamidino-2-phenylindole (DAPI) (Vectashield, Vector Laboratories, Burlingame, California). Negative controls (primary antibody omitted) were included with every antibody-binding experiment. Additional controls were performed with unrelated monoclonal antibodies to ensure specificity. The sections were viewed and photographed under a light microscope (Eclipse E800, Nikon) equipped with a digital SPOT camera (Micro Video Instruments, Avon, Massachusetts).

#### **LIGHT AND TRANSMISSION ELECTRON MICROSCOPY**

The corneas were fixed in a solution of 2% paraformaldehyde and 2% glutaraldehyde in a vehicle of 1.3 M sodium phosphate buffer containing 0.05%  $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$ , pH 7.3, at  $4^{\circ}\text{C}$  for 12 to 24 hours. The specimens were then washed twice with the buffered fixative vehicle for 15 minutes at room temperature and stored at  $4^{\circ}\text{C}$  in the fixative vehicle until they were processed.

For processing, the corneas were bisected and a 1.5-mm strip was removed from the center of each cornea. This strip was again bisected, and the fragments were placed in the primary fixative vehicle prior to secondary fixation. Secondary fixation was carried out in 1%  $\text{OsO}_4$  in 1.0 M phosphate buffer, pH 7.3, for about 45 minutes at room temperature, followed by three washings in the phosphate-buffered fixative vehicle and dehydration in a graded ethanol series. The transition from 100% ethanol to epoxy was mediated by two changes of propylene oxide. An epoxy medium of Spurr's formulation<sup>47</sup> was used for infiltration and embedding. The fragments were mounted in flat molds and hardened at  $70^{\circ}\text{C}$  for 24 hours before sectioning.

Both 1- $\mu\text{m}$ -thick light microscopic sections and ultra-thin TEM sections were cut for viewing. The light microscopy sections were stained with 50% modified Richardson's stain (1% methylene blue and 1% azure II in 1% sodium borate solution diluted 1:1 with 1 M dibasic sodium phosphate solution at pH 8.5), and the TEM sections were mounted on grids coated with polyvinyl butyral (Pioloform, Sigma-Aldrich Corp, St Louis, Missouri)<sup>48</sup> and stained with saturated aqueous uranyl acetate and Reynold's<sup>49</sup> lead citrate.

Evaluation was performed by light microscopy (Nikon E600, Melville, New York) and TEM (model PW6020, CM10 transmission electron microscope, Royal Philips Electronics NV, Eindhoven, The Netherlands).

#### CELL COUNTING FOR QUANTITATION OF TUNEL ASSAYS AND IMMUNOCYTOCHEMISTRY ASSAYS

Six corneas with each surgical procedure were used for counting at each time point; in some groups one of the corneas could not be counted owing to sectioning artifact. In each cornea, all of the cells in seven nonoverlapping, full-thickness columns extending from the anterior stromal surface to the posterior stromal surface were counted by a single observer.<sup>18</sup> The diameter of each column was that of a 400× microscope field. The columns in which counts were performed were selected at random from the central cornea of each specimen. As previously reported,<sup>18</sup> this procedure was used to allow quantitative comparisons to be made between the corneas with PRK and LASIK procedures, which demonstrate healing responses at different depths.

Data were analyzed with statistical software (StatView 4.5, Abacus Concepts, Berkeley, California). Variations were expressed as standard errors of the mean (SEM). Statistical comparisons between the groups were performed using analysis of variance (ANOVA) with the Bonferroni-Dunn adjustment for repeated measures. All statistical tests were conducted at an alpha level of 0.01 because of the large number of statistical tests performed.

#### RESULTS

##### TUNEL-POSITIVE (PRESUMED APOPTOTIC) CELLS IN THE STROMA AFTER PRK AND LASIK

TUNEL-positive cells were detected in the corneal stroma at 4, 24, and 72 hours and 1 week after all three laser surgical procedures: PRK for low (-4.5 D) myopia (Figure 5), PRK for high (-9.0 D) myopia (Figure 6), and LASIK for high myopia (-9.0 D) (Figure 7). In the PRK corneas, the TUNEL-positive cells were located in the superficial stroma. In the LASIK corneas, the TUNEL-positive cells were typically scattered anterior and posterior to the lamellar interface created by the microkeratome cut. Thus, the depth of the band of TUNEL-positive cells in the stroma relative to the epithelium in the LASIK corneas was related to the thickness of the flap (Figure 7). A few of the LASIK corneas showed occasional TUNEL-positive cells in the more anterior stroma of the flap, away from the interface (Figure 7E, F, H, I). Through the 72-hour observation point, all corneas examined in all surgical groups—PRK and LASIK—showed at least some TUNEL-positive cells, but by 1 week after surgery, some corneas in each group had TUNEL-positive cells in the stroma and some did not. In the PRK groups, especially the -9.0 diopter PRK group, occasional TUNEL-positive cells were noted at 1 and 3 months after surgery in the superficial stroma. (An example of this can be seen in Figure 6I). These cells were rare, and with

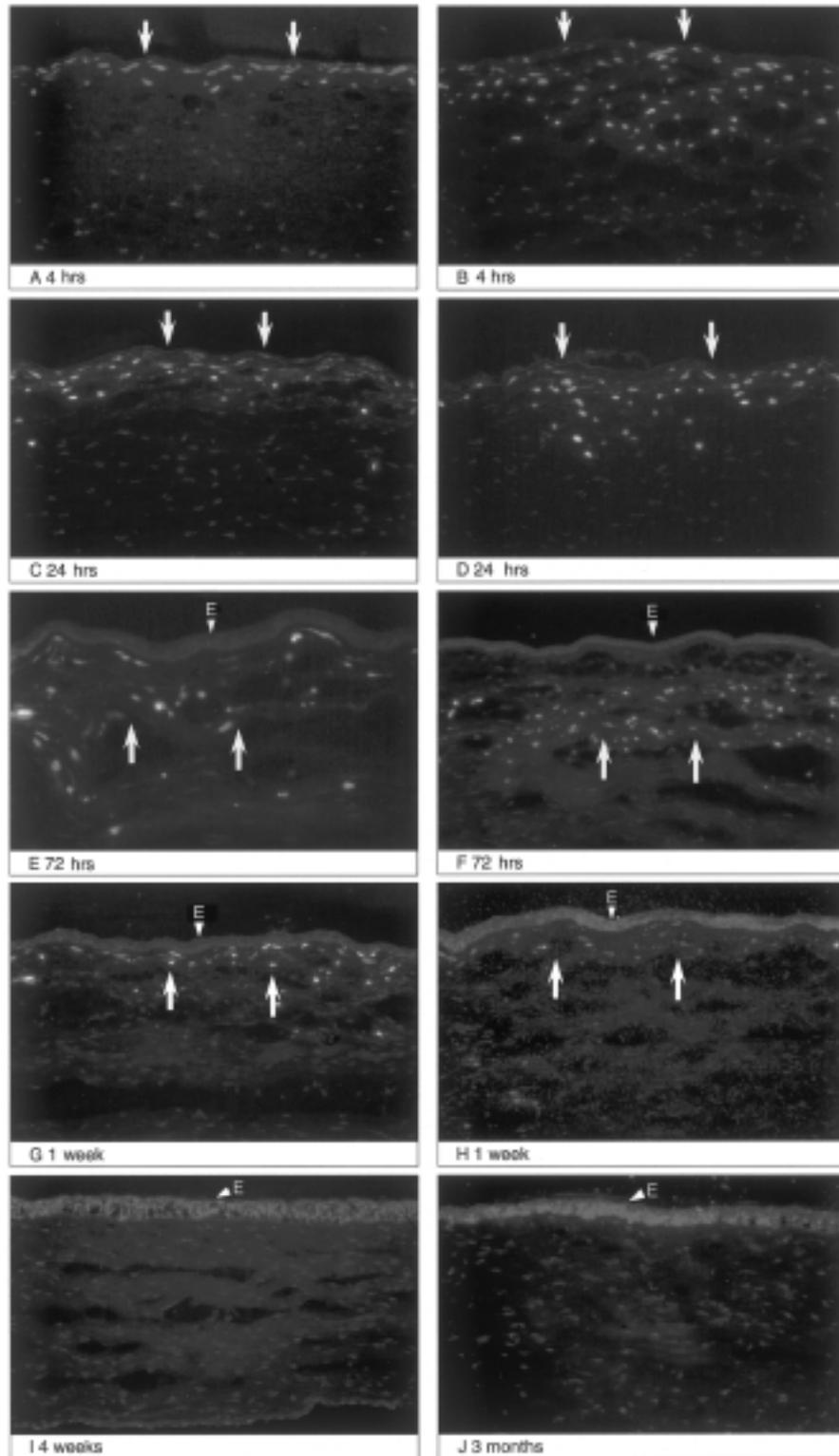
quantitation the means remained near zero.

Figure 8 shows the numbers of TUNEL-positive cells per 400× microscopic field column through the central corneal stroma at each time point from 4 hours to 3 months after PRK and LASIK. Significant numbers of TUNEL-positive stromal cells were counted in the central corneas of all three groups only at 4, 24, and 72 hours after surgery. At each of these three observation points, TUNEL-positive cells in the central cornea were most numerous in the high-myopia PRK group, fewer in the low-myopia PRK group, and fewest in the LASIK group. Although some TUNEL-positive cells were still seen at 1 week in all three groups, the numbers were small and differences between the groups were not significant. Only occasional TUNEL-positive cells were seen at 4 weeks or 3 months after surgery in each of the groups. Many of these later time point specimens in each of the three groups had no TUNEL-positive cells.

##### KI-67-POSITIVE (MITOTIC) CELLS IN THE STROMA AFTER PRK AND LASIK

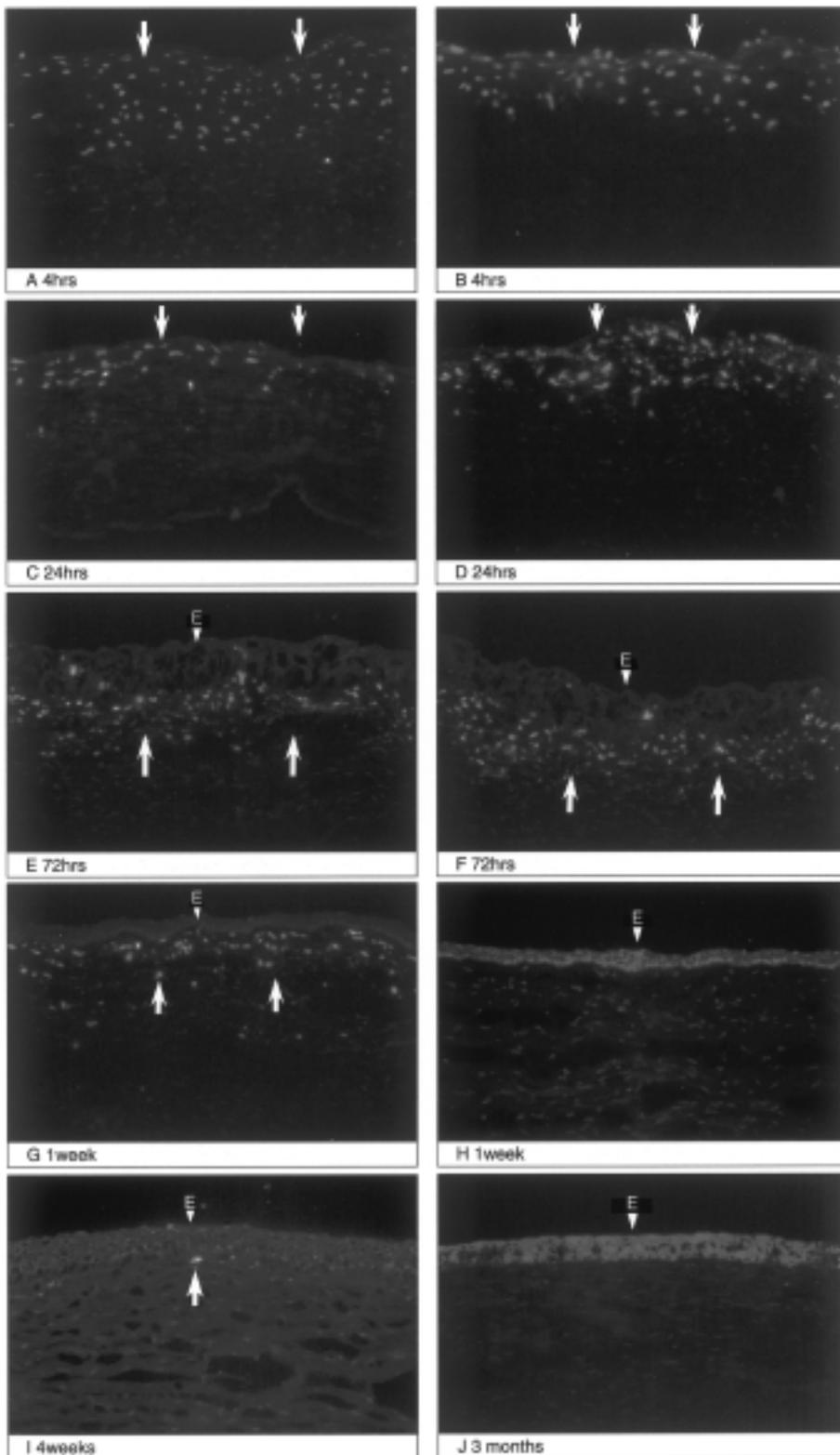
Figure 9 shows immunocytochemistry for the Ki-67 antigen associated with mitosis in the rabbit corneas that underwent PRK for low and high myopia or LASIK for high myopia. In the PRK corneas, the Ki-67-positive (mitotic) cells tended to localize in a band extending across the stroma posterior to the zone where the TUNEL-positive cells had been seen earlier. In the LASIK corneas, the Ki-67-positive (mitotic) cells were found largely in the areas anterior and posterior to the lamellar interface.

Figure 10 shows the numbers of Ki-67-positive (mitotic) cells per 400× microscopic field column through the central corneal stroma at each time point from 4 hours to 3 months after PRK and LASIK. In both the low- and high-myopia PRK corneas, significant numbers of Ki-67-positive cells were seen as early as 4 hours after surgery. In the low-myopia PRK corneas, mitosis continued to increase through 24 hours, maintained a steady rate through 72 hours, declined somewhat by 1 week, and returned to baseline (control) levels by 4 weeks after surgery. In the high-myopia PRK corneas, mitotic activity was sharply increased at 24 hours, about threefold that seen in the low-myopia PRK corneas; by 72 hours, however, the level of Ki-67-positive cells was similar to that seen in the low-myopia PRK group. By contrast, very few mitotic cells were seen in the LASIK corneas until 72 hours after surgery, when the numbers peaked at about the same levels seen in the low- and high-myopia PRK corneas at that observation point. At 4 weeks and 3 months after surgery, the numbers of mitotic cells were not significant in any of the three groups. Overall, the numbers of mitotic cells were highest in the high-myopia



**FIGURE 5**

Central cornea of rabbit eyes after PRK for low myopia (-4.5 D) assayed for apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). Typical results for each time point are shown: 4 hours (A, B), 24 hours (C, D), 72 hours (E, F), 1 week (G, H), 4 weeks (I), and 3 months (J) after surgery. TUNEL-positive stromal cells (arrows) are seen in all specimens from 4 hours to 72 hours. By 1 week some specimens show TUNEL-positive stromal cells (G, H) and some do not (not shown). Very few, if any, TUNEL-positive stromal cells are visible at 4 weeks or 3 months after surgery in a particular specimen (I, J). Soon after epithelial healing, epithelial cell layer (arrowhead labeled "E") is largely negative for apoptosis (E, F, G). TUNEL-positive epithelial cells begin to appear in epithelium 1 week after surgery (H) and increase in numbers thereafter (I, J), consistent with normal levels of apoptosis that occur in corneal epithelium during maturation (magnification x200) (also see Figure 6).



**FIGURE 6**

Central corneas of rabbit eyes after PRK for high myopia (-9.0 D) assayed for apoptotic cells by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). Typical results for each time point are shown: 4 hours (A, B), 24 hours (C, D), 72 hours (E, F), 1 week (G, H), 4 weeks (I), and 3 months (J) after surgery. As with the low-myopia PRK corneas shown in Figure 5, TUNEL-positive stromal cells (arrows) are seen in all high-myopia PRK specimens from 4 hours to 72 hours (A-F). By 1 week, some specimens show TUNEL-positive stromal cells (G), and some do not (H). Rare TUNEL-positive cells (arrow in I) were visible in anterior stroma immediately beneath epithelium (arrowhead labeled "E") at 4 weeks (I) and 3 months (J) after surgery (magnification x200).

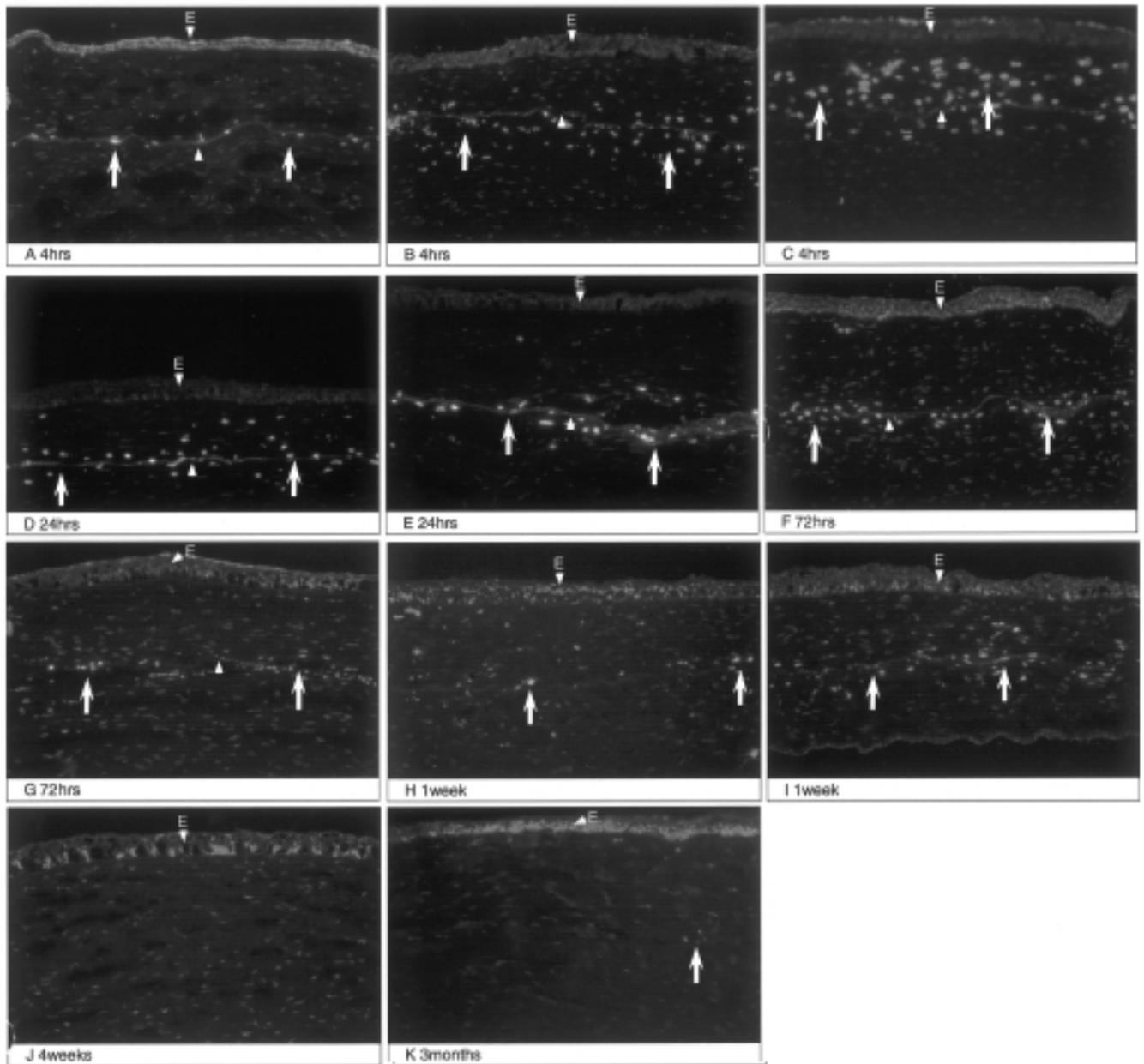


FIGURE 7

Central corneas from rabbit eyes after LASIK for high myopia (-9.0 D) assayed for apoptotic cells by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). Typical results for each time point are shown: 4 hours (A, B, C), 24 hours (D, E), 72 hours (F, G), 1 week (H, I), 4 weeks (J), and 3 months (K) after surgery. In sections where interface between anterior flap and posterior stroma is visible (upward pointing arrowhead; A-G), apoptotic keratocytes (arrows) are seen to be distributed above and below lamellar microkeratome cut. As with both the high- and low-myopia PRK corneas in Figures 5 and 6, TUNEL-positive stromal cells are visible in all LASIK specimens from 4 hours to 72 hours (A-G). Most LASIK specimens have fewer TUNEL-positive cells in stroma (A, D, E, F, G, H) than the PRK corneas at the same time point, although a few had more (B, C, I). At 1 week after surgery, some specimens had no TUNEL-positive cells (not shown). Very few, if any, TUNEL-positive cells are seen at 4 weeks and 3 months after surgery; however, a possibly TUNEL-positive cell (arrow) is visible in at least one specimen (K) at 3 months. Note that location of apoptotic cells in cornea, in terms of stromal depth relative to epithelium (arrowhead labeled "E"), is determined directly by thickness of LASIK flap (magnification x200).

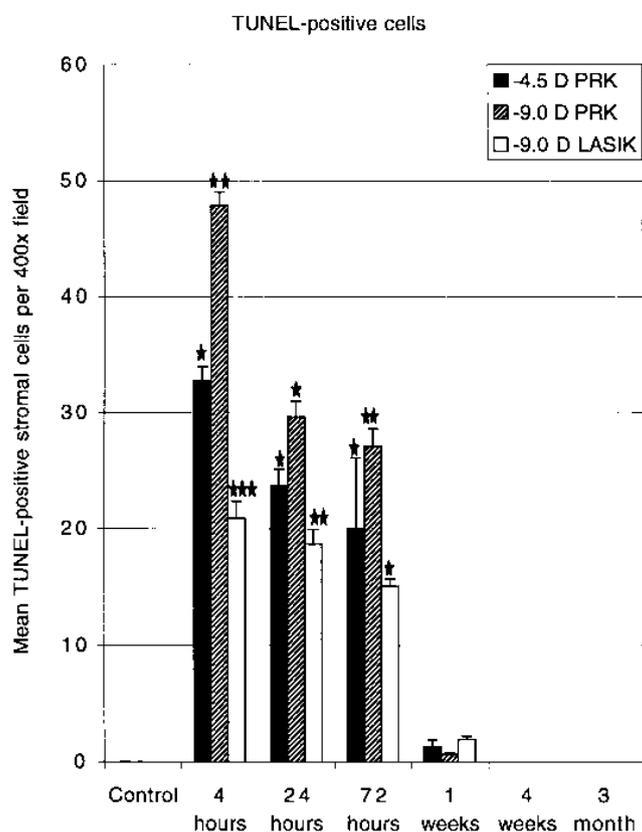


FIGURE 8

Numbers of TUNEL-positive cells in stroma after -4.5 diopters (D) PRK, -9.0 D PRK, and -9.0 D LASIK for myopia. Plotted values are numbers of cells counted in seven nonoverlapping, full-thickness columns (diameter of  $\times 400$  microscope field) extending from anterior stromal surface to posterior stromal surface. Five or six corneas were counted for each surgical procedure at each time point. Error bars represent standard error of mean. Bars marked with \*, \*\*, or \*\*\* indicate values significantly different from control ( $P < .01$ ). At a given time point, bars marked with different numbers of asterisks (\*, \*\*, and \*\*\*) indicate values significantly different from each other.

PRK corneas, lower in the low-myopia PRK corneas, and least in the LASIK cornea. Temporally, mitosis occurred earlier and persisted longer in the PRK corneas, compared with the LASIK corneas.

#### ALPHA SMOOTH-MUSCLE ACTIN-POSITIVE CELLS (MYOFIBROBLASTS) IN THE STROMA AFTER PRK AND LASIK

No alpha-SMA-positive stromal cells were seen in any of the corneas of the three groups at 4, 24, or 72 hours after surgery (not shown). Figure 11 shows immunocytochemistry for SMA associated with myofibroblasts in the central cornea of rabbit eyes that underwent PRK for high myopia. Significant numbers of alpha SMA-positive cells in the central cornea were seen only in this group. Some of the low-myopia PRK corneas had rare SMA-positive cells in the superficial stroma at 1 week, 4 weeks, and 3 months after surgery (not shown). In the high-myopia

PRK corneas, only a few SMA-positive cells were detected immediately beneath the epithelium 1 week after surgery (Figure 11, top); however, SMA staining of stromal cells was considerably increased at 4 weeks after surgery (Figure 11, middle) and persisted, albeit at a somewhat diminished level, through the end of the 3-month observation period (Figure 11, bottom). Few, if any, SMA-positive cells were detected in the central corneas of any of the LASIK eyes at any time point. A few SMA-positive cells were seen in the periphery near the edge of the flap in LASIK corneas at 1 week and 4 weeks after surgery (not shown). No such cells were seen in the 3-month LASIK corneas.

Figure 12 shows the numbers of SMA-positive cells in the stroma per  $400\times$  microscopic field column through the central corneal stroma at each time point when staining was detected (1 week to 3 months) after PRK and LASIK. No staining was detected in the stromal cells of control corneas. A few SMA-positive cells were detected in the low-myopia PRK corneas at 1 and 4 weeks and 3 months, but the numbers of these cells did not reach statistical significance, compared with controls, at any of these observation points. By contrast, the high-myopia PRK corneas showed significant staining for myofibroblasts at 4 weeks and only slightly less staining at 3 months. In the LASIK group, the mean numbers of SMA-positive cells in the central cornea were not significantly different from control values at any time point.

#### TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy confirmed that the majority of dying stromal cells observed at 4 hours after PRK or LASIK showed the classic signs—cell shrinkage, chromatin condensation, and formation of apoptotic bodies (Figure 13A and B)—consistent with apoptosis, as noted in previous studies.<sup>13,14</sup> At 4 hours after surgery, all of the dying cells appeared to be keratocytes.

At 24 hours after surgery, some cells still showed morphologic changes consistent with apoptosis, but most of the dying cells had an appearance more consistent with necrosis (Figure 13C and D). By this time, large numbers of inflammatory cells had invaded the stroma, including many polymorphonuclear leukocytes and somewhat fewer monocytes/macrophages (Figure 13C, D, E, F). Many of these inflammatory cells were noted near other necrotic-appearing cells (Fig 13C and D). In some cases, the inflammatory cells appeared to be engulfing the dead cells. The inflammatory cells were most numerous in the high-myopia PRK corneas but were also seen in the low-myopia PRK corneas and the LASIK corneas. It was not possible to perform reliable quantitation of the inflammatory cells with TEM.

At 24 and 72 hours and 1 week after surgery, it was

often difficult to determine the identity of cells undergoing apoptosis or necrosis. Some appeared to be keratocytes or myofibroblasts derived from keratocytes, while others appeared to be polymorphonuclear leukocytes or monocytes/macrophages. In the PRK corneas, the necrotic and apoptotic cells were noted in the superficial stroma. In the LASIK corneas, most of the necrotic or apoptotic cells were located near the lamellar interface at a stromal depth that varied with flap thickness.

At 4 weeks and 3 months after surgery, there continued to be rare inflammatory cells. Some of the cells could be identified as probably polymorphonuclear cells or monocytes. In some cases, these cells appeared to be engulfing other cells that appeared to have died (Figure 13G). Some of the dead cells appeared to have undergone apoptosis (Figure 13G). In others, the mechanism of cell death was not clear and could have been apoptosis or necrosis. Some of the dead cells appeared to be myofibroblasts or keratocytes.

The high-myopia PRK corneas showed numerous living cells with prominent intracellular filaments and notable rough endoplasmic reticulum in the anterior stroma beneath the epithelium (Figure 13H, I, J). These cells were found in the same location where the SMA-positive cells had been detected with immunocytochemistry and were, therefore, believed to be myofibroblasts. Very few such cells were detected in the low-myopia PRK corneas, and none were noted in the LASIK corneas.

## **DISCUSSION**

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This study represents the largest and most detailed investigation of the stromal cellular response to corneal surgery in any species. Previous studies have examined limited time intervals (4 hours or less after surgery) or have used methods such as *in vivo* confocal microscopy that are not able to detect cell death, quantitate cell proliferation, or determine cell type. Previous studies have also generally included too few animals to allow for statistical analyses. Additionally, none have looked at multiple aspects of the cellular response in the same corneal specimens over a long interval of time after surgery.

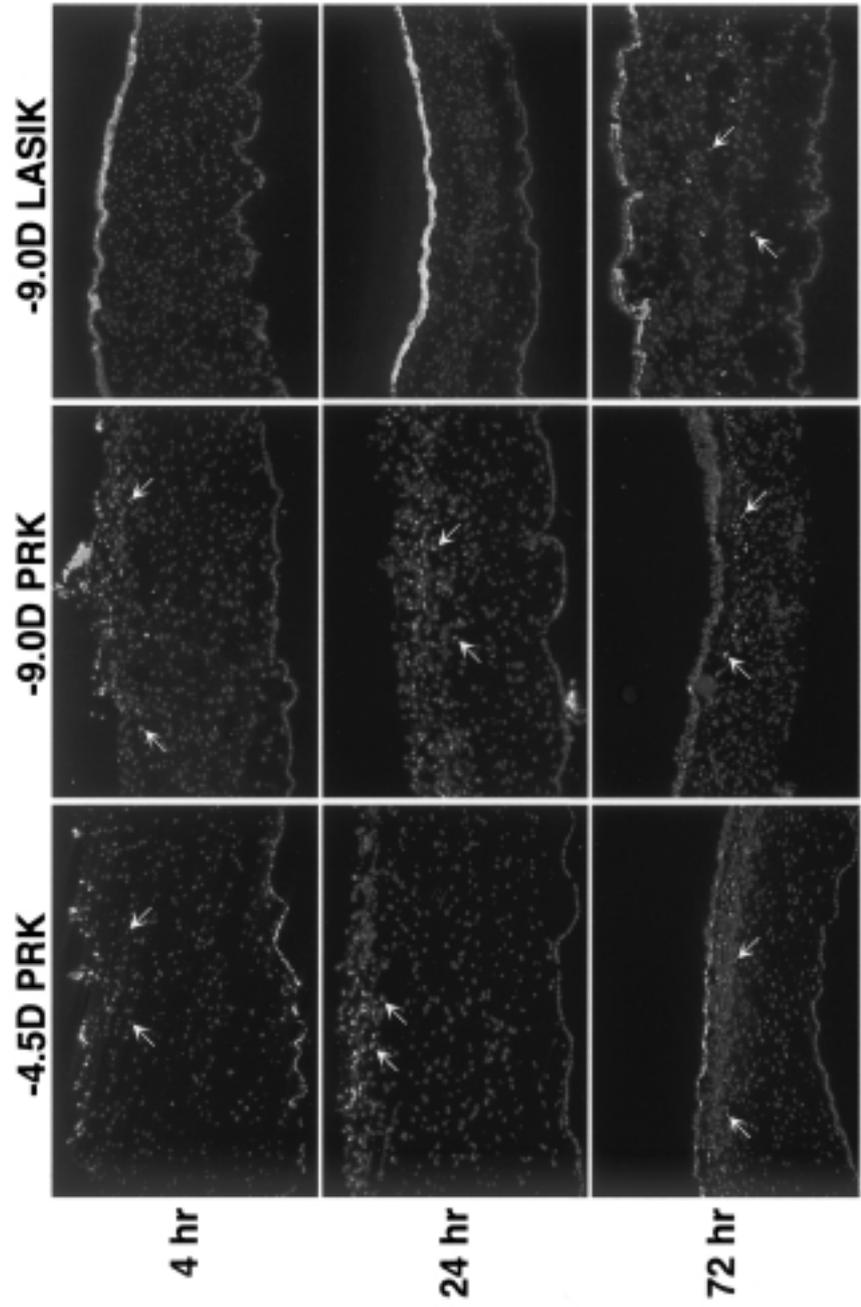
The goals of the present study were to determine whether there are quantitative differences in the cellular responses in the corneal stroma after PRK for low and high myopia and whether there are both qualitative and quantitative differences in the wound-healing response after PRK for high myopia and LASIK for high myopia. The results of this investigation provide strong evidence to support both hypotheses.

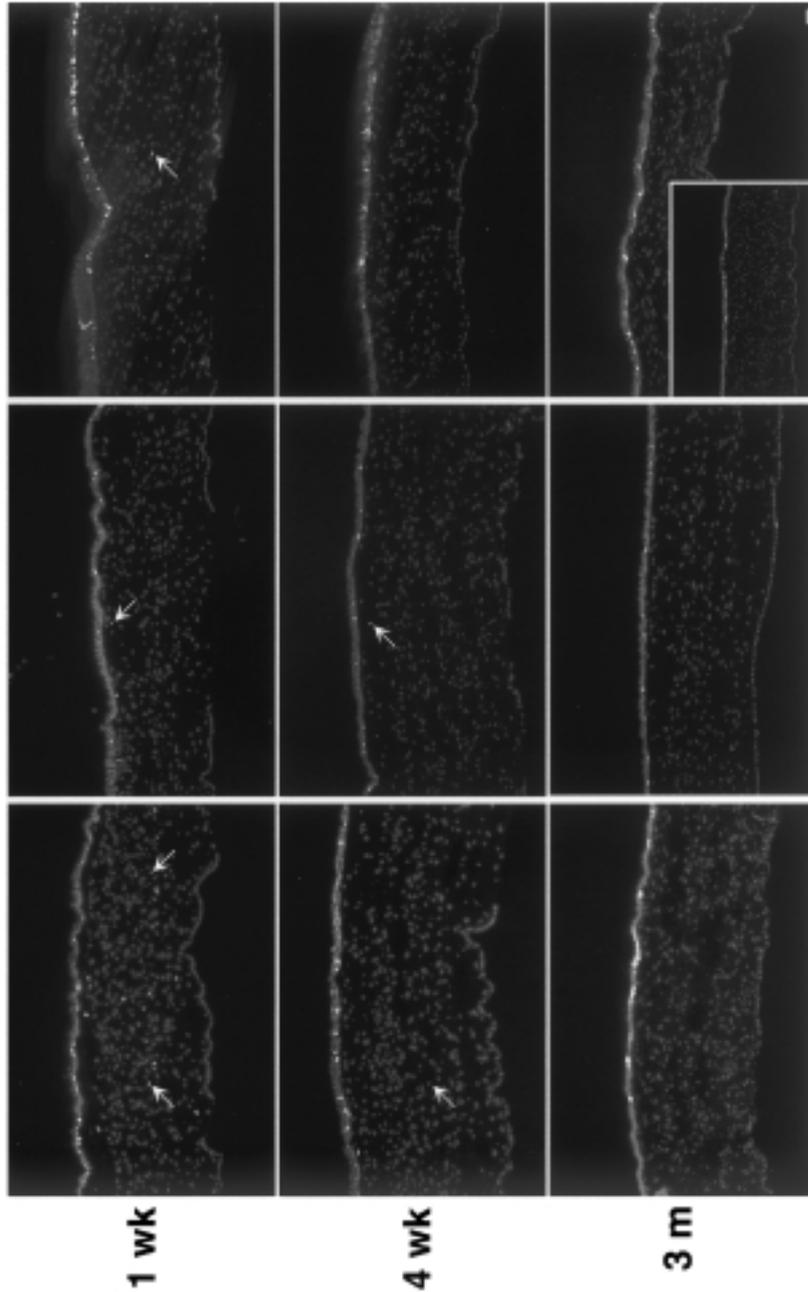
First, quantitative analysis showed statistically significant differences between low- and high-myopia PRK corneas in terms of keratocyte apoptosis (TUNEL-posi-

tive cells), keratocyte proliferation (Ki-67-positive cells), and myofibroblast cell density (SMA-positive cells). Each of these cellular responses is important in corneal wound healing, and each response was significantly greater in the high-myopia PRK corneas than in the low-myopia PRK corneas at every time point examined, from 4 hours to 1 week after surgery. These quantitative differences in the cellular responses likely underlie the clinical differences in outcome and complications such as regression and stromal haze that are seen after mechanical epithelial scrape, PRK for low myopia, and PRK for high myopia in the clinical setting.

Visually significant regression is most commonly noted after PRK for high myopia (>5 to 6 D in humans), consistent with our findings of higher levels of keratocyte apoptosis, stromal cell necrosis, keratocyte proliferation, and myofibroblast transformation. Visually significant haze is also more typically found after PRK for high myopia. The time course and localization of SMA-positive staining in the high-myopia PRK rabbit corneas is consistent with the timing and localization of dense haze that are noted in some human corneas after large PRK corrections. This correlation supports the hypothesis of Jester and coworkers<sup>28-30</sup> that myofibroblasts underlie the development of stromal haze after PRK.

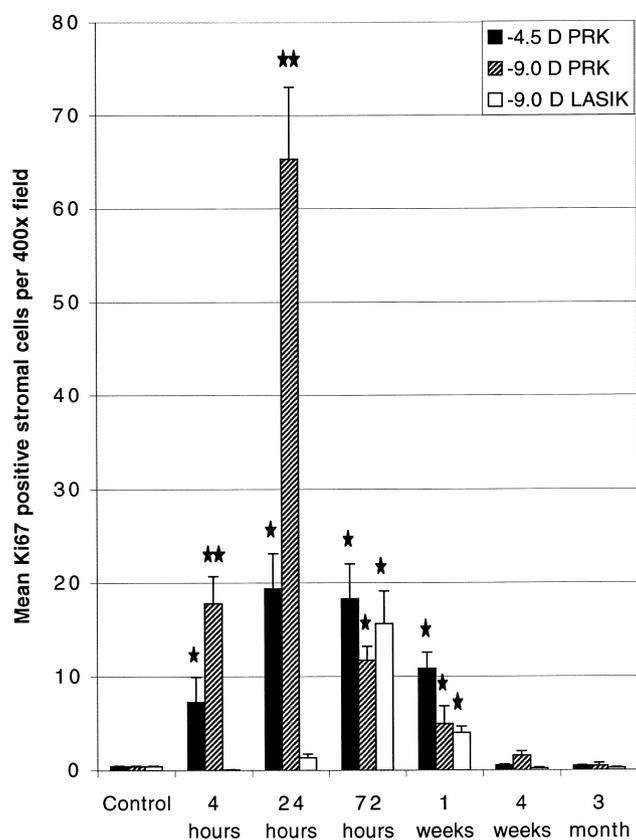
Second, there were both qualitative and quantitative differences between the high-myopia PRK corneas and the high-myopia LASIK corneas in terms of the cellular responses in the stroma. Significantly greater numbers of apoptotic keratocytes, proliferating keratocytes, and myofibroblasts were seen in the -9.0 D PRK corneas, compared with the -9.0 D LASIK corneas. Furthermore, most of the TUNEL-positive cells in the LASIK corneas were located near the lamellar interface between the flap and the underlying bed, and therefore the thickness of the LASIK flap tended to determine the depth at which apoptosis and necrosis occurred. Thus, with a thin flap, the keratocyte apoptosis response was more superficially located in the stroma (Figure 7C), similar to the location of the response noted in the PRK corneas. Also in the LASIK corneas, significant keratocyte proliferation was not detected until 72 hours after surgery. By contrast, high levels of mitosis were detected at 4 hours after surgery in both the low- and high-myopia PRK corneas. Figure 10 shows that the total number of mitotic cells after surgery would likely be much greater for eyes undergoing PRK for high myopia, compared with eyes undergoing LASIK for high myopia. Mitosis is thought to give rise to the majority of wound-healing stromal cells, such as corneal fibroblasts and myofibroblasts, which in turn participate in stromal remodeling through the production and reabsorption of collagen, production of glycosaminoglycans, and other functions.





**FIGURE 9**

Immunocytochemistry to detect mitosis-associated antigen Ki-67 in corneal stroma. Cells positive for Ki-67 are stained green. Cells stained with propidium iodide counterstain appear red. In unoperated control corneas (inset), stromal cells undergoing mitosis are rare. At 4 hours after surgery, some stromal cell mitosis (arrows) is detectable in both the low- and high-myopia PRK groups, but mitotic cells are not seen in the LASIK corneas. At 24 hours, both PRK groups show increased mitosis (arrows), with larger numbers of mitotic cells visible in high-myopia PRK corneas; in these corneas, mitotic cells are located in a band beneath area where keratocyte apoptosis was noted earlier, deeper in central stroma and more superficially in peripheral stroma (not shown). Only a few mitotic stromal cells are seen at 24 hours in LASIK corneas (none in the image presented). At 72 hours and 1 week, mitotic stromal cells (arrows) are seen in both the low- and high-myopia PRK corneas. The 72-hour and 1-week LASIK corneas have mitotic cells, but they are at low levels relative to low or high PRK. After 1 week, stromal mitosis declines in all groups until, by 4 weeks, both the low- and high-myopia PRK corneas and the LASIK corneas have very few mitotic stromal cells. As would be expected, many epithelial cells stain for mitosis in the three groups at time points where epithelium is intact (magnification x200).



**FIGURE 10**

Numbers of Ki-67-positive cells in stroma after -4.5 diopter (D) PRK, -9.0 D PRK, and -9.0 D LASIK for myopia. Plotted values are numbers of cells counted in seven nonoverlapping, full-thickness columns (diameter of 400x microscope field) extending from anterior stromal surface to posterior stromal surface. Five or six corneas were counted for each surgical procedure at each time point. Error bars represent standard error of the mean. Bars marked with \* or \*\* indicate values significantly different from control ( $P < .01$ ). At a given time point, bars marked with different numbers of asterisks (\* and \*\*) indicate values significantly different from each other.

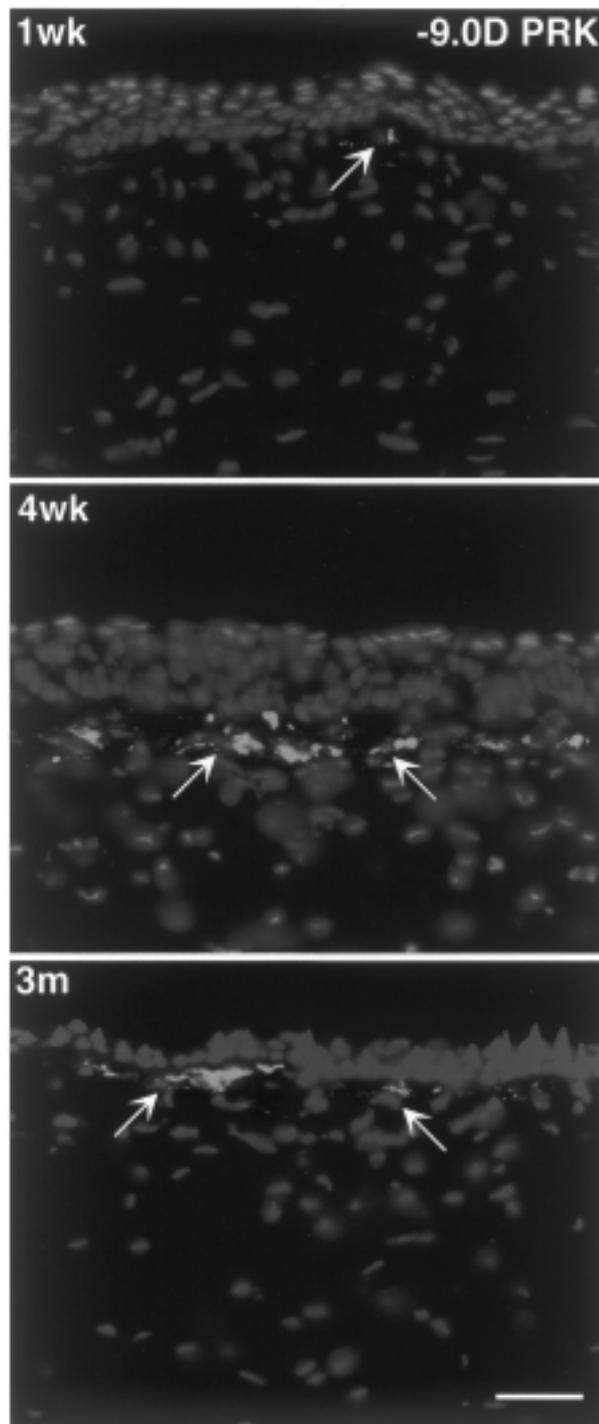
The underlying factors that delay keratocyte mitosis after LASIK, compared with PRK, are uncertain. Previous studies suggested that cytokines from the injured epithelium, such as PDGF, may have a role in triggering mitosis and migration of keratocytes after corneal injury.<sup>23-25</sup> In that there is far less epithelial injury in LASIK than in PRK, lower concentrations of these epithelial mitogens may be found in the stroma following LASIK, compared with PRK.

SMA-positive myofibroblasts were detected at significant levels in the central cornea only in the -9.0 D PRK group. Some myofibroblasts were detected in the superficial central stroma of the -4.5 D PRK corneas and near the lamellar interface in the periphery of -9.0 D LASIK corneas, but the numbers did not reach statistical significance at any time. Myofibroblast density in the -9.0 D PRK corneas determined by SMA staining peaked at 4

weeks after surgery, at which time electron microscopy showed cells with prominent rough endoplasmic reticulum and intracellular filaments consistent with myofibroblast morphology in the same locations in the anterior stroma where the SMA-positive cells had been identified by immunocytochemistry. The delayed appearance of these cells, proximity of these cells to the overlying epithelium, and their absence from corneas with the same level (-9.0 D) of LASIK correction suggests that the epithelium may have an important role in generating and maintaining myofibroblasts.

The numbers of myofibroblasts declined in the high-myopia PRK corneas between 4 weeks and 3 months after surgery. At least two possibilities could account for this phenomenon. One possibility is that some of the myofibroblasts transdifferentiate into keratocytes that do not express alpha-SMA. A second possibility is that the myofibroblasts slowly undergo apoptosis. Our detection of apoptotic cells in the anterior stroma of corneas with numerous myofibroblasts by means of both the TUNEL assay and electron microscopy supports the latter mechanism for disappearance of the myofibroblasts over time. However, our findings cannot exclude the possibility that some myofibroblasts transdifferentiate to keratocytes. It is possible that cytokines (such as transforming growth factor beta) released from the overlying epithelium act to maintain the myofibroblast phenotype,<sup>28,30,31</sup> and that the release of these factors diminishes over time as the cornea returns to a normal state. This idea is supported by the persistent localization of myofibroblasts only beneath the epithelium. Thus, we hypothesize that myofibroblasts are derived from dividing keratocytes that invade the stroma after injury, but that persistence of myofibroblasts over time requires cytokine input from the epithelium. Further support for this theory is provided by the disappearance of the transient SMA-positive cells found in the periphery near the flap-stroma interface in LASIK corneas. When present, these cells are located a considerable distance from the peripheral epithelium, and so are likely to be less affected by epithelial-derived factors.

In this study, TEM provided important new insights into the corneal wound-healing response after PRK and LASIK. At 4 hours after surgery, we saw that the majority of dying cells in the corneal stroma after PRK or LASIK were undergoing apoptosis, with chromatin condensation and formation of membrane-bound apoptotic bodies (Figure 13A and B). By contrast, at 24 hours (Fig 13C and D), 72 hours, and 1 week after PRK and LASIK, many of the dying cells had a morphology more consistent with that of necrosis, a disorganized form of cell death in which the cells appear to disintegrate, releasing their cellular contents into the surrounding tissues. Some of the cells detected at the later time points were undergoing



**FIGURE 11**

Immunocytochemistry to detect alpha smooth-muscle actin (SMA) myofibroblast-associated antigen in the corneal stroma. SMA-positive cells are stained green. Cells stained with propidium iodide counterstain appear red. High-myopia PRK corneas first show rare SMA-positive cells 1 week after surgery (top); greatest density of stained cells is seen at 4 weeks (middle), but numbers are still significant at 3 months after surgery (bottom). In these corneas, SMA-stained cells are located in anterior stroma immediately beneath epithelium. Few, if any, SMA-positive cells are detected in central cornea after PRK for low myopia or LASIK at any time after surgery, although LASIK corneas have some SMA-positive cells in peripheral stroma at site of perforation of epithelium by microkeratome blade at 1 week and 4 weeks after surgery (not shown) (magnification x400).

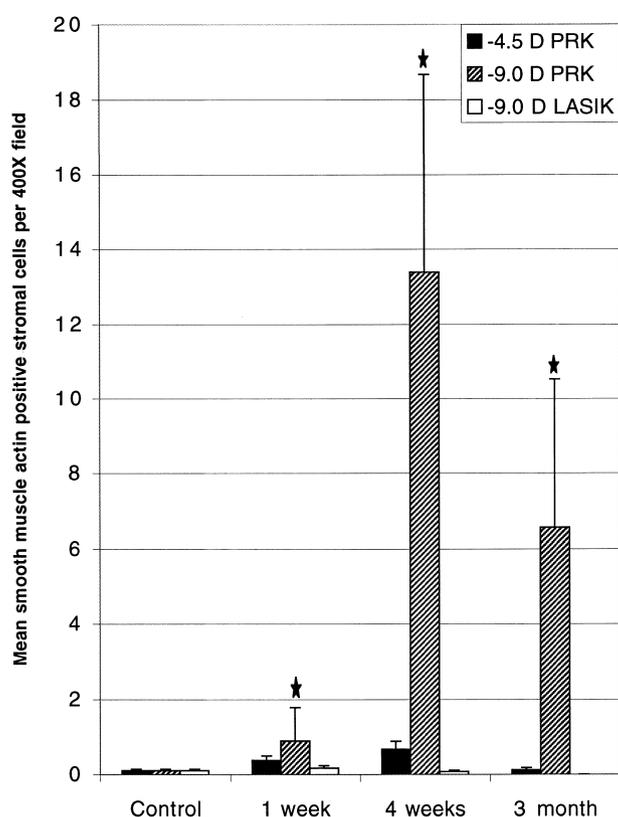


FIGURE 12

Numbers of alpha smooth muscle actin-positive cells in the central corneal stroma after -4.5 diopter (D) PRK, -9.0 D PRK, and -9.0 D LASIK for myopia. Plotted values are numbers of cells counted in seven nonoverlapping, full-thickness columns (diameter of 400x microscope field) extending from anterior stromal surface to posterior stromal surface. Five or six corneas were counted for each surgical procedure at each time point. Error bars represent standard error of mean. Bars marked with \* indicate values significantly different from control ( $P < .01$ ).

apoptosis, consistent with the findings of Gao and coworkers,<sup>21</sup> but apoptotic cells were not as common as cells undergoing necrosis. The levels of cellular necrosis were highest at 24 hours after surgery and decreased thereafter in all three groups. More necrotic cells were seen in the high-myopia PRK corneas than in the low-myopia PRK or LASIK corneas. Cells dying by either apoptosis or necrosis included keratocytes, polymorphonuclear leukocytes, and monocytes. In many cases, however, necrotic cells could not be identified by type because of the disrupted cellular morphology.

Few dying stromal cells were detected by TEM in any of the groups at 4 weeks after surgery. Of those that were seen, almost all were found in the anterior stroma of the -9.0 D PRK corneas (Figure 13G), and many were undergoing apoptosis, as determined by both electron microscopy and the TUNEL assay.

Comparison of the TUNEL assay and electron microscopy results at 24 hours, 72 hours, and 1 week

suggests that some cells that appeared to be necrotic by TEM may have also showed TUNEL-positive staining, since there were many TUNEL-positive cells, but only a relatively few cells with morphologic changes characteristic of apoptosis seen by TEM at these time points. We cannot be certain, however, since the TUNEL assay sampled many more cells than could be examined by electron microscopy. Also, the DNA fragmentation that is identified by the TUNEL assay does occur during necrosis, although usually in a more random manner that tends to make these cells invisible to the TUNEL assay. Previous studies have noted that in some situations, necrotic cells are falsely detected by the TUNEL assay.<sup>50</sup> This highlights the importance of using methods such as TEM to confirm the type of cell death detected by the TUNEL assay in a particular experimental system.<sup>50</sup>

It was difficult to identify the types of cells that underwent apoptosis or necrosis during the interval from 24 hours to 1 week after PRK or LASIK in this study. Previous studies have shown that in the early wound-healing phase (up to 4 hours after PRK or LASIK), the apoptotic cells are virtually entirely keratocytes.<sup>13,18,21</sup> However, it seems unlikely that the same keratocytes that begin apoptosis prior to 4 hours after injury are still present in the same location in the cornea a week later, since complete disappearance of the associated chromatin and the organelle-containing apoptotic bodies has been observed by 8 to 48 hours after surgery using electron microscopy.<sup>13</sup> Thus, the cells that are seen undergoing apoptosis at later time points are probably different cells; some could be keratocytes, corneal fibroblasts, or myofibroblasts<sup>26-28</sup> that migrate into the area from the peripheral and posterior cornea after injury. This migration could account for the disappearance of the SMA-positive myofibroblasts from the peripheral cornea between 1 week and 4 weeks after surgery in the LASIK group. This study found that some of these cells are polymorphonuclear leukocytes and monocytes/macrophages that are attracted to the wound-healing area.<sup>43,51</sup>

Transmission electron microscopy also showed large numbers of living inflammatory cells appearing by 24 hours after PRK and LASIK. Some of these inflammatory cells are still present, and in increased numbers compared to control corneas, at 4 weeks and 3 months after low- or high-correction PRK. To a lesser extent, some of these inflammatory cells are still present at 4 weeks and at 3 months after LASIK. These inflammatory cells included polymorphonuclear leukocytes and monocytes/macrophages. Recent studies suggest that chemokines such as monocyte chemoattractant and activating factor (MCAF) and granulocyte colony-stimulating factor (G-CSF) produced by the keratocytes during the wound-healing response attract these cells.<sup>51</sup> In the present study,

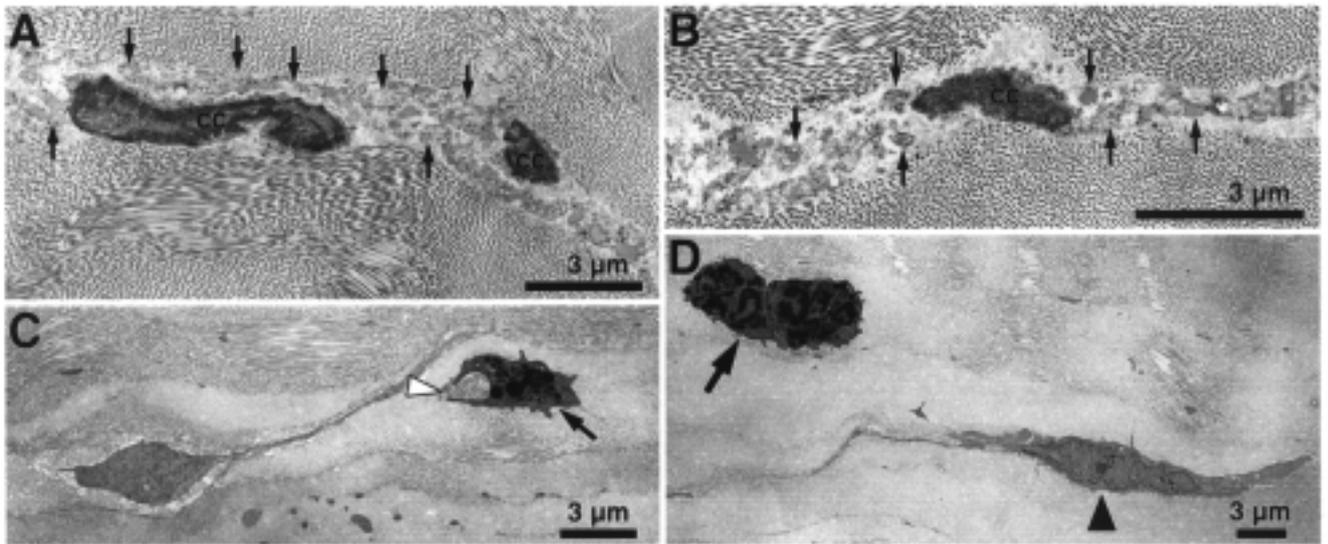


FIGURE 13A THROUGH D

Transmission electron microscopy (TEM) 4 hours after PRK or LASIK. At 4 hours after PRK for high or low PRK (A, magnification  $\times 1,500$ ) or LASIK (B, magnification  $\times 800$ ), dying cells show morphologic features consistent with apoptosis, including chromatin condensation (CC) and the formation of apoptotic bodies (arrows). At 24 hours after PRK for high myopia, TEM found cells that had cellular morphology more consistent with necrosis than apoptosis. C, Polymorphonuclear leukocyte (arrow) engulfing cellular debris (arrowhead) adjacent to a necrotic keratocyte in anterior stroma (magnification  $\times 2,950$ ). D, Polymorphonuclear leukocyte (arrow) adjacent to a necrotic-appearing keratocyte (arrowhead) in anterior stroma (magnification  $\times 3,900$ ). Findings were similar, but with fewer apoptotic or necrotic cells and inflammatory cells in the low PRK or LASIK group.

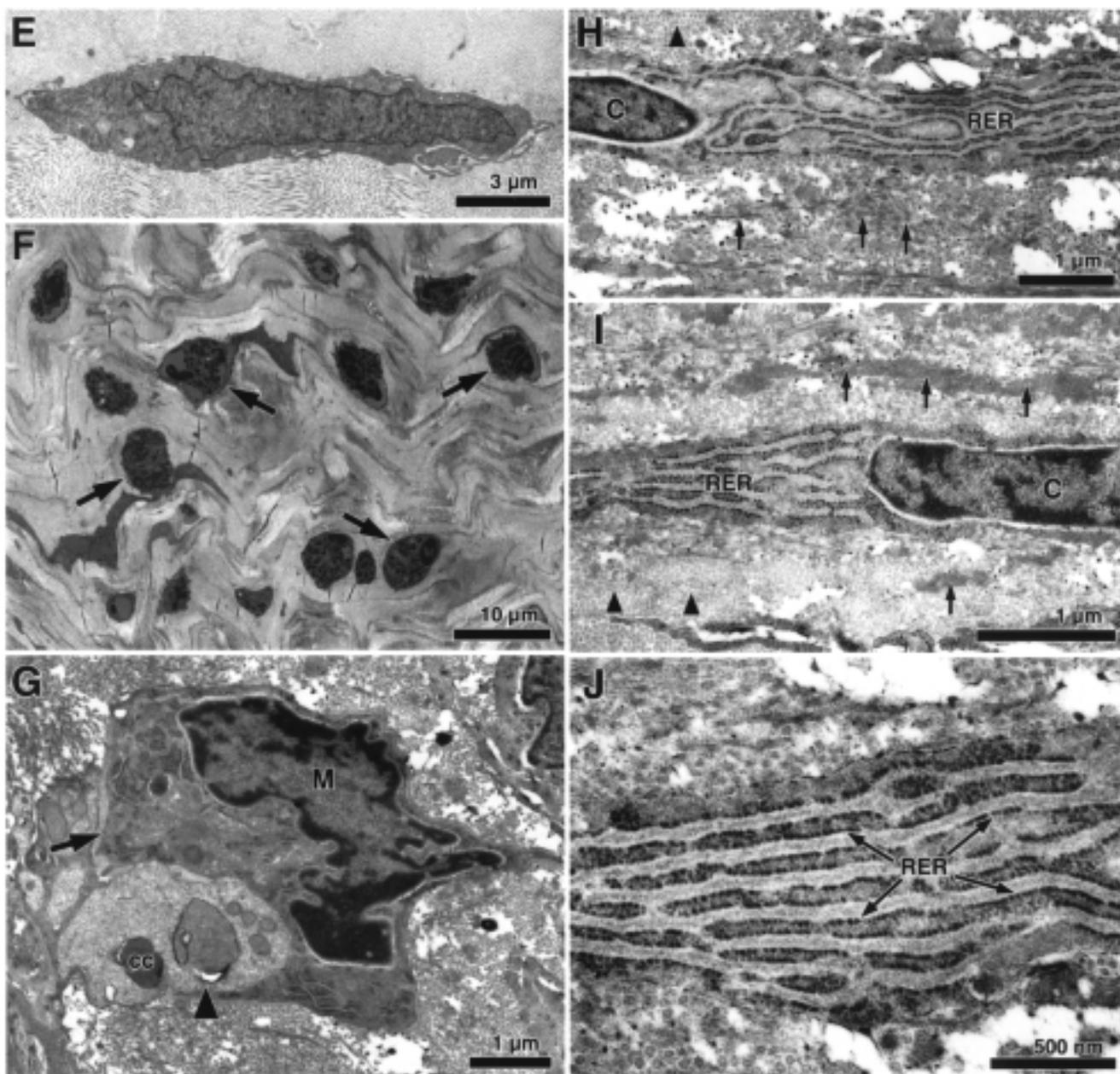
inflammatory cells were often seen in proximity to cells that appeared to be undergoing apoptosis or necrosis; in some cases the inflammatory cells were engulfing the dead cells (Figure 13C, D, G). The timing of the appearance of the inflammatory cells and detection of apoptotic and necrotic cells in the stroma suggests that the inflammatory cells themselves may trigger necrosis of some stromal cells at time points later than 4 hours. Further work is needed to elucidate these complex cellular interactions.

Within each surgical group, the magnitude of keratocyte apoptosis at the 4-hour time point appeared to correlate with the overall level of keratocyte proliferation and myofibroblast density in the stroma. Thus, the -9.0 D PRK corneas had the most TUNEL-positive cells in the stroma at 4 hours and the most Ki-67-positive, proliferating keratocytes and SMA-positive myofibroblasts at the later time points. In contrast, the LASIK corneas had the fewest TUNEL-positive cells at 4 hours after surgery and the fewest Ki-67-positive, proliferating cells and myofibroblasts at later time points. These correlations support the idea that the early keratocyte apoptosis response occurring after epithelial injury is a determinant of the overall stromal wound-healing response in the cornea.<sup>14,18,52</sup>

Considerable variation was seen among the eyes in terms of stromal cell apoptosis, keratocyte proliferation, and numbers of myofibroblasts at a given time point in a given surgical group. Figure 7 shows the variability in keratocyte apoptosis in three eyes at 4 hours after LASIK. These differences are similar to those noted clinically between the eyes of different patients, as well as between

the two eyes of a single patient, after PRK or LASIK.<sup>53-55</sup> This variability has several potential causes. One is biological variability in the healing response between individuals. Another is the unavoidable variation in surgical procedure. For example, there may be differences in the amount of cytokine-containing epithelial debris tracked into the interface by the microkeratome blade or efficiency of interface irrigation from eye to eye in LASIK.<sup>13-18,21</sup> Whatever the specific causes, it is likely that this variability in the wound-healing response ultimately leads to a significant proportion of the variability in outcome between patients undergoing refractive surgical procedures.

Localization of the wound-healing response in the cornea after PRK and LASIK is also likely to be an important determinant of clinical differences between PRK and LASIK for high myopia. Patients who undergo PRK for high myopia are more likely to experience regression and to develop corneal haze after surgery than those who undergo LASIK.<sup>53-55</sup> Regression after both PRK and LASIK is thought to result from a combination of epithelial hyperplasia and/or stromal remodeling.<sup>43-45,52,56</sup> Both of these processes are thought to be modulated via cytokine-mediated communication between the wound-healing cells of the stroma and the overlying epithelium.<sup>56</sup> It is likely that such interactions would be more pronounced in corneas that have undergone PRK, where the interacting cells are in immediate proximity to one another (Figure 5 and Figure 6), compared with LASIK corneas, in which the epithelial cells and stromal wound-healing cells are typically separated by a zone of normal stroma (Figure 7).



**FIGURE 13E THROUGH J**

Transmission electron microscopy. E, A monocyte in stroma at 24 hours after high PRK (magnification x1,500). F, Lower magnification of anterior stroma shows many polymorphonuclear leukocytes in anterior stroma at 24 hours after high PRK in one specimen (magnification x900). G, At 4 weeks after high PRK a monocyte/macrophage (M) is seen engulfing remnants of another cell (arrow) with condensed chromatin (CC) suggestive of apoptosis. Arrow indicates cellular membrane for monocyte/macrophage that delineates this cell from dead cell. Specific cell type of dead cell is uncertain (magnification x4,500). At 4 weeks after high PRK (H, magnification x4,200; I, magnification x5,000; J, magnification x8,000), numerous cells with large amounts of rough endoplasmic reticulum (RER), indicating a metabolically active cell with high-protein synthesis, were noted in anterior stroma. These are likely myofibroblasts. In H and I, arrowheads indicate regular collagen bundles and arrows indicate disorganized matrix material that likely includes collagen, glycosaminoglycans, and other substances. These areas of disorganized matrix material would likely be associated with corneal opacity, as would the myofibroblast cells themselves. Normal chromatin pattern of nucleus (C) is seen in H and I.

The appearance of a strong healing response with scarring at the edge of the flap in LASIK cases is also consistent with this theory. A stronger healing response with scarring is also typical of LASIK flap complications resulting when the microkeratome exits in the central cornea.<sup>57</sup> We hypothesize that this localization of the response is a major reason for the difference between PRK and LASIK in the treatment of high myopia.

The difference in myofibroblast cell density in PRK for high myopia compared with PRK for low myopia also has clinical relevance. Significant stromal haze is much more likely to occur after PRK for high myopia.<sup>58,59</sup> The author has never seen clinically significant haze in a cornea that underwent PRK for less than 5 D of myopia in more than 2,500 eyes (unpublished data, 2001). Jester and coworkers<sup>28,30</sup> suggested that loss of transparency is attributable to changes in crystallin expression in myofibroblasts that populate the stroma after PRK. Our results demonstrate a much higher density of myofibroblasts in the high-myopia PRK corneas compared with the low-myopia PRK corneas (Figure 11 and Figure 12), which is consistent with this hypothesis. The timing of the appearance of the myofibroblasts after PRK is also consistent with the clinical observation that haze may begin as early as a few weeks after PRK, but typically intensifies between 1 and 3 months after the procedure.<sup>58,59</sup> Finally, localization of myofibroblasts also suggests a role in PRK-associated haze. At the slit lamp, haze is most commonly noted immediately beneath the epithelium in humans and rabbits.<sup>58-60</sup>

## SUMMARY

The observations made in this study regarding the cellular responses associated with PRK and LASIK support our hypothesis that the early keratocyte apoptosis response is a good target for controlling later events in the wound-healing cascade.<sup>52</sup> Although electron microscopy revealed later cellular necrosis, apoptosis was the earliest stromal event noted after corneal injury or surgery. It remains our working hypothesis that blocking early keratocyte apoptosis could lead to diminished cellular apoptosis and necrosis, keratocyte proliferation, and myofibroblast generation later in the wound-healing process. To date, efforts to pharmacologically control early keratocyte apoptosis have not been successful<sup>50</sup>; however, studies are ongoing to identify the magic bullet that can regulate this phenomenon and its sequelae. Corneal surgeons have long sought the capacity to pharmacologically regulate the wound-healing response to clinical advantage, and early intervention in the wound-healing process still seems to be the most promising strategy. Once the early phase of keratocyte apoptosis has been initiated, the subsequent events that include late stromal cell apoptosis and necrosis, kera-

toocyte proliferation, and the generation of myofibroblasts in the stroma are highly variable and, therefore, likely to be difficult to control.

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*Analysis of the Keratocyte Apoptosis, Keratocyte Proliferation, and Myofibroblast Transformation*

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