HISTOLOGICAL CHANGES AND WOUND HEALING RESPONSE FOLLOWING NONCONTACT HOLMIUM: YAG LASER THERMAL KERATOPLASTY

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ABSTRACT

Purpose: To evaluate acute histological changes and the induced wound healing response in corneal tissue following noncontact holmium:YAG laser thermal keratoplasty (LTK).

Methods: LTK using 10 pulses and a range of radiant energies was performed on 3 human corneas one day prior ro their removal at penetrating keratoplasty. Rabbit corneas were treated with 10-pulse and 5-pulse LTK and followed for up to 3 months. Tissues were studies with light and transmission electron microscopy and immunohistochemistry.

Results: The amount of acute tissue injury increased with increasing pulse radiant energy. In human corneas, changes in the irradiated zones included epithelial cell injury and death loss of fine filamentous structure in Bowman's layer, disruption of stromal lamallae, and keratocyte injury and death. In the rabbit corneas, similar acute changes were noted. By 3 weeks, epithelial hyperplasia and stromal contraction were present. Wound healing in the rabbit corneas included repair of the epithelial attachment complex, keratocyte activation, synthesis of type I collagen, partial restoration of stromal keratan sulfate and type VI collagen, and retrocorneal membrane formation. Compared to 10-pulse treatments, 5-pulse treatments produced less acute tissue injury and had more rapid restoration of normal stromal architecture.

Conclusion: Noncontact LTK produces acute epithelial and stromal tissue changes and in rabbit corneas stimulates a brisk wound healing response. These changes could contribute to postoperative regression of induced refractive correction. Further work is required to determine if reductions in the magnitude of acute tissue injury and induced wound healing response will enhance the efficacy and stability of LTK.

INTRODUCTION

BACKGROUND

Lans[1] in 1898 first demonstrated the use of heat to induce corneal steepening by the application of electrocautery to the peripheral cornea of the

rabbit. He coagulated the cornea at the 6-mm optical zone with an arc length of 90° and induced 6 diopters (D) of astigmatism with regression to 3 D at 3 months. Comparing these results with tissue resection, he found cautery to be more effective but less predictable and less stable. In 1900, Terrien[2] reported on his use of cautery to correct the severe astigmatism in Terrien's marginal degeneration. In 1914, Wray[3] successfully used cautery to treat a patient with 6 D of hyperopic astigmatism. O'Connor[4] in 1933 reported on his treatment of a patient with high myopic astigmatism (preoperative refraction, -10.00+8.00 x 130) with corneal cautery. One day postoperatively, the refractive error had dramatically improved to -3.00 D sphere; 10 years later it was -5.00 D sphere. O'Connor concluded that corneal cautery was beneficial in selected cases of high astigmatism.

The modern era of keratoplasty began in 1964, when Stringer and Parr[5] reported the shrinkage temperatures of corneal collagen (55°C to 58°C) and scleral collagen (61°C to 62°C). In the mid-1970s many investigators explored the use of thermal keratoplasty as a therapeutic alternative to penetrating keratoplasty to treat keratoconus.[6-10] In 1973, Gasset and colleagues[6] reported on their treatment of keratoconus with thermal keratoplasty using a heated hand-held metal probe, a "thermokeratophore," to heat the ectatic cornea to 115°C, causing it to flatten. They reported excellent 1-year results with this technique in five keratoconus patients who were intolerant of contact lenses. Aquavella and coworkers[9] described a case of successful treatment of corneal hydrops. These and other reports of successful thermal keratoplasty raised the expectation that it might reduce the need for corneal transplants in keratoconus patients intolerant of spectacles and contact lenses, but subsequently these treatments were found to have a high incidence of regression and were compromised by poor predictability.[10] Other complications included delayed epithelial healing,[11] recurrent corneal erosions, vascularization, corneal scarring, instability of corneal curvature, aseptic stromal necrosis, bullous keratopathy, and fibrinous iritis with hypopyon.[12,13] Although thermal keratoplasty for keratoconus fell into disuse after these reports, Itoi[14] in 1981 reported that only 7% of 750 keratoconus patients with thermal keratoplasty needed subsequent corneal transplantation. He advocated that because of shortage of corneal donors in Japan, a trial of thermal keratoplasty should be performed prior to penetrating keratoplasty. Interestingly, some corneal surgeons in the United States still use thermal keratoplasty to flatten the cone at the time of penetrating keratoplasty to facilitate trephination.

In the early 1980s, Rowsey and coworkers[15,16] used a 1.6-MHz radio frequency probe (the Los Alamos Probe) to induce stromal collagen

shrinkage in an attempt to perform thermal keratoplasty in a more controlled fashion. However, in 1987 this effort was abandoned because of problems with scarring and poor predictability.[17]

RECENT ADVANCES IN THERMAL KERATOPLASTY

The advent of radial thermokeratoplasty, first developed by Fyodorov[18] in the Soviet Union in 1981, spurred new interest in thermal keratoplasty and was the first systematic attempt to use it to treat hyperopia. His original procedures consisted of using a probe to make superficial thermal applications to the peripheral cornea. In 1984, he developed a retractable thermal probe consisting of a 34-gauge nichrome wire in order to obtain deeper stromal heating. The nichrome wire was designed to penetrate the cornea to a depth of 85% to 90% and reached temperatures of 600°C for 0.3 seconds. The coagulations were made in a radial pattern with eight rows of three or four applications, each up to a premarked optical zone.[18] Clinical trials using this method demonstrated high initial corrections[19,20] but were still plagued with moderate to severe regression in the first few months following treatments.[21] Drawbacks with radial thermokeratoplasty include the extreme temperatures produced, the nonuniform heating within the corneal stroma, and the need to penetrate corneal tissue with a foreign body.[22] Because of substantial regression of effect and poor predictability, interest in radial thermokeratoplasty has largely subsided, and attention is now focused on more controlled heat-delivery systems to induce stromal collagen shrinkage .

A critical advance in the field of thermal keratoplasty was the utilization of infrared laser sources to deliver controlled quantities of thermal energy with exquisite precision to the corneal stroma. In 1979, Mainster[23] described a theoretical model for using lasers in thermal keratoplasty, emphasizing that lasers would deliver heat more uniformly throughout the stroma while at the same time prevent excessive heating of the epithelial and endothelial layers.

The carbon dioxide (CO_2) laser with a wavelength of 10.6 μm was used to produce stromal heating, but the corneal curvature changes were transient, presumably because most of the infrared radiation from the CO_2 laser was absorbed at the superficial depth of 0.01 to 0.02 mm in the cornea.[24-26] Kanoda and Sorokin[27] used a 1.54-μm yttrium-erbium-glass (Yt-Er-glass) laser in an effort to produce deeper thermally induced collagen shrinkage. They reported correction of over 3 D of hyperopia with ongoing low-grade regression at 12 months following treatment. Although no data concerning endothelial damage were reported, the high penetration depth of approximately 1 mm achievable by this laser led other

researchers to look for a more suitable wavelength. Horn and coworkers,[28] using a cobalt:magnesium fluoride laser that was tunable from wavelengths of 1.85 μm to 2.25 μm, reported corneal curvature changes in rabbits of up to 8 D with stability for at least 1 year. Subsequently, Koch and colleagues[29] reported results using a continuous-wave hydrogen fluoride chemical laser with a wavelength of 2.61 μm for simulated correction of myopia in rabbits and a rhesus monkey. With selected treatment parameters, they achieved stable corrections with no evidence of corneal scarring at follow-up of 3 months in rabbits and 1 year in the rhesus monkey.

In 1990 Seiler and coworkers[30] published the results of laser thermal keratoplasty (LTK) on four blind human eyes using a pulsed holmium:yttrium-aluminum-garnet (Ho:YAG) laser with a wavelength of 2.06 μm. They achieved hyperopic changes up to 5 D that were stable for 4 months and hypothesized that selection of the appropriate laser wavelength was critical to obtain safe and effective thermal keratoplasty. The 2.06-μm wavelength of the Ho:YAG laser had a penetration depth in corneal tissue of 300 to 400 μm, which, when appropriately delivered, would be ideal for achieving deep controlled coagulation zones with minimal damage to adjacent tissues.[31,32] Seiler and coworkers reported that the focused Ho:YAG laser beam produced a cone-shaped profile as opposed to the cylindrical temperature profile produced by the hot needle used for radial thermokeratoplasty. This led to more pronounced shrinkage of collagen fibrils in anterior stroma than in the posterior stroma, which they believed produced greater initial refractive effect and long-term stability.[30] Seiler[33] subsequently reported 1-year results on hyperopic corrections of 2 to 4 D in 20 partially sighted and sighted eyes. There was regression of approximately 1.5 D in the first 6 months with refractive stability between 6 and 12 months.[33] These studies indicated that for the short follow-up interval, moderately stable corneal curvature changes could be achieved if stromal heating of appropriate magnitude and depth could be accomplished.

One of the significant advantages of the Ho:YAG laser is that it uses solid-state technology, which is relatively inexpensive to manufacture and maintain and may provide increased safety and ease of use. Currently, two principal Ho:YAG laser delivery systems are being investigated for LTK: (1) contact probe types manufactured by Summit Technology (Waltham, Mass)[32] and by Technomed (Baesweiler, Germany) and (2) a noncontact device manufactured by Sunrise Technologies (Fremont, Calif).[34] The contact mode permits sequential delivery of laser pulses into individual premarked spots using a hand-held fiberoptic probe. The noncontact mode device (Fig 1) allows simultaneous delivery of laser energy in a symmetri-

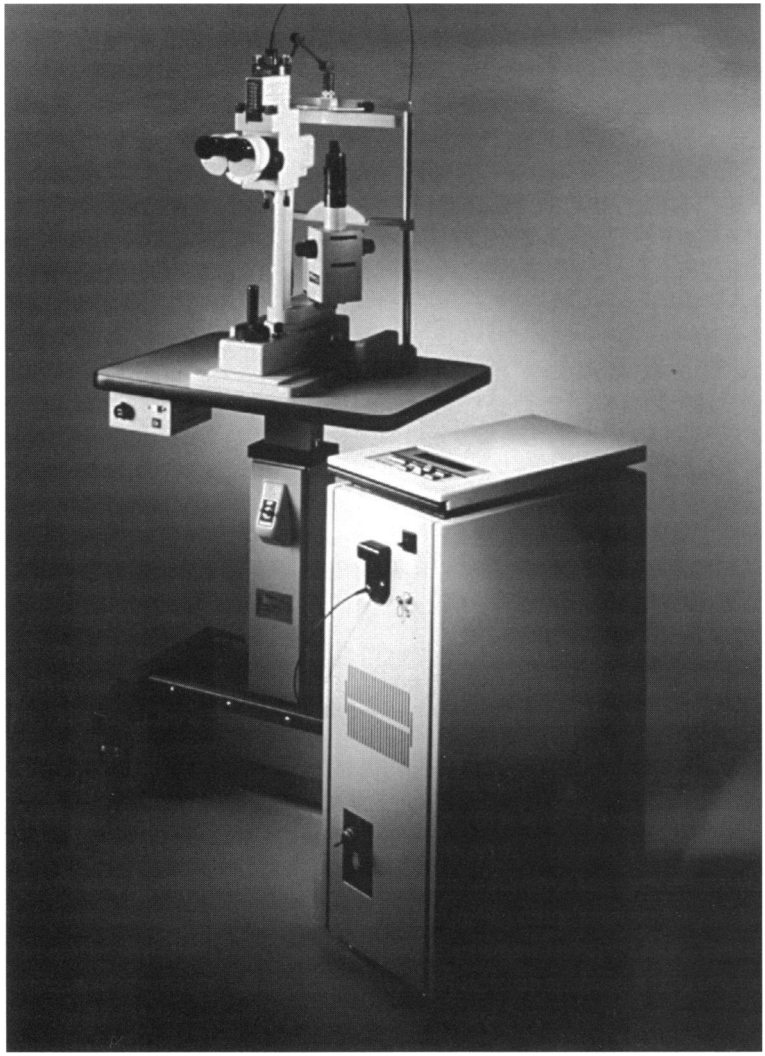

FIGURE 1A
Noncontact holmium: YAG (Ho:YAG) laser (Sunrise Technologies, Inc., Fremont, Calif). Laser is contained in floor unit on right. Laser light is delivered by fiberoptic cable to slit-lamp delivery system on the left.

cal pattern of 8 spots imaged on the cornea using a slit-lamp delivery system. The Summit and Sunrise devices are sponsored by their respective manufacturers under US Food and Drug Administration Investigational Device Exemptions, and these clinical trials are currently in progress.

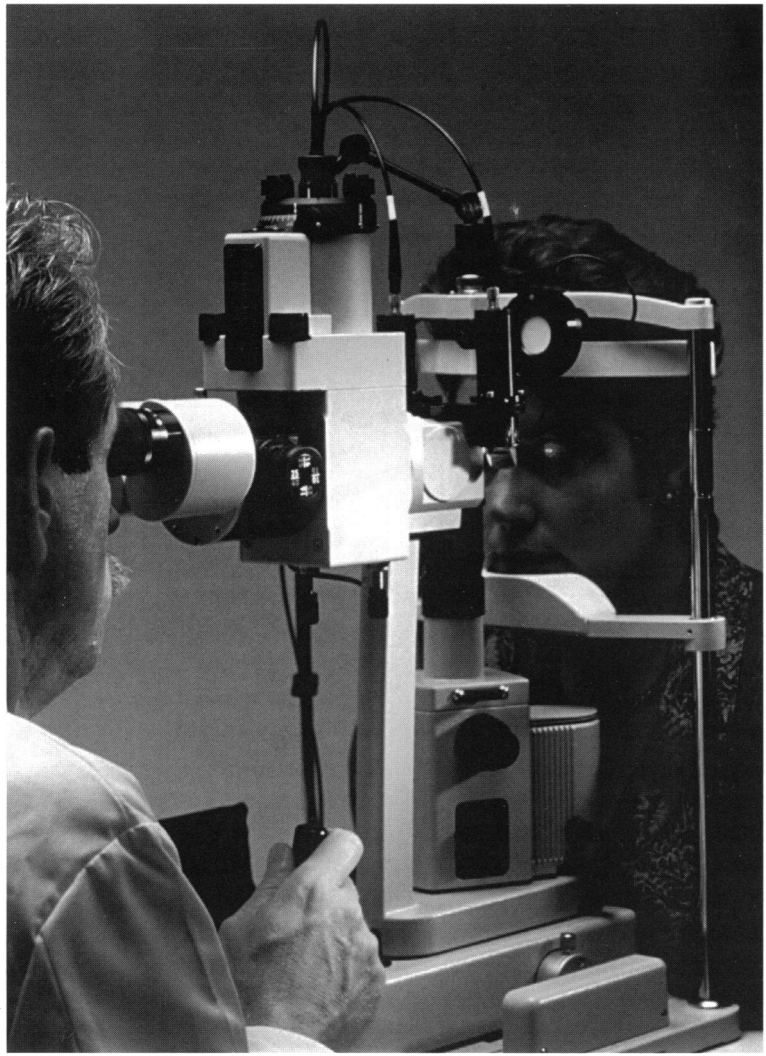

FIGURE 1B
Slit-lamp delivery system of noncontact Ho: YAG laser.

NONCONTACT LASER THERMAL KERATOPLASTY

Clinical studies with the Sunrise noncontact Ho:YAG device began about 3 years ago, and a growing body of clinical work demonstrates the promise of this device as a refractive surgical modality. The device parameters are described in the first section of Materials and Methods.

Ariyasu and coworkers[35] performed initial safety studies on 10 blind

eyes of human subjects. Modest curvature changes were produced, and there were no significant complications. Depending on the treatment parameters, they noted conical-shaped corneal stromal opacities extending up to 75% stromal depth.

Two-year follow-up results have been reported on the initial clinical treatments of sighted eyes.[36] One eye each of 17 patients was treated. Treatment parameters included simultaneous delivery of eight Ho:YAG laser spots in a symmetric octagonal array with a centerline diameter of 6 mm, 10 pulses of laser light at 5-Hz pulse repetition frequency, and pulse radiant exposures of 7.9 to 9.2 J/cm^2 (corresponding to pulse energies of 159 to 199 mJ). In the 15 eyes with 2-year follow-up, mean uncorrected distance Snellen visual acuity improved from 20/125-1 preoperatively to 20/50-2. The mean change in spherical equivalent of subjective manifest refraction was -0.79 ± 0.74 D. Eleven of these 15 eyes (73%) had a mean refractive correction of -1.1 D (range, -0.38 to -2.63 D); regression between 14 days and 2 years was 0.2 D. Four eyes (27%) had no persistent refractive correction (within ± 0.25 D). Mean induced refractive astigmatism was 0.18 D. None of the eyes lost two or more lines of spectacle-corrected distance visual acuity. The amount of refractive correction at 2 years postoperatively was correlated to the treatment pulse energy and the volume of the opacified corneal tissue observed immediately after treatment. Although most patients achieved stable refractive corrections by 3 months postoperatively, a small number of patients experienced regression up to 18 months.

In the United States, 28 eyes of 28 patients were treated in the Phase IIa study (monitored by the Food and Drug Administration).[37] Follow-up was 1 year. Two treatment patterns were used: (1) one ring, 10 pulses, 8 spots, and a 6-mm treatment zone (Fig 2A) and (2) two rings at 6- and 7-mm zones, each with 10 pulses, 8 spots, with a 22.5° offset between the rings (Fig 2B). Pulse radiant exposures ranged from 8.5 to 10.2 J/cm^2 (corresponding to pulse energies of 208 to 242 mJ). Mean refractive corrections were 0.6 ± 0.3 D in patients treated with one ring and 1.6 ± 0.6 D in the two-ring group. There was no mean regression in the one-ring group between 6 and 12 months nor in the two-ring group between 9 and 12 months. However, again a small number of patients had continuous regression throughout the first year.

Other treatment parameters have been explored, particularly the use of smaller numbers of pulses with higher treatment energies. Two regimens currently under investigation in a variety of clinical sites are 5- and 7-pulse treatments. One-year follow-up on 22 patients treated with 5 pulses, 240 mJ, and two rings at 6.5 and 7.5 mm showed less initial correction than the Phase IIa 10-pulse treatments (eg, at 1 month 2.4 D versus 3.2 D

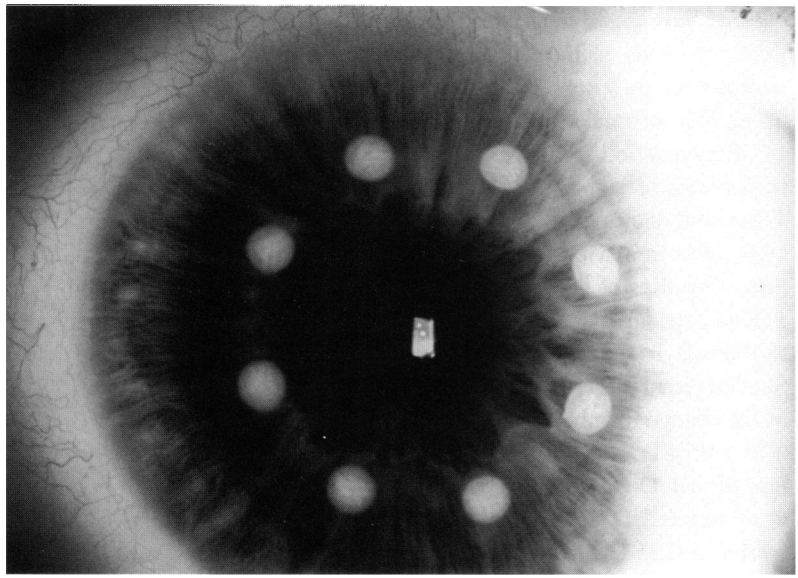

FIGURE 2A
Slit-lamp photographs of human corneas 1 week following noncontact Ho:YAG laser treatment using one ring at 6 mm.

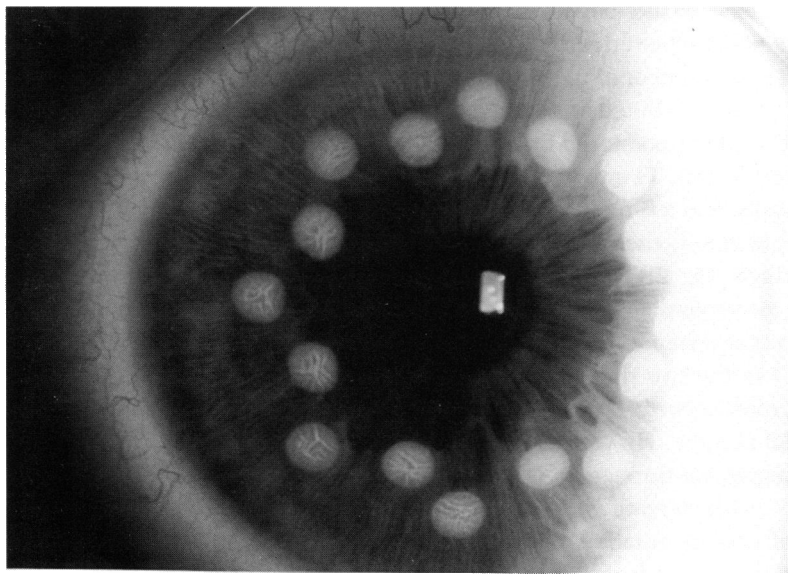

FIGURE 2B
Slit-lamp photographs of human corneas 1 week following noncontact Ho:YAG laser treatment using two rings at 6 and 7 mm. Note opacification and corrugated surface of treated epithelium.

in the Phase IIa group; R. Villarreal, T. Kohnen, M. Berry, and D. Koch, personal communication). However, at 1 year the mean refractive correction was 1.3 D, which is comparable to the 1.6-D correction in the Phase IIa series, considering the slightly smaller correction anticipated with the larger treatment zone in the patients receiving 5 pulses.

MECHANISM OF LASER THERMAL KERATOPLASTY

LTK is based on the principle of heat-induced modification of corneal collagen at approximately 55°C to 60°C.[5] At this temperature the helical structure of the tropocollagen collapses owing to dissociation of interpeptide hydrogen bonds between the procollagen fibers, partial unwinding of the triple helix, cross-linking between amino acid moieties, and changes in hydration.[38-40] Although the application of heat dissociates the interpeptide bonds, the cross-linkage between tropocollagen molecules remains intact, and the contraction of these linked molecules leads to shortening of the collagen fiber.[41] This thermal processing of the collagen helical structure causes it to shrink maximally to one third its original size; the amount of shrinkage is based on the mechanical properties of the surrounding tissue. The objective of a successful LTK treatment is to heat the stromal collagen sufficiently to induce these molecular changes while maintaining the structural integrity of the surrounding tissue and the endothelium.[41]

The ultimate success of LTK is likely to be determined by at least four critical parameters:

1. Collagen Shrinkage Temperatures

The shrinkage temperature for human collagen, beginning at about 55°C, lies within a relatively narrow range. This temperature allows collagen shrinkage without destroying collagen fibrils. Allain and colleagues[42] reported that thermally contracted collagen begins to relax if the temperature is increased to 78°C; the temperature threshold for relaxation rises with increasing tissue age owing to an increase in the number of thermally stable cross-linking bonds.[43] Overheating the collagen could therefore be counterproductive.

2. Tissue Resistance and Elasticity

The amount of induced refractive change depends in part on tissue resistance to collagen shrinkage[42]; greater initial corrections are achieved with less rigid corneas. On the other hand, tissue elasticity or mechanical creep may contribute to regression by compensating for thermally induced changes through stretching and relaxation of adjacent tissues. Preliminary clinical data suggest that LTK is not as stable in patients younger than 40

to 45 years (M. Berry, personal communication), and mechanical tissue factors such as elasticity may account for this finding.

3. Keratocyte Response

On the basis of evidence from other refractive surgical procedures, it is probable that keratocyte injury leads to keratocyte activation and an induced wound healing that plays a major role in postoperative regression. Activation of keratocytes with keratocytic proliferation, new collagen synthesis, collagen remodeling, and the production of new glycosaminoglycans may contribute to the post-LTK wound healing response. By studying the wound healing response in rabbit corneas exposed to CO_2 laser radiation, McCally and colleagues[44] found that keratocyte injury occurs when stromal collagen is heated to 79°C. (A similar damage threshold of 75°C ± 4° was noted for rabbit epithelial and endothelial cells treated with the CO_2 laser,[45] and 79°C was again found to be the damage threshold for rabbit epithelium treated with the thulium:YAG laser.[46]) Presumably, treatment parameters must be designed to maximize the amount of collagen shrinkage while minimizing keratocyte and other tissue injury that might hinder long-term stability following LTK. However, the histologic changes and wound healing response following LTK are unknown.

4. Stability of Corneal Collagen

Independent of the postoperative wound healing changes, normal homeostatic replacement of treated collagen by newly synthesized collagen might cause regression of the refractive effect. The half-life of corneal collagen is likely to be many years. Smelser and colleagues[47] reported that collagen turnover in the cornea is a slow process, with a half-life of 10 years or more. Lass and coworkers[48] used proline radiolabelling to investigate wound healing in corneal transplants in rabbits and found a moderately high turnover in collagen in the graft periphery within the first 20 days postoperatively with no significant change thereafter. They also found that little change occurred centrally. The stability or longevity of collagen treated with LTK is unknown, as is the postoperative rate of new collagen synthesis. Additional studies are needed to evaluate long-term collagen turnover in corneas treated with LTK.

STUDY GOALS

Successful outcomes in LTK obviously depend on achieving adequate initial corrections and controlling postoperative regression, with the latter being the major hurdle in developing this technology for routine clinical use. If regression is at least in part caused by wound healing, a fundamental understanding of the tissue changes and responses is required. The pur-

pose of this study was to explore the acute histopathologic changes induced by LTK in human corneas and to evaluate the wound healing response to LTK in rabbits, using histopathology and immunohistochemistry.

This study was conducted in two phases. The first was an evaluation of histologic changes and wound healing response following 10-pulse LTK with a range of pulse radiant exposures; the tissues studied were (1) human corneas scheduled to undergo penetrating keratoplasty 24 hours later and (2) corneas of New Zealand white rabbits.

The second study, again using New Zealand white rabbits, was designed to assess two factors: (1) the wound healing response following LTK using 5 instead of 10 pulses and (2) the role of epithelial cell loss alone in generating a wound healing response. The LTK parameters were selected because *in vitro* studies in cadaver eyes had shown that 5-pulse treatments resulted in corneal curvature changes similar to those produced using 10 pulses; in addition, these parameters are under investigation in clinical trials. In the original sighted-eye study and the Phase IIa study, a few patients lost fixation during the 2-second 10-pulse treatments. This resulted in nonuniform laser application with irregular enlargement of the treatment spots. By decreasing the pulses from 10 to 5 and thereby shortening the treatment from 2 seconds to 1 second, treatment variability due to patient movement could be reduced. In terms of laser energy delivered, this is also a gentler treatment with a total energy of 1,200 to 1,300 mJ, compared with a minimum of 1,600 mJ with 10-pulse treatments. We therefore wished to determine if this treatment modality resulted in less tissue injury and subsequent wound healing response. To evaluate the effect of epithelial defects on the stromal wound healing response, additional corneas were treated with epithelial debridement in a similar pattern as the LTK treatments.

MATERIALS AND METHODS

DEVICE

The treatments were performed with a repetitively pulsed Ho:YAG laser with a slit-lamp delivery system (Fig 1). Laser parameters included a 2.13-μm wavelength; about 600-μm spot size; 250-μsec pulse duration; and 5-Hz pulse repetition rate. The Ho:YAG laser is contained in a relatively small floor unit, and laser light is transmitted via a fiberoptic cable to a polyprismatic beam-splitting optical tower delivery system in the slit-lamp biomicroscope. The polyprism breaks the laser light into 8 beams in a symmetric octagonal array. To align the Ho:YAG beams, there are 8 red helium neon (wavelength, 633 nm) laser tracer beams. Calibrated green helium neon (wavelength, 543 nm) laser beams are used to focus the laser on the surface of the cornea.

10-PULSE STUDY

Treatment Parameters

The human and rabbit corneas received a total of 10 pulses with a treatment duration of 2 seconds. Pulse radiant exposures F_p (units, J/cm^2) were varied as noted below; for the human corneas we selected F_p values ranging from below to above those used in the first sighted-eye human clinical trials (7.3 to 9.2 J/cm^2),[10] whereas for the rabbit corneas we used F_p values at and above the upper end of those used clinically.

F_p values were calculated from the relation

$F_p = E_p / A$,

where E_p is the pulse energy per spot (units, J),

and $A = \pi r^2$ is the spot area (units, cm^2); r is the spot radius.

In the first human cornea experiment, the spot diameter was calculated to be 562 µm, whereas in all other experiments, the spot diameter was calculated to be 597 µm.

These spot diameters are effective values D_{eff} calculated from the relation

$D_{eff} = D_{400} (E_{p,total}/E_{p,400})½$,

where D_{400} is the diameter of a 400-µm aperture,

$E_{p,total}$ is the total energy per spot,

and $E_{p,400}$ is the energy transmitted through the 400-µm aperture.

Human Corneas

Three human corneas were treated under topical anesthesia 1 day before undergoing penetrating keratoplasty. The criterion for inclusion was moderate corneal edema secondary to Fuchs' dystrophy. Exclusion criteria included previous corneal surgery, previous corneal inflammation, and advanced corneal edema with bullous epithelial changes. Each cornea was treated with the Ho:YAG laser using four pairs of spots at four different F*p* values: 5.8, 7.4, 9.3, and 11.4 J/cm^2 for the first cornea; 7.7, 8.5, 9.3, and 11.0 J/cm^2 for second; 9.0, 10.2, 11.4, and 12.6 J/cm^2 for the third. The corneas were harvested 1 day following treatment and immediately immersed in Optisol (Chiron IntraOptics, Irvine, Calif), stored over ice, and shipped by overnight express service to the National Vision Research Institute Laboratory. Upon receipt, the corneas were appropriately fixed, embedded in plastic, and prepared for light microscopy (LM) and transmission electron microscopy (TEM).

Rabbit Corneas

The right eyes of six fully anesthetized New Zealand white rabbits were treated with the Ho:YAG laser; the treatment pattern consisted of 6 spots each at 9.0 J/cm^2 in the temporal half of the cornea and 10.9 J/cm^2 in the nasal half. The spots were separated by at least 1 mm from each other and

by a minimum distance of 2 mm from the limbus. The animals were euthanized immediately or at 24 hours, 1 week, 3 weeks, 2 months, and 3 months after treatment. The corneas were removed, and a small wedge was dissected from the periphery of the corneal button between the 9.0-J/cm^2 and 10.9-J/cm^2 treatment spots to orient each dosage. The tissues were placed in Optisol on ice and sent by overnight mail for submission for histology. Upon receipt, the tissues were examined under the dissecting microscope and dissected into four portions, each containing three treatment spots. The left corneas served as controls; these were also divided into four portions. The tissues were then prepared for either LM, TEM, or immunohistochemical studies.[11-13]

5-PULSE STUDY

One eye each of nine New Zealand white rabbits was treated. Six corneas were irradiated with the Ho:YAG laser using 8 spots at a 6.5-mm zone, 5 pulses, and pulse radiant exposures of 9.9 and 10.7 J/cm^2 (corresponding to pulse energies of 240 mJ or 260 mJ) (three corneas each). Three corneas received eight epithelial wounds (using a diamond burr) in the same pattern as the LTK spots, and one cornea (the left cornea of the rabbit treated with 240 mJ and followed for 12 weeks) received neither laser treatment nor a burr wound and was used as a control. The rabbits were fully anesthetized during the treatments.

The animals were euthanized and the eyes enucleated either immediately following treatment, at 1 week, or at 12 weeks. The corneas were dissected from the whole globes, placed in Optisol on ice, and sent by overnight mail for submission for histology. Upon receipt, the corneas were removed from the Optisol, examined under a dissecting microscope, and dissected, using a fresh scalpel blade, into three pieces. Tissues were processed for LM and TEM[49-51] and immunohistochemistry (Table I).[52-60]

MICROSCOPY

Dissected tissue samples from each treated cornea (human and rabbit) were processed for LM and TEM using methods previously described.[50] Briefly, samples were fixed in 0.1 M cacodylate-buffered 2% paraformaldehyde/2.5% glutaradehyde, pH 7.3, 300 mOsm, overnight at 4°C and postfixed in 2% osmium tetroxide, in 0.1 M cacodylate buffer. The tissues were then dehydrated in graded ethanol solutions from 50% to 100%, followed by two changes in 100% propylene oxide, and embedded in Polybed/812 (Polysciences, Inc, Warrington, Pa). Sections were cut on a Reichert ultracut microtome (Reichert-Jung, Austria) into 1-μm-thick sections, stained with Mallory's azure II-methylene blue, and counter-

stained with basic fuschin, for LM. For TEM, 70- to 90-nm sections were cut and then stained 10 minutes with uranyl acetate and 10 minutes with lead citrate.[51] Sections were examined on an Olympus Vanox light microscope (Olympus Optical Co Ltd, Japan) and a Hitachi HU 12A electron microscope (Hitachi Ltd, Tokyo, Japan).

During tissue processing in the 10-pulse study, the section from the rabbit cornea that had been taken 1 week after laser treatment was lost to further use during the embedding process owing to hydration of the resin component dodecenyl succinic anhydride. An attempt to retrieve the

TABLE I: ANTIBODIES USED IN IMMUNOHISTOCHEMICAL ANALYSIS

PROTEIN/TEST/ SOURCE	ANATOMIC LOCATION	ROLE IN WOUND HEALING
Beta 4 integrin/ MAB 3E1/Gibco[52,53]	Hemidesmosomes of basal epithelial cells	Lost during epithelial cell migration, reappears with formation of epithelial attachment complexes[52-54]
Type IV collagen/ PAB/Southern Biotechnology Associates	Epithelial basement membrane, Descemet's membrane	Appears in basement membrane during wound healing[52,53,55]
Type VII collagen/ MAB 185/Gibco	Anchoring fibrils of attachment complex, epithelium/stroma	Lost during epithelial migration, reappears with formation of epithelial attachment complexes[52,53,56]
Procollagen type I (carboxy)/MAB/ Chemicon	Collagen synthesizing cells	Present only in cells that are actively synthesizing type I collagen; identifies activated keratocytes[53,57]
Keratan sulfate/ MAB 5D4/ICN	Stromal matrix	Found in normal stroma, but with reduced sulfation in healing stroma and thus reduced response to certain antibodies[58,59]
Type VI collagen/ MAB/Chemicon	Stromal matrix	Found in normal stroma and at reduced levels in healing rabbit stroma[60]

MAB, monoclonal antibody; PAB, polyclonal antibody (goat).

frozen section for microscopy was unsuccessful because the freezing process had destroyed too much ultrastructure for satisfactory results.

IMMUNOFLUORESCENCE

The remaining rabbit tissues were frozen in O.C.T. compound (Miles Inc, Naperville, Ill), and 6-μm cryostat sections were mounted on gelatin-coated slides, air-dried, and stored at -4°C overnight. Before staining, sections were fixed in cold acetone, air-dried 5 minutes, and rehydrated in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA-PBS). Primary antibodies were incubated with the sections under humidified conditions for 1 hour at 37°C followed by three washes of BSA-PBS. Fluorescein isothiocyanate (FITC)–conjugated secondary antibodies were then introduced onto the sections followed by incubation, as previously described. After three washes in PBS, the slides were covered with glycerol-buffered *p*-phenylenediamine, coverslipped, examined, and photographed by epifluorescence microscopy on an Olympus BHTU light microscope, fitted with a reflected light fluorescence attachment (Olympus Optical Co Ltd, Japan). Although some micrographs of the 1-week corneal results in the 10-pulse study were lost during processing and therefore cannot be shown, observations of the antibody staining were recorded and are reported here.

The antibodies used for the 10-pulse study, their sources, the corneal locations of the proteins, and the changes expected during wound healing are shown in Table I. For the 5-pulse study, we studied procollagen type I and keratan sulfate because our interest was in the extent of disruption of normal glycosaminoglycans and the amount of new collagen and glycosaminoglycan synthesis stimulated by epithelial defects and by the 5-pulse treatments.

Secondary antibodies labeled with FITC were obtained from Sigma Chemical Co (St Louis). In the 10-pulse study, the paired, untreated corneas served as individual controls for each animal. In the 5-pulse study, the three burr corneas and the one untreated cornea served as controls. Immunohistochemical procedures were also performed in the absence of the primary antibody to test for nonspecific binding of the secondary antibodies.

RESULTS

10-PULSE STUDY

Human Corneas: Light and Transmission Electron Microscopy

LM was performed on human corneas treated at 7.7, 8.5, 9.0, 9.3, 10.2, 11.0, 11.4, and 12.6 J/cm^2. In the zone of treatment, all corneas showed cone-shaped swelling that was widest anteriorly and tapered toward the endothelial surface (Fig 3A). Several distinctive histologic changes were

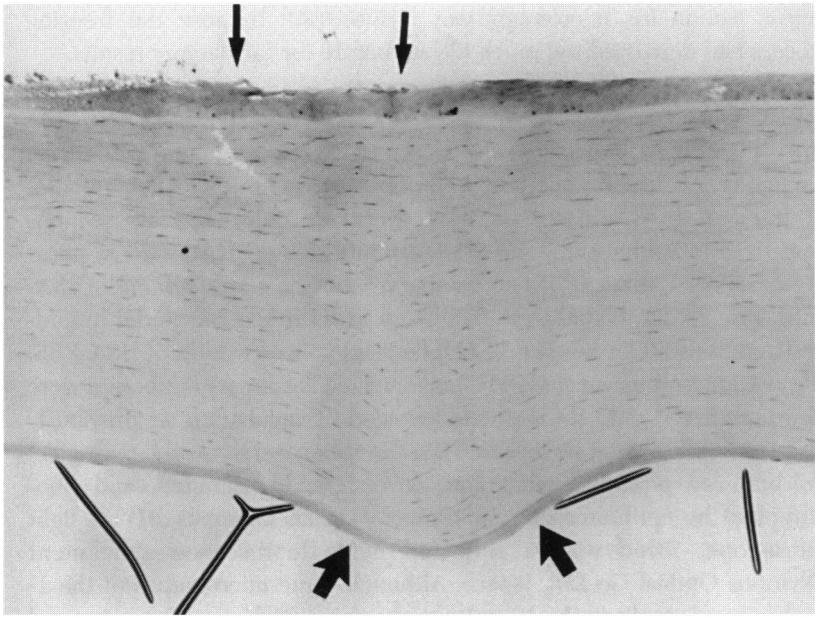

FIGURE 3A

Human cornea, 7.7 J/cm^2. Note epithelial injury (small arrows) and corneal swelling in zone of treatment (large arrows). Stroma in this region has been altered and has a nonuniform staining pattern (light microscopy, x 37.5).

present in the treated regions. In all specimens, there was epithelial sloughing and increased staining of the remaining epithelial cells. Changes in Bowman's layer ranged from increased stain uptake at low F_p to loss of its structural definition and thinning at the higher levels (Fig 3B). Keratocytes in the anterior stroma were fragmented and reduced in size and number; the degree and depth of these changes increased with increased F_p. Stromal lamellae were disorganized in the anterior one quarter of the stroma at low F_p; this extended to two-thirds depth at high F_p. Altered stromal staining ranged from one-half to full depth. Up to 10.2 J/cm^2, no endothelial changes were evident. The endothelium and Descemet's membrane were inadvertently removed in the 11.0-J/cm^2 specimen; endothelium was absent in the treatment sites in the cornea treated at 11.4 J/cm^2, presumably owing to the laser treatment. In the 12.6-J/cm^2 specimen, small, basophilic aggregates were randomly dispersed throughout the cells.

Ultrastructural analysis by TEM was performed on regions treated at 7.7, 9.0, 9.3, 10.2, 11.0, and 11.4 J/cm^2. As expected, increasing tissue damage was seen with increasing radiant exposures. The epithelium in the treated region was damaged, and the linear structure of the basement

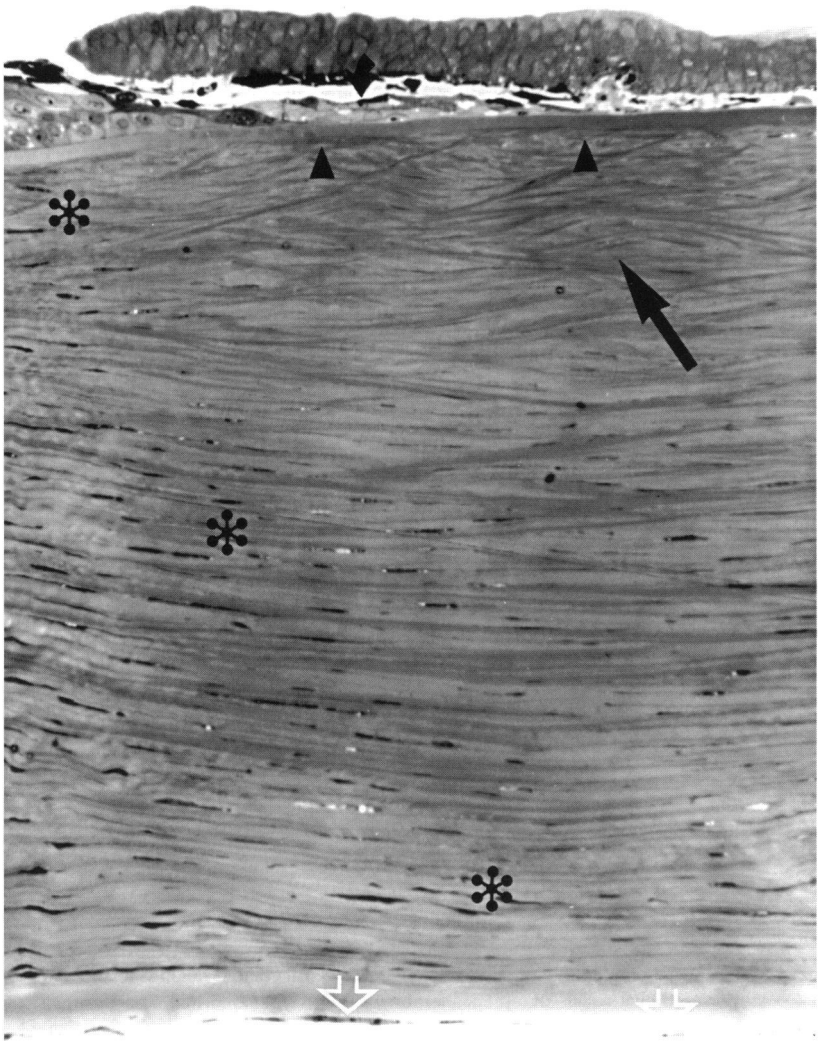

FIGURE 3B

Human cornea, 12.6 J/cm^2. Squamous-shaped epithelial cells have migrated beneath sloughing sheet of epithelium (small arrow). Posteriorly, Bowman's layer (arrowheads) has been thinned; stromal lamellae are somewhat distorted (large arrow) with changes in stain differentiation (region to right of asterisks). Continuous endothelium (open arrows) is visible (light microscopy x 110).

membrane was disrupted at all radiant exposures. At 8.5 J/cm² and above, there was loss of the fine filamentous structure in Bowman's layer (Fig 4A). Stromal lamellae were splintered and irregularly organized with randomly distributed electron dense particulate matter and splitting of individual fibrils into subfibrillar structures (Fig 4, B and C). The depth of these changes ranged from approximately one third at low F_p to two thirds at high F_p. There was evidence of keratocyte injury throughout the treated stroma in all corneas, although the extent of injury increased with increased F_p (Fig 4D). At 7.7 J/cm², endothelial cells appeared to be normal. In the 9.3- to 10.2-J/cm² corneas, endothelial cells appeared to be injured. No endothelial cells were seen in the 11.0- and 11.4-J/cm² specimens.

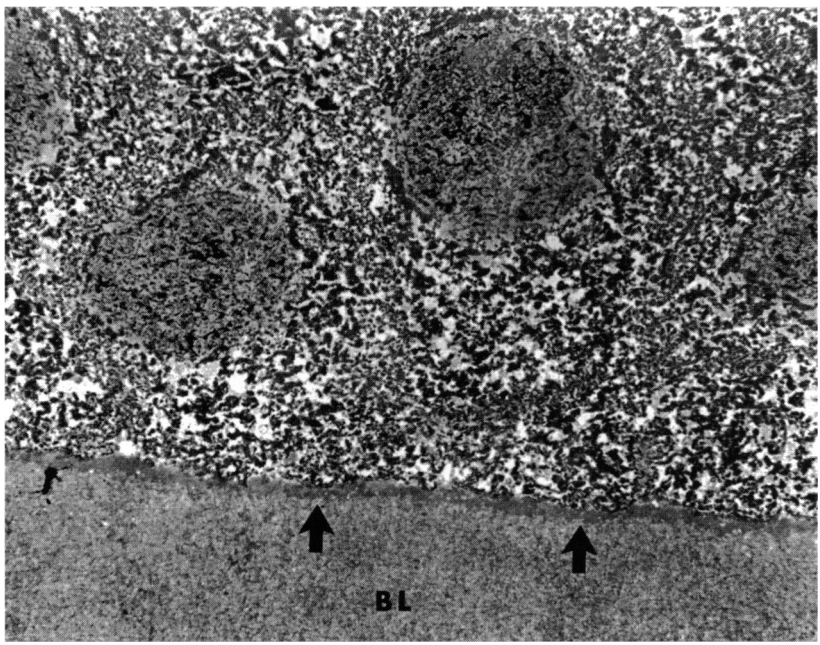

FIGURE 4A

Human cornea, 9.3 J/cm², epithelium and Bowman's layer. All cellular detail is lost in damaged posterior epithelial cells. Basement membrane (arrows) is abnormal, and Bowman's layer (BL) has lost its fine filamentous appearance (transmission electron microscopy, x 6,000).

Rabbit Corneas: Light and Transmission Electron Microscopy

Corneal responses immediately and 24 hours following LTK at 9.0 J/cm², as seen by LM and TEM, consisted of marked stromal swelling in the anterior and posterior region in a similar configuration as seen in the

human specimens, alterations in stromal matrix, and epithelial, keratocytic, and endothelial damage (Fig 5A). The laser effects appeared more pronounced in the 10.9-J/cm^2 specimens. Inflammatory cells were present in the damaged epithelium and endothelium at 24 hours after treatment.

At 3 weeks following laser treatment at 9.0 J/cm^2, a hyperplastic epithelium was present and new basement membrane was beginning to form. Keratocyte activation was observed, indicated by increased keratocyte density and size and by increased rough endoplasmic reticulum (Fig 5B). Stromal contraction replaced corneal swelling at 3 weeks. There was endothelial cell loss and initiation of retrocorneal membrane formation only under the treatment sites, confirming the endothelial damage noted in the earlier specimens.

At 2 and 3 months following treatment at 10.9 J/cm^2, stromal contraction and epithelial hyperplasia were still evident (Fig 5C). A more normal-appearing basement membrane underlay the hyperplastic epithelium, keratocytes remained activated, and the stromal matrix appeared disorganized. Retrocorneal membranes were present between Descemet's membrane and an endothelial monolayer (Fig 5D).

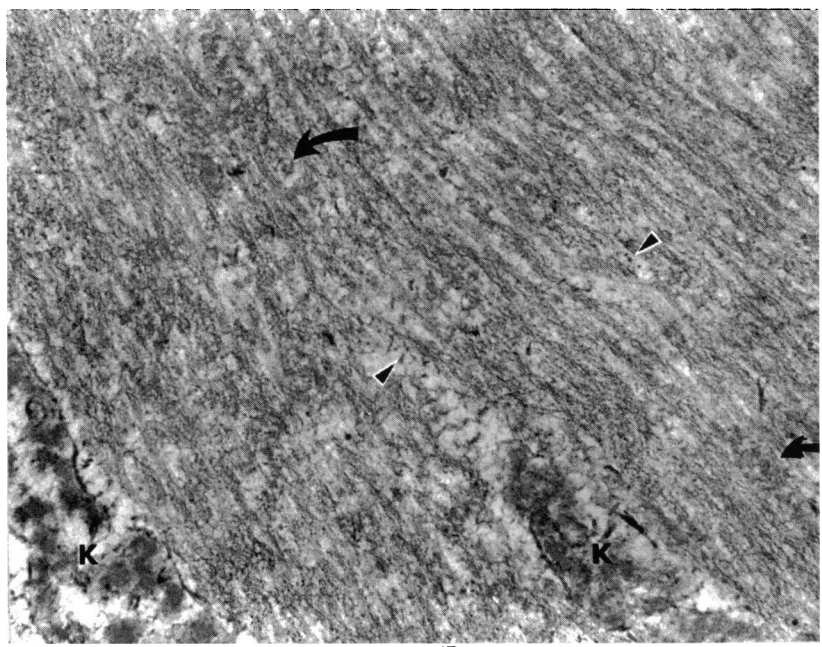

FIGURE 4B

Human cornea, 9.0 J/cm^2, anterior stroma. Collagen fibrils (arrows) have been split into subfibrillar particles; electron dense focal irregularities (arrowheads) are dispersed among fragmented fibrils. Keratocytes (K) appear as electron dense amorphous bodies (transmission electron microscopy, x 3,000).

FIGURE 4C

Human cornea, 9.0 J/cm², anterior stroma. Lamellae are splintered (asterisks), widely separated by ground substance (arrow). Keratocytes (K) have lost all definition (transmission electron microscopy, x 6,300).

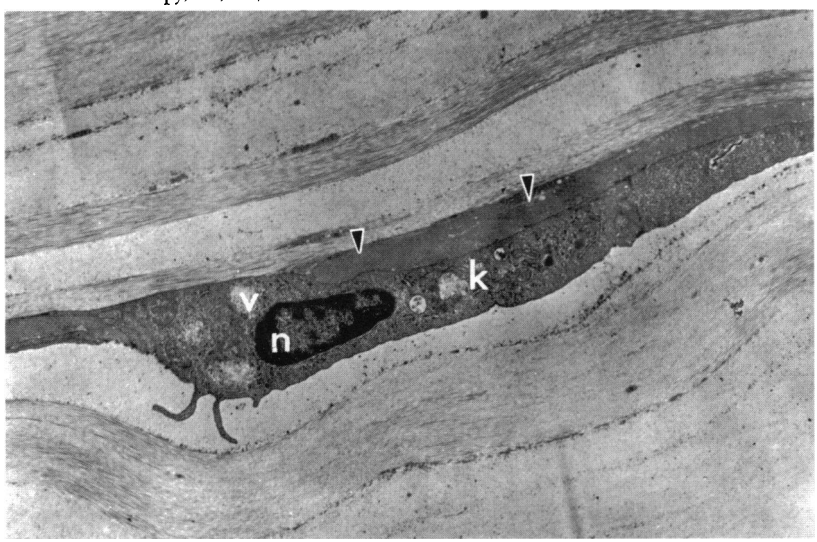

FIGURE 4D

Human cornea, 7.7 J/cm², central stroma. Lamellar structure is well defined. Much of intracellular structure of keratocyte (k) has been lost, although some organelles can be distinguished. Nuclear (n) chromatin is condensed peripherally, and vacuoles (v) are seen in cytoplasm. Amorphous material (arrowheads) surrounding keratocyte is often seen in normal corneas (transmission electron microscopy, x 9,600).

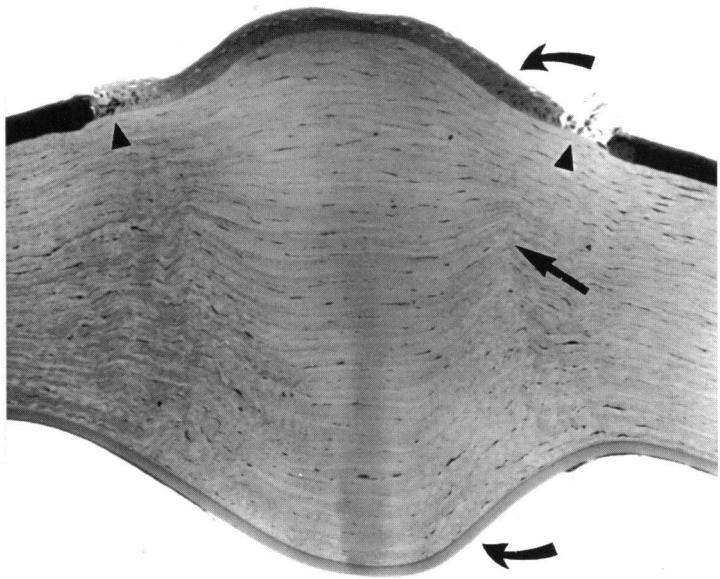

FIGURE 5A

Rabbit cornea, 9.0 J/cm², immediately after laser thermal keratoplasty. Treatment site is characterized by anterior and posterior corneal swelling (curved arrows), epithelial damage with cell loss at treatment edges (arrowheads), changes in extracellular matrix extending into posterior stroma, as indicated by differential staining in stroma (arrow), and loss of endothelial cells (light microscopy, x 80).

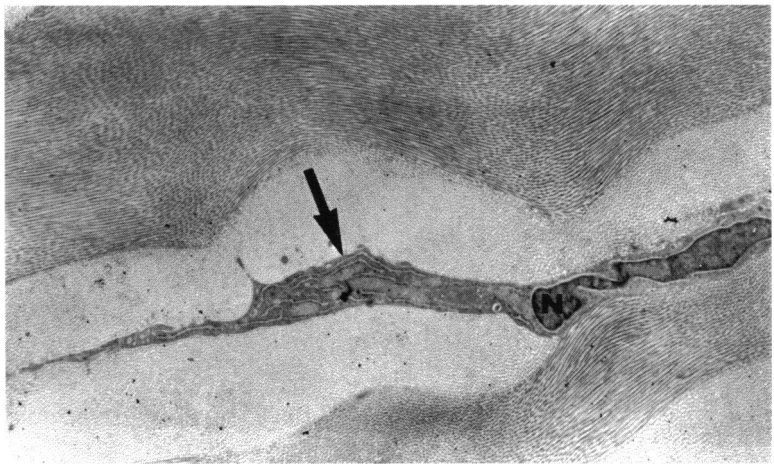

FIGURE 5B

Rabbit cornea, 9.0 J/cm², 3 weeks after laser thermal keratoplasty. Increased rough endoplasmic reticulum (arrow) and well-dispersed nuclear chromatin (N) indicate keratocyte activation (transmission electron microscopy, x 7,200).

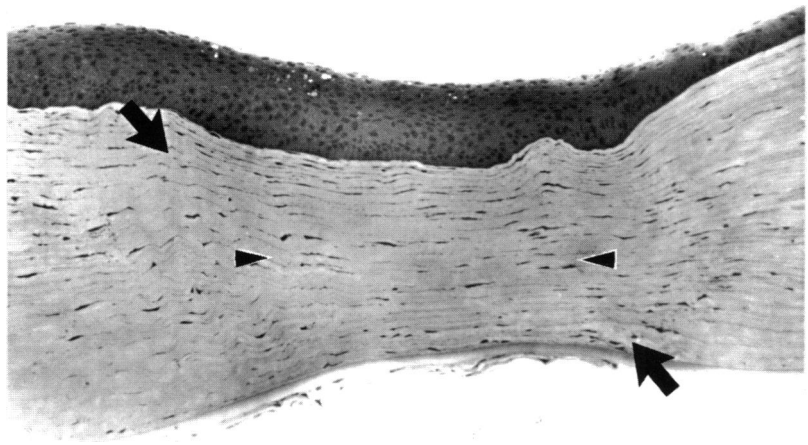

FIGURE 5C

Rabbit cornea, 10.9 J/cm², 2 months after laser thermal keratoplasty. In treated region, there is marked epithelial hyperplasia, and keratocyte density is increased (arrowheads). Anterior stromal lamellae slope downward and posterior stromal lamellae and Descemet's membrane bend upward (arrows), giving treated region a contracted appearance (light microscopy, x 80).

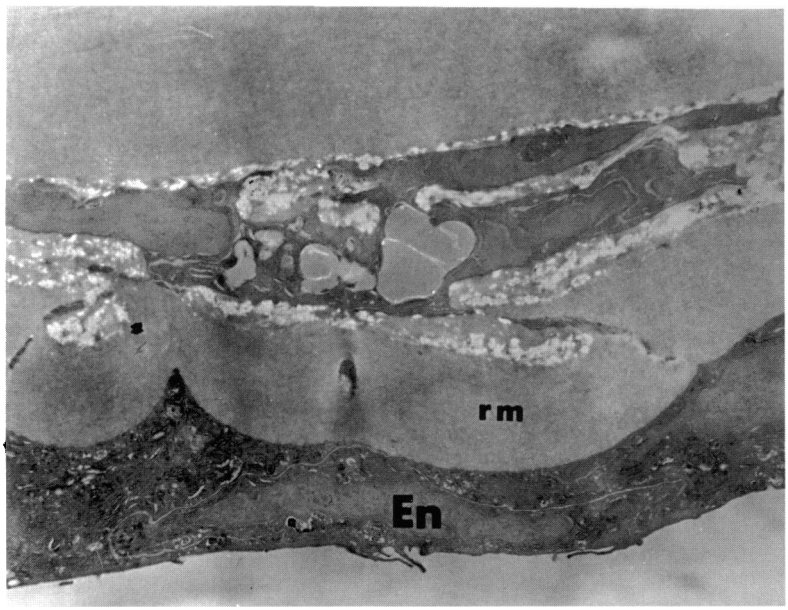

FIGURE 5D

Rabbit cornea, 10.9 J/cm², 3 months after laser thermal keratoplasty. Enlarged endothelial cells (En) lie posterior to retrocorneal membrane (rm) (transmission electron microscopy, x 5,400).

TABLE II: 10-PULSE STUDY: IMMUNOHISTOCHEMICAL STAINING OF RABBIT CORNEAS

COMPONENT	SITE OR FUNCTION	IMMEDIATE	1 DAY	1 WK	3 WK	2 MO	3 MO
Beta 4 integrin	Hemidesmosomes	0	0	↓	↓	✔	✔
Type IV collagen	Basement membrane	0	0	↓	✔	✔	✔
Type IV collagen	Stroma	0	↑	↑	↑	↑	↑
Type VII collagen	Anchoring fibrils	0	↓	↓	↓	✔	✔
Procollagen type I	Collagen synthesis	0	0	↑	↑↑	↑↑	↑
Keratan sulfate	Glycosaminoglycans	↓	↓/0*	NA	↓	↓	↓
Type VI collagen	Stroma	0	0	0/↓†	↓	↓	↓

0, absent; ↓, below normal; ✔, present at normal or near normal levels; ↑, above normal; ↑↑, markedly elevated; NA, data not available.

* Decreased in 9.0 J/cm² specimen and absent in 10.9 J/cm² specimen.
† Absent in 9.0 J/cm² specimen and descreased in 10.9 J/cm² specimen.

Rabbit Corneas: Immunohistochemistry (Table II)
Immunohistochemical studies substantiated the results seen by LM and TEM. Except as specifically noted, the immunohistochemical staining patterns were similar for the 2 pulse radiant exposures.

Epithelial basement membrane and attachment complexes, as defined by the associated antigens beta 4 integrin (hemidesmosomes), type IV collagen (basement membrane), and type VII collagen (anchoring fibrils), were disrupted by the initial treatment and were not seen immediately after treatment. Beta 4 integrin staining did not appear in the 24-hour specimens either (Fig 6A), but at 1 and 3 weeks (Fig 6B), beta 4 integrin staining appeared as a thin, discontinuous line. Beta 4 integrin distribution at 2 months and 3 months (Fig 6C) still retained some irregularities and discontinuities, showing that mature reconstruction of the hemidesmosomes was not completed, as seen in the control cornea (Fig 6D).

Staining for type IV collagen in the basement membrane was not seen at 24 hours after laser treatment, although normal staining of Descemet's membrane was retained. Basement membrane staining for type IV began to appear by 1 week, and at 3 weeks and beyond, type IV staining was linear and somewhat thicker than normal (Fig 7A). In the control rabbit corneas, type IV staining was seen as a narrow linear stain along the basement membrane with no staining in the stroma (Fig 7B); type IV staining was also seen in Descemet's membrane of the control corneas. Type IV staining of Descemet's membrane appeared broader and more irregular than normal at 2 and 3 months owing to the presence of retrocorneal membranes in the same region.

At 24 hours in the 10.9-J/cm^2 treatment and at 1 week in the 9.0-J/cm^2 treatment, stromal staining for type IV collagen began to appear. This stromal staining seemed to peak at about 3 weeks and then diminished in intensity, but was still present even 3 months after treatment.

Staining for type VII collagen, a major constituent of the anchoring fibrils of the attachment complex, reappeared in the 24-hour specimens as a faint, discontinuous stain (Fig 8A). Staining was still discontinuous in the 1- and 3-week samples. By 2 and 3 months, type VII distribution appeared continuous (Fig 8B) but was more irregular than that seen in the normal cornea, particularly in the 10.9-J/cm^2 specimen. Type VII staining was also found in the retrocorneal membrane, a new finding that suggests an epithelial nature of these cells (Fig 8C).

Procollagen staining, which was not seen in the control, was absent immediately and at 24 hours after treatment. Procollagen staining began to appear at 1 week, becoming quite intense and widely distributed throughout the treated region at 3 weeks (Fig 9A) and 2 months. Three

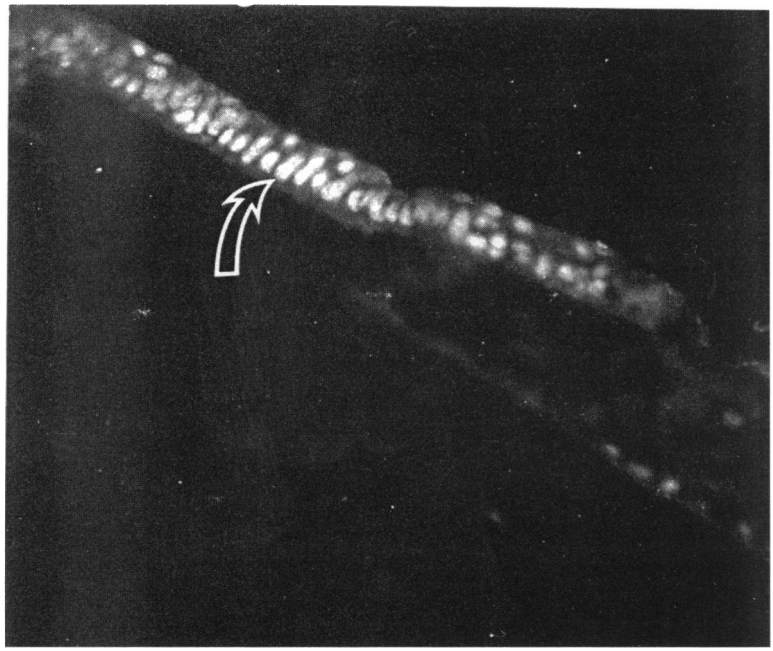

FIGURE 6A

Rabbit cornea, immunohistochemical staining for beta 4 integrin, 9.0 J/cm^2. At 24 hours, no signal was seen in treated region; only autofluorescence of epithelial nuclei (curved arrow) can be seen (x 75).

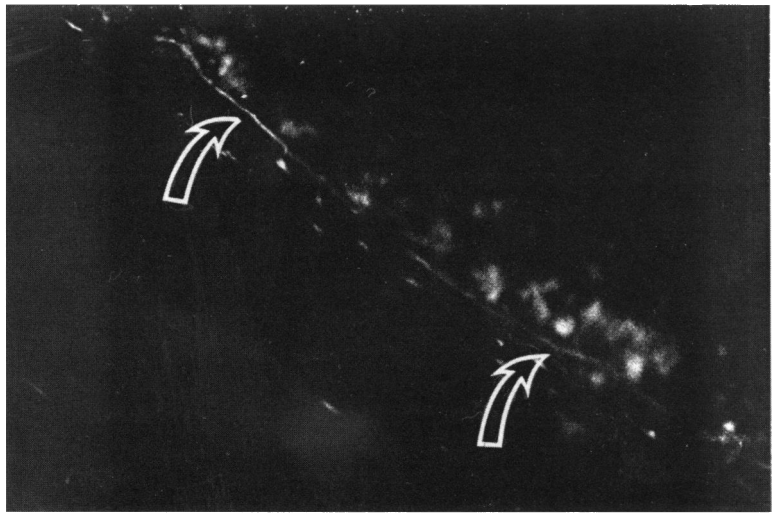

FIGURE 6B

Rabbit cornea, immunohistochemical staining for beta 4 integrin, 9.0 J/cm^2. At 3 weeks reformation of epithelial attachment complexes has begun as indicated by thin discontinuous stain (curved arrows) posterior to basal epithelium (x 75).

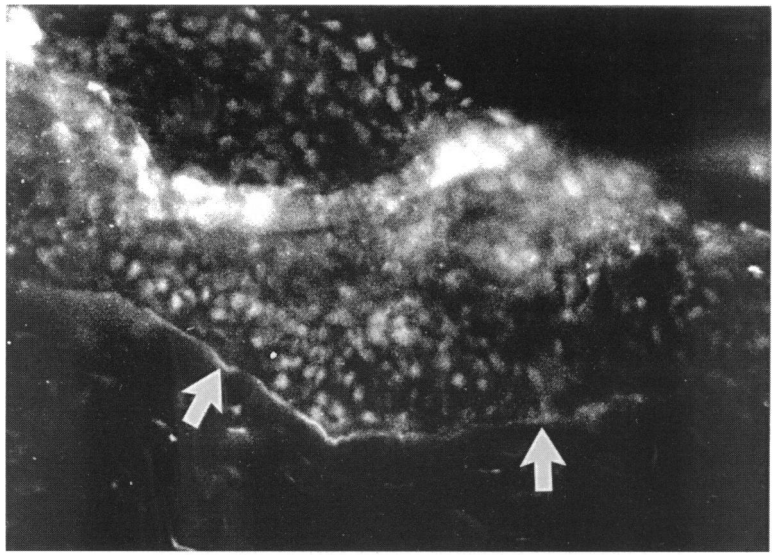

FIGURE 6C
Rabbit cornea, immunohistochemical staining for beta 4 integrin, 9.0 J/cm^2. At 3 months, staining pattern is linear but still somewhat thin and irregular (arrows), suggesting partial restoration of epithelial cell attachment complexes. Autofluorescence of epithelium and poor focusing distort linear pattern (x 75).

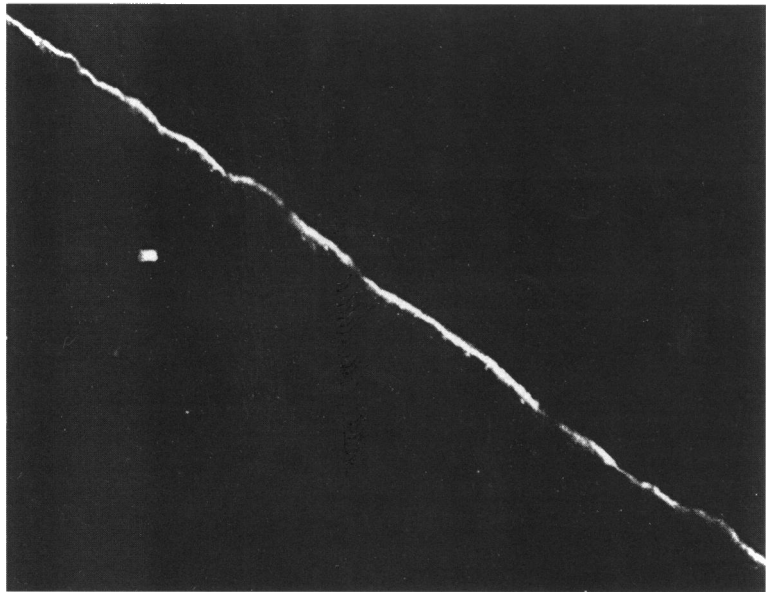

FIGURE 6D
Rabbit cornea, immunohistochemical staining for beta 4 integrin, 9.0 J/cm^2. Control cornea shows continuous stain pattern (x 75).

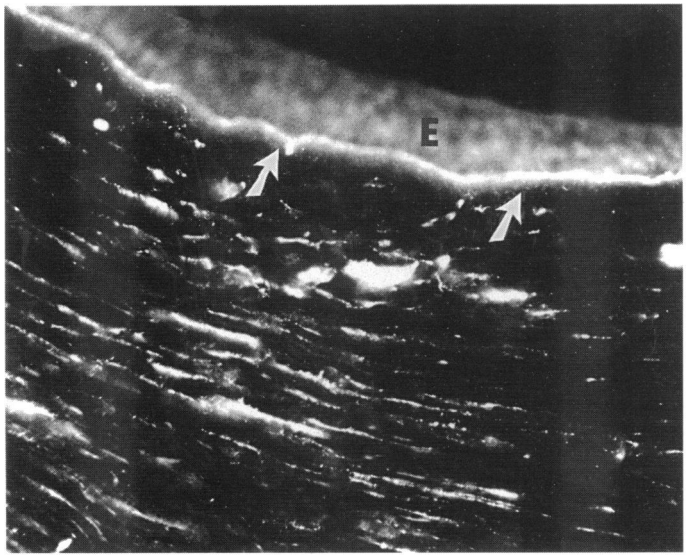

FIGURE 7A

Rabbit cornea, immunohistochemical staining for type IV collagen, 9.0 J/cm². At 3 weeks. staining for type IV collagen was detected in basement membrane (arrows) as thick linear stain, and in stroma, stain was distributed in lamellar pattern (E: epithelium) (x 75).

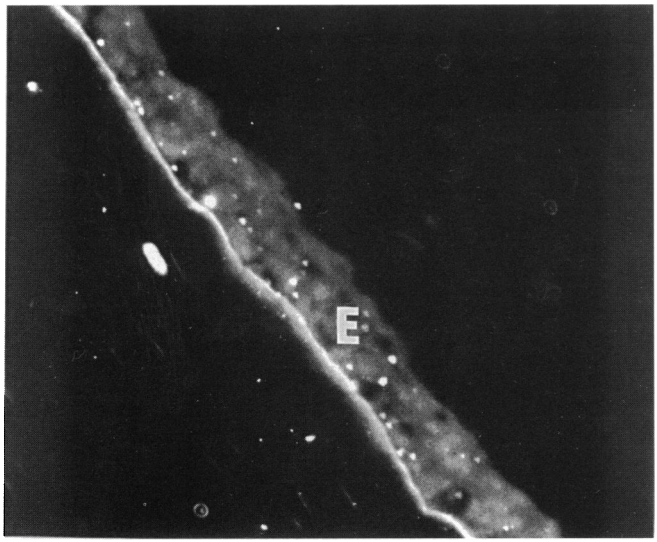

FIGURE 7B

Rabbit cornea, immunohistochemical staining for type IV collagen, 9.0 J/cm². Control corneas showed type IV collagen staining as linear stain in basement membrane (E: epithelium) (x 75).

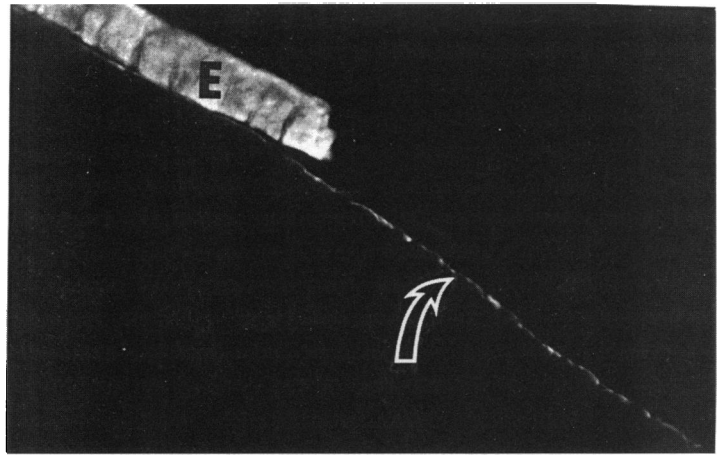

FIGURE 8A

Rabbit cornea, immunohistochemical staining for type VII collagen, 10.9 J/cm². Twenty-four hours after laser treatment, faint staining pattern (arrow) can be seen along bare anterior stroma (E: epithelium) (x 75).

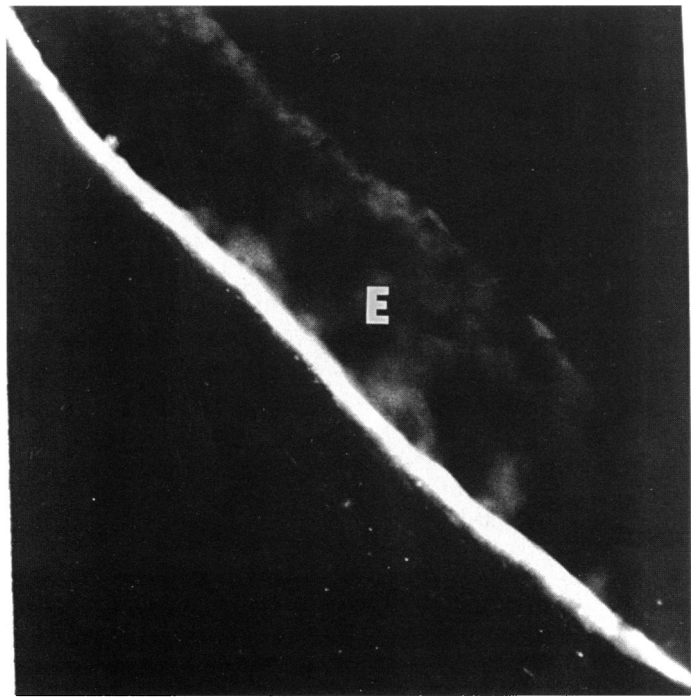

FIGURE 8B

Rabbit cornea, immunohistochemical staining for type VII collagen, 10.9 J/cm². Strong continuous staining can be seen at 3 months (E: epithelium) (x 75).

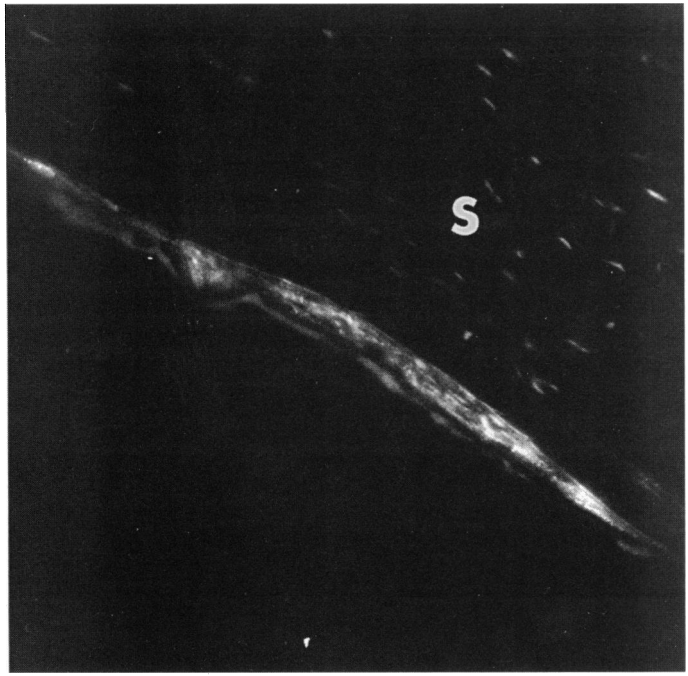

FIGURE 8C
Rabbit cornea, immunohistochemical staining for type VII collagen, 10.9 J/cm². At 3 months, there is some staining for type VII collagen in retrocorneal membrane (S: stroma) (x 150).

months after treatment, procollagen staining was still present, although somewhat less intense than seen in the earlier samples (Fig 9B).

Keratan sulfate proteoglycan is a normal constituent of the cornea, and the antibody used in these experiments evenly stained throughout the entire depth of the normal cornea. Immediately after laser treatment, the response to this antibody was lost, indicating alteration of this proteoglycan. At the lower F_p of 9.0 J/cm², staining began to reappear within 24 hours. As already noted, the 1-week specimen was lost. By 3 weeks, stain was increased in the 9.0-J/cm² specimen (Fig 10A) and appeared in the stromal regions treated at 10.9 J/cm². Staining for keratan sulfate increased during the period tested, but even after 3 months of healing, did not reach the intensity seen in the normal corneas (Fig 10B).

Staining for type VI collagen was seen throughout the normal rabbit stroma. There was no staining immediately and at 24 hours after treatment. In the 9.0-J/cm² specimen, there was also no staining at 3 weeks, and the 2- and 3-month specimens had only mild staining that was markedly reduced compared with the control cornea. In the 10.9-J/cm² specimen,

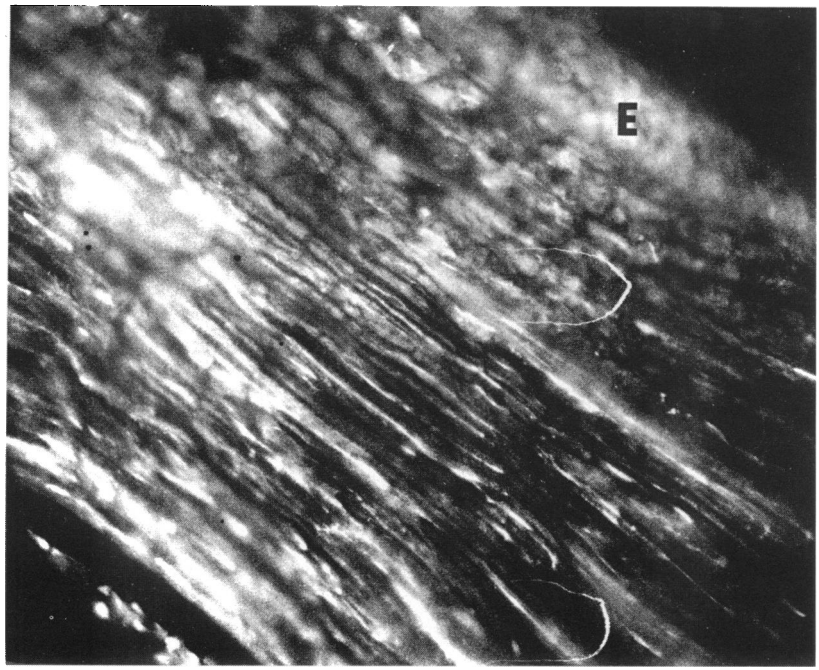

FIGURE 9A
Rabbit cornea, immunohistochemical staining for procollagen type I, 9.0 J/cm². At 3 weeks procollagen staining was widely distributed throughout treated area, indicating keratocyte activation and collagen synthesis (E: epithelium) (x 75).

FIGURE 9B
Rabbit cornea, immunohistochemical staining for procollagen type I, 9.0 J/cm². Three months after treatment, distribution had markedly decreased (x 150).

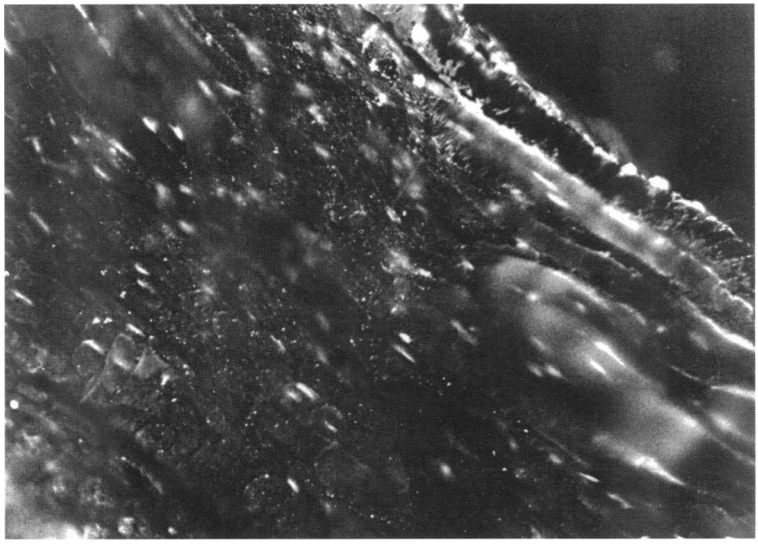

FIGURE 10A
Rabbit cornea, immunohistochemical staining for keratan sulfate, 9.0 J/cm^2. Three weeks after laser treatment, stain distribution for keratan sulfate is still markedly diminished (x 75).

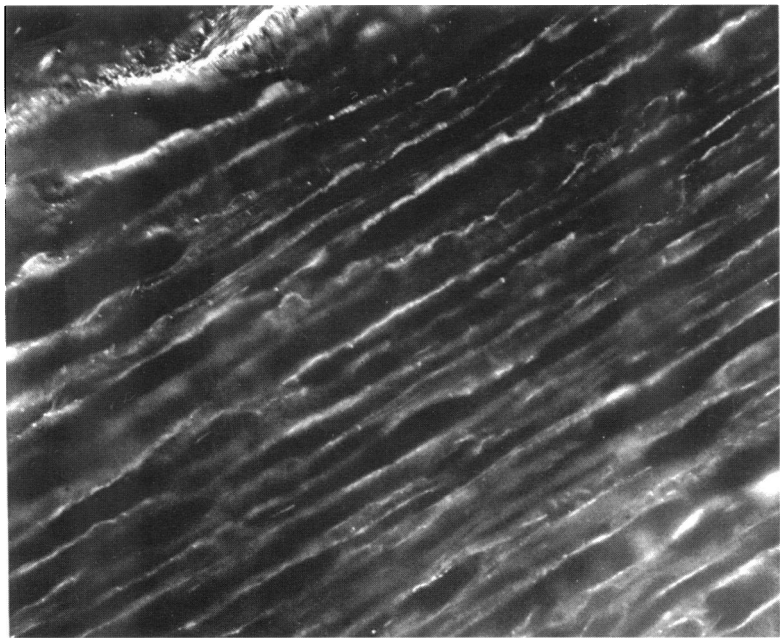

FIGURE 10B
Rabbit cornea, immunohistochemical staining for keratan sulfate, 9.0 J/cm^2. Control cornea shows even distribution of keratan sulfate through entire depth of cornea (x 75).

there was focal staining in the mid-stroma at 3 weeks; by 2 and 3 months there was diffuse stromal staining that was still reduced in intensity compared with the control.

5-PULSE STUDY

Light and Transmission Electron Microscopy

Immediately after wounding a rabbit cornea with a burr, LM observations (Fig 11) confirmed loss of corneal epithelium in the area of the wound and slight stromal swelling compared with normal cornea. TEM showed no disruption of the underlying stroma. At 1 week following the burr injury, re-epithelialization over the site of the wound had occurred. LM and TEM observations showed no stromal histopathologies in this cornea. At 12 weeks following the burr wound, no epithelial or stromal structural abnormalities were observed at the site of the injury by LM and by TEM.

Unless otherwise specified, the LM and TEM micrographs demonstrated similar changes for the Ho:YAG laser treatments at 9.9 J/cm^2 and

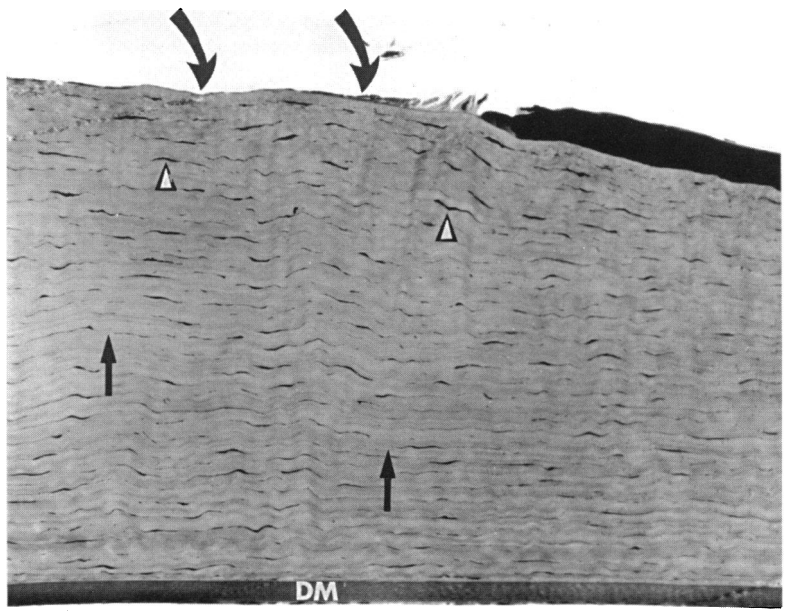

FIGURE 11

Rabbit cornea, burr wound. Corneal epithelium at site of burr wound has been removed to level of basement membrane (curved arrows), which appears to be intact. Corneal stroma under burr injury appears slightly swollen compared with nontreated control cornea. Stroma contains well-organized lamellae of collagen (arrows) and numerous keratocytes (arrowheads). Descemet's membrane (DM) appears to be intact and is continuous under wounded and adjacent nonwounded areas (light microscopy, x 37.5).

10.7 J/cm^2. Immediately after treatment, epithelial damage and stromal swelling were seen at the site of the laser treatment (Fig 12A). Stromal swelling was greatest anteriorly and tapered posteriorly. By TEM, stromal changes included disruption of collagen lamellae, loss of definition of collagen fibrils, and damaged keratocytes with fragmented nuclei and cytoplasm (Fig 12, B and C). Laser-induced changes extended primarily to mid-stroma; TEM showed little or no disturbance of the posterior stroma (Fig 12, C and D).

One week after Ho:YAG laser treatment, LM showed that re-epithelialization had occurred. The epithelium formed a continuous layer over the area of the wound, with no evidence of hyperplasia. Stromal swelling

FIGURE 12A

Rabbit cornea, 10.7 J/cm^2, immediately after laser thermal keratoplasty. Micrograph suggests that only anterior portion of stroma is distorted by swelling (long curved arrows). Corneal epithelium (arrowheads) is damaged over this area and forms interface with undamaged adjacent cells (arrows). Descemet's membrane (short curved arrows) is intact and continuous under site of laser wound (light microscopy, x 37.5).

FIGURE 12B
Rabbit cornea, 10.7 J/cm², immediately after laser thermal keratoplasty. Anterior corneal stroma is disrupted with loss of definition of collagen fibers (arrows). Fragmented, damaged keratocyte is present (arrowheads) (transmission electron microscopy, x 9,600).

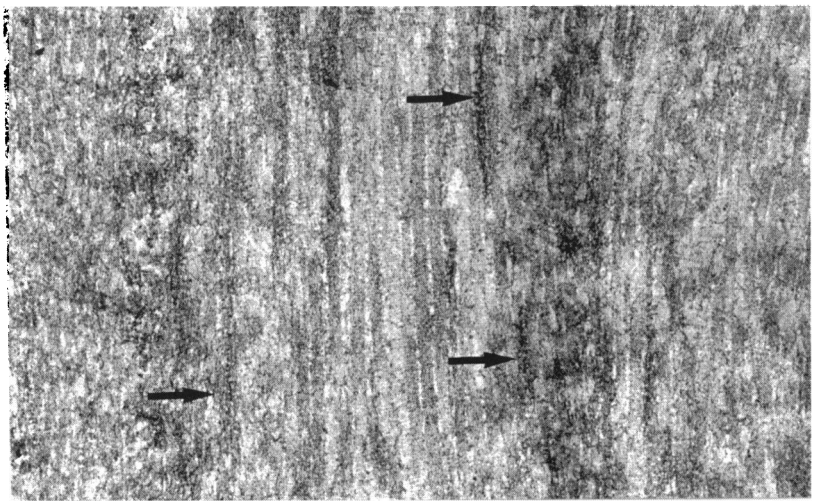

FIGURE 12C
Rabbit cornea, 10.7 J/cm², immediately after laser thermal keratoplasty. Collagen alterations are confirmed at higher magnifications, where loss of fiber definition is more prominent (arrows) (transmission electron microscopy, x 48,000).

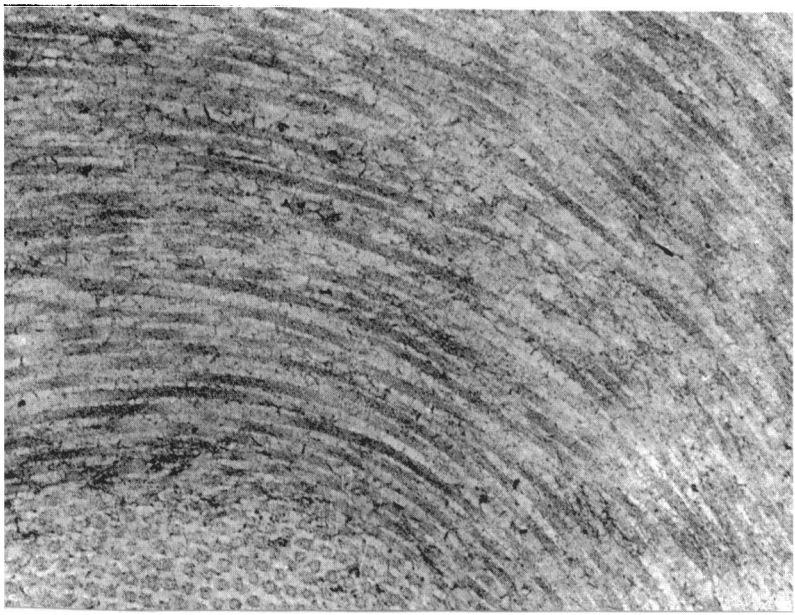

FIGURE 12D
Rabbit cornea, 10.7 J/cm², immediately after laser thermal keratoplasty. Stroma in the posterior portion of cornea under area of laser treatment shows no disruption to lamellae, which contain normal intact banded collagen fibers (transmission electron microscopy, x 48,000).

was diminished compared with the corneas immediately following laser treatment. LM showed increase in keratocyte density at the wound margins, whereas the central regions showed loss of keratocytes (Fig 13A). By TEM, keratocytes in the treatment area demonstrated numerous mitochondria and rough, endoplasmic reticulum, indicating keratocyte activation (Fig 13B). In the anterior stroma, disorganized collagen fibers were still evident. Retrocorneal membranes were observed below the area of stromal damage between Descemet's membrane and the endothelium.

At 12 weeks following Ho:YAG laser treatment, areas of epithelial hyperplasia and stromal thinning were evident, particularly in the 10.7-J/cm² specimen. Activated keratocytes were still present in the area of stromal damage, suggesting that remodeling and collagen synthesis were occurring (Fig 14A). This was confirmed by TEM, which showed extensive stromal remodeling characterized by the presence of well-ordered deposition of collagen bundles in these areas (Fig 14B). Retrocorneal membranes were present in these corneas under the wound areas, between Descemet's membrane and the corneal endothelium.

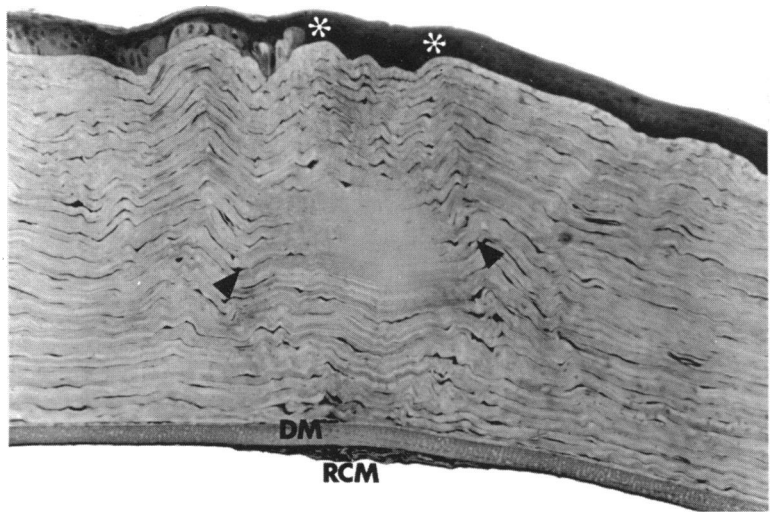

FIGURE 13A

Rabbit cornea, 10.7 J/cm², 1 week after laser thermal keratoplasty 10.7 J/cm². Damaged corneal epithelium has been replaced over area of laser wound (*) to form continuous layer. Keratocytes appear to be absent from central region of laser-treated corneal stroma. Activated keratocytes (arrowheads) can be seen at wound margin between damaged and non-wounded stroma and also above Descemet's membrane (DM) in area of retrocorneal membrane (RCM) (light microscopy, x 37.5).

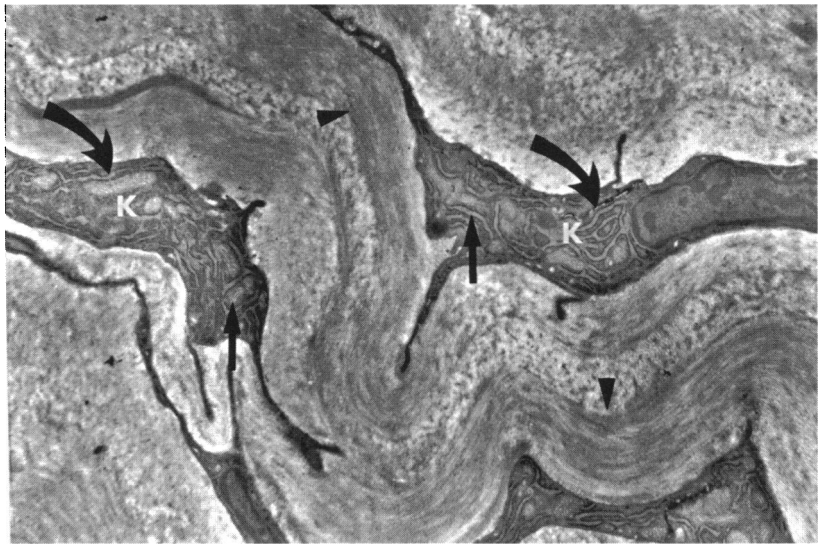

FIGURE 13B

Rabbit cornea, 9.0 J/cm², 1 week after laser thermal keratoplasty. Activated keratocytes (K) are present in anterior stroma, interspersed between bundles of collagen fibers (arrowheads); they contain extensive rough endoplasmic reticulum (curved arrows) and numerous mitochondria (arrows) (transmission electron microscopy, x 9,600).

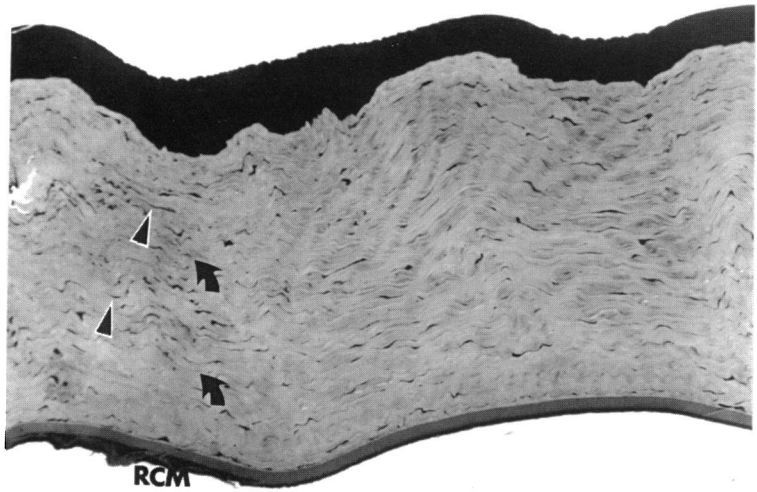

FIGURE 14A

Rabbit cornea, 10.7 J/cm^2, 12 weeks after laser thermal keratoplasty. There is epithelial hyperplasia over laser-treated region. Collagen bundles in treated stroma (curved arrows) appear to be substantially remodeled, with little evidence of stromal damage or swelling. Activated keratocytes (arrowheads) are present in this area and above Descemet's membrane at site of retrocorneal membrane (RCM) (light microscopy, x 37.5).

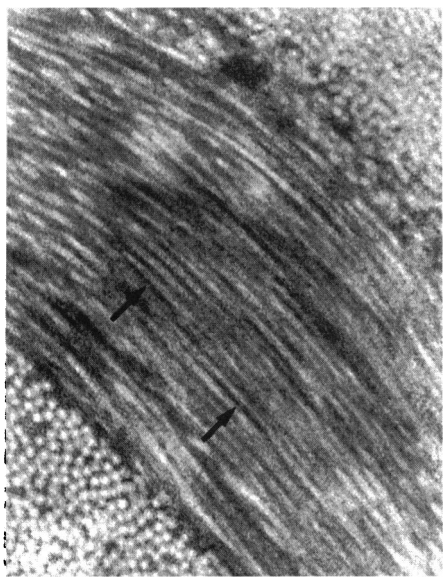

FIGURE 14B

Rabbit cornea, 10.7 J/cm^2, 12 weeks after laser thermal keratoplasty. In laser-treated stroma, collagen fibers are well organized (arrows) (transmission electron microscopy, x 48,000).

Immunohistochemistry (Table III)
Staining for procollagen was not observed in the control unwounded rabbit corneal stroma. In corneas receiving a burr wound, some procollagen staining was seen immediately (Fig 15A) but not at 1 week nor at 12 weeks.

Staining for procollagen was absent immediately following Ho:YAG laser treatment (Fig 15B). At 1 week following laser treatment, procollagen staining was observed at the stromal wound margin, in the area of damaged stroma, and occasionally in the adjacent untreated stromal tissue (Fig 15C). At 12 weeks, procollagen staining was slightly reduced compared with the corneas at 1 week (Fig 15D).

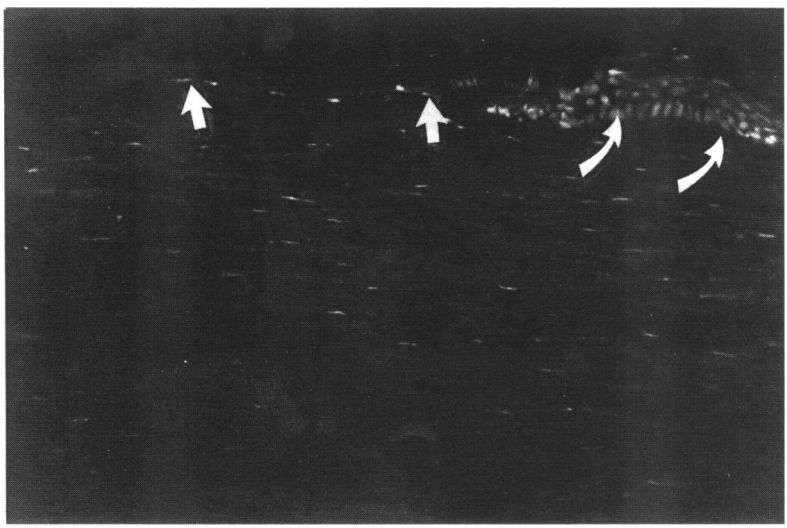

FIGURE 15A
Rabbit cornea, immunohistochemical staining for procollagen type I. Burr wound, immediately after treatment. Staining for procollagen occurs under and adjacent to epithelial burr injury (arrow) but not in surrounding stroma. Fluorescent signal seen in adjacent intact corneal epithelium may be due to autofluorescence (curved arrows) (x 40).

The primary antibody for keratan sulfate stained evenly throughout the stroma in the untreated cornea (Fig 16A). Normal staining was also observed in corneas with the burr wound immediately and at 1 and 12 weeks after LTK.

Immediately after laser treatment, staining for keratan sulfate was reduced in the wound area, indicating alteration in either the chemistry or the configuration of the keratan sulfate proteoglycan (Fig 16B). One week following laser treatment, keratan sulfate staining remained decreased in

TABLE III: 5-PULSE STUDY: IMMUNOHISTOCHEMICAL STAINING OF RABBIT CORNEAS

COMPONENT		IMMEDIATE	1 WK	12 WK
Procollagen:	Control	0	0	0
	Burr	↑	0	0
	LTK	0	↑↑	↑
Keratan Sulfate:	Control	✔	✔	✔
	Burr	✔	✔	✔
	LTK	0	↓	✔

0, absent; ↓, below normal; ✔, present at normal or near normal levels; ↑, above normal; ↑↑, markedly elevated; LTK, laser thermal keratoplasty; NA, data not available.

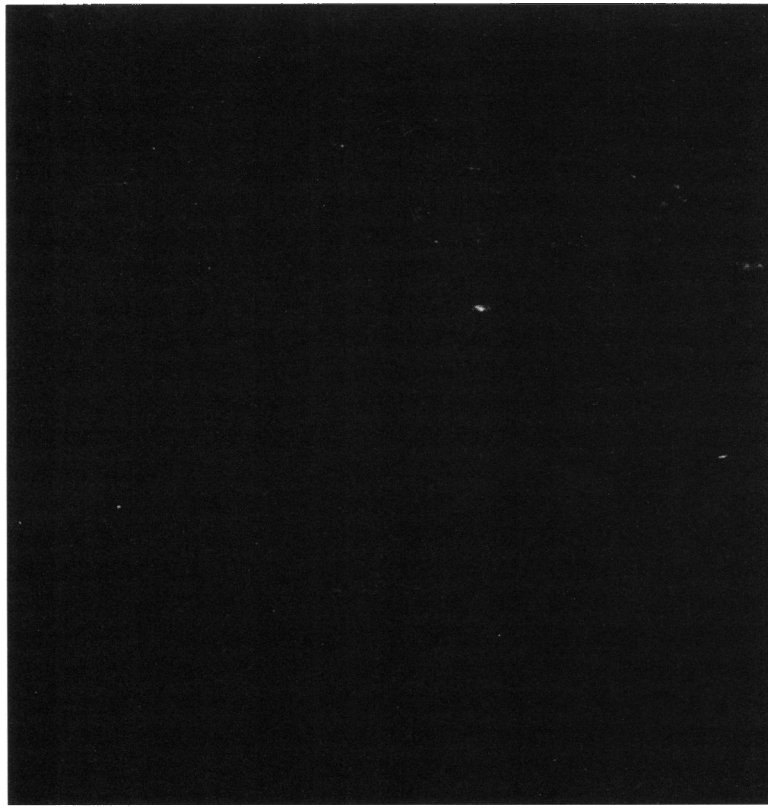

FIGURE 15B
Rabbit cornea, immunohistochemical staining for procollagen type I, 9.9 J/cm², immediately after laser thermal keratoplasty. Procollagen staining is not present in area of stromal wound (x 40).

the area of the stromal treatment (Fig 16C). By 12 weeks following laser treatment, keratan sulfate staining had increased in the treated areas and was comparable to staining in the control and adjacent untreated stroma (Fig 16D).

DISCUSSION

This study demonstrates that Ho:YAG LTK induces a broad spectrum of histologic changes in human and rabbit corneas and stimulates a moderate wound healing response in rabbit corneas. While some of these changes are desirable (*ie*, necessary to produce a refractive change), others may contribute to postoperative regression. The results of each study are initially discussed separately.

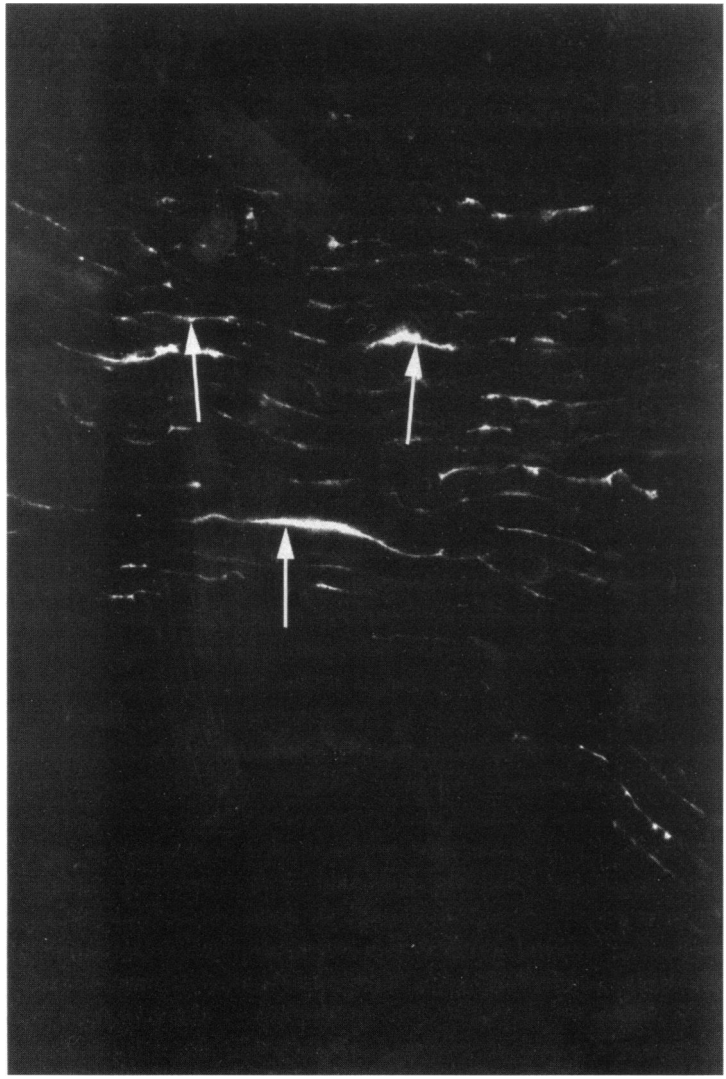

FIGURE 15C

Rabbit cornea, immunohistochemical staining for procollagen type I, 10.7 J/cm², 1 week after laser thermal keratoplasty. Keratocytes staining for procollagen (arrows) are present in anterior region of laser-treated stroma and absent from posterior stroma (x 40).

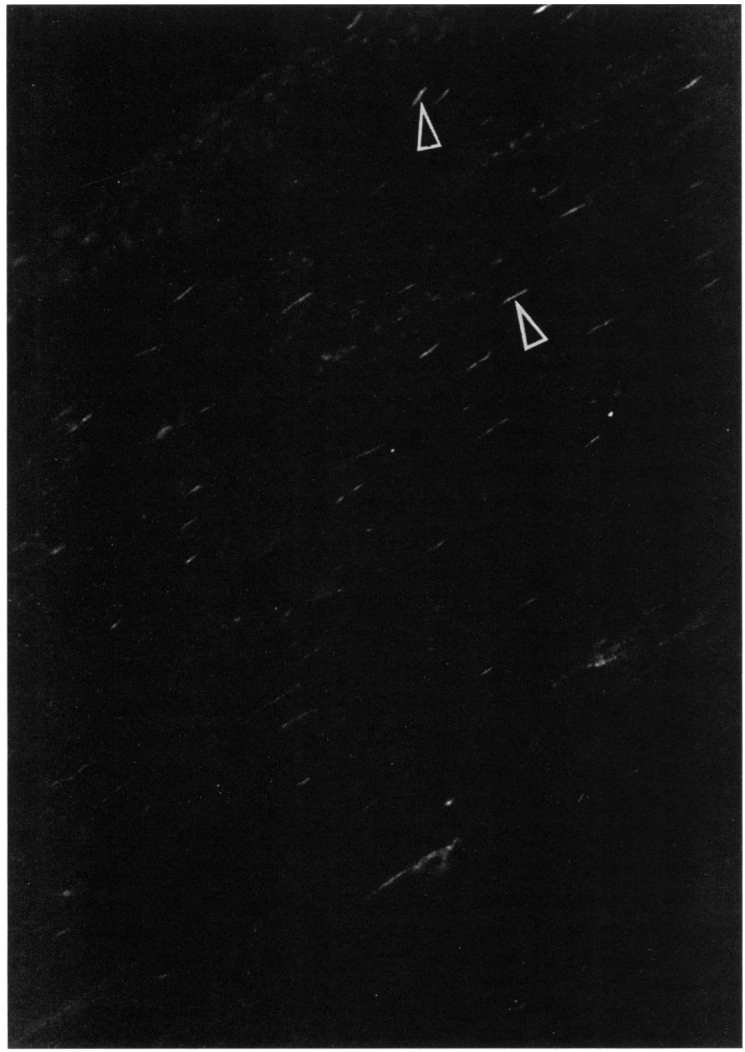

FIGURE 15D

Rabbit cornea, immunohistochemical staining for procollagen type I, 9.9 J/cm^2, 12 weeks after laser thermal keratoplasty. Procollagen staining is present in keratocytes throughout area of laser-treated stroma (arrowheads) but not in adjacent unwounded stroma (not shown) (x 40).

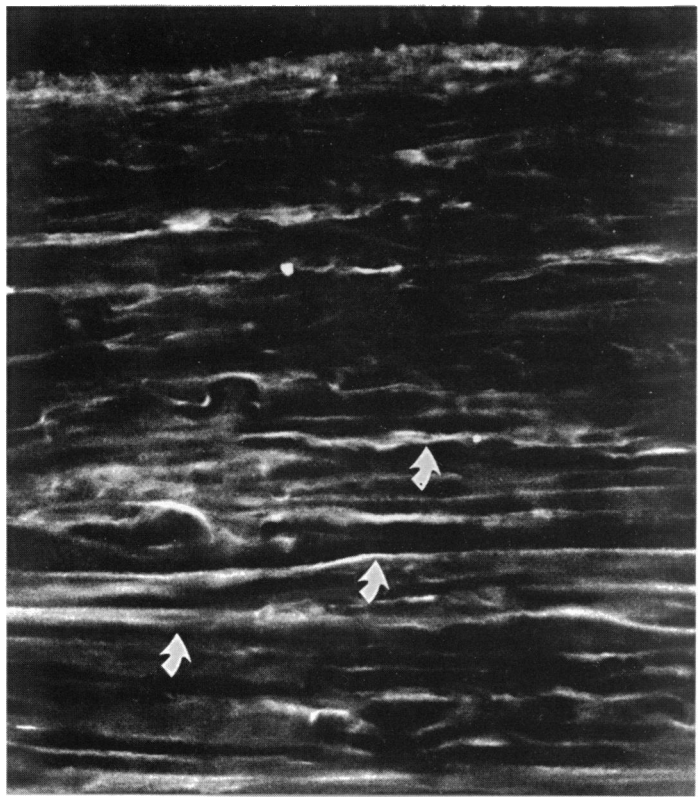

FIGURE 16A
Rabbit cornea, immunohistochemical staining for keratan sulfate, control cornea. Keratan sulfate is evenly distributed (arrows) throughout matrix of normal, rabbit corneal stroma (x 40).

10-PULSE STUDY

Human Corneas

In the human corneas examined 1 day following treatment, there was evidence of marked epithelial and basement membrane injury. This finding was anticipated, since clinically the treatment induces immediate epithelial opacification, and some patients have epithelial defects in some of the treated zones 1 day later.[36,37] At low-pulse radiant exposures (F_p =7.7 J/cm^2), Bowman's layer was unaffected; marked changes were present only at the highest F_p levels. Anterior stromal lamellae were disorganized, and the degree of disorganization and amount of posterior extension increased with increasing F_p values. Likewise, increasing amounts of keratocyte cell death were noted with increasing energies, again extending progressively

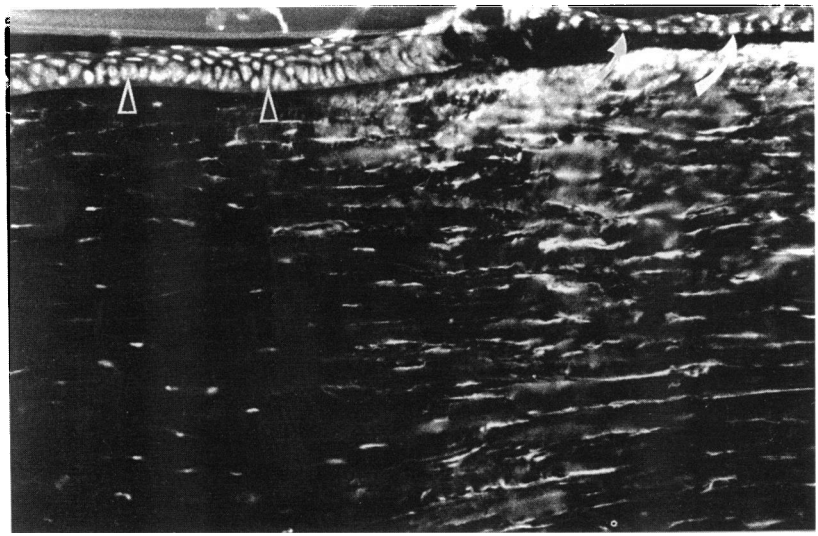

FIGURE 16B
Rabbit cornea, immunohistochemical staining for keratan sulfate, 10.7 J/cm², immediately after laser thermal keratoplasty. Keratan sulfate staining is absent in area of laser treatment but is retained in adjacent nontreated stromal matrix. There is intense autofluorescence of nuclei of damaged epithelium over area of laser treatment (arrowheads) and adjacent undamaged epithelium (curved arrows) (x 40).

deeper into the posterior stroma. Finally, endothelial injury was evident at all but the lowest energy levels.

Rabbit Corneas

The acute histopathologic changes in the rabbit corneas were generally similar to those seen in the human tissue. In the human cornea there was swelling in the posterior region only, whereas the rabbit corneas had anterior and posterior stromal swelling, perhaps owing to the absence of Bowman's layer. Greater amounts of posterior stromal and endothelial injury were noted in the rabbit corneas, no doubt in part because the rabbit cornea is approximately 350 μm thick centrally, compared with 650 to 700 μm in the Fuchs' dystrophy corneas used in this study. Other species-specific structural variations, such as the absence of Bowman's layer in the rabbit, might have contributed to the more severe endothelial response to laser treatment seen in the rabbit.

In the rabbit, the initial phase of epithelial recovery from the laser treatment included the invasion of inflammatory cells. By 3 weeks the epithelium was hyperplastic, and by 2 months TEM showed relatively nor-

FIGURE 16C
Rabbit cornea, immunohistochemical staining for keratan sulfate, 10.7 J/cm², 1 week after laser thermal keratoplasty. Staining for keratan sulfate (arrows) is still diminished in stroma in area of laser wound compared with adjacent, posterior stroma (x 40).

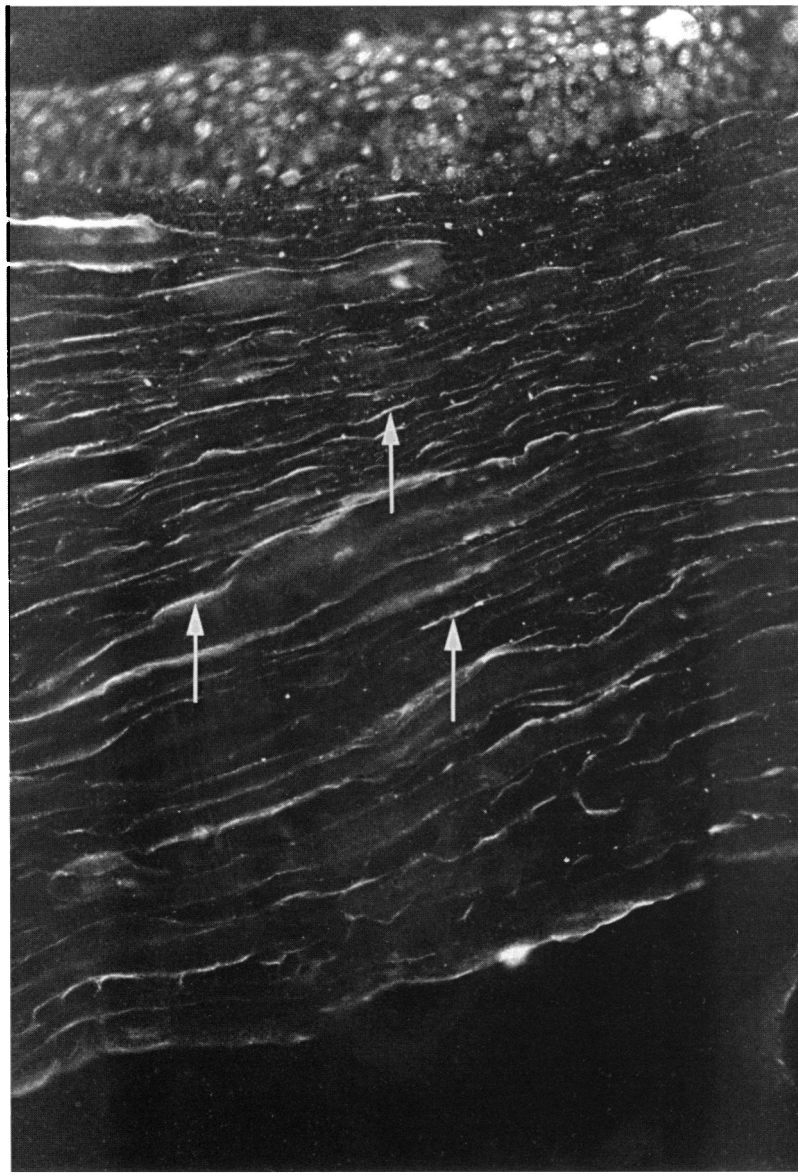

FIGURE 16D

Rabbit cornea, immunohistochemical staining for keratan sulfate, 9.9 J/cm^2, 12 weeks after laser thermal keratoplasty. Staining for keratan sulfate (arrows) is present throughout area of laser-treated stroma and is similar to that seen in adjacent nontreated stroma. Nuclear staining of corneal epithelium is probably autofluorescence (x 40).

mal basement membrane. Immunohistochemical staining for hemidesmosomes (beta 4 integrin), basement membrane (type IV collagen), and anchoring fibrils (type VII collagen) showed gradual restoration to normal levels by 2 months, apparently by the same process of healing that is seen in human and rabbit corneas after epithelial removal by abrasion,[55,56] keratectomy,[61] or excimer laser photorefractive keratectomy (PRK).[53,62-64] In this process, separated segments of basement membrane and attachment complexes are first formed under the new epithelium, and then, as healing progresses, are filled in with more segments, forming a continuous linear distribution of the components.[56] However, even at 3 months, the staining patterns were irregular, indicating that normal morphology was not fully restored. This could predispose to recurrent epithelial erosions in the treatment sites, which to my knowledge have not been reported in any Ho:YAG LTK clinical trials to date.

The laser treatment produced marked acute stromal changes in the rabbit corneas, as indicated by modified tissue staining and stromal edema. Factors contributing to the edema could include breakdown of the epithelial barrier, chemical alterations of the stromal proteoglycans and collagens, and localized endothelial dysfunction. TEM showed substantial changes in the ultrastructure of the collagen fibers and the lamellae.

By 3 weeks, stromal thinning had replaced stromal swelling. This change is presumably responsible for at least a portion of the refractive change produced by LTK. For correction of hyperopia, local stromal thinning and associated collagen fiber shrinkage in the mid-periphery increase the slope from the center to these mid-peripheral points, thereby increasing corneal curvature. This effect is smoothed (and possibly blunted) by epithelial hyperplasia over the treatment site, which extends centrally.

Acutely, the treatment appeared to have damaged the keratocytes, but by 1 week they were replaced by activated keratocytes synthesizing new collagen, as indicated by staining for procollagen.[57] Increased keratocytic activity was still evident at 3 months. This was an expected response to the laser energy. Similar changes can be seen following excimer laser PRK.[62,64,65]

The loss of staining for keratan sulfate and type VI collagen was consistent with previous findings associated with healing stroma,[58,59,62,64,66,67] although Malley and coworkers[66] noted normal levels of Type VI collagen in monkey corneas following excimer laser PRK and mechanical keratectomy. The initial loss was probably due to chemical modifications of existing molecules. Recovery of staining was presumably due to synthesis of new molecules by activated keratocytes, as has been noted for type VI collagen in rabbit corneas following wounding[68] and for keratan sulfate in

monkey corneas following excimer laser PRK.[62] The appearance of type IV collagen throughout the treated stroma has been seen in keratoconus[69] and in excimer laser–treated human corneas during wound healing.[67] This suggests that keratocytes share with epithelial and endothelial cells the capability of synthesizing type IV collagen.

There was also a marked endothelial proliferative response in the rabbit corneas. Interestingly, type VII staining was seen in the retrocorneal membrane, suggesting an epithelial origin of these cells. Considering the human endothelial response to other forms of injury (eg, cataract surgery), it is highly unlikely that retrocorneal membranes will form in humans, although endothelial cell injury was present at the base of the treated zones in sites receiving high radiant exposures. If all endothelial cells were lost in the treated regions using an 8-spot pattern with a 0.6-mm spot diameter, this would represent at most a 1.6% endothelial cell loss. The zone of cell loss is likely much smaller, considering the tapering conical shape of the induced stromal change.

5-PULSE STUDY

Rabbit Corneas

As anticipated, we observed in the 5-pulse LTK corneas similar but less extensive morphologic changes than were seen following the 10-pulse treatments. The epithelium was again acutely injured by the laser treatment, and there was complete re-epithelialization by 1 week. As with the 10-pulse corneas, however, there was evidence of epithelial hyperplasia at 12 weeks.

Corneal stromal damage included swelling, keratocyte injury, and damage to collagen fibers, most prominent anteriorly and tapering posteriorly. Unlike the corneas treated with 10 pulses, the 5-pulse specimens showed little or no evidence of posterior stromal injury. Activated keratocytes were present 1 and 12 weeks following laser treatment. The collagen fibers appeared to be repaired by 12 weeks.

Keratocyte activation in the laser-treated regions of the stroma was confirmed by the presence of procollagen staining. The presence of activated keratocytes at 1 and 12 weeks following laser treatment suggests that stromal remodeling and wound healing were ongoing throughout this time. The immunohistochemical staining indicated that keratan sulfate proteoglycan was altered in the area of the stromal wound immediately after laser treatment. At 1 week, the reactivity of the antibody in the wounded stroma remained diminished compared with the surrounding unwounded stroma, indicating the presence of abnormal stromal matrix. In contrast to the 10-pulse corneas, there appeared to be more complete

restoration of normal staining by 12 weeks.

In contrast to the 10-pulse study, in which there was endothelial cell damage immediately following laser treatment, the corneas treated with 5 pulses had no evidence of acute endothelial injury. However, the presence of retrocorneal membranes at 1 and 12 weeks following laser treatment suggests that some stimulus to endothelial cell proliferation was produced, even though no cell damage was apparent.

Rabbit corneas receiving the burr injury showed initial loss of epithelial cells but were re-epithelialized by 7 days following wounding. Stromal changes consisted only of modest stromal swelling noted immediately after treatment.

The survival of keratocytes in the burr study was anticipated. The corneas in this study were immersed within 30 minutes into Optisol, where they remained for about 24 hours until fixated. Szerenyi and coworkers[70] showed that epithelial defects in rabbit corneas produce an almost immediate reduction in the density of anterior stromal keratocytes, which is followed by keratocytic hyperplasia and increased mitotic activity for up to 8 days. The mechanism of keratocyte loss was felt to be stromal edema and increased stromal pressure. Subsequent studies from the same laboratory demonstrated that the keratocyte dropout produced by epithelial cell defects can be prevented by administration of Optisol every 15 minutes[71] or by placing an Optisol-soaked collagen bandage lens on the cornea.[72] Our use of corneal storage media undoubtedly blunted the normal attrition of keratocytes produced by epithelial cell defects, both in the burr and LTK corneas.

The immediate appearance of procollagen after the burr injury suggests rapid keratocyte activation, which was possible because the burr wound did not appear to damage the stroma or its cellular elements and because of keratocyte preservation by the corneal storage media, as previously noted. By contrast, the absence of an immediate staining for procollagen in the LTK corneas suggests that the keratocytes were initially too damaged to produce new collagen; evidence for procollagen synthesis at 7 days indicates keratocyte recovery, migration of unwounded cells into the damaged areas, or both.

Staining for keratan sulfate remained unchanged in the stroma of corneas receiving the burr injury, confirming the absence of stromal damage in these corneas.

Several conclusions can be drawn from the 5-pulse study. First, it is evident that an active stromal wound healing response following LTK is produced by thermal stromal injury and does not require epithelial cell loss per se. There was evidence of new stromal collagen synthesis imme-

diately after the burr injury, but this had subsided by 1 week, which is in marked contrast to the delayed but prolonged new collagen synthesis seen following LTK. This study has not, however, eliminated the possibility that thermally damaged epithelium might enhance the magnitude or alter the time course of stromal wound healing. There are at least two differences between mechanical debridement and LTK-induced epithelial injury: (1) After debridement, presumably most damaged cells are removed, whereas thermally damaged epithelial cells remain in place until they slough or are replaced, and (2) it is conceivable that epithelial cytokines and growth factors released following thermal injury may vary quantitatively and/or qualitatively relative to those produced following mechanical epithelial abrasion. The 10-pulse study demonstrated that the epithelial attachment complex is incompletely healed at 3 months, so long-term epithelial stimulus of keratocytic activity cannot be ruled out. However, the marked stromal wound healing response following LTK relative to the negligible response to the burr injury suggests that the primary causative factor is direct stromal injury by the laser.

Second, this study indicates that the 5-pulse treatments produce shallower stromal changes and less endothelial cell injury than do 10-pulse treatments. The likelihood of producing endothelial cell injury in humans following 5-pulse LTK is therefore negligible, considering the over 50% greater thickness of the human cornea. It is unclear if the shallower stromal penetration produced by 5-pulse treatments will enhance or reduce the long-term refractive changes, relative to the 10-pulse treatment regimen. Clinical studies are required and indeed are under way to assess this. However, on the basis of the marked difference in histologic changes following 5- versus 10-pulse LTK, consideration should be given to eliminating the 10-pulse regimen from further clinical investigation, assuming comparable refractive efficacy.

Third, this study provides further evidence that regression following LTK may in part be attributable to epithelial smoothing or filling in of peripherally steepened regions of the cornea. This has not yet been demonstrated in human patients, but epithelial hyperplasia as a cause of regression has previously been seen following epikeratophakia[73] and excimer laser PRK.[74,75] If this does contribute to regression in humans, treatments could be designed to provide a broader, smoother transition between the central and peripheral cornea.

Finally, the 5-pulse study again suggests that LTK can stimulate brisk stromal wound healing with replacement of thermally altered collagen and glycosaminoglycans by newly synthesized molecules and that the impact of this response must be investigated further as a potentially major cause of postoperative regression.

OTHER RESEARCH

Two other published reports describe histologic findings following noncontact LTK using an Ho:YAG laser. Moreira and coworkers[70] treated rabbit corneas with a Ho:YAG laser with one spot, F_p values of 5 to 21 J/cm^2, and a spot size of 410 µm. They noted a range of tissue changes similar to those noted in the current study, although an anterior chamber inflammatory response was seen at extremely high F_p (25 J/cm^2).

Ren and coworkers[77] studied acute histologic findings on one monkey cornea. Treatment parameters, which differed markedly from those in the current study, included 8 spots in a 5-mm annulus, F_p = 8 J/cm^2, 25 pulses at 1 Hz, and spot size of 300 µm. The investigators noted damage to endothelial cells and denaturation of keratocytes within the treated area. They also treated five cat corneas with follow-up of 3 months. The treatment parameters included 8 spots in a 3-mm annulus, F_p = 15.6 J/cm^2, 1 pulse, and spot size of 300 µm. At 3 months they noted laser-induced stress lines and a region of altered stroma with increased keratocyte density, suggestive of ongoing wound healing. The histopathologic findings following 5- and 10-pulse LTK are obviously consistent with theirs, despite the differences in treatment parameters. I am unaware of any clinical studies performed with Moreira's or Ren's laser parameters.

I believe that it is important to recognize the possible limitations of the rabbit model in assessing the nature of the wound healing response in humans. Wound healing is presumably much more vigorous in rabbits, and qualitative as well as quantitative differences may exist.[78,79] Ideally, one should examine human tissue more than 24 hours following LTK, but this tissue is more difficult to obtain. Treatment of keratoplasty patients requires a central pattern of laser spots to avoid the graft-host junction; this reduces vision and thus is not likely to be tolerated for prolonged periods. In addition, the pool of suitable candidates is small, since advanced corneal pathology could alter or obscure the laser-induced changes. Human excimer laser specimens have been derived from two pools of patients not available to LTK: (1) corneas harvested at penetrating keratoplasty following failed excimer laser phototherapeutic keratectomy (PTK),[63,67,80-82] and (2) tissue scraped from the anterior surface of human corneas following either PRK or PTK.[83,84] It will likely take years to obtain a large number of human corneal LTK specimens, particularly those with long intervals between LTK treatment and accession for histopathological examination.

CONCLUSION

Treatment of the cornea with the Ho:YAG laser leads to acute structural changes and loss of certain tissue components. In the human cornea, these

changes extend to variable depths as a function of the treatment parameters. Marked epithelial and anterior stromal changes are produced, but there is minimal risk of clinically significant endothelial cell loss. In the rabbit, the thermally damaged components are replaced through healing processes similar to those observed after other types of corneal wounding. Previous studies of corneal wound healing have shown that healing continues over many months; differences from normal appearances have been seen in healing excimer laser PRK corneas for up to 18 months.[62,63,65,82] In the rabbit corneas in this study, the basement membrane, epithelial attachment complexes, stromal matrix, keratocytes, Descemet's membrane, and endothelium had not fully returned to normal after 3 months of healing, although the extent of injury, magnitude of response, and rate of recovery of tissue components suggest that 5-pulse treatments are gentler than 10-pulse treatments.

I believe that there are many unresolved questions that arise from this study:

1. Does thermal epithelial damage increase keratocyte injury and the subsequent wound healing response? As previously mentioned, two factors might be important: (1) keratocyte loss caused by the epithelial defect and (2) cytokines and growth factors released by thermally injured epithelial cells. If these factors are important, then the current delivery system might be suboptimal, since by necessity the greatest amount of heating occurs in the epithelium. Smithpeter and coworkers[85] have demonstrated that epithelial injury can be minimized or even prevented by using a continuous-wave Ho:YAG laser and a contact probe with a heat sink. Further studies of this delivery system are warranted.

2. Is the acute postoperative inflammatory response of sufficient magnitude to increase initial injury and the subsequent wound-healing response? Kenyon and coworkers[86] have shown that stromal ulceration in alkali-burned rabbit corneas can be prevented by blocking ingress of polymorphonuclear leukocytes. In the 10-pulse LTK-treated corneas, although inflammatory cells migrated into the treated epithelial regions, no surface ulceration was seen. Nevertheless, it is possible that hydrolytic enzymes diffused into the stroma, contributing to keratocyte injury and stromal matrix degradation. Reducing the inflammatory response might decrease the amount of acute injury and thereby enhance the stability of the thermally induced change. However, considering the relative minimal nature of the inflammatory response in the 10-pulse study and the absence of such a response in the 5-pulse study, it is unlikely that this is a clinically important factor in the corneal response to LTK.

3. Does epithelial hyperplasia contribute to postoperative regression?

This was clearly evident by 3 weeks in the rabbit corneas; to what extent does it occur in human corneas, and what is its refractive impact? Clinical studies of epithelial thickness following LTK are required to explore this issue. As previously mentioned, documentation of a role of epithelial hyperplasia in promoting regression would necessitate development of new treatment patterns, presumably designed to create larger transition zones between centrally steepened and peripherally unchanged regions.

4. How important is stromal wound healing in the regression of the desired refractive change? What is the nature, magnitude, and time course of stromal wound healing in the human cornea? The stromal thinning seen in the rabbit corneas by 3 weeks indicates that LTK produces persistent local contraction. New collagen and ground substance might replace or fill in interstices of the thermally modified collagen: either could alter curvature by thickening the stroma or by counteracting more distal effects that might have been produced by contraction of the thermally treated collagen. There is ongoing controversy regarding the nature of the wound healing response in human corneas following excimer laser PRK. Citing their own findings in evaluating tissue scraped from the surface of human PRK corneas, Corbett and coworkers[87] asserted that the human wound healing response following PRK consists largely of the synthesis of new glycosaminoglycans. On the other hand, animal PRK studies indicate that, as reported here following LTK, wound healing consists of synthesis of both new collagen and glycosaminoglycans[62,65,66,88-90] and that this new tissue is a form of corneal scar tissue that differs chemically and ultrastructurally from normal corneal tissue. Likewise, several studies have shown that new collagen is produced following PTK in human corneas;[67,80-82] however, a potential complicating factor in these cases is, of course, the effect of the underlying corneal pathology on the postoperative healing response. Ascertainment of the nature and impact of the wound healing response following LTK in humans is required before therapeutic strategies can be devised.

5. Is keratocyte cell death per se an important contributor to wound-healing effect, or is the postoperative keratocytic hyperplastic and synthetic response caused by other factors? If keratocyte death is a major contributor to postoperative wound healing, then treatment parameters must be devised to minimize or eliminate initial keratocyte injury. From the previously mentioned work of Stringer and Parr[5] and McCally and coworkers,[44-46] one can hypothesize that ideal LTK treatments should elevate corneal temperature to the narrow range of 60°C to 75°C: above the shrinkage temperature of corneal collagen and below the temperature that is lethal to keratocytes.

6. Do initial alterations in glycosaminoglycans and their subsequent turnover and replacement contribute to postoperative regression? What is the thermal damage threshold for glycosaminoglycan injury? Do by-products of thermally injured glycosaminoglycans stimulate keratocytic synthetic activity?

7. Is there a potential role for postoperative administration of corticosteroids or other inhibitors of wound healing? If human wound healing consists essentially of only glycosaminoglycan synthesis, then corticosteroids may provide little benefit.[87] If collagen synthesis is important, however, then corticosteroids or other inhibitors of collagen synthesis might aid in preserving postoperative refractive stability.[74,91-94]

Results of clinical studies of LTK to date suggest that it may be capable of correcting up to 2 D of hyperopia in presbyopic patients. Answering these questions offers new opportunities to enhance the procedure parameters and postoperative regimen to augment the induced refractive change, enhance the stability, and extend the applications of Ho: YAG LTK.

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