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Detection of Corneal Fibrosis by Imaging Second Harmonic–Generated Signals in Rabbit Corneas Treated with Mitomycin C after Excimer Laser Surface Ablation

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Abstract

Purpose—Recent studies have shown that confocal imaging of second harmonic–generated (SHG) signals can detect corneal collagen organization. The purpose of this study was to assess whether SHG signals can detect differences in corneal fibrosis after excimer laser surface ablation (photorefractive keratectomy [PRK]).

Methods—Rabbits received 9-D PRK in one eye followed by treatment with either mitomycin C (MMC) or vehicle. Corneal haze was measured by in vivo confocal microscopy before and 2, 4, 8, and 12 weeks after surgery. Animals were then killed and corneas were evaluated by visible and nonlinear confocal microscopy.

Results—PRK induced significant haze in vehicle-treated corneas that peaked at 2 weeks and remained elevated at 12 weeks after surgery. MMC treatment significantly ($P = 0.05$) reduced corneal haze at 2 weeks and was essentially normal by 12 weeks. Imaging of SHG signals in vehicle-treated eyes showed an anterior layer of collagen forming a honeycomb network blending into a dense mat of irregularly arranged collagen fibers that overlaid normal orthogonally arranged collagen lamellae. MMC treatment showed normal collagen organization at the surface. Fibrotic tissue was associated with a high cell density and alignment of intracellular actin filaments with collagen fiber bundles. In MMC-treated eyes, an anterior acellular zone overlaid a sparsely populated stroma containing isolated and enlarged keratocytes.

Conclusions—Imaging of SHG signals provides a sensitive means for detection of corneal fibrosis after surface ablation and can be used to assess the effects of antifibrotic therapy on corneal healing after refractive surgery.

Subepithelial corneal fibrosis or haze remains the primary deterrent to surface refractive laser treatment in patients with higher myopia. Haze formation after photorefractive

keratectomy (PRK) may be associated with variable degrees of visual compromise and less refractive predictability. On a molecular level, there is a production of disorganized collagen in the anterior stroma and an increase in cellular density that is responsible for haze formation.¹ Other than phakic intraocular lens,² PRK remains the only option for certain patients with higher myopia in whom the production of a LASIK flap may result in residual stromal bed thickness that is too low, risking mechanical instability and keratoectasia.

The use of confocal microscopy has allowed detailed study of in vivo stromal wound healing after PRK, including the role of myofibroblast generation in haze production.^{3–5} Various clinical factors, including depth of ablation, smoothness of stromal surface after ablation, epithelial removal techniques, and epithelial healing, likely contribute to the degree of haze formation.^{6–9} Moreover, various corneal medications and corneal wound healing modulators have been investigated for the treatment of haze.^{10–13} The use of prophylactic mitomycin C (MMC) in surface excimer ablation has gained significant popularity and has yielded consistent results in preventing haze.^{14–19}

MMC belongs to a family of antitumor quinolone antibiotics.²⁰ It functions as a powerful alkylating agent by inhibiting DNA synthesis.^{20,21} MMC is used as a potent chemotherapeutic agent by inhibiting the proliferation of any cell it enters in sufficient concentration and triggering apoptosis.^{22–24} On molecular and cellular levels, the primary mechanism of action of MMC in the cornea remains unclear. The predominant effect of MMC in haze prevention is suggested to be at the level of blocked replication of keratocytes and apoptosis of any myofibroblast progenitors in the anterior stroma.^{19,25–27} The long-term safety profile of MMC on the cornea, appropriate dosing, and exposure times are still under investigation. Morales et al.²⁸ suggest that there may even be a long-term endothelial cell dropout with the use of MMC.

SHG signals from tissues can be created when two near-infrared photons interact with collagen fibers to create a single visible light photon half the wavelength of the excitation light.^{29,30} With multiphoton confocal microscopy using femtosecond lasers, SHG imaging has recently been expanded to include studies of actinomycin and tubulin.³¹ Strong corneal SHG signals can be used to quantitatively determine the orientation of collagen fibrils with the polarization dependence of the SHG signal.^{32,33} With this technology, the three-dimensional collagen organization of the cornea^{34–37} and the effect of photoablation³⁸ and laser-induced optical breakdown³⁹ can be studied.

The purpose of our study was to determine whether SHG signal imaging microscopy could be used to study differences in corneal fibrosis and haze production induced by excimer surface ablation of rabbit corneas treated with and without MMC.

Methods

Rabbits

Ten New Zealand White rabbits were used in this study. Rabbits were anesthetized with intramuscular injection of xylazine (10 mg/kg; Phoenix Pharmaceutical, St. Joseph, MO) and ketamine hydrochloride (50 mg/kg; Phoenix Pharmaceutical) before in vivo confocal

microscopy and surgery. After the induction of anesthesia, the right eye of each rabbit was topically anesthetized with one drop of 0.5% proparacaine hydrochloride (Bausch & Lomb, Tampa, FL). At the end of the study, rabbits were humanely killed by intravenous injection of sodium pentobarbital (100 mg/kg; Abbott Laboratories, Chicago, IL). All procedures were approved by the University of California at Irvine Institutional Animal Care and Use Committee and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. One animal died in the vehicle-treated group following anesthesia after the 8-week examination.

Excimer Laser Photorefractive Surgery and Mitomycin C Treatment

Rabbits were randomly assigned to two groups of five rabbits each at the time of surgery to receive either 0.02% MMC or vehicle (phosphate-buffered saline, pH 7.4). Animals were then prepared for surgery and anesthetized, and an 8-mm diameter region covering the central cornea of the right eye was marked using an 8-mm trephine (Bausch & Lomb). The surface epithelium was then removed with the use of a corneal knife (Tooke; Bausch & Lomb). The rabbit was then placed under an excimer laser (LaddarVision; Alcon, Fort Worth, TX) and received a 6-mm diameter, 9-D spherical myopic photorefractive correction. Immediately after surgery, small slivers of surgical sponge (Bausch & Lomb) were soaked in 0.02% MMC or vehicle. Sponges were then placed on the corneal surface for exactly 10 seconds. The eye was then immediately rinsed with 5 mL PBS, and rabbits were placed on a pad and allowed to recover. After surgery, eyes received topical gentamicin sulfate (Akorn, Buffalo Grove, IL) three times a day for 3 days and intramuscular buprenorphine (Buprenex; 0.1 mg/kg; Reckitt Benckiser Healthcare, Hull, UK) twice daily for 3 days as needed for relief of pain.

In Vivo Confocal Microscopy

In vivo confocal microscopy was performed under anesthesia 1 week before surgery and 2, 4, 8, and 12 weeks after surgery. Corneal epithelial thickness, stromal thickness, and corneal haze were measured using a method previously described.⁴⁰ Briefly, in vivo confocal microscope examinations were performed using a tandem scanning confocal microscope (TSCM; Tandem Scanning Corporation, Reston, VA) with a 24× surface-contact objective (numerical aperture, 0.6; working distance, 1.5 mm), encoder mike controller (Oriol 18011; Oriol, Stratford, CT) for focal plane control, and a camera (MTI VE-1000; Dage MTI, Michigan City, IN). One drop of 2.5% hydromethyl-cellulose (Gonak, Akorn, Buffalo Grove, IL) was placed on the tip of the objective as a coupling gel. All camera settings were kept constant throughout the experiment. For each examination, repeated through-focus data sets were obtained from the central corneal region to identify the thinnest stromal region or center of the photoablated stromal surface. Three to five through-focus data sets were then collected around this region for measurement of epithelial thickness, stromal thickness, and haze using in vivo confocal microscopy software developed for these measurements.⁴¹

Ex Vivo Confocal Microscopy

Corneas were fixed immediately after euthanatization by anterior chamber perfusion with 2% paraformaldehyde in phosphate-buffered saline, pH 7.4. Corneas were removed and stored in fixative overnight. Corneal blocks (2 × 2 mm) were then cut from the central

region and stained en bloc with nucleic acid stain (Syto59; Molecular Probes, Eugene, OR) and phalloidin (Alexa Fluor 488 phalloidin; Molecular Probes) in 50% TD buffer (0.5% dimethyl sulfoxide, 0.5% Triton X, 2.5% dextran 40 in PBS, pH 7.4) overnight to identify nuclei and cell cytoskeleton, respectively. Specimens were placed on a microscope (Axiovert 200; Zeiss, Jena, Germany) and imaged with a laser confocal microscope system (LSM 510 META; Zeiss) equipped with a mode-locked titanium/sapphire laser (Chameleon; Coherent, Santa Clara, CA). The fluorescent signal detection from phalloidin and nucleic acid stain (Syto59; Molecular Probes) was obtained using the 488-nm and 633-nm laser lines of the argon and red helium-neon lasers, respectively. From the same optical plane, collagen organization of the corneas was visualized by second harmonic imaging microscopy. SHG signals to detect collagen have been used to identify the three-dimensional organization of the stromal collagen lamellae in humans, rabbits, and mice.³⁴ Briefly, SHG signals were generated using 800-nm infrared pulses to obtain maximum SHG emission signal at 400 nm. SHG forward-scattered signals passing through the tissue were collected using an 0.8-NA condenser lens with a narrow band-pass filter (400/50) placed in front of the transmission light detector. Backward-scattered SHG signals were detected using 370 to 440 nm band filter of the laser scanning microscope detector (LSM 510 META; Zeiss).

Statistical Analysis

From individual through-focus scans, average epithelial thickness, stromal thickness and haze measurement were calculated for each eye at each time point and were used for statistical analysis (Sigma Stat version 3.11; Systat Software Inc, Point Richmond, CA) with two-way repeated-measures ANOVA and Bonferroni multiple comparisons using a control group. The group average and SD were also calculated for each time and were used to plot changes (Sigma Plot version 9.01; Systat Software Inc.). We used a sample size of five eyes per group, with a power of 0.95 and $\alpha = 0.05$ to detect a 50% reduction in haze.

Results

Changes in Epithelial Thickness, Stromal Thickness, and Corneal Haze

At baseline, all corneas appeared normal and showed a scattering of light predominantly from the surface epithelium (Epi), the basal lamina, and the corneal endothelium (Endo) in *xz* reconstructions of in vivo confocal microscopy through-focusing data sets (Fig. 1A). Light scattering from the corneal stroma (Fig. 1A, Str-double-headed arrow) was localized in *xy* plane images (Fig. 1B) to occasional corneal nerves (arrowhead) and stromal cell nuclei (small arrows). Two weeks after PRK surgery, vehicle-treated eyes showed thinning of the corneal stroma (Fig. 1C, Str-double headed arrow) with marked light scattering at the stromal surface (arrowhead). Images through the light-scattering region detected a high density of stromal cells immediately below the corneal epithelium (Fig. 1D). In 0.02% MMC-treated corneas at 2 weeks, the stroma appeared slightly thinner than vehicle-treated corneas (Fig. 1E, Str-double-headed arrow), and light scattering from the stromal surface was greatly diminished (arrowhead). Images through the anterior stroma showed a diffuse light scattering from the stroma with occasional enlarged stromal cell nuclei (Fig. 1F, arrows). No changes in the corneal endothelium were detected (not shown).

In vivo confocal microscopy through focusing measurements of epithelial thickness, stromal thickness, and corneal haze are summarized in Table 1 and Figure 2. For epithelial thickness (Fig. 2A), vehicle-treated corneas showed a slight but not significant decrease 2 weeks after surgery, followed by a significant increase in thickness at 12 weeks, when the epithelium was $10\text{ }\mu\text{m}$ thicker than at baseline ($54 \pm 5\text{ }\mu\text{m}$ compared with $44 \pm 2\text{ }\mu\text{m}$; $P = 0.05$). MMC-treated corneas showed a significant increase of $15\text{ }\mu\text{m}$ in epithelial thickness as early as 2 weeks after surgery compared with baseline values and remained significantly thickened for the duration of the study ($P = 0.05$). As a group, the corneal epithelial thickness was significantly thicker in MMC-treated corneas than in vehicle-treated corneas after surgery ($P = 0.04$), particularly at week 2 and week 4 ($P = 0.05$).

For stromal thickness (Fig. 2B), both groups showed significant stromal thinning at 2 weeks after PRK that averaged $60\text{ }\mu\text{m}$ and $82\text{ }\mu\text{m}$ for vehicle- and MMC-treated corneas, respectively. Although MMC corneas appeared to show a greater amount of thinning, this difference was not significant ($P = 0.055$). For corneal haze, vehicle-treated corneas showed a significantly ($P = 0.05$) elevated level of light scattering that peaked at 2 weeks and remained significantly elevated until 12 weeks after PRK (Fig. 2C). By comparison, MMC-treated corneas showed significant light scattering 2 weeks and 4 weeks after PRK ($P = 0.05$) but were not significantly different from baseline at 8 and 12 weeks. As a group, the level of light scattering in MMC-treated corneas was significantly lower than in vehicle-treated corneas after PRK ($P = 0.001$).

Detection of Corneal Fibrosis by SHG Imaging

SHG imaging microscopy of vehicle-treated excimer laser photoablated corneas showed a highly irregular organization of collagen fibers immediately underlying the corneal epithelium (Fig. 3A). This irregular organization at the most superficial level of the stroma appeared to form a honeycomb-like scaffolding (arrows) that surrounded clusters of basal epithelial cells identified by phalloidin and nucleic acid stain (Syto59; Molecular Probes; Fig. 3B, arrows). These collagen scaffolds appeared to extend more deeply into the cornea and to merge with the underlying collagen to form thin, irregularly arranged collagen bundles (Fig. 3A, arrowheads). Below the epithelium, the thin collagen bundles formed a highly irregular and interwoven collagen network with no particular organizational pattern (Fig. 3C). Although the thickness of this collagen layer varied between samples, the random organizational pattern was a consistent and characteristic feature that allowed for accurate determination of the thickness of this layer. Below this region, normal-appearing stromal collagen lamellae with parallel bundles of collagen fibers were detected, with regions of more irregular collagen from the region above appearing to extend between some lamellae (Fig. 3D, asterisk). In MMC-treated corneas, the anterior stroma underlying the basal corneal epithelium appeared to contain disrupted normal corneal stromal collagen lamellae of varying thickness (Fig. 3E, arrow). Occasional lamellae appeared to end abruptly (asterisk) in regions immediately adjacent to the overlying corneal epithelium, as identified in the same optical plane by phalloidin and nucleic acid stain (Syto59; Molecular Probes; Fig. 3F, asterisk). Below this stromal-epithelial interface, the lamellar organization appeared to be normal and contained collagen fibers organized into broad lamellar bands (not shown).

Reconstruction of three-dimensional data sets along the xz plane showed that the irregular collagen organization in the vehicle-treated eyes formed a layer of fibrotic tissue above the unablated corneal stroma (Fig. 4A, top arrows). The average shrinkage-adjusted thickness of this region was $44.47 \pm 1.57 \mu\text{m}$ ($n = 3$), as measured from the basal epithelial cells to the interface between the irregular collagen network and the normal-appearing stromal lamellae. This region also appeared to contain a higher density of stromal cell nuclei detected in cross-section (Fig. 4B, top arrows) and in optical sections through the irregular collagen network (Figs. 4C, 4D, same optical plane). The number of nuclei in a single optical plane through this region averaged $686 \pm 104 \text{ cells/mm}^2$ ($n = 3$). MMC-treated corneas showed no irregular collagen above the stromal interface in three-dimensional reconstructions along the xz plane (Fig. 5A); however, immediately below the corneal epithelium, a region apparently devoid of stromal cell nuclei was detected (Fig. 5B, double-headed arrow). Optical sections through this region showed normal broad stromal lamellae with wide parallel bands of collagen fibers (Fig. 5C) and occasional stromal cells that appeared isolated, with enlarged nuclei (Fig. 5D, arrows), together with a few small cells, possibly macrophages (arrowheads). Although no fibrosis was detected in this experiment, disrupted collagen fiber organization at the epithelial stromal interface was detected and averaged $9.07 \pm 3.70 \mu\text{m}$ in thickness. The cell density in these regions was also markedly below that observed in the vehicle-treated corneas and averaged $62 \pm 10 \text{ cells/mm}^2$ ($n = 3$).

Although MMC-treated corneal wounds showed no evidence of myofibroblasts, vehicle-treated corneas showed occasional areas where fibroblasts with abundant actin filament staining were detected 3 months after PRK (Fig. 6). These regions were located below the corneal epithelium (Figs. 6A, 6D, arrowhead) and immediately overlying regions of corneal fibrosis (Figs. 6A, 6D, brackets). SHG optical section through these regions showed small bundles of collagen fibers extending out from the corneal stroma, under the basal epithelial cells (Fig. 6B, 6E, arrows). Interestingly, these collagen fibers appeared to coalign with the actin filament bundles (Figs. 6C, 6F, arrows). Similar coalignment of collagen fibers and actin filament bundles have been noted in anti-type I collagen-stained corneal wounds that also showed colocalization with fibronectin fibrils and $\alpha5\beta1$ integrin.³

Discussion

Previous investigators have imaged SHG signals microscopically to study the normal corneal collagen structure of humans and other species,^{34,36,37} to identify differences between normal corneal structure and keratoconic corneas,^{35,42} and to identify the dynamic effects of pressure on the collagen organization of the lamina cribrosa.⁴³ In this article, we report for the first time the microscopic imaging of SHG signals obtained from regions of subepithelial fibrosis induced by excimer laser surface ablation of the rabbit cornea. At the epithelial/stromal interface, SHG images identified a unique pattern of collagen fibers that were organized into thin bundles surrounding clusters of basal corneal epithelial cells and forming a honeycomb-like collagen scaffold. These thin collagen fiber bundles appeared to be continuous with deeper collagen fiber bundles that formed a highly irregular, interwoven network of relatively thin collagen bundles. This pattern persisted deeply into the cornea to an average depth of $44.47 \mu\text{m}$, where there was an abrupt transition to a highly organized arrangement of collagen that formed wide bundles of orthogonally arranged fibers, similar to

what has been previously reported for normal rabbit corneal stroma.³⁴ Importantly, MMC treatment, which was shown to markedly inhibit corneal haze after PRK by in vivo confocal microscopy, blocked the formation of this irregular collagen network, indicating that this pattern was deposited by fibrotic healing of PRK wounds. Together these findings indicate that microscopic imaging of SHG signals provides a highly sensitive method for detecting and measuring corneal fibrosis and the potential effects of antifibrotics on the wound-healing response.

Collagen-generated SHG signals in tissues were first detected in 1996 by Guo et al.⁴⁴ and represent one of three types of multiphoton interactions that occur when high-energy, pulsed femtosecond laser beams are focused into biological tissues. A more familiar multiphoton interaction is two-photon-excited fluorescence (TPEF), which was first reported for the detection of fluorescence-labeled cells.⁴⁵ TPEF differs from single-photon fluorescence in that excitation is achieved with low-energy, near infrared light providing improved depth penetration of the excitation light into tissues with decreased photobleaching and cellular toxicity normally associated with higher energy UV and visible light excitation. TPEF has been used to noninvasively detect living cells in the intact cornea without previous labeling by exciting autofluorescent cytoplasmic molecules, such as NADH/FAD, which normally excite with ultraviolet light under single-photon conditions.^{46,47} A third interaction involves the absorption of multiple photons to sufficiently excite molecules to a very high energy state that leads to laser-induced optical breakdown (LIOB).⁴⁸ LIOB creates very small diameter (<10 μm) cavitation bubbles with minimal collateral damage that has been used in the cornea to generate cleavage planes for resection of corneal tissue to form highly reproducible and uniform LASIK flaps and penetrating keratoplasty buttons.^{49,50} Most recently, SHG, TPEF, and LIOB have been used in living rabbits to image corneal collagen and stromal cells before and after cutting lamellar flaps, demonstrating the clinical feasibility of microsurgery at the cellular level.^{51,52} Based on our current findings, coupling microscopic imaging of SHG and TPEF signals with LIOB may provide a clinically useful approach to dissecting fibrotic membranes in the future.

It is clear that the use of femtosecond lasers to produce SHG signals has opened the door to detailed study of collagen organization in the eye under various conditions. In this study, a correlation between collagen organization and cellular behavior was identified by combining SHG imaging with visible confocal microscopy under ex vivo conditions. Specifically, regions of subepithelial fibrosis were associated with dramatic increases in the number of keratocytes and myofibroblasts in the anterior stroma. Interestingly, and as shown previously,³ coalignment of actin filament bundles with the collagen fibers was observed. These findings suggest that the collagen deposited by myofibroblasts is organized and aligned along intracellular actin filament bundles, presumably through cell matrix interactions involving integrin cell membrane receptors and extracellular matrix fibronectin. The irregular organization of collagen within fibrotic tissue may then be related to the random organization of myofibroblasts within the wound, as suggested earlier by Cintron et al.⁵³ in studies comparing embryologic stromal development to wound fibrosis and by Petroll et al.⁵⁴ in biomechanical studies of actin filament assembly in wound-healing

fibroblasts in situ. Future study of the spatial and temporal organization of collagen during wound healing may provide important insight into this process.

Another remarkable finding in the present study was the observation that MMC dramatically reduced by approximately 90% the number of stromal cells in the anterior cornea. These findings are consistent with earlier studies showing decreased keratocyte density after MMC treatment of PRK wounds in the rabbit.^{19,25} MMC is increasingly used as a modulator of corneal fibrosis after PRK. Clinically, MMC is a potent inhibitor of haze formation,^{16,55} making it a very attractive adjunct in the treatment of patients with high myopia, who are at highest risk for postoperative haze. The use of a potent alkylating agent such as MMC after PRK is thought to inhibit haze by preventing the regeneration of keratocytes and the possible formation of myofibroblasts within the wound.¹⁹ Although it is generally accepted that direct application of MMC induces apoptosis of keratocytes and myofibroblasts,^{17,19} the mechanism underlying the prolonged absence of keratocyte repopulation remains unknown. In the present study, visible confocal three-dimensional reconstructions of PRK wounds treated with MMC identified isolated, enlarged cells that occasionally appeared multinucleated. The appearance of these cells suggests sublethal effects of MMC that may underlie the lack of proliferative response noted for keratocytes after PRK and MMC treatment.¹⁹ Clearly, future studies must evaluate the dose effects of MMC on keratocyte proliferation and differentiation to corneal myofibroblasts.

In conclusion, SHG signal imaging is an exciting new technology with important applications to the study of collagen organization in the cornea during development, disease, and wound healing. Microscopic imaging of SHG provides a means to quantitatively measure corneal fibrosis and, therefore, can be used to objectively assess the effects of various antifibrotic therapies on wound healing. It also has potential in vivo applications because it is noninvasive and does not require staining or processing of tissue.

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Figure 1.

In vivo confocal microscopic images of rabbit corneas at baseline (**A, B**) and 2 weeks after PRK in eyes treated with vehicle (**C, D**) and 0.02% MMC (**E, F**). Images are taken from three-dimensional data and represent sections along the *xz* (**A, C, E**) and *xy* (**B, D, F**) planes. (**A, B**) Baseline light scattering is limited to the superficial corneal epithelium (Epi) and endothelium (Endo) with light scattering from the stroma (Str; *double-headed arrows*) localized to stromal nerves (*arrowhead*) and stromal cell nuclei (*arrows*). (**C, D**) PRK in vehicle-treated corneas lead to a reduction in stromal thickness (Str; *double-headed arrow*) and marked light scattering (*arrowhead*) in the anterior stroma that was associated with increased numbers of stromal cells. (**E, F**) MMC-treated corneas showed increased stromal thinning (Str; *double-headed arrow*), reduced anterior stromal light scattering (*arrowhead*), and enlarged stromal nuclei (*arrows*).

Figure 2.

Graph showing changes in epithelial thickness (A), stromal thickness (B), and corneal haze (C) in vehicle-treated (*closed circles*) and 0.02% MMC-treated (*open circles*) corneas. There were significant differences in the epithelial thickness and corneal haze between vehicle- and MMC-treated corneas. P values are based on two-way repeated-measures ANOVA. (*Significant difference [$P = 0.05$] between MMC and control based on Bonferroni multiple comparisons test.)

Figure 3.

Vehicle-treated (**A–D**) and 0.02% MMC-treated (**E, F**) corneas 3 months after PRK evaluated by SHG imaging of forward-scattered (*cyan*) and backward-scattered (*magenta*) signals (**A, C–E**). Tissues were also stained with FITC phalloidin (*green*) and nucleic acid stain (*red*) and were evaluated by confocal fluorescence microscopy (**B, F**). (**A, B**) Underlying the corneal epithelium, collagen bundles formed a honeycomb-like scaffold (**A**, *arrows*) that surrounded clusters of basal corneal epithelial cells (**B**, *arrows*). Collagen bundles also appeared to combine and extend more deeply into the anterior stroma (**A**, *arrowheads*). (**C, D**) Below the epithelium, collagen bundles formed an irregular, disorganized network (**C**) suggesting fibrotic tissue that overlaid and insinuated into the normal stromal lamellar pattern (**D**, *asterisk*). (**E, F**) The anterior stroma of MMC-treated corneas show disrupted but otherwise normal-appearing stromal lamellae (**E**, *arrows*) that in areas appeared to abruptly terminate (**E**, *asterisk*) at regions adjacent to the corneal epithelium (**F**, *asterisk*), suggesting transection during excimer photoablation.

Figure 4.

Vehicle-treated cornea evaluated by SHG imaging microscopy (**A, C**) of forward (*cyan*) and backward (*magenta*) signal combined and confocal fluorescence microscopy (**B, D**) showing FITC-phalloidin (*green*) and Syto59 (*red*) in the same optical plane. (**A, B**) *xz* section shows interface (*arrows*) between irregular, disorganized fibrotic collagen (**A**) with increased stromal cell density (**B**). (**C, D**) *xy* plane taken through the fibrotic region indicated in (**A, B**, *arrowhead*) shows disorganized collagen matrix (**C**) with numerous stromal cells (**D**).

Figure 5. 0.02% MMC-treated cornea evaluated by SHG imaging microscopy (**A**, **C**) of forward (cyan) and backward (magenta) signal combined and confocal fluorescence microscopy (**B**, **D**) showing FITC-phalloidin (green) and Syto59 (red) in the same optical plane. (**A**, **B**) xz plane showing absence of anterior stromal fibrosis (**A**) and loss of anterior stromal cells (**B**, double-headed arrow). xy plane taken through the a cellular region (**A**, **B**, large arrowhead) shows intact stromal collagen lamellar architecture underlying the stromal surface (**C**) and the presence of a few stromal cells with greatly enlarged nuclei (**D**, arrows) intermingled with a few small cells (**D**, arrowheads).

Figure 6.

Two separate regions of active fibrosis (**A–C** and **D–F**) in vehicle-treated corneas evaluated by backward-scattered (*magenta*) SHG imaging microscopy and confocal fluorescence microscopy of FITC-phalloidin (*green*) and Syto59 (*red*). *xz* projections (**A**, **D**) show fibroblasts with intense phalloidin staining (*arrowhead*) between basal epithelial cells and anterior stromal regions of fibrosis (*brackets*). *xy* optical planes show collagen fibers (**B**, **E**, *arrows*) coaligned with actin filaments (**C**, **F**, *arrows*).

Table 1

Changes in Epithelial Thickness, Stromal Thickness, and Haze

Treatment	Time (wk)	Sample Size	Epithelial Thickness (μm)			Stromal Thickness (μm)			Corneal Haze (UAUC)		
			Mean	SD	P *	Mean	SD	P *	Mean	SD	P *
Vehicle	0	5	44	2	—	335	15	—	52	9	—
	2	5	41	6	—	275	17	0.05 [‡]	4511	801	0.05 [‡]
	4	5	47	7	—	287	34	0.05 [‡]	3271	539	0.05 [‡]
	8	5	51	2	—	295.5	15	0.05 [‡]	2155	412	0.05 [‡]
0.02% MMC	12	4 [†]	54	5	0.05 [‡]	294	22	0.05 [‡]	1470	737	0.05 [‡]
	0	5	41	3	—	317	17	—	43	12	—
	2	5	56	7	0.05 ^{‡,§}	235	21	0.05 [‡]	1759	801	0.05 ^{‡,§}
	4	5	53	4	0.05 ^{‡,§}	256	36	0.05 [‡]	1140	663	0.05 ^{‡,§}
	8	5	54	5	0.05 [‡]	257	9	0.05 [‡]	653	273	0.05 [§]
	12	5	53	3	0.05 [‡]	282	18	0.05 [‡]	215	63	0.05 [§]

* Two-way repeated-measures ANOVA; multiple comparisons versus control group (Bonferroni *t*-test).[†] One animal died after week 8 following anesthesia.[‡] Comparison with baseline.[§] Comparison with vehicle.