Corneal Surface Ablation by 193 nm Excimer Laser and Wound Healing in Rabbits

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The 193 nm argon fluoride excimer laser was used to ablate a 6 mm diameter area of the central rabbit cornea under various conditions of power, beam configuration and exposure time. High repetition rates or prolonged exposures produced charring and prevented rapid epithelial wound closure. Endothelial vacuolization, reduction in density, and displacement of cell material into Descemet's layer resulted in these experiments. A beam of low and uniform power intensity (40 pulses per second, 100 seconds at 23 mJ/cm²) reduced stromal damage, cellular infiltration, and epithelial irregularities including punctate staining and cell exfoliation. Epithelial reheating occurred within two days. Basal lamina and hemidesmosomes were reformed by one week. Endothelial damage was not detected. Excimer laser ablation may allow removal of superficial dystrophies or scars, followed by rapid healing from normal corneal reparative processes. Invest Ophthalmol Vis Sci 30:90-98, 1989

Short-wavelength excimer lasers were first introduced to ophthalmology by Trokel et al.1 These lasers remove surface molecules by disruption of molecular bonds with little heat transfer to underlying tissue.2-6

Excimer lasers have been used to produce keratectomy incisions.6-13 The far ultraviolet 193 nm argon fluoride (ArF) laser produced deep cuts in stromal collagen with sharply delineated, compacted edges. Advantages of the ArF laser, including minimal tissue interaction, control of etch depth and ablative efficiency, have been previously described.14 Light radiation at 193 nm shows less mutagenic potential than longer ultraviolet wavelengths.15,16

Potential uses of this tool in ophthalmology are numerous. Excimer lasers can modify refraction by removing tissue so as to change the curvature of the central cornea.17-20 Serdarevic and coworkers have ablated the cornea to remove a yeast infection in an experimental model.21 Corneal ablation might also improve treatment of dystrophies of the epithelium and Bowman's layer, including Cogan's (map, dot, fingerprint) dystrophy, Meesmann's (juvenile epithelial) dystrophy, and Reis-Bücklers dystrophy.22

In this report we present results of our experiments in refining the ArF excimer laser output for ablation of the cornea and wound healing in rabbits.

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Materials and Methods

We used 4-10 lb New Zealand White rabbits, which were treated in accordance with the ARVO Resolution on the Use of Animals in Research. For in vitro study of deep stromal ablations, freshly enucleated rabbit eyes were used. In later studies, rabbits were fully anesthetized before laser exposure using intramuscular xylazine (Rompun®, Farbenfabriken Bayer GmbH, Leverkusen, W. Germany) (6 mg/kg) and ketamine HCl (Ketaset®, Bristol Laboratories, Syracuse, NY) (25 mg/kg). Additionally, they received topical proparacaine 0.5% for local anesthesia. Sacrifice of all animals was by overdose of sodium pentobarbital in the marginal ear vein.

Epithelial wounds were measured daily until closure with application of Fluor-I-Strips® to the tear film. Each cornea was observed using a slit lamp with fluorescein excitation filter. Photographic transparencies were taken at X1 magnification and measured by a reticle. Wound size was calculated as the average of vertical and horizontal measurements, accurate to 0.2 mm. Eyes were examined for signs of conjunctival, corneal, or iris inflammation and irritation using the method of McDonald and Shadduck.23 Ocular refraction was measured by streak retinoscopy.

Specular endothelial micrographs were taken in situ using a Keeler-Konan specular microscope based on the principle of Maurice.24 The ×30 objective cone was wetted with BSS® before application. Photographs were taken on transparency film with a transposed 0.1 mm square reticle grid at 3 days and at 1 month after ablation in order to determine endothelial cell density and appearance. Five grids per
cornea were counted and averaged for cell density. Corneal thickness at the center of the wound was measured using the internal optics of the specular microscope.

Excised corneas were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and dehydrated in an acetone series. Scanning electron microscope specimens were dried by the critical point method using carbon dioxide as the transition fluid, coated with gold-palladium, and examined at 20 kV. Transmission electron microscopy specimens were postfixed with 1% osmium tetroxide, embedded in epoxy resin, sectioned, stained with lead citrate and uranyl acetate to provide electron density, and viewed at 60 kV.

Laser Parameters

The lasers used in this study were Lambda Physik (Göttingen, W. Germany) Model 102 or Model 103 charged with argon and fluorine. These devices emit at a wavelength of 193 nm with a bandwidth less than ±1 nm. Output is in discrete pulses of 15 nanosecond duration. Pulses may be delivered singly or as a gated (timed) pulse train at repetition rates adjustable up to 200 Hz. Maximum energy per pulse is 150 mJ. The beam envelope emitting from the laser is approximately rectangular with a horizontal axis of 22 mm length by 4 mrad divergence and a vertical axis of 7 mm length by 2 mrad divergence. The raw beam intensity profile is multimode (greater than 10^6 modes).

No attempts were made to smooth the output intensity profile other than those described below. The laser was operated at maximum output for all experiments.

Energy per pulse was measured at the plane of corneal incidence just prior to and immediately after each series of exposures. The pulse-to-pulse energy variation was measured to be less than ±10%. Energy measurements were made with a calibrated Gentech (Plattsburg, NY) ED-500 calorimeter using a Tektronix (Beaverton, OR) Model 7834 oscilloscope. The beam from the excimer laser was found to be non-uniform in cross-section (see Results). A Wratten Neutral Density #3 gelatin filter was therefore used as an ablative test target. The energy profile of the beam thus produced an optical density profile in the filter which became a permanent record.

Deep Stromal Ablation (Method 1)

Experiments on freshly enucleated eyes were done in order to evaluate the ability of endothelium to function physiologically following deep, 80% depth ablation of the corneal tissue (see Acknowledgment).

The rectangular laser output was directed by a 45° front surface mirror and converged by a 15 mm focal length plano-cylindrical lens to a 7 × 7 mm image on the rabbit cornea (Fig. 1A). Pulse energy was estimated to be 150 mJ/cm², or 74 mJ/cm² over the square target. The laser was pulsed at 10 Hz and the number of pulses required to perforate an enucleated eye was determined. The cutting rate was about 1 μm per pulse, and was dependent on exact corneal position. The exposure was then reduced by 20% in order to ablate two corneas to 80% of original thickness. Thinned corneas were mounted in perfusion chambers at 34°C. Silicone oil on the ablated front surface prevented anterior fluid movement and Dakstein-Maurice media was perfused across the endothelial surface. Corneal thickness was measured for four hours.

Tangential Ablation (Method 2)

Experiments were performed to evaluate tissue removal by a laser beam directed tangential to the corneal refractive surface. An animal was positioned...
Fig. 2. Gelatin neutral density filter images of 193 nm excimer laser beam. (A) Beam focussed to 7 x 7 mm square image (Methods 1 and 3). A vertical pattern of surface irregularity occurs as the result of internal reflection in the cylinder lens. Central beam power also varies widely. (B) Divergent normal beam collected at the exit tube (Method 4). After travelling 340 cm the expanded and attenuated beam becomes more uniform.

vertically on a lab jack and the eye was fixed motionless with a Thornton ring. The image of the rectangular laser cavity, concentrated by a spherical lens, formed an intense cutting beam at the cornea (Fig. 1B). The beam was focussed 0.25 mm below the corneal epithelial tangent. This procedure was done in six rabbits.

Convergent Ablation (Method 3)

An eye shield was fabricated in the shape of a contact lens with a 6 mm central opening to prevent the deposition of debris by the cutting beam. The shield was of highly polished aluminum to minimize heating during exposure, and had an internal curve of 8 mm radius to fit the rabbit cornea. A single drop of pilocarpine 2% was administered 1 hr before the procedure to induce pupillary miosis, facilitating optical centration over the iris. The shield was then placed on the cornea and temporarily fixed by two edges to bulbar conjunctiva using cyanoacrylate glue.

Excimer laser exposure at 178 mJ/cm² per pulse (50 mJ per pulse through the 6 mm diameter shield aperture), 10 Hz for 5 seconds, 2.5 mJ total, was made using lenses and a mirror (Fig. 1C). A 7 X 7 mm square pattern was cast over the 6 mm diameter aluminum target. This procedure was done in eight rabbits.

Divergent Ablation (Method 4)

The beam was allowed to expand by divergence to a size of approximately 75 mm by 12 mm over a path length of 340 cm (Fig. 1D). This allowed the selection of a portion of the beam with relatively even power density (Fig. 2B). An aperture limited this broad beam to 8 mm diameter just before striking the eye at the 6 mm diameter aluminum shield, as in Method 3. A He-Ne laser aiming beam was added coaxially with the ablative beam, using a quartz plate at a 45° angle in the beam path. Shielded eyes were then aligned to a

Table 1. Specular microscopy results for Method 4 (divergent beam)

<table>
<thead>
<tr>
<th>Healing time/exposure</th>
<th>Corneal thickness</th>
<th>Endothelial density</th>
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<tr>
<td></td>
<td>Treated</td>
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<tr>
<td>Three days</td>
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<td>100 sec</td>
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<td>360 μm</td>
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<td>200 sec</td>
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<tr>
<td>100 sec</td>
<td>400 μm</td>
<td>370 μm</td>
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<td>200 sec</td>
<td>410 μm</td>
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* N.R.—Not readable due to corneal turgescence.
Fig. 4. Light micrographs after laser ablation and epithelial healing. (A) Epithelium is five to seven layers thick peripheral to ablation. (B) Thinned epithelium of four to five cell layers over irregular stroma at center of cut. (C) Stromal swelling is typical of the edge of a deep cut. Epithelium is ten to 13 cell layers thick at edge of wound. Keratocytes are present in large numbers subjacent to epithelium. (D) Epithelium thickens slightly to nine layers at edge of shallow ablation, resulting in maintenance of smooth profile at the tear film surface.

portion of the laser beam selected for uniform power output.

Pulse fluence equalled 23 mJ/cm² (6.5 mJ through the 6 mm diameter shield aperture). Exposures were as follows: Long exposure—40 Hz, 200 seconds, 52 Joules (three rabbits); Short exposure—40 Hz, 100 seconds, 26 Joules (six rabbits).

Results

Beam Profile

The neutral density filter target decreased in thickness in proportion to excimer laser ablation. This provided a profile of the x-y energy distribution of the laser beam.

Methods 1 and 3 showed uneven intrabeam structure as evidenced by the pattern of dark and light areas (Fig. 2A). Particulate debris adhered to the edge of the target following ablation, as previously described for polymethyl methacrylate.²

Beam x-y profile uniformity was smoothed after travelling unconverted through a 340 cm air path by Method 4 (Fig. 2B). The energy density was greatly reduced by both beam spread and air attenuation (50% over the 340 cm path).
Method 2, using tangential ablation, showed uneven epithelial re healing since the laser beam cut through the edges of the tissue more rapidly than the center. The endothelium was disrupted, as indicated by specular microscopy and by swelling and turgescence of the corneas within a few hours of the procedure. Epithelial re healing was complete by 3 days in all cases.

Fig. 5. Transmission electron micrographs showing cut area immediately after ablation. (A) Undulating stroma and uneven cutting observed in some areas of ablation by nonsmoothed beam. (B) Cut surface demonstrating single lamellar layer of corneal stroma.

Observations In Vivo

Method 1 resulted in ablation of approximately 1 μm per pulse, producing a square stromal bed with a coarse granular texture. An 80 μm thickness of stroma remained covering the endothelial cell layer. Endothelial fluid pumping maintained corneal thickness ±2 μm over a period of 4 hr, indicating physiological function. Endothelial cells had a normal hexagonal appearance. Epithelial wound healing was not observed in this experiment.

Fig. 6. Transmission electron micrographs showing epithelial regeneration and new basal lamina after 4 weeks. (A) Irregular basal lamina and hemidesmosome formation over irregular stromal cut as in Figure 5A. (B) Basal lamina and epithelium appear near normal over smooth area of stromal ablation as in Figure 5B.
Method 3, using a converging beam, produced a granulated surface immediately following treatment. Epithelial margins were round and well defined, and wound closure was complete by 48 hr. By 1 month corneas were nearly transparent and normal endothelial appearance and thickness were restored (see Table 1). Corneal clarity was restored by 3 months.

Method 4, 200-second-long exposure, using 340 cm of air for divergence, provided a low-energy beam. The bare stroma had an opalescent texture and a yellow cast. Three days were required for epithelial closure. Corneas were 175% of normal thickness at 3 days, preventing endothelial density measurement. Corneal cloudiness was still apparent at the 4 week examination.

Method 4, 100-second short exposure, produced corneas which were slightly opalescent until wetted by tears, and which had no visible coloration. Epithe-
Fig. 9. Transmission electron micrographs of endothelial cells. (A) Descemet's layer shows inclusion of densely staining material. (B) Endothelial intracellular border shows large irregular vacuolar inclusions.

Histology

The epithelium in the peripheral corneal areas was normal in appearance, with a total thickness of five to seven cell layers (Fig. 4A). The central area of a shallow ablation had four to five layers of cells after one week (Fig. 4B). When the ablation had a steep edge, as in Method 3 and in Method 4—200 seconds, stromal swelling occurred, as seen in Figure 4C. Endothelium thickened over the edge, and remained thickened to over ten cell layers in the bed of the ablation. When slight thinning of stroma occurred at the edge of the ablation, the epithelium thickened to nine to 11 layers and filled in the defect to maintain a smooth, unbroken epithelial surface (Fig. 4D). Increased numbers of keratocytes were seen at the wound margins in such cases.

Electron Microscopy

Tissues fixed immediately after laser ablation showed complete removal of the epithelium and basal lamina, leaving a surface defined by stromal lamellae of collagen fibrils. Method 3, convergent ablation, left an irregular surface with an undulating border exposing collagen fibrils (Fig. 5A). Method 4—100 seconds, divergent ablation, produced a more even surface, often cutting parallel to the fibrils of a single lamella (Fig. 5B). Collagen immediately below the ablated edge appeared structurally normal.

Reformation of a fibrillar, densely staining basal lamina adjacent to epithelium occurred in all cases by 1 week. Hemidesmosomes were seen along the basal surface of epithelial cells, indicating that full attachment had occurred. In Figure 6A, resulting from a focussed laser cut by Method 3, the juncture remained wavy and irregular in appearance. Figure 6B shows the appearance of the central area of ablation by normal distance Method 4—100 seconds. The appearance of the epithelial surface by scanning electron microscopy 1 month after ablation was dependent on the configuration of the stromal surface underneath. Epithelium overlying irregular stroma from Method 3 showed exfoliation of superficial cells over the ablated area (Fig. 7A). Figure 7B shows the normal appearance of epithelium over smooth stroma after ablation by Method 4.

Keratocytes just beneath the ablation surface often contained vacuoles with densely staining inclusions (Fig. 8A). One month after laser ablation by Method 3 large numbers of keratocytes were visible in large numbers subjacent to the ablation (Fig. 8B). These cells had more than normal amounts of endoplasmic reticulum. Keratocytes did not increase markedly in numbers during the first 3 months after ablation by Method 4.

Method 3 caused a deposition of densely staining material in the posterior Descemet's layer in half the specimens observed. By 3 weeks, this line had separated from the endothelium by about 0.5 μm, (Fig.
By 3 months the distance had increased to nearly 1 μm. Endothelial cells sometimes had enlarged cisternae of rough endoplasmic reticulum, irregular in outline, between 0.5 and 1 μm in longest dimension (Fig. 9B). Method 4 did not result in any apparent inclusions in Descemet's layer or in abnormal endothelial cell vacuolization.

Discussion

We have shown by a series of experiments the refinement of a 193 nm excimer laser to ablate corneal tissue and promote wound healing. The ablation depth can be controlled to an accuracy of several microns, allowing more precise removal of stroma, and a more adequate base for epithelial rehaling, than mechanical means. Laser beam energy and distribution, repetition rate and time of exposure are interactive variables which determine the characteristics of wound healing. Tissue damage due to heating occurs from high repetition rates at high energy per pulse, or from extended total exposure times. The roughness of the ablated stromal surface determines the amount of inflammatory cellular infiltration and the irregularity of epithelium.

Endothelial cell loss and the presence of abnormal stained material in Descemet's layer may be related to high pulse energy resulting in production of shock waves. Endothelial cell density was reduced at 3 days in rabbits but not at 1 month. This observation is consistent with the findings of Van Horn et al. that rabbit corneal endothelium can rehale within 10 to 17 days. Monkey eyes may be a good model for humans, since primates have no capacity for regeneration of endothelial cells, and therefore sustain permanent reduction in cell number from such damage.

Reis-Bücklers Syndrome is an opacification of the cornea involving the superficial stroma, the basal lamina, and the epithelium. Cogan's (map, dot, fingerprint) dystrophy consists of intermittently appearing intraepithelial cysts of widely variable size, with basement membrane protruding into the epithelial cellular layers or becoming absent altogether. Recurrent erosion can be caused by fingernails, plant leaves and branches which damage epithelial basal lamina and prevent normal epithelial adhesion. Treatment of these conditions may require lamellar keratectomy or corneal "stripping" of epithelium. Superficial corneal ablative technique may be useful in these and other cases where it is desirable to completely remove the epithelium and underlying basement membrane, leaving a surface which can be rapidly rehaled.

Key words: excimer laser ablation, wound healing, epithelium, endothelium, superficial dystrophy

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