Unscheduled DNA Synthesis Following Excimer Laser Ablation of the Cornea In Vivo

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The amount of unscheduled DNA synthesis (UDS) produced in the cells adjacent to excimer laser ablations in the cornea was compared for 193-nm and 248-nm laser wavelengths. UDS is interpreted to indicate the process of excision repair of pyrimidine dimers formed in DNA. 193-nm laser ablation did not produce a statistically significant difference in the amount of UDS as compared to a negative control (diamond-knife corneal incision). However, 248-nm laser ablation did produce a highly statistically significant difference in the amount of UDS as compared to both the negative control (P < 0.001) and the 193-nm laser irradiation (P < 0.001). Other forms of DNA damage (single-strand DNA chain breaks and DNA-protein crosslinks) are not measureable by UDS and need to be investigated in the evaluation of the oncogenic potential of 193-nm laser ablation. Invest Ophthalmol Vis Sci 28:287-294, 1987

Rare gas-halide excimer lasers are high-power sources of ultraviolet radiation. Recently, 193-nm radiation from an argon-fluoride (ArF) excimer laser has been shown to produce precise etching of polymer films and clean incisions in biologic materials, in particular the cornea, skin, and aorta.1-5 In the eye, experimental work has centered upon the use of 193-nm radiation and 248-nm radiation from a krypton-fluoride (KrF) excimer laser to produce corneal incisions.3,6-8 Comparisons of the two wavelengths in terms of the quality of the lesions produced and of the adjacent zone of tissue damage have been reported.3,7,8 However, none of these studies have investigated damage to the DNA of the corneal cells adjacent to the region of excimer laser ablation.

Ultraviolet radiation has been demonstrated to be both a mutagen and a carcinogen.9-12 One significant form of DNA damage that is known to occur is the generation of cyclobutyl pyrimidine dimers after exposure to 254-nm irradiation.9,13,14 Living cells use an excision repair process to correct this damage.12,13-19 The technique of autoradiography is commonly used to quantify unscheduled DNA synthesis (UDS), which is interpreted to be an indicator of the excision repair process.20-24

Materials and Methods

Animals

Ten 1-kg Dutch pigmented rabbits were used (20 eyes) in these experiments. Prior to irradiation or diamond knife incision, the rabbits were anesthetized with ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (4 mg/kg). In addition, topical proparacaine hydrochloride (0.5%) was applied to the cornea. The corneas of the anesthetized rabbits were protected from abrasions and were examined visually for scratches or cloudiness before use. All laboratory work adhered to the ARVO Resolution on the Use of Animals in Research.

Irradiation

The corneas were irradiated with a Questek series 2000 excimer laser (Questek Inc.; Bedford, MA) at wavelengths of 193 nm (ArF gas fill, 15 ns pulse duration) or 248 nm (KrF gas fill, 20 ns pulse duration). An articulated arm delivery system, which couples the laser output with a Topcon SL-5D slit lamp (Topcon Instrument Corp., America, Paramus, NJ), was used to aim and focus the laser beam on the rabbit cornea. A helium-neon laser was aligned to be colinear with the excimer beam; both laser beams were transmitted through the delivery system and shaped with mirrors into a 76-μm wide slit, which was focused on the cor-
ne. The He-Ne laser allowed accurate positioning and focusing prior to ablation with the excimer laser.

The excimer laser energy per pulse delivered to the cornea was measured with a joulemeter (Gentec ED 200, Ste-Foy, Québec, Canada), whose output was connected to a Tektronix Model 2235 oscilloscope (Tektronix; Beaverton, OR). The area of excimer laser irradiation was determined by making a series of single-pulse ablations on a piece of developed photographic film. The beam width and length were measured from the film with a digital micrometer and microscope. For both wavelengths a beam size of 76 μm × 4.80 mm and an energy of 1.45 mJ resulted in a fluence of 400 ± 40 mJ/cm² per pulse. All ablations were performed at a repetition rate of 30 Hz. A single excimer laser ablation was made in the center of the cornea. Eight eyes were ablated with 193-nm and eight eyes with 248-nm laser output. For both wavelengths, two eyes were ablated with 60 pulses, two eyes with 180 pulses, two eyes with 300 pulses, and two eyes with 420 pulses.

Two eyes were cut with a micrometer diamond knife (Metico, Inc., Clearwater, FL) at depths of 300 μm and 450 μm to serve as negative controls (ie, no ultraviolet irradiation). Two eyes were exposed to a UV-germicidal lamp containing three Sylvania GI5T8 bulbs (Sylvania, Danvers, MA) (emitting primarily at 254 nm) at an irradiance of 5.60 × 10⁻⁴ W/cm² to serve as positive controls. One eye received a total radiant energy dose of 0.50 J/cm², and the other eye received a total dose of 1.0 J/cm².

Autoradiography

Immediately following excimer laser ablation, the eye was enucleated and the entire cornea dissected. The corneas were temporarily floated in MEM (Gibco; St. Lawrence, MA) at 37°C. The rabbits were killed following enucleation with a lethal injection of pentobarbital.

The entire corneas were incubated in MEM with ³H-thymidine (New England Nuclear; Boston, MA; final activity 20 µCi/ml, specific activity 6.7 Ci/mmol) at 37°C and 7% CO₂ for 2 hr. The mean time interval between excimer laser ablation of the cornea and the start of incubation in ³H-thymidine was 1½ hr. The tissue was prepared following an established method. ²⁵ Following incubation, the corneas were rinsed thoroughly in PBS (Gibco) and fixed in a 2% paraformaldehyde-2.5% glutaraldehyde-0.1 M sodium cacodylate buffer solution. The fixed tissue was dehydrated through a graded ethanol series, grossly cut down to a size of 8 mm × 8 mm, and embedded in JB-4 glycol methacrylate. From the embedded corneal block, tissue sections at a thickness of 2–3 μm were cut perpendicular to the lesion. At least 15 sections per cornea were cut from the middle third of the ablated area.

The slides were dip-coated with Kodak Nuclear Track Emulsion Type (Eastman Kodak; Rochester, NY) NTB-2 and stored in light-proof boxes at 4°C for an exposure interval of 2 wk. The dip-coated tissue sections were developed with Kodak D-19 developer, fixed with Kodak Rapid Fixer, and neutralized with Perma Wash. The developed slides were stained with Stevenol’s blue and destained with dH₂O and 95% ethanol for greatest contrast.

Cell Counting

The counting zone in all sections was defined as including all cells within 100 μm of each edge of the ablation, producing a rectangular box encompassing the lesion. Thus, the minimum size of the counting zone would be 200 μm width × 100 μm depth (in the case of no lesion). For irradiations in which a portion of the cornea was ablated, the size of the counting zone would increase congruently. For cornea, the tissue absorption length at 248 nm is 47 μm and at 193 nm is 3.70 μm. ⁶ The 100-μm width of the counting zone includes two absorption lengths of 248 nm light; 86.5% of the 248-nm radiation and 100% of the 193-nm radiation would be absorbed within this area. The 254-nm germicidal lamp irradiated the entire cornea without tissue ablation, so an area 200 μm wide and 100 μm deep in the center of the cornea was taken to be the counting zone.

All epithelial cells and keratocytes (mature fibroblasts in the stroma of the cornea) within the counting zone of each prepared section were counted with the exception of grossly damaged cellular fragments bordering the lesion edge. These cellular fragments represent ablated debris and could not be reliably counted. An average of 2300 cells per cornea were counted, including 1350 epithelial cells and 950 keratocytes. Background labeling was defined by cells far (>2000 μm) from the lesion and in all sections was found to be 2 or fewer intranuclear silver grains. Sparingly labeled cells (SLCs) were defined as those cells containing between 3 to 15 intranuclear grains, and they were readily distinguishable from heavily labeled cells (HLCs) containing more than 15 intranuclear grains per cell. ²¹±²⁴

All counting was done with the slide identification masked and was performed in a random order by the same investigator. A one-sided student’s t-test was performed to evaluate differences in the mean percent of SLCs for each group of eyes.

Results

Negative Control: Diamond Knife Incision

The two eyes subjected to a diamond knife corneal incision were exposed to no UV irradiation at all. Only
0.29 ± 0.12% (mean ± 1 standard deviation) of the epithelial cells and 0.22 ± 0.12% of the keratocytes were sparsely labeled. The presence of HLCs (indicating replication of DNA during S-phase of the cell cycle) close to the incision demonstrates tissue viability (see Fig. 1).

Positive Control: UV-Germicidal Lamp Irradiation

254-nm UV irradiation is known to cause DNA damage through the production of pyrimidine dimers. For both eyes irradiated with the UV-germicidal lamp taken together, the mean percent of epithelial SLCs was 18.95% ± 2.58% and 4.78 ± 0.57% for the keratocytes. In this case, the substantial proportion of SLCs is interpreted to indicate the excision repair of pyrimidine dimers formed in the DNA as a result of UV irradiation (see Fig. 2). The presence of some HLCs (primarily found in the basal portion of the epithelial cell layer) demonstrated that the tissue remained viable. A student's t-test indicated a highly statistically significant difference in the percent of SLCs between the positive control and the negative control for both the epithelial cells (P = 0.008) and the keratocytes (P = 0.006).

193-nm Laser Ablation

The eight eyes ablated with 193-nm laser (fluence of 400 mJ/cm²) received between 60 and 420 pulses of laser irradiation, corresponding to a total incident energy dose ranging between 24 and 171 J/cm². The 193-nm lesions had clean edges and contained a narrow zone of damage adjacent to the ablation of approximately one-cell width in the epithelial layer. HLCs were observed quite close to the lesion in some of the sections, indicating viable cells replicating their DNA with no apparent thermal damage (see Fig. 3). No correlation was found to exist between total 193-nm incident energy and the density of SLCs within the
No correlation was found to exist between total 248-nm energy received (holding fluence constant and varying the number of pulses delivered) and the density of SLCs in the counting zone for either the epithelial cells or the keratocytes. A higher total incident-energy dose produced a deeper lesion, a larger counting zone, and a corresponding greater total number of SLCs. However, the density of SLCs throughout the counting zone was not increased. Taken together as a group, the mean percent of epithelial SLCs was 18.79 ± 1.71% and of keratocyte SLCs was 4.99 ± 3.19%. Comparing these eyes to the negative control diamond-knife incised eyes, the student’s t-test indicates a highly statistically significant difference in the two groups for the epithelial cells ($P < 0.001$) and a statistically significant difference in the two groups for the keratocytes ($P = 0.05$).

In addition, there was a highly statistically significant difference between the 193-nm group and the 248-nm group of eyes for both the epithelial cells ($P < 0.001$).

Fig. 3. 193-nm laser ablation. Note HLC close to lesion edge (arrow), indicating a viable cell replicating its DNA with no apparent irradiation-associated damage. Ablation depth is 120 μm, produced with 180 pulses at 400 mJ/cm² (original magnification, ×800).

counting zone. For all eight eyes together, the mean percent of epithelial SLCs was 0.92 ± 0.53% and of keratocyte SLCs was 0.15 ± 0.11%. Comparing these eyes to the diamond knife negative control, the student’s t-test indicates that the difference in the mean percent of SLCs is outside of the 95% confidence interval and thus is not statistically significant for either the epithelial cells ($P = 0.085$) or the keratocytes ($P = 0.27$).

248-nm Laser Ablation

The eight eyes subjected to 248-nm laser ablation of the cornea also received between 60 and 420 laser pulses (fluence of 400 mJ/cm²). The lesions produced by this wavelength typically had more jagged edges and an apparent zone of thermal damage extending two to three cell widths from the lesion in the epithelial cell layer. An increased number of SLCs adjacent to this zone of thermal damage was readily noticeable (see Fig. 4).

Fig. 4. 248-nm laser ablation. Note the SLCs adjacent to the ablation (arrows), indicating unscheduled DNA synthesis (excision repair of pyrimidine dimers formed in the DNA). Ablation depth is 80 μm, produced with 180 pulses at 400 mJ/cm² (original magnification, ×800).
and the keratocytes ($P = 0.001$). The total number of sparsely labeled cells (SLCs) and total counted cells for each of the four groups of eyes are presented in Table 1. All of these results are summarized in the bar graph in Figure 5.

### Discussion

Ultraviolet laser radiation has recently shown promise as a technique for removing tissue with a high degree of precision and with minimal thermal damage to adjacent structures. Experimental studies of excimer laser ablation have been performed using 193-nm (ArF) and 248-nm (KrF) radiation in the cornea, crystalline lens, and skin and using 308-nm (xenon chloride) radiation in iris, experimental vitreous membranes, and aortic wall.3,4,27,28 Moreover, the use of 266-nm radiation from a frequency-quadrupled neodymium-YAG laser for corneal ablation has also been suggested. For laser ablation of a nonvascularized surface tissue, such as the cornea, 193-nm radiation seems to be the most desirable wavelength. In comparative studies of excimer laser ablation of the cornea, we found that 193-nm radiation produced the most precise cuts, when compared with incisions made with 248-nm radiation.7,8 When examined by transmission electron microscopy, a narrow zone of damaged tissue (0.1–0.3 μm) was seen immediately adjacent to the tissue removed by the laser.7,8 Ablation with 248-nm radiation produced incisions with ragged edges and with a wider and more severe zone of damage in the adjacent stroma.7,8 These studies have suggested that the excimer laser may have potential use in corneal surgery or in other applications where a highly controlled incision into the outer structure of the eye is necessary.

Clinical acceptance of ultraviolet laser ablation, however, requires an assessment of potential ultraviolet-induced carcinogenesis and mutagenesis. UV radiation is known to cause the formation of DNA photoproducts, mutation, transformation, and cell death. This is of particular concern in the case of 248-nm radiation, since this wavelength lies near a peak of DNA absorption at 260 nm (corresponding to the absorption of the purine and pyrimidine bases of the nucleic acids).29 Damage to DNA in cells adjacent to and remote from the area of ablation is of concern. Cells adjacent to the region of the ultraviolet laser ablation are subject to indirect irradiation due to scattering of incident photons. Although the fluence of this scattered ultraviolet radiation is below the threshold for ablation, the energy of single scattered photons at these wavelengths (6.4 eV at 193 nm and 5.0 eV at 248 nm) may be great enough to cause one or more possible forms of DNA damage.9 The present study was performed to compare the amount of DNA damage that is repaired by unscheduled DNA synthesis (UDS) in the corneal epithelial and stromal cells surrounding corneal incisions produced by 193-nm and 248-nm excimer laser ablation performed in vivo.

### Table 1. Total number of sparsely labeled cells (SLCs) and total counted cells for the four experimental groups*

<table>
<thead>
<tr>
<th></th>
<th>Epithelial cells</th>
<th>Keratocytes</th>
<th>Total cells in counting zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>193-nm irradiation</td>
<td>94</td>
<td>12</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>10,265</td>
<td>8141</td>
<td>18,406</td>
</tr>
<tr>
<td>248-nm irradiation</td>
<td>2094</td>
<td>346</td>
<td>2440</td>
</tr>
<tr>
<td></td>
<td>11,146</td>
<td>6952</td>
<td>18,098</td>
</tr>
<tr>
<td>Diamond knife incision</td>
<td>8</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>(negative control)</td>
<td>2750</td>
<td>2158</td>
<td>4908</td>
</tr>
<tr>
<td>254-nm germicidal rays</td>
<td>871</td>
<td>88</td>
<td>959</td>
</tr>
<tr>
<td>(positive control)</td>
<td>598</td>
<td>1851</td>
<td>6449</td>
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</table>

* SLCs/Total Cells = %SLC.
Ultraviolet-induced DNA alterations may include the production of cyclobutyl pyrimidine dimers, single-strand DNA chain breaks, and DNA-protein cross links. The type of photoproduction that predominates clearly depends upon the energy (and hence the wavelength since $E = h\nu = hc/\lambda$) of incident photons and upon the presence of a cellular chromophore for that wavelength. The most significant effect of UV irradiation of DNA is the production of cyclobutyl pyrimidine dimers (at 220–320 nm), particularly the thymidine dimer. Adjacent pyrimidines in one strand of the DNA molecule are covalently linked by their 5,6-carbon atoms to form a cyclobutane ring. This linkage causes a local deformation in the DNA structure that is thought to present at least a temporary block to the process of replication. Failure of repair or misrepair of dimers has been implicated as important factors in solar oncosogenesis in humans.

In mammalian cells the excision repair process appears to be the most important mechanism for the repair of thymidine dimers. Unscheduled DNA synthesis (UDS), as detected by autoradiography, is interpreted to represent the process of excision repair. A distinction is made between cells that are sparsely labeled (SLCs, 3–15 intranuclear grains per cell) and heavily labeled cells (HLCs, >15 intranuclear grains per cell). SLCs are considered to have undergone UDS, whereas HLCs have undergone semiconservative replication of their DNA in the S-phase of the cell cycle.

We used the technique of autoradiography to measure the amount of UDS occurring in the cells adjacent to corneal incisions produced by two wavelengths of ultraviolet laser irradiation. The results were compared with both a negative control (a diamond knife corneal incision, no UV irradiation) and a positive control known to produce pyrimidine dimers (254-nm germicidal lamp). The 193-nm laser ablations did not produce DNA damage as measured by this UDS assay. There was not a statistically significant difference in the percentage of SLCs in either the epithelial cells or the keratocytes as compared to the negative control. In contrast, the 248-nm laser ablations produced a highly statistically significant amount of DNA damage repaired by the excision repair process as compared to both the negative control and the 193-nm laser ablations.

In the case of 248-nm ablation, there was no relationship observed between total laser irradiation dose delivered to the cornea and the density of SLCs. This lack of a dose-response relationship has been described in other published reports. It is possible that the density of pyrimidine dimer production may be related to the intensity of incident laser irradiation rather than to total incident energy.

It is useful to examine possible chromophores of the cornea in order to explain observed differences in DNA damage at 193-nm and 248-nm laser ablations. At 248 nm, possible chromophores are the nucleic acids (DNA and RNA). All bases in nucleic acids absorb well between 230 and 290 nm (due to the 260-nm absorption peak of the purines and pyrimidines), approximately 10 to 20 times greater than proteins in the same spectral region. Nucleic acids absorb even more strongly at 193 nm than at 248 nm. At 193 nm, a primary chromophore may be the peptide bonds in collagen, with an absorption maximum in the region of 180–190 nm. Another potential chromophore of importance may be glycosaminoglycans, with absorption peaks around 190 nm and no significant absorption at 248 nm. A possible explanation for the absence of UDS at 193-nm laser ablation of the cornea may be cytoplasmic and interstitial shielding of the nuclear DNA from incident 193-nm photons by these chromophores.

Ultraviolet-induced corneal tumors have been reported in rats, mice, and hamsters upon chronic exposure to a broad band source of ultraviolet light (a mercury arc lamp emitting radiation from 220–760 nm for 5 days per wk for 1 yr). However, primary neoplasms of the human cornea are not a common event. Corneal tumors almost always represent secondary extensions of lesions that are primary in the bulbar conjunctiva and limbus. Xeroderma pigmentosum, an autosomal recessive disease, is an example of a pathological condition characterized by defective repair of DNA damaged by ultraviolet radiation. The defect stems from the absence of an enzyme required for the excision repair of UV-induced pyrimidine dimers in DNA. The conjunctiva and cornea often are severely involved in the disease process and may exhibit a spectrum of changes, including inflammation, edema, vascularization, scarring, opacification, and epithelial neoplasia. Excimer ablation of the cornea represents an extremely intense ultraviolet exposure that the eye normally does not receive. It is pertinent to note that 248-nm laser ablation of the cornea produced pyrimidine dimers in the DNA, whereas 193-nm laser ablation did not.

DNA damage induced by 248-nm irradiation may have important consequences.

Although a significant difference in the amount of pyrimidine dimer formation produced by 193-nm and 248-nm laser irradiation of the cornea has been observed in this study, other forms of DNA damage may occur that are not measurable by UDS. In particular, single-strand DNA chain breaks and DNA-protein crosslinks may be produced. Single-strand breaks, if they occur, are likely to be “two-hit” phenomena because the energy of 193-nm photons (6.4 eV) is slightly less than the nucleic acid ionization potential in aqueous solution (7.5–8.0 eV). DNA-protein crosslinks may occur following the production of highly reactive-free radical intermediates formed by the absorption of UV irradiation. Future investigations will be performed comparing 193-nm laser irradiation with known positive and negative controls for these forms of DNA damage. Such studies will be important in the evaluation of the oncogenic potential of 193-nm laser ablation.

In addition to the direct comparison of UDS occurring as a result of 193-nm and 248-nm laser ablation, this study has helped to demonstrate the usefulness of the cornea as a model system for gaining insight into the biologic consequences of ultraviolet laser tissue ablation. The epithelial cell layer of the cornea in particular appears to be an excellent model for evaluating ultraviolet laser mutagenesis. The 4–5 cell-deep epithelial layer is avascular, so bleeding is not a problem during ultraviolet corneal ablation. The cells of the epithelial layer have a very rapid turnover rate, with a cell cycle time of approximately 7 days. As a result, damage to these active cells may be more readily apparent. Lastly, because the cornea is a surface organ, laser mutagenesis experiments can be readily performed in vivo, allowing the physiological tissue response to laser irradiation to occur. It will be informative to evaluate the mutagenicity of other ultraviolet laser wavelengths, such as 266-nm (frequency-quadrupled Nd:YAG), 308-nm (XeCl excimer), and 351-nm (XeF excimer) with the corneal epithelial layer.

Key words: cornea, excimer laser, ultraviolet radiation, unscheduled DNA synthesis, mutagenesis

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