DNA Damage Induced by 193-nm Radiation in Mammalian Cells

Irene E. Kochevar, Agnes A. Walsh, Howard A. Green, Margaret Sherwood, Alice G. Shih, and Betsy M. Sutherland

ABSTRACT

The contribution of DNA damage to the effects of 193-nm excimer laser radiation on mammalian cells in culture was studied in order to evaluate the mutagenic potential of this UV wavelength in vitro. Two approaches were taken: measurement of pyrimidine dimer-specific endonuclease-sensitive sites/megabase and comparison of the 193-nm radiation-induced cytotoxicity in normal versus DNA repair-deficient cells. The formation of pyrimidine dimer-specific endonuclease-sensitive sites/megabase was inversely related to the thickness of the cytoplasm over the nuclei of normal human fibroblasts (NHF) and Chinese hamster ovary cells. The results of these measurements and a calculation of the absorption coefficient of cytoplasm indicate that each 1 Mm of cytoplasm will protect 1% of DNA from 193-nm radiation by overlying cytoplasm.

The reduction in colony-forming ability induced by 254-nm, 193-nm, and X-ray radiation was measured in NHF, xeroderma pigmentosum (group A) cells, and ataxia telangiectasia cells. Xeroderma pigmentosum (group A) cells were 16.5 times more sensitive to 254-nm radiation but only 3.5 times more sensitive to 193-nm radiation than NHF cells, indicating that cylobutylylpyrimidine dimers were not the major lethal lesion formed at 193 nm. AT cells were 3.4 times more sensitive to X-rays than NHF cells, but these cell types were almost equally sensitive to 193-nm radiation, indicating that 193 nm did not induce the same type of lethal lesions as X-rays.

INTRODUCTION

High-intensity, short-pulse UV radiation from an excimer laser is currently being evaluated for corneal surgery (1, 2). In these treatments, a very small volume of tissue is removed with each 13-nsec pulse of 193-nm radiation, thus permitting controlled reshaping of the cornea. Because UV radiation is well known to be a mutagen and carcinogen, concern has been expressed over the medical use of high intensity 193-nm radiation. Studies of mutations induced in vitro by 193-nm radiation have yielded conflicting results. In two studies, 193-nm radiation did not cause mutations to 6-thioguanine resistance in CHO cells using fluences of up to 85 J/m² (3) or 250 J/m² (4). In one of these studies, mutations to ouabain resistance in CHO cells were detected after exposing the cells to fluences of up to 250 J/m² of 193-nm excimer laser radiation (4). The reason for the difference between these two studies is not clear. The current study was initiated in order to understand better the interaction of 193-nm radiation with cellular DNA and to help evaluate the potential mutagenic hazard of 193-nm radiation in vivo.

There are few reports of damage to cellular DNA by 193-nm radiation. Sister chromatid exchanges were detected after exposure of CHO cells to 193-nm radiation (4). The results of studies of 193-nm radiation-induced effects on bacteria and yeast suggested that DNA photoproducts were involved in the responses (5, 6). However, the DNA photoproducts probably resulted from the laser-induced UV fluorescence that was produced from the medium (7). Unscheduled DNA synthesis was not detected either in skin or in cornea in cells adjacent to regions ablated with 193-nm radiation (8, 9). Several studies have shown that 193-nm radiation is less cytotoxic per incident photon than 254-nm radiation (3, 4, 10), and we have postulated that the mechanism for cell killing by 193-nm radiation mainly involves damage to proteins in cellular membranes (11).

DNA absorbs strongly at 193 nm; the extinction coefficient for DNA is about twice as great at 193 nm as at 260 nm (12). Radiation at 193 nm induces strand breaks in viral DNA (13). The ratio of strand breaks to cyclobutylylpyrimidine dimers formed by irradiating plasmid DNA in solution is higher at 193 nm than at 254 nm (14). In cells, nuclear DNA will be partially shielded from 193-nm photons by the absorption of other chromophores, mainly proteins in the cytoplasm and cellular membranes. Absorption of 254-nm radiation by cellular chromophores reduces the number of photons reaching the center of the nucleus by about 50% (15). Shielding of the DNA by other cellular chromophores is expected to be much more pronounced at 193 nm than at 254 nm, because the maximum in the absorption of peptide linkages is at about 190 nm.

We report here the results of studies designed to estimate the relative amount of 193-nm radiation that reaches the nuclear DNA in mammalian cells and the involvement of the 193-nm radiation-induced DNA photoproducts in the mechanism for cytotoxicity.

MATERIALS AND METHODS

Cells. Human cells were obtained from the Coriell Institute (Camden, NJ). NHF, 05659B, were employed at passages 6–20. Two strains of XPA skin fibroblasts (02990A, called XPA-1, and 05509A, called XPA-2) were employed at passages 4–15. AT fibroblasts (GM02052A) were used at passages 10–15. The cells were routinely maintained in Eagle's minimal essential medium supplemented with 20% fetal calf serum, 1% penicillin and streptomycin, 1% l-glutamine, and 1% nonessential amino acids (GIBCO). CHO cells (Dr. L. Gerweck, Massachusetts General Hospital) were maintained in McCoy's 5A medium with 10% fetal calf serum and 1% penicillin and streptomycin.

Excimer Laser Radiation. Cells were rinsed with HBSS (GIBCO) without Ca²⁺ or Mg²⁺, drained, and irradiated with a Lambda Physik EMG103 excimer laser at 193 nm (pulse width = 13 nsec) at 2 or 3 Hz. The laser beam was passed through a 5-mm diameter aperture, attenuated, and then defocused with a quartz lens to illuminate fully a 10-cm-diameter plastic tissue culture dish mounted vertically. This position allows remaining buffer, which absorbs at 193 nm, to drain away. The dish was rotated at 24 rpm to ensure uniform irradiation. The energy per pulse, measured behind the lens with a Gentec ED-200 energy meter, was 3.3 mJ. The area irradiated was 78.5
end of the nuclear membrane and one from the center of the nuclear membrane (Fig. 1). Ten cells from three different blocks for each cell type were measured.

Unscheduled DNA Synthesis. CHO cells were grown to a subconfluent layer on glass microscope slides for 24 h. Prior to irradiation, the cells were washed with HBSS without Ca\(^{2+}\) and Mg\(^{2+}\). Cells were exposed to 0, 2, 4, 6, or 10 J/m\(^2\) 254-nm radiation or to 40 or 80 J/m\(^2\) 193-nm radiation in the same manner as described above. The cells were then incubated for 24 h in complete medium plus 10 \(\mu\)Ci/ml \([\text{H}]\)thymidine, after which the slides were washed with HBSS and allowed to dry. The slides were then fixed in acetone for 10 min and allowed to dry. All subsequent steps were done under a Kodak no. 2 filter lamp in a dark room. The slides were dipped in NTB2 emulsion (diluted 1:1 with water, 45°C; Kodak), and kept in a paper safe with desiccant overnight. After storage in black boxes at \(-70°C\) for 1 week, the slides at room temperature were dipped in Kodak D-19 developer, rinsed for 2 min in lukewarm water, dipped in Kodak fixer for 5 min, and rinsed in warm water for 10 min before staining with hematoxylin and eosin. Grains on cells were counted using oil immersion. One hundred cells were counted at each fluence. Cells containing between 0 and 50 grains were counted.

Cytotoxicity Assays. The cytotoxicity of 193- or 254-nm and X-ray radiation was assessed by a clonogenic assay. Cells were detached with trypsin, counted, serially diluted, and plated on 10-cm-diameter plastic tissue culture dishes. The inocula of cells were varied for each fluence or radiation exposure based on the anticipated survival. Usually there were 5–50 surviving colonies/dish after exposure. Three or four dishes were used for each fluence. Prior to exposure, the dishes were allowed to stand for 4–5 h at 37°C to permit attachment. For UV radiation exposures, the medium was then removed and the cells were rinsed twice with HBSS without Ca\(^{2+}\) or Mg\(^{2+}\) and were irradiated without buffer. X-Ray exposures occurred through the growth medium. After the irradiation, fresh medium was added and the cells were incubated 7 to 14 days before the colonies were scored. The colonies were fixed with methanol and stained with 0.1% Crystal Violet for 5 min. The effect of radiation on the clonal growth was expressed as a percentage of survival relative to unirradiated sham-exposed controls. At least two experiments were performed for each condition. The sensitivity of each cell type to radiation is expressed as \(D_{50}\) values (the UV fluence or X-ray dose required to reduce survival to 37% of the unirradiated control).

RESULTS

Endonuclease-sensitive Sites in Cells Exposed to 193-nm and 254-nm Radiation. The frequency of pyrimidine dimer-specific ESS is a measure of the number of UV photons reaching the nuclear DNA. NHF cells were exposed to 0–360 J/m\(^2\) 193-nm radiation and to 1 J/m\(^2\) 254-nm radiation in the same manner as for cytotoxicity experiments, except that the plates were cooled on ice to inhibit DNA repair. For comparison, CHO cells were also exposed to higher fluences of 193 nm (0–1000 J/m\(^2\)), because the \(D_{50}\) for these cells is higher. CHO cells were also exposed to 254-nm radiation (1 J/m\(^2\)).

ESS were detected in both cell types after exposure to 193-nm radiation, indicating that 193-nm photons penetrate to the nucleus. However, the average frequency of ESS/megabase per incident photon was about 5-fold greater for the NHF than for the CHO cells (Table 1). Also, radiation at 254 nm produced more ESS/megabase than an equivalent number of photons at 193 nm in both cell types. For example, approximately the same frequency of ESS/megabase was detected in NHF cells exposed to 1 J/m\(^2\) 254-nm radiation or to approximately 100 times the number of photons (120 J/m\(^2\)) of 193-nm radiation.

Unscheduled DNA Synthesis in 193-nm- and 254-nm-irradiated Cells. To further compare 193- and 254-nm radiation-induced DNA damage, UDS was measured in CHO cells. The cells were exposed to 193-nm and 254-nm radiation in the same

\[ N = \left[ L^{-1}(\text{endo}) - L^{-1}(\text{endo}) \right]/1000 \]

where \(L^{-1}(\text{endo})\) is the number average molecular length of the DNA sample treated with UV endonuclease and \(L^{-1}(\text{endo})\) is the number average molecular length of the corresponding untreated sample.

Cytoplasm Thickness. CHO and NHF cells were grown to a subconfluent layer on glass coverslips. Cells were washed 3 times in Dulbecco's phosphate-buffered saline (GIBCO), fixed in 4% glutaraldehyde, post-fixed in 2% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded by inversion of Beem capsules containing Epon 812. Thin sections of the cells in cross-section were collected on single hole-slot grids, stained with uranyl acetate and lead citrate, and viewed with an electron microscope (model CM 10; Philips, Inc., Eindhoven, The Netherlands).

The distance between the nuclear membrane and the plasma membrane on the apical surface of each cell was measured on the electron microscope. Three measurements/cell were obtained, one from each end of the nuclear membrane and one from the center of the nuclear membrane (Fig. 1). Ten cells from three different blocks for each cell type were measured.
DNA DAMAGE BY 193-nm RADIATION

Table 1 Pyrimidine dimer-specific endonuclease-sensitive sites after exposure of NHF and CHO cells to 193-nm or 254-nm radiation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ESS/megabase</th>
<th>NHF</th>
<th>CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>No UV</td>
<td>0.4 ± 0.8*</td>
<td>2.4 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>193 nm, J/m²</td>
<td>9.2 ± 1.9</td>
<td>7.8 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>16 ± 3</td>
<td>20 ± 7</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>26 ± 8</td>
<td>26 ± 8</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>11 ± 2</td>
<td>8.7 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>14 ± 4</td>
<td>14 ± 4</td>
<td></td>
</tr>
<tr>
<td>360</td>
<td>17 ± 3</td>
<td>18 ± 4</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SE of at least three electrophoresis assays.

manner as for the cytotoxicity determinations. The mean number of grains/nucleus was calculated. UDS was induced in a fluence-dependent manner. When CHO cells were exposed to fluences which caused 10–15% cell killing (4 J/cm² at 254 nm and 40 J/cm² at 193 nm), the mean number of grains/nucleus was approximately 1.6-fold higher using 254-nm radiation. When fluences causing 45–55% cell killing (9 J/cm² at 254 nm and 80 J/cm² at 193 nm), the ratio was 3.4. Thus, the amount of UDS produced/J of UV radiation delivered was much greater using 254-nm than 193-nm radiation. The mean number of grains/nucleus per J at 254 nm was 1.0–1.4, whereas for 193 nm this value was only 0.049–0.063, a factor of over 20 difference.

Cytoplasmic Thickness. Our results indicate that the thickness of cytoplasm over the nuclear DNA may strongly influence the effect of 193-nm radiation of cells. Electron microscopy was used to directly measure this dimension for NHF and CHO cells (Fig. 1). The results are given in Table 2. The cytoplasmic layer was about twice as thick over the center of the nucleus of CHO cells than over NHF nuclei (1.09 versus 0.52 μm). The difference was less when measurements of the center and at each side were averaged, although the thickness was still 50% greater in the CHO cells. As can be seen from Fig. 1, the cytoplasmic thickness over the center of the nucleus probably represents the average thickness best and will be used in the calculations discussed below.

Cytotoxicity of 254-nm (Germicidal) and Ionizing Radiation to Human Fibroblast Cells. To evaluate the contribution of DNA damage to 193-nm radiation-induced cytotoxicity, the effects of 254-nm radiation and X-rays on cells deficient in DNA repair were compared to the sensitivity of these cells to 193-nm radiation. First, the inhibition of the colony-forming ability
of NHF, XPA-1, and AT cells was determined after exposure to varying fluences of 254 nm radiation. As expected from previously published studies (20-22), the XPA-1 cells were substantially more sensitive to 254-nm radiation than were the NHF or AT cells (Fig. 2). The survival curves for the NHF and AT cells showed an initial shoulder before the exponential decrease in cell survival. As shown in Table 3, the $D_{70}$ values for the XPA-1 and AT cells were, respectively, 16.5 and 1.3 times lower than the value for the NHF cells.

The same three cell types were exposed to varying doses of X-rays, and colony-forming ability was assayed. The AT cells were the most sensitive to X-ray damage, with a $D_{20}$ value of 0.07 Gy (Fig. 3). The NHF and XPA-1 cells were 3.4- and 2.3-fold, respectively, less sensitive than AT cells (Table 3).

**Cytotoxicity of 193-nm Excimer Laser Radiation to Human Fibroblast Cells.** Survival curves for NHF, AT, and two XPA cell lines after exposure to 193-nm radiation are plotted from the results of clonogenic assays in Fig. 4. For all the cell types, 193-nm radiation caused less cell killing than 254-nm radiation at the same fluence. It should be noted that comparisons between wavelengths should be based on the number of photons/area causing the effect rather than the fluence, because of the differences in number of photons/J at each wavelength. There are about 1.3-fold more photons/J at 254 nm than at 193 nm. However, this effect is very small compared to the differences in cytotoxicity caused by these two wavelengths, and cytotoxicity is usually reported in terms of fluence. Therefore, our data will be reported in terms of fluence in order to facilitate comparison with other literature on UV effects on cells.

The AT cells and NHF cells showed almost the same sensitivity to 193-nm radiation below 40 J/m$^2$ (Fig. 4). For NHF cells, a shoulder was seen before the initial linear portion. All of the survival curves, except the curve for AT cells, showed at least two regions, a linear region at low fluences and a region with a decreasing slope at higher fluences. Although 193-nm radiation was more cytotoxic to the XPA cells than to the NHF cells, it is significant that this difference is not as great as that found using 254-nm radiation. The $D_{70}$ values for the XPA-1 and XPA-2 cells were lower by factors of only 3.5 and 5.6, respectively, than those for NHF cells, compared to a factor of 16.5 for XPA-1 cells obtained using 254-nm radiation. This result indicates that the mechanisms for cell killing differ at the two wavelengths. If 193-nm radiation-induced the same type of lethal damage to cells as X-rays, the AT cells should be more sensitive to 193 nm than are NHF cells. Table 3 shows that this is not the case; the ratios of $D_{70}$ values for NHF and AT cells were almost identical (1.2 at 193 nm and 1.3 at 354 nm).

**DISCUSSION**

The overall goal of this investigation was to understand the interaction of 193-nm radiation with cells, and particularly with...
nuclear DNA, in order to assess the in vivo mutagenic and carcinogenic potential of this wavelength. The first question asked was whether 193-nm photons penetrated into the nucleus of cells and were absorbed by DNA. Our data indicate that 193-nm radiation is absorbed by nuclear DNA; DNA photoproducts were measurable as pyrimidine dimer-specific ESS (Table 1), and unscheduled DNA synthesis was detected. The data obtained from each of these measurements indicated that, for the same number of photons incident on the cells, 254-nm radiation had a greater effect than 193-nm radiation.

Previous studies suggested that nuclear DNA is shielded from 193-nm photons by the strong absorbance of proteins in the cellular membranes, cytoplasm, and nucleus at 193 nm (3, 11). This is a reasonable hypothesis, because the peptide bond of proteins and the aromatic amino acids have high extinction coefficients ($7 \times 10^4$ and $1.9-4.4 \times 10^4$ M$^{-1}$ cm$^{-1}$, respectively) and the peptides are present at high concentrations. However, direct measurements of the 193-nm absorption of cytoplasm and membranes are not available. Qualitatively, our results suggest that shielding of the nucleus from 193-nm photons may explain the lower ESS/megabase value in CHO cells compared to NHF cells, because the cytoplasm layer over the nucleus is thicker for CHO cells than for NHF cells (Table 2). An estimate of the shielding of nuclear DNA by cytoplasmic protein can be calculated from the concentration of protein in mammalian cells. Proteins comprise 18% of the total cell weight; thus, the amino acid concentration is 1.2 M, assuming an average molecular weight for amino acids in protein of 150. With the extinction coefficients given above and assuming that 10% of the protein amino acids are aromatic, a calculation using the Beer-Lambert law yields 94% absorption/1 $\mu$m in cells. The attenuation of radiation penetrating cells can also be expressed in terms of an absorption coefficient, $\alpha$, as shown in Eq. 1.

$$\frac{F}{F_0} = e^{-\alpha t}$$  \hspace{1cm} (1)

where $F =$ fluence reaching a particular point in cells; $F_0 =$ fluence incident on plasma membrane; $\alpha =$ absorption coefficient in $\mu$m$^{-1}$; and $t =$ thickness of the absorbing layer in $\mu$m.

From the calculation above, $\alpha =$ 2.8 $\mu$m$^{-1}$ for cytoplasm and can be used to estimate the percentage of photons penetrating to varying depths in cells. For example, the thicknesses of the cytoplasm for CHO and NHF cells (Table 2) allow averages of 6 and 25%, respectively, of the incident photons to be transmitted to the surface of the nucleus. The factor of about 4 difference in transmitted photons is similar to the factor of 5 difference in frequency of ESS/megabase per incident photon between CHO and NHF cells (Table 1). Thus, it appears that, given the approximations used in this calculation, absorption of 193-nm radiation by protein in the cytoplasm can account for reduction of the yields of DNA photoproducts.

Because cells in tissue usually have $\geqslant 2$ $\mu$m of cytoplasm between the plasma membrane and the nucleus, the incident 193-nm photons will be attenuated by the cytoplasm by more than 99% before they reach the nuclear envelope. A combination of low penetration of 193-nm photons into cells and the low quantum yield for dimers (14) may account for the lack of UDS in tissue exposed to 193-nm radiation (8, 9).

The only direct measurement available for absorption of 193-nm photons by tissue is for cornea, where $\alpha$ was reported to be 0.27 $\mu$m$^{-1}$ (23). From studies of 193-nm ablation of cornea, $\alpha$ can be estimated to be 0.75 $\mu$m$^{-1}$ (24) or 1.23-1.57 $\mu$m$^{-1}$ (25). All of these values for $\alpha$ are lower than our calculated value. The very low content of aromatic amino acids in collagen may account for the low $\alpha$ in cornea. Despite the low $\alpha$ for cornea, UDS was not observed after exposure of cornea to 193-nm radiation (8).

The second major question addressed in this study concerned the contribution of DNA photoproducts to the cytotoxicity of 193-nm radiation to normal cells. Our results indicate that neither cyclobutylpyrimidine dimers nor DNA strand breaks are the major lethal lesions in normal cells exposed to 193-nm radiation, although the dimers may contribute to the 193-nm radiation-induced killing of XPA cells.

Based on the measurements discussed above, 254-nm radiation should be much more cytotoxic than 193-nm radiation to fibroblasts if cyclobutylpyrimidine dimers are the lethal lesions for both wavelengths. For NHF cells, 1 J/cm$^2$ of 254 nm produced 17 ESS/megabase and extrapolation of the data in Table 1 for 193 nm yields approximately 0.2 ESS/megabase at 1 J/cm$^2$. Thus, the ratio of $D_{\gamma}$ values for 254-nm to 193-nm radiation should equal approximately 85, if cyclobutylpyrimidine dimers are the lethal lesions. However, the results in Table 3 indicate that the ratio of $D_{\gamma}$ values for NHF cells exposed to 254- and 193-nm radiation is only 3.4. This comparison suggests that a mechanism other than DNA photodamage is responsible for 193-nm radiation-induced cytotoxicity in normal cells. The ratio of $D_{\gamma}$ values for the XPA cells is higher, about 16, indicating that pyrimidine dimers may contribute to the cytotoxicity of 193 nm to these cells.

An additional comparison also supports this conclusion. If dimers do not contribute to cell killing using 193-nm radiation, the $D_{\gamma}$ values for XPA and NHF cells should be about the same (assuming that they differ only in their ability to repair dimers). The data in Table 3 indicate that the $D_{\gamma}$ values differ by a factor of about 3.5. Taken together, these results indicate that the majority of 193-nm radiation-induced cytotoxicity does result from unrepaired dimers in normal cells and that, even in cells deficient in dimer repair, dimers are not the sole lethal lesions.

The possible contribution of ionizing radiation-like damage to 193-nm radiation-induced cytotoxicity was evaluated because the quantum yield for DNA single-strand breaks is roughly 10-fold greater at 193-nm than at 254-nm (14). The AT cells used in this study were about 3.4 times more sensitive to X-rays than NHF cells (Table 3). If DNA strand breaks are major photoproducts in 193-nm-irradiated cells and 193 nm radiation causes X-ray-type lethal damage (26), the ratio of $D_{\gamma}$ values should be about 3.4. As shown in Table 3, the AT and NHF cells were almost equally sensitive to 193-nm radiation. Thus, X-ray-type lethal damage is not induced by 193-nm radiation even in cells deficient in repair of these lesions. This result is consistent with our earlier report that alkali-labile sites are not detectable even at fluences of 193-nm radiation severalfold higher than the $D_{\gamma}$ for CHO cells (11).

In summary, this study shows that the cytoplasm of cells efficiently protects nuclear DNA from 193-nm radiation, that this protection plus the lower quantum yield for cyclobutylpyrimidine dimer formation at 193-nm reported previously (14) sharply reduces the risk of mutagenesis and carcinogenesis from exposure to 193-nm radiation during surgical procedures, and that the DNA photoproducts investigated do not contribute
the cytotoxicity of 193-nm radiation to DNA repair-proficient cells.

ACKNOWLEDGMENTS

Discussions with Katheryn D. Held and Thomas Flotte (Massachusetts General Hospital) and Franz Hillenkamp (University of Münster) are gratefully acknowledged.

REFERENCES


DNA Damage Induced by 193-nm Radiation in Mammalian Cells

Irene E. Kochevar, Agnes A. Walsh, Howard A. Green, et al.