Differential Effect of UV-B and UV-C on DNA Damage in L-132 Cells

Takayuki SATO,^a Naoto OKU,^a Eri IIDA,^a Kenji KAWAGUCHI,^a Kenzo YAMANAKA,^b Toshio MORI,^c and Shoji OKADA^{*,a}

Department of Radiobiochemistry, School of Pharmaceutical Sciences, University of Shizuoka,^a Yada, Shizuoka 422, Japan, Department of Biochemical Toxicology, College of Pharmacy, Nihon University,^b Funabashi, Chiba 274, Japan, and RI Center, Nara Medical University,^c Nara 634, Japan. Received November 9, 1995; accepted January 8, 1996

Ultraviolet radiation is known to induce skin cancer. The induction of DNA damage caused by UV-B and UV-C was investigated using cultured L-132 cells. DNA strand breaks assayed by the alkaline elution procedure occurred in a dose-dependent manner, the extent of the strand breaks were inversely well correlated with the number of viable L-132 cells after 24 h incubation. About a 10-fold dose of UV-B irradiation was required to induce a similar degree of strand breaking to that induced by UV-C. Similarly about a 10-fold dose of UV-B was required to produce a similar amount of pyrimidine dimers, such as cyclobutane-type dimers and pyrimidine-(6-4)-pyrimidone photoproducts, which were determined by ELISA using the specific monoclonal antibody, to that produced by UV-C. Strand breaks induced by UV-B, however, were not fully repaired in viable cells remaining after incubation of cells for a longer period of time, although UV-C-induced strand breaks were repaired in a time-dependent manner. Furthermore, an experiment with a cell-free system, where the induction of strand breaks by repair enzymes did not take place, indicated that UV-B caused significantly more direct DNA strand breaks than that caused by one-tenth the dose of UV-C. The data shown here suggest that UV-B-induced DNA damage is mediated, at least in part, *via* a different mechanism from the UV-C induced one.

Key words UV-B; DNA damage; strand break; UV; pyrimidine dimer

Solar UV irradiation is thought to induce carcinogenic, mutagenic and killing effects on mammalian cells through generation of photolesions and/or DNA strand breakage. DNA photolesions such as cyclobutane dimers and pyrimidine-(6-4)-pyrimidone photoproducts are considered to be the major photoproducts caused by UV irradiation.^{1,2)} The most efficient wavelength for the induction of these types of damage is about 260 nm, a wavelength in the UV-C region.^{3,4)} This type of damages, in general, is mainly repaired by an excision repair system.⁵⁾ Xeroderma pigmentosum is a human genetic disorder with an autosomal recessive mode of inheritance. This gene product is involved in the nucleotide excision repair system, particularly in damage recognition and incision processes.⁶⁾

The particular concern of solar UV irradiation caused by depletion of the stratospheric ozone layer is related to irradiation by near ultraviolet wavelengths, *i.e.*, UV-B (290–320 nm) and UV-A (320–400 nm), since very little UV-C reaches humans due to atmospheric shielding. Furthermore, carcinogenic and mutagenic effects of UV-B and UV-A have been observed.^{7–9)} UV-B is also reported to enhance the aggressiveness of human cutaneous melanoma in terms of its proliferation and metastatic potential.¹⁰⁾ Therefore, we determined the DNA damage caused by UV-B in comparison with that by UV-C by monitoring DNA strand breaks, pyrimidine dimer formation and cellular toxicity in L-132 human alveolar type 2 cells. Furthermore, to evaluate the action of UV causing direct DNA strand breaks, naked DNA was irradiated with UV. This system could eliminate the strand breaks caused by enzymatic repair processes.

MATERIALS AND METHODS

Cell Culture L-132 cells were cultured in Eagle's MEM

(Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS). The cells (1×10^6 per 9-cm² dish) were cultured for 24 h, with a doubling time of about 21 h, to monolayers in humidified air with 5% CO₂ at 37 °C. DNA in the cells was labeled by incubation for another 24 h with [methyl-³H]thymidine (37 kBq/dish).

UV Irradiation UV-B or UV-C irradiation was performed by exposing monolayer cultures in dishes without lids to a UV-B (312 nm) irradiation apparatus (ATTO Co., HP-30M, Tokyo; using a cut-off filter to guarantee the elimination of shorter wave-lengths in the UV-C range) or a germicidal lamp (Toshiba, Tokyo). The fluence rates were determined with a UVX Radiometer (UVP, Inc. San Gabriel, CA). Both UV-B and UV-C irradiation was carried out for various periods of time at a dose rate of 1 J/m² s.

Alkaline Elution of DNA DNA strand breaks were determined by the alkaline elution method as described previously¹¹⁾ and which is based on the method originally described by Kohn *et al.*¹²⁾ In brief, after UV irradiation, cells were harvested and applied to a polycarbonate filter (2.0 μm pore size, Nuclepore, Costar Co. Cambridge, MA). After the cells were lysed with proteinase K in the presence of 2% SDS, they were washed with 20 mM EDTA (pH 10.0), then the DNA was eluted with buffer (pH 12.1) at a flow rate of 0.05 ml/min. Fractions were collected at 60 min intervals for 10 h, and the radioactivity of each fraction and that remaining on the filter were counted in a liquid scintillation counter.

In a cell-free system, ³H-labeled cells were harvested, lysed with SDS containing proteinase K, and washed with 20 mM EDTA (pH 10.0) applied to the filter. Then DNA on the filter in a wet condition was irradiated with UV and washed with EDTA, before performing the alkaline elution procedure indicated above.

* To whom correspondence should be addressed.

Detection of Pyrimidine Dimers by ELISA L-132 cells prepared as described above, except for the [methyl- ^3H]thymidine labeling, were irradiated with UV-B or UV-C, and then lysed with SDS containing proteinase K. Total DNA was extracted with phenol and purified with ethanol. Cyclobutane dimers and (6-4)photoproducts were detected by ELISA with specific monoclonal antibodies as described previously.¹³⁻¹⁵ In brief, polyvinylchloride flat-bottom microtiter plates (Dynatec, Alexandria, VA) precoated with 1% protamine sulfate (Sigma) were incubated with isolated DNA in PBS at 37°C for 20 h after denaturation of DNA. The plates were dried and washed 5 times with PBS containing 0.05% Tween 20 (PBS-T), prior to incubation with 4% FBS in PBS at 37°C for 30 min to avoid non-specific binding of the antibody in PBS-T.

A hundred microliters of monoclonal antibody was added to each well and the plates were incubated at 37°C for 30 min. Then the plates were washed with PBS-T and incubated with 100 μl goat anti-mouse IgG conjugated with peroxidase in PBS at 37°C for 30 min. The plates were washed with PBS-T and with citrate-phosphate buffer, pH 5.0. Then, 100 μl of a solution containing 0.04% *o*-phenylene diamine and 0.007% H_2O_2 in citrate-phosphate buffer was added to each well. After 30 min incubation at 37°C, 2 M H_2SO_4 was added to stop the reaction and the absorbance at 492 nm was measured using a microplate reader (Corona, MTP-120). The mean value of 4 wells was calculated.

Determination of Cytotoxic Action of UV L-132 cells (1×10^5 cells/well) were seeded on 96-well plates and incubated for 2 d. The sub-confluent cells were irradiated with UV and incubated for another 24 h. The density of viable cells was determined as described previously.¹⁶ In brief, cells were stained with 0.5% crystal violet in methanol-water (4:1) for 15 min, and washed with water. Then the dye was eluted with 33% acetic acid to determine the absorbance at 630 nm on a microplate reader.

RESULTS

DNA Single Strand Breaks Induced by the Irradiation of UV-B or UV-C on L-132 Cells In order to determine the DNA-damaging ability of UV-B and UV-C, L-132 cells were irradiated with different doses of UV prior to alkaline elution. As shown in Fig. 1, both UV-B and UV-C caused dose-dependent strand breaks on L-132 cellular DNA, although the dose of UV-C was one-tenth that of UV-B needed to cause a similar degree of strand breaks: For example, the alkaline elution profile of 1000 J/m² UV-B irradiation is almost equivalent to that of 100 J/m² UV-C irradiation. The percentage of viable L-132 cells 24 h after UV-irradiation is shown in Fig. 2. Similarly to strand breaks-induction, a 10-fold dose of UV-B irradiation was required to induce a similar degree of cell killing to that caused by UV-C; a 37% viable dose of UV-B was about 1000 J/m² and that of UV-C was about 100 J/m².

To examine the pyrimidine dimer formation caused by UV irradiation, cyclobutane dimers and (6-4)photoproducts in UV-irradiated cells were detected semiquantitatively by ELISA. Figure 3 shows the relative amounts of both types of pyrimidine dimer in UV irradiated cells. Again, about 10-fold dose of UV-B irradiation was required to induce a similar degree of both types of pyrimidine dimer formation to that caused by UV-C. The reduction of both dimers after incubation was similar in both UV-B- and UV-C-irradiated cells, indicating that the dimers were repaired in both UV-B- and UV-C-irradiated cells in a similar way (Fig. 4).

Differential Effect of UV-B and UV-C on DNA The data shown above indicate that UV-B and UV-C cause DNA damage by a similar mechanism which involves pyrimidine dimer formation, and the efficiency of UV-B is about one-tenth that of UV-C. Furthermore, since the strand breaks caused by UV irradiation were markedly enhanced in the presence of aphidicolin, an inhibitor of DNA polymerase α , δ and ϵ , but not in the presence of dideoxythymidine, an inhibitor of DNA polymerase β

Fig. 1. UV-Induced DNA Strand Breaks in L-132 Cells

DNA strand breaks in L-132 cells induced by UV-B (a) or UV-C (b) were determined by alkaline elution assay as described in Materials and Methods. a) $\square$, control; $\bullet$, 500 J/m² UV-B; $\circ$, 1000 J/m²; $\blacktriangle$, 2000 J/m²; $\blacksquare$, 5000 J/m². b) $\square$, control; $\bullet$, 10 J/m² UV-C; $\circ$, 100 J/m²; $\blacktriangle$, 500 J/m²; $\blacksquare$, 1000 J/m².

Fig. 2. Cytotoxic Effect of UV on L-132 Cells

L-132 cells were irradiated with UV-B (a) or UV-C (b) at the indicated dose. Then cells were further incubated at 37°C for 24 h. Viable cell density was measured at 630 nm absorbance after viable cell staining with crystal violet as described in Materials and Methods. Dashed line indicates 37% survival rate.

Fig. 3. Pyrimidine Dimer Formation Following UV Irradiation

L-132 cells were irradiated with various doses of UV-B (a, c) or UV-C (b, d). Then the cyclobutane dimers (a, b) and (6-4) photoproducts (c, d) produced were detected by ELISA as described in Materials and Methods. The amounts of pyrimidine dimers are expressed as the absorption at 492 nm which correlates with the amount of dimers, although values are not comparable between two types of pyrimidine dimers.

Fig. 4. Repairing Pyrimidine Dimers after UV Irradiation

After irradiation of L-132 cells with 1000 J/m² of UV-B (●) or 100 J/m² of UV-C (▲), the cells were incubated to repair the pyrimidine dimers. The amounts of cyclobutane dimers (a) and (6-4) photoproducts (b) were determined by ELISA as described in Materials and Methods, and are expressed as the absorption at 492 nm.

(Fig. 5), UV-induced strand breaks seem to occur mainly by the excision repair system. Therefore, we next determined strand-break repair after UV irradiation using the alkaline elution assay. The initial doses of UV-B and UV-C used for the repair experiments were 1000 and 100 J/m², respectively. As shown in Fig. 6, strand breaks caused by UV-C irradiation were repaired during post-incubation. However, strand breaks caused by UV-B irradiation remained even after a given repair time.

The Repair kinetics of strand breaks were biphasic for both UV-B and UV-C: Fast repairing, which may relate mainly to excision repair including DNA polymerase α , δ and ϵ systems, and which was indicated in Fig. 5, occurred in a similar manner in both UV-B- and UV-C-irradiated cells. However, slow repair occurred only in UV-C-irradiated cells (Fig. 6). These data suggest that strand breaks caused by UV-B involve, at least in part, ones that are very difficult to repair such as double strand breaks

Fig. 5. Effect of DNA Polymerase Inhibitors on UV-Induced Strand Breaks

L-132 cells were treated with DNA polymerase inhibitors for 1 h each before and after irradiation with 1000 J/m^2 UV-B (a) or 100 J/m^2 UV-C (b). DNA strand breaks in viable L-132 cells were determined by alkaline elution assay as described in Materials and Methods. Without UV irradiation: $\square$, control; $\triangle$, $25 \mu\text{g/ml}$ aphidicolin with 10 mM hydroxyurea; $\diamond$, 10 mM dideoxythymidine with $1 \mu\text{M}$ methotrexate and $50 \mu\text{M}$ adenosine. With UV irradiation: $\circ$, without inhibitors; $\blacktriangle$, $25 \mu\text{g/ml}$ aphidicolin with 10 mM hydroxyurea; $\blacklozenge$, 10 mM dideoxythymidine with $1 \mu\text{M}$ methotrexate and $50 \mu\text{M}$ adenosine.

Fig. 6. Repair of DNA Strand Breaks after UV Irradiation

After irradiation of L-132 cells with 1000 J/m^2 UV-B (a) or 100 J/m^2 UV-C (b), these cells were incubated to repair the strand breaks for selected times. DNA strand breaks in viable L-132 cells were determined by alkaline elution assay as described in Materials and Methods. $\square$, control without UV irradiation; $\circ$, no repairing time; $\bullet$, 2 h incubation; $\blacktriangle$, 4 h incubation; and $\blacksquare$, 16 h incubation.

of DNA.

To consider such a possibility, we attempted to investigate the effect of UV irradiation on DNA under conditions where no enzymatic repair takes place (Fig. 7). UV irradiation was performed for naked DNA, and strand breaks were observed in this cell-free system. The number of strand breaks induced by UV-B was significantly large compared with that produced by one-tenth the dose of UV-C.

DISCUSSION

DNA strand breaks on L-132 cells were observed following irradiation with UV-B as well as UV-C. The number of strand breaks increased dose-dependently for

both types of UV, as far as the doses tested were concerned. About a 10-fold dose of UV-B, however, was required to cause the same degree of strand breaks following irradiation by UV-C. This ratio was also displayed with respect to the toxic effect of UV and the formation of pyrimidine dimers. In fact, both cyclobutane dimers and (6-4)photoproducts are known to be responsible for UV-induced cytotoxic action^{17,18} and mutation^{19,20} in mammalian cells. Therefore, the induction of strand breaks by UV-B and UV-C might be, at least in part, through the same mechanism, *i.e.*, excision repair following pyrimidine dimer formation.

However, the present experiment also shows that UV-B caused DNA strand breaks in another way, in addition to the mechanism above. The strand breaks caused by

Fig. 7. UV-Induced DNA Single-Strand Breaks in the Cell-Free System

³H-Labeled-DNA was obtained from L-132 cells as described in Materials and Methods, and irradiated with UV-B (a) or UV-C (b) just prior to alkaline elution assay. a) □, control; ●, 200 J/m² UV-B; ▲, 500 J/m²; ■, 1000 J/m². b) □, control; ●, 20 J/m² UV-C; ▲, 50 J/m²; ■, 100 J/m².

UV-B remained to some extent after incubation for a long period of time, suggesting that UV-B causes severe DNA strand breaks which seem to be unreparable, except for pyrimidine dimer formation. UV-C-induced photolesions are actually known to be repaired in a few hours.¹⁾ Other results suggest that UV-B causes non-dimer damage to human T-lymphocytes,²¹⁾ which is consistent with the observation presented here, although the cytotoxic action of UV-B was not great compared with that of UV-C at the doses used for forming similar amounts of pyrimidine dimers.

To confirm the observation that UV-B causes severe DNA damage, an experiment involving direct irradiation of UV-B was performed and this was found to cause marked DNA strand breaks which were not readily reparable. Strand breaks in cell-free systems were distinct from those preceded by excision repair, and the strand breaks might be a non-pyrimidine dimer lesion. Hydroxyl radicals generated by UV-B are one of candidates for causing these non-enzymatic strand breaks. Another possibility is that hydroxyl radicals cause alkaline labile defect(s) on DNA other than strand breaks which are then detected as strand breaks in the alkaline elution assay. In either case, this damage might not be repaired by DNA polymerase α , δ and ϵ but by polymerase β , an error-prone repair.²²⁾ If so, this could explain the carcinogenicity and mutagenicity of UV-B.

Further study is required to identify the unreparable lesion and to elucidate the mechanism for generating such a lesion by UV-B irradiation.

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