Use of a Highly Sensitive Assay to Analyze the Excision Repair of Dimer and Nondimer DNA Damages Induced in Human Skin Fibroblasts by 254-nm and Solar Ultraviolet Radiation

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ABSTRACT

The excision repair of nondimer DNA damages induced in normal human skin fibroblasts exposed to the Mylar-filtered UV produced by a fluorescent sunlamp was investigated. This work was accomplished through the development of a modification of the bromodeoxyuridine photolysis assay that greatly increases the sensitivity of this assay. This enhancement in sensitivity was achieved through use of alkaline elution to measure the DNA strand breakage produced by the photolysis of bromodeoxyuridine incorporated into the DNA through excision repair. Using this modified bromodeoxyuridine photolysis assay, it was found that the solar UV-induced nondimer DNA damages appear to have been repaired by a short patch repair mechanism in which a small number of nucleotides (two to four) were inserted into the repaired site. This is in contrast to the long patch repair process involved in the excision of cyclobutane pyrimidine dimers in which approximately 40 nucleotides were inserted into each repaired region.

INTRODUCTION

The excision repair of dimers, the most common photoprotein induced in the DNA of cells exposed to 254-nm UV, has been studied extensively (1, 2). In contrast to the relative wealth of information as to the repair of dimers, little is known about the mechanism by which other types of UV-induced DNA damages are repaired. For cells exposed to 254-nm UV, these nondimer photoproducts are produced at a very low level. However, the yield of these damages, compared to dimers, increases markedly in cells exposed to UV wavelengths in the 290- to 400-nm region (3-6). This range is of interest, because it represents the UV wavelengths present in sunlight reaching the earth’s surface (7). The UV region is of particular importance due to evidence that this component of sunlight is responsible for the induction of most skin cancers (8). For cells exposed to these solar UV wavelengths, the yield of various nondimer photoproducts such as thymine glycols (3), DNA-protein cross-links (5), and DNA strand breaks (4), to dimers, is as much as 1000-fold higher than in 254-nm-irradiated cells. In addition, the results of numerous experiments have demonstrated that nondimer DNA damages play a critical role in the induction of a variety of biological effects, including neoplastic transformation, in cells exposed to solar UV wavelengths (9-20). It is also important to note that photorepair appears to be an important and rapid process in human skin in vivo (21-23). Hence, it is possible that, during sunlight irradiation, dimers are induced by the mid-UV wavelengths (290 to 320 nm) and then photorepaired through the combined action of DNA photolyase and the near-UV and visible component (320 to 600 nm; 24). The net result, therefore, of an exposure to sunlight may be the induction primarily of nondimer photoproducts. This point is often overlooked in the interpretation of the results from photocarcinogenesis experiments utilizing mice which are capable of photorepair only during the early neonatal period (25). It is also worth noting that active oxygen species may play a role in tumor promotion (26, 27), and that solar UV-induced nondimer damages appear to be produced principally by photodynamic mechanisms mediated by endogenous photosensitizers involving the conversion of oxygen to reactive states (28). In addition, the near-UV wavelengths present in sunlight appear to be effective in tumor promotion (29). Hence, it is of importance to study the induction and repair of nondimer DNA damages produced by solar UV wavelengths due to their possible role in the initiation and promotion of skin cancers.

The purpose of the present study was to investigate the excision repair of solar UV-induced nondimer DNA damages using the BrdUrd photolysis assay (30). It has been demonstrated, using this assay, that DNA damages are generally repaired by either a short patch excision repair mechanism in which 2 to 4 nucleotides are inserted into the repaired region or by a long patch mechanism in which approximately 50 to 200 nucleotides are inserted into the repaired site (31-36). Through the development of a modification, which greatly enhances the sensitivity of the BrdUrd photolysis assay, it was determined that the nondimer DNA lesions induced in cells exposed to solar UV wavelengths are repaired by a short patch mechanism as opposed to the long patch process involved in dimer repair.

MATERIALS AND METHODS

Cells and Culture Conditions. Normal human fibroblasts were derived from a skin biopsy (37) and grown in Dulbecco’s modified Eagle’s medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (HyClone, Logan, UT), L-glutamine at 0.4 mg/ml, penicillin at 100 units/ml, and streptomycin at 0.1 mg/ml (GIBCO). Cultures were incubated at 37°C in a 7.5% CO₂ humidified atmosphere, and the medium was changed 2 times per week. Under these conditions the cell number doubled every 1 to 2 days.

Labeling and Irradiation Conditions. Human fibroblasts were plated in 100-mm tissue culture dishes (Coming Glass Works, Corning, NY) at...
a density of 10^4 cells/cm^2, and either [methyl-^3H]thymidine was added to a final concentration of 0.05 μCi/ml (20 Ci/mmol; New England Nuclear, Boston, MA) or [2-^14C]thymidine was added to a final concentration of 0.04 μCi/ml (40 mCi/mmol). The cells were grown for 3 days, the medium was replaced with fresh nonradioactive medium containing 0.5% fetal calf serum (unless otherwise indicated), and the cultures were incubated an additional 3 days. The cultures were then washed twice with PBS (8.0 g NaCl, 0.2 g KCl, 1.15 g Na2HPO4, and 0.2 g KH2PO4 per liter H2O), covered with PBS, and exposed either to 254-nm UV produced by four GE G15T8 germicidal lamps at a fluence rate of 0.35 W/m^2 or the solar UV produced by two FS40 Westinghouse fluorescent sunlamps at a fluence rate of 5.5 W/m^2. For the solar UV irradiations, the cultures were covered with a sheet of 5000 Mylar to eliminate wavelengths shorter than approximately 310 nm (17). Dosimetry was performed as previously described (38). Following the irradiations, the PBS was replaced with medium containing 0.5% serum and 0.1 ml BrdUrd for the ^14C-labeled cells and medium with 0.1 ml dThd for the ^3H-labeled cells. Following a 24-h incubation, the cells were washed twice with PBS, removed from the culture dishes, and for each DNA-damaging treatment, the ^3H- and ^14C-labeled cells were mixed and suspended in PBS containing disodium EDTA (0.2 mg/ml) at a concentration of 2 x 10^4 cells/ml. Twenty-five ml of this cell suspension were then placed in a 75-cm^2 culture flask (Coming) in an ice water bath at 1°C and exposed to the UV produced by the sunlamps which acted as the photolytic source. For these irradiations, the Mylar was not used, and the polystyrene culture flask top functioned as a filter to eliminate wavelengths shorter than about 300 nm (17). The sunlamps provided a source of 300- to 320-nm UV which caused the photolysis of BrdUrd incorporated into the DNA through repair. The photolytic cross-section for this photolytic source was 0.0061 breaks per BrdUrd residue per kJ per m^2. For each photolytic fluence, a 5-ml sample of cells was removed from the flask and lysed on a 25-mm, 2-μm pore size polycarbonate filter (Nucleospore Corp., Pleasanton, CA) with a 2% solution of sodium dodecyl sulfate (Gailard-Schlesinger, Carleplace, NY), 0.1 g glycine, and 0.02 g EDTA, pH 10. The lysis solution was allowed to flow through the filter by gravity. Two ml of lysis solution containing proteinase K (0.5 mg/ml; Scientific Products, Dallas, TX) were then placed on the filter followed by the elution solution of tetrapropylammonium hydroxide (RSA Corp., Ardsley, NY), 0.1 M glycine, and 0.02 M EDTA, pH 10. The elution solution was pumped at 0.035 to 0.045 ml/min for 15 h. Five fractions were collected at 3-h intervals. Upon completion of the elution, the fractions were made isovolumetric with water when necessary, and 10 ml of Aquassure (New England Nuclear) were added. Filters were processed as previously described (39). All fractions were counted in a Packard Tri-Carb liquid scintillation counter.

The number of breaks induced by BrdUrd photolysis was calculated from the following equation:

\[ \text{Breaks/dalton induced by BrdUrd photolysis} = 8.1 \times 10^{-10} \left( \frac{S_{\text{UV, dThd}} - S_{\text{BrdUrd, dThd}}}{S_{\text{300 rad γ-rays}} - S_{\text{300 rad γ-rays, dThd}}} \right) \]

where S equals the logarithm of the slope of the best-fit line for each elution profile determined by linear regression using a least-squares analysis (40). Previous measurements have shown that a γ-ray dose of 300 rads induces 8.1 x 10^{-10} single strand breaks per dalton of DNA (41). The specified variable, \( S_{\text{UV, dThd}} \), refers to cultures irradiated with UV, incubated in BrdUrd- or dThd-containing medium, and exposed to a particular photolytic fluence, while \( S_{\text{BrdUrd, dThd}} \) refers to similarly treated cells, but not exposed to the photolytic treatment.

The number of repaired sites and the patch size were calculated from a best-fit line through the data points following the equation \( B = N(1 - e^{-n\psi}) \), where \( B \) is the number of breaks induced by BrdUrd photolysis, \( N \) is the number of repaired sites, \( n \) is the average number of BrdUrd residues incorporated into each repaired site, \( \psi \) is the cross-section for photolysis, and \( F \) is the fluence of photolytic light (30). The best-fit line was computed using the SAS nonlinear regression procedure (42) utilizing the Marquardt method (43). The endonuclease-sensitive site assay was performed as previously described (44).

### RESULTS

Development of the Modified BrdUrd Photolysis Assay. A preliminary set of experiments was performed in which cells were exposed to solar UV in which it proved impossible to detect photolysis of BrdUrd incorporated into the DNA through the repair of nondimer damages (Chart 1b). In contrast, the repair of 254-nm-induced dimer damage at equitoxic levels was easily measured (Chart 1a). This provided the first indication that the repair of dimer and nondimer damages differed and stimulated as an initial goal the development of a modification of the BrdUrd photolysis assay so as to enhance the sensitivity of this technique. The method chosen to accomplish this goal was to utilize alkaline elution to measure the DNA strand breaks produced by BrdUrd photolysis. Alkaline elution provides much greater sensitivity for the measurement of DNA strand breakage compared with sucrose gradients which are used in the original assay.

For the first set of experiments in the development of this modified assay, ^14C-labeled cells were incubated for 24 h in medium containing 0.1 ml BrdUrd while ^3H-labeled cells were incubated in medium containing 0.1 ml dThd. The cells were removed from the culture dishes, mixed, suspended in PBS, and exposed to 1.6 kJ/m^2 from the photolytic source which corresponds to a photolytic fluence of 0.01 breaks/BrdUrd residue.

The purpose of this experiment was to measure the level of strand breaks produced in cells that were not exposed to a DNA-damaging agent and therefore did not incorporate BrdUrd into DNA through repair. Hence, the strand breaks induced are the result of the photolytic treatment exclusive of BrdUrd photolysis. These cells were then subjected to alkaline elution, and it can be observed from the elution profiles shown in Chart 2 that a much higher level of strand breakage, 8 breaks/10^10 daltons,
series of experiments has been performed using serum-arrested cells in which it has been demonstrated that these cells are capable of performing excision repair in a normal fashion and a rate comparable to cells grown in medium with 10% serum (53–56). In order to determine whether this length of incubation was sufficient to cause a complete blockage of DNA synthesis, the serum-arrested cells were grown an additional 24 h in medium containing [3H]dTd. The amount of radioactivity incorporated into trichloroacetic acid-precipitable material in 108 of these cells was only about 300 dpm, whereas the level measured in 108 cells grown in medium containing 10% serum for this period was approximately 24,000 dpm. Hence, semiconservative DNA synthesis was essentially eliminated by culturing the cells in the 0.5% serum medium. Serum-arrested cells were incubated in medium containing BrdUrd or dThd at a concentration of 0.1 mM, exposed to a photolytic fluence of 0.01 break/BrdUrd, and subjected to alkali elution. As shown in Chart 2, approximately the same level of breaks, 2 breaks/1010 daltons, was produced in both the BrdUrd- and dThd-incubated cells.

**Measurement of Repair in 254-nm and Solar UV-irradiated Cells.** 14C- and 3H-labeled cells were serum arrested and exposed to either 0.5 J/m2 of 254-nm UV, 5.0 J/m2 of 254-nm UV, or 50 kJ/m2 of UV produced by a fluorescent sunlamp in which the beam was passed through 5000 Mylar in order to eliminate the dimer-inducing short wavelength component (<310 nm) of this lamp. The surviving fraction for cells exposed to 5.0 J/m2 of 254-nm UV was 0.44 ± 0.21, while the survival of cells treated with 50 kJ/m2 of solar UV was 0.36 ± 0.18. Hence, these represent approximately equitoxic treatments. However, assuming dimers are the lethal photoproducts induced in 254-nm-irradiated cells, it is clear that damages other than dimers are responsible for the killing of cells exposed to solar UV, because it was not possible to detect dimers in cells exposed to 50 kJ/m2 of solar UV (Chart 3). The assay used to measure dimers was the endonuclease-sensitive site assay for which the minimum level of detection in these experiments was about 3 sites/109 daltons or the yield of dimers induced by a 254-nm fluence of about 1 J/m2.

For each treatment, the 14C-labeled cells were incubated for...
24 h in medium containing BrdUrd, and the $^3$H-labeled cells, in medium with dThd. At the end of this period, the cells were suspended in PBS and exposed to a range of photolytic fluences. The elution profiles for several treatments are shown in Chart 4, and the photolytic profiles are exhibited in Chart 5. In each case, the number of breaks induced by BrdUrd photolysis is equal to the number of breaks produced in BrdUrd-incubated cells minus the number of breaks produced in dThd-incubated cells. As shown in Chart 5a, the photolytic profile for cells exposed to 0.5 J/m$^2$ of 254-nm UV is curvilinear, which is indicative of damage that is repaired through the action of a long patch repair mechanism. It was determined that 70 ± 30 damages/10$^{10}$ daltons were repaired and that 40 ± 20 nucleotides were inserted into each repaired region. An important feature of these results is the demonstration that the modified BrdUrd photolysis assay possesses a high level of sensitivity in that it is possible to measure repair in cells following an essentially nontoxic treatment with a DNA-damaging agent. This is of added importance, because an assumption made in the analysis of data from BrdUrd photolysis experiments is that the distribution of repaired sites is random. This appears to be true at low levels of damage, but it is not correct for cells exposed to 254-nm fluences 6 J/m$^2$ or greater (57).

The photolytic profile for cells exposed to 5.0 J/m$^2$ of 254-nm UV is shown in Chart 5b. It was only possible to use very low photolytic fluences for these cells because the maximum level of strand breaks that can be accurately measured using alkaline elution is about 50 breaks/10$^{10}$ daltons. Hence, the results on the initial linear portion of the photolytic profile could be obtained. However, using the patch size calculated for cells exposed to the lower 254-nm fluence, the number of repaired sites in cells exposed to 5.0 J/m$^2$ was determined to be approximately 700 ± 40/10$^{10}$ daltons or a 10-fold increase compared with cells irradiated with 0.5 J/m$^2$.

The photolytic profile shown in Chart 5c for cells exposed to 50 kJ/m$^2$ of solar UV differs markedly from the response of cells exposed to an equitoxic treatment with 5.0 J/m$^2$ of 254-nm UV. First, the level of BrdUrd photolysis is lower for the solar UV-irradiated cells even though the photolytic fluences used were more than 10-fold higher than for cells exposed to 254-nm UV. One possible explanation may be that the solar UV exposure inactivated the cellular repair systems. Therefore, cells were exposed to 50 kJ/m$^2$ of solar UV followed by treatment with 5.0 J/m$^2$ of 254-nm UV, or the order of these exposures was reversed. In either case, approximately the same level of BrdUrd photolysis and therefore repair was detected, indicating that the solar UV exposure did not inhibit repair (Chart 5b). The other feature of the photolytic profile shown in Chart 5c is that it is linear with no indication of a plateau, even though relatively high photolytic fluences were used. It is not possible from this response to determine the precise patch size, but this type of photolytic profile is indicative of a short patch repair mechanism in which less than 20 nucleotides are inserted into a repaired region. However, the patch size can be estimated using the action spectrum for the induction of thymine glycols (3) and values obtained for the production of these photoproducts in sunlamp-irradiated cells. Using these data, it is possible to estimate that about 400 thymine glycols per 10$^{10}$ daltons are induced by 50 kJ/m$^2$ of solar UV. Assuming that most of these lesions are repaired during the 24-h incubation following irradiation (58), a patch size of 3 ± 1 nucleotides can be calculated for solar UV-irradiated cells. It should also be noted that a certain portion of these breaks may have been induced through the photolysis of BrdUrd incorporated via the repair of a very small

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D. Mitchell, personal communication.
number of dimers which may have been induced by the solar UV irradiation.

DISCUSSION

A modification of the BrdUrd photolysis assay was developed in which the sensitivity of this technique was greatly enhanced. This was accomplished through use of alkaline elution to measure the DNA strand breaks induced by BrdUrd photolysis. This assay was used to investigate the repair of nondimer DNA damages induced by solar UV in comparison with the repair of 254-nm-induced dimers. The results of these experiments indicate that the repair of these nondimer damages takes place via a short patch repair process, whereas dimer repair is the result of a long patch type of repair.

The value calculated for patch size associated with the removal of dimers, 40 nucleotides, is close to the value obtained using the CsCl density shift technique (59–63) and is lower than the 100 to 200 nucleotides generally determined for patch size using the BrdUrd photolysis assay utilizing sucrose gradient sedimentation to measure the level of strand breaks induced by BrdUrd photolysis. The discrepancy between the values obtained for these two methods appears primarily to stem from underestimates that are consistently made in the level of breaks calculated for the high photolytic fluences used in the BrdUrd photolysis assay (31–35). This problem arises because the procedure followed in this assay is to expose cells to a range of photolytic fluences, lyse the cells on sucrose gradients, and sediment the DNA under one set of sedimentation conditions, so that the number of breaks induced in BrdUrd- and dThd-incubated cells can be determined from one sucrose gradient. The difficulty with this approach is that the DNA from BrdUrd-incubated cells exposed to a high photolytic fluence barely sediments into the gradient, leading to an underestimate in the value for strand breaks induced by BrdUrd photolysis. Because the calculation for patch size is very sensitive to values determined for these high photolytic fluences, the underestimates in the levels of strand breakage lead to an overestimate in patch size. This problem was solved, however, by Kantor and Setlow (36) through the use of different sedimentation conditions for each sample, so that the DNA always sedimented in the middle of the gradient regardless of the level of breaks induced. Using this approach, the patch size for dimer repair was calculated to be about 50 nucleotides. It was also found in these experiments that the value for repaired sites was similar to the number obtained using the endonuclease-sensitive site assay. Cognizant of this problem, great care was taken in the calculation of the data presented in this paper, particularly the level of strand breakage induced by high photolytic fluences.

An additional finding of this work is that a higher level of breaks was induced in the labeled parental strand by photolytic exposure of unirradiated cells incubated in full growth medium containing BrdUrd compared with cultures grown in the presence of dThd (Chart 2). Hence, these excess breaks are not produced as a result of the photolysis of BrdUrd incorporated into the DNA through repair. This raises a note of caution as to the origin of the low level of breaks induced by high photolytic fluences in cells treated with certain DNA-damaging agents following incubation in full growth BrdUrd-containing medium without hydroxyurea (64). An added complication to this problem is that the extent of semiconservative DNA synthesis and therefore the level of breaks induced in BrdUrd-incubated cells depends both on the DNA-damaging agent and also the dose of the agent that was used to treat the cells.

An interesting comparison can be made between the results described in this paper and the effect of solar UV-induced nondimer damages on semiconservative DNA synthesis. It has been demonstrated that the effect on DNA synthesis of these nondimer damages is to cause primarily an inhibition of replication initiation as is also observed in γ-irradiated cells, while the effect of dimers is principally a blockage of replication chain elongation (38). In an analogous fashion, the nondimer damages induced by solar UV and the DNA lesions produced by ionizing radiation (32) are both repaired by a short patch repair mechanism. Possibly, this is a reflection of the high yield of DNA damages induced by solar UV such as thymine glycols, DNA-protein cross-links, and DNA strand breaks that are also produced in cells exposed to ionizing radiation (3–6). In addition, these results are also consistent with the finding that cells from individuals with the disease xeroderma pigmentosum, which are deficient in the excision repair of dimers, are hypersensitive to 254-nm UV, whereas they display about the same level of sensitivity to wavelengths in the 325- to 400-nm region as do normal human cells (15).

As a final note, it should be emphasized that the BrdUrd photolysis assay is useful in the investigation of the repair of a variety of DNA damages. Hence, through use of this modified technique, which greatly enhances the sensitivity of the BrdUrd photolysis assay, it should be possible to examine excision repair in cells treated with a wide range of DNA-damaging agents at the low dose levels that are encountered in typical environmental exposures.

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REFERENCES


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