UV Light-induced DNA Damage and Tolerance for the Survival of Nucleotide Excision Repair-deficient Human Cells*

Satoshi Nakajima†, Li Lan‡, Shin-ichiro Kanno‡, Masashi Takao‡, Kazuo Yamamoto§, Andre P. M. Eker§, and Akira Yasui∥

From the †Department of Molecular Genetics, Institute of Development, Aging and Cancer, Tohoku University, 980-8575 Sendai, the ‡Department of Biomolecular Sciences, Graduate School of Life Sciences, Tohoku University, 980-8578 Sendai, Japan, and the §Department of Cell Biology and Genetics, Erasmus University Medical Centre, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands

DNA damage can cause cell death unless it is either repaired or tolerated. The precise contributions of repair and tolerance mechanisms to cell survival have not been previously evaluated. Here we have analyzed the cell killing effect of the two major UV light-induced DNA lesions, cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone photoproducts (6-4PPs), in nucleotide excision repair-deficient human cells by expressing photolyase(s) for light-dependent photorepair of either or both lesions. Immediate repair of the less abundant 6-4PPs enhances the survival rate to a similar extent as the immediate repair of CPDs, indicating that a single 6-4PP lesion is severalfold more toxic than a CPD in the cells. Because UV light-induced DNA damage is not repaired at all in nucleotide excision repair-deficient cells, proliferation of these cells after UV light irradiation must be achieved by tolerance of the damage at replication. We found that RNA interference designed to suppress polymerase ζ activity made the cells more sensitive to UV light. This increase in sensitivity was prevented by photorepair of 6-4PPs but not by photorepair of CPDs, indicating that polymerase ζ is involved in the tolerance of 6-4PPs in human cells.

DNA lesions cause cell killing and mutations if they are not adequately repaired. Exposure of cells to UV light radiation results in formation of the two most common lesions, the cyclobutane pyrimidine dimer (CPD)1 and the 6-4 pyrimidine-pyrimidone photoproduct (6-4PP) into a XP-A cell line, XP12ROSV, a CPD photolyase gene (CPDphr). We isolated cells showing the effects of CPDs and 6-4PPs, we introduced into a XP-A cell line, XP12ROSV, a CPD photolyase gene (CPDphr) derived from a marsupial (Potorous tridactylis, rat kangaroo) and/or a 6-4PP photolyase gene (6-4PPphr) derived from a higher plant (Arabidopsis thaliana). We isolated cells showing a stable high expression of either one or both of the photolyases. Using these cell lines, we show the influence of each lesion on cell survival and the role of polymerase ζ (pol ζ) for bypass of 6-4PPs in vivo.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—XP12ROSV, an SV40-transformed cell line derived from an XP-A patient, is mutated in the XPA gene and possesses no detectable NER activity (10). Cells were cultured in Eagle’s minimum essential medium containing 10% fetal calf serum. For the expression of photolyase genes in the XP-A cell line, the cDNA of a CPD photolyase gene (CPDphr) derived from the rat kangaroo P. tridactylis (11) and the cDNA of a 6-4PP photolyase gene (6-4PPphr) derived from A. thaliana (12) were used. Because the A. thaliana gene contains putative signal sequences for transport into mitochondria and chloroplasts at the amino-terminal region,5 57 nucleotides encoding 19 amino acids from the putative start codon were deleted in the expression construct. cDNA for each gene was introduced into the vector

---

*This work was supported by a grant-in-aid for Scientific Research on Priority Area 12143201 (to A. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed. Tel.: 81-22-717-8465; Fax: 81-22-717-8470; E-mail: ayasui@idac.tohoku.ac.jp.

‡The abbreviations used are: CPD, cyclobutane pyrimidine dimer; 6-4PP, 6-4-pyrimidine-pyrimidone; NER, nucleotide excision repair; pol, polymerase; siRNA, short interfering RNA; TLS, translesion synthesis; XP-A, xeroderma pigmentosum A.

§S. Nakajima, K. Yamamoto, and A. Yasui, unpublished data.
poligonucleotides were synthesized (Japan BioService) and then used for geneticin and/or 2 trifugation, and suspended in extraction buffer A (50 mM Tris-HCl, pH 7.5, 0.3 mM KCl, 2 mM dithiothreitol, protease inhibitor, and 0.1% Triton X-100). These cell suspensions were sonicated, an equal volume of extraction buffer B (50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, protease inhibitors, and 0.1% Triton X-100) was added, and the suspension was then centrifuged. The supernatant was used for Western blotting. Antibodies against CPDs or 6-4PPs photolyases were raised against purified recombinant proteins of each photolyase produced in Escherichia coli.

Measurement of CPDs and 6-4PPs—The rate of repair of CPDs and 6-4PPs was measured by an enzyme-linked immunosorbent assay using the monoclonal antibodies TD-1 (specific for CPDs) and 6AM2 (specific for 6-4PPs) (14). Cells were grown until confluent, washed twice with Hanks’ buffer, and irradiated with 20 J/m² UV light (0.87 J/m²/s at 254 nm). Irradiation was followed immediately by illumination with white fluorescent lamps at room temperature for the time indicated. The light used for illumination was a Toshiba FL20SS fluorescent light, and cells were exposed from the bottom of the dishes through a box made of transparent poly(methyl methacrylate) plate and filled with water to absorb the heat generated by the light. Other illumination conditions have been described previously (11). After illumination, cells were harvested and genomic DNA was extracted using an Easy-DNA kit (Invitrogen). Genomic DNA was then treated with RNase A, extracted with phenol and phenol-chloroform, precipitated and washed with ethanol, and resuspended in TE (10 mM Tris-HCl and 1 mM EDTA, pH 7.5) buffer. Genomic DNA at 30 or 300 ng/ml was used to detect CPDs and 6-4PPs, respectively.

UV Light Survival—Exponentially growing fibroblasts were plated at 1.5–3 × 10⁴ cells per 100-mm Petri dish and cultured for 5–12 h. Cells were then washed twice with Hanks’ buffer and irradiated with UV light (254 nm) at 0.05 or 0.25 J/m²/s. Immediately after irradiation, plates were filled with Hanks’ buffer and illuminated with visible light for 90 min or kept in the dark at room temperature. After illumination, the Hanks’ buffer was removed and fresh medium was added. Cells were cultured for 12–14 days. Colonies were fixed and stained with 0.3% crystal violet in methanol, and the number of colonies was counted.

Preparation of Short Interference RNA (siRNA) for pol ζ and Transfection—Double-stranded siRNA of 19-mer with a protruding 3’-TT sequence (total 21-mer) was synthesized and purified by a Silencer transfection kit (Ambion). 5’-AAATGTCGGAGCCAACCTCAG-3’ and 5’-AATCTGACTGGTGGTCGACATCTCGTCTC-3’ oligonucleotides were synthesized (Japan BioService) and then used for pol ζ siRNA synthesis. The position of siRNA corresponded to the coding region (nucleotides 9176–9185) of the pol ζ catalytic subunit REV3 gene (GenBank™ accession number AF071798) (15). Cells were plated at 2 × 10⁵ cells per 60-mm Petri dish and cultured overnight. Cells were transfected with 20 nM siRNA for pol ζ by using OligofectAMINE (Invitrogen). After 40 h these cells were subjected to the UV light survival assay.

Reverse Transcription PCR—Total RNA was extracted from cells by the High Pure RNA isolation kit (Roche Applied Science). cDNAs were synthesized using the First Strand cDNA synthesis kit (Roche Applied Science). The reverse transcription PCR primers were designed as 5’-CCCTCTTACCTCTATGTTCC-3’ and 5’-TGTAGGATGTTAGGAATATGC-3’, which amplify the coding region of polymerase ζ (nucleotides 71 to 485). Quantitative reverse transcription PCR was performed by using Light Cycler (Roche).

RESULTS

Light-dependent Repair of UV Light-induced Lesions in Human Cells—We established human XP-A cell lines expressing either a marsupial CPD photolyase or a plant 6-4PP photolyase. The cell line expressing CPD photolyase was further transfected to produce a cell line expressing both CPD and 6-4PP photolyases. By immunofluorescence microscopy we found that >95% of the cells express either or both of the photolyases (not shown). Fig. 1 presents a Western blotting analysis showing the expression of each photolyase in the established cell lines. Using these cell lines, we analyzed the light-dependent decrease of CPDs and 6-4PPs by antibodies against each lesion (Fig. 2). Cells were irradiated with 20 J/m² UV-C light and exposed to visible light. In cells expressing the CPD photolyase alone, the amount of CPDs decreased to ~10% after 2 h of illumination. In contrast to CPDs, in cells expressing 6-4PP photolyase almost all 6-4PPs disappeared after 2 h of illumination. In cells expressing both photolyases, both CPDs and 6-4PPs disappeared more rapidly than in cells expressing either photolyase. 6-4PPs disappeared almost completely after 60 min, and CPDs disappeared after 2 h of light illumination as well. These data indicate that almost all CPDs and 6-4PPs are accessible to the foreign photolyases. The presence of either lesion may influence the repair of the other lesion.

Fig. 3A shows survival of UV light-irradiated cells with and without visible light illumination. The repair of each UV lesion by either photolyase contributes to survival increase. After UV light illumination and light illumination, cells expressing both photolyases have a survival level slightly higher than that of XP-A cells expressing wild-type XPA cDNA (Fig. 3B). To compare the effects of photorepair between CPD and 6-4PP photolyases, the fluence decrement was used, which is defined as the UV light dose whose effect is annulled by light exposure and corresponds to the effect of repair on survival increase (16). The fluence decrements of the cells irradiated with 0.5 and 0.75 J/m² are similar after the repair of CPDs and 6-4PPs (Fig. 3). Immediately after UV light irradiation and light illumination, cells expressing both photolyases have a survival level slightly higher than that of XP-A cells expressing wild-type XPA cDNA (Fig. 3B). To compare the effects of photorepair between CPD and 6-4PP photolyases, the fluence decrement was used, which is defined as the UV light dose whose effect is annulled by light exposure and corresponds to the effect of repair on survival increase (16). The fluence decrements of the cells irradiated with 0.5 and 0.75 J/m² are similar after the repair of CPDs and 6-4PPs (Fig. 3). Thus, immediate repair of the less abundant 6-4PPs enhances the survival rate to a similar extent as that of CPDs, indicating that a single 6-4PP is severalfold more toxic than a CPD lesion in NER-deficient human cells.

Suppression of Polymerase ζ Activity—Cells expressing each photolyase could repair one type of damage by photoreactivation, whereas the other photoprotein must be tolerated for surviving. One of the most available pathways for this damage tolerance is TLS. As for CPD, pol ζ can bypass CPDs with high efficiency and accuracy. In the case of 6-4PP, a previous in vitro study of translesion synthesis proposed that pol η or pol ε incorporates deoxynucleotides opposite the 3’-base of 6-4PP and that pol ζ functions as an extender at mispaired sites (17). Because CPDs and 6-4PPs are induced by UV-C light and
FIG. 2. Removal of UV light-induced lesions from genomic DNA of the cells by illumination with visible light. A, XP-A/vector cells. B, XP-A/CPDphr cells. C, XP-A/6-4PPphr cells. D, XP-A/CPDphr + 6-4PPphr cells. The amounts of CPD (squares) or 6-4PP (circles) lesions relative to those of non-illuminated cells are shown. The UV light dose was 20 J/m².

FIG. 3. Effect of light illumination on the survival of UV light-irradiated XP-A cell lines. A and B, UV light survival with and without light illumination. The cell lines tested were XP-A/vector (triangles), XP-A/cXPA (inverted triangles), XP-A/CPDphr (squares), XP-A/6-4PPphr (circles), and XP-A/CPDphr + 6-4PPphr (diamonds). Open and filled symbols show survival with and without light illumination for 90 min, respectively. C, fluence decrements after maximum photorepair for XP-A/CPDphr and XP-A/6-4PPphr cells after UV light irradiation with 0.5 or 0.75 J/m². For fluence decrements see “Results.”

FIG. 4. Influence of RNA interference designed for pol ζ. A, amount of mRNA of pol ζ. The expression levels of mRNA in XP-A/vector, XP-A/CPDphr, and XP-A/6-4PPphr cells without and with siRNA treatment (filled and open bars, respectively) are shown. B, survival of siRNA-untreated or -treated (closed triangles and closed diamonds, respectively) XP-A/vector cells. C, survival of siRNA-untreated or -treated (squares and diamonds, respectively) XP-A/CPDphr cells without or with light illumination (closed or open symbols, respectively) D, survival of siRNA-untreated or -treated (circles and diamonds, respectively) XP-A/6-4PPphr cells without or with light illumination (closed or open symbols, respectively).

DISCUSSION

We have established human cell lines in which either UV light-induced CPD or/and 6-4PP can be repaired by visible light illumination after UV light irradiation. Because our cell lines express high levels of either or both photolyases, we were able to analyze the effects of almost complete repair of each or both lesions at any time after UV light irradiation; this has not been possible in previous studies (18, 19). First of all, we found that most CPDs and 6-4PPs in human cells are accessible and are repaired by the respective foreign photolyases during illumination with visible light. Because 6-4PPs are produced predominantly in linker DNA, possibly due to the higher flexibility of linker DNA, and because CPDs are produced in nucleosome core DNA as well (20, 21), the access of repair enzymes to CPDs in the nucleosome may be hampered by the presence of core proteins (22). However, we found that ~90% of CPDs are repaired by CPD photolyase only within 2.5 h after UV light irradiation of 20 J/m² (Fig. 2). Similar results have been reported in mini-chromosomes of budding yeast and are ex-
explained by the mobility of nucleosomes (23). A slight movement of DNA around core proteins may expose DNA lesions and make them accessible to a repair enzyme (24, 25). The relatively slower repair of CPDs compared with that of 6-4PPs in Fig. 2D may be explained by the severalfold more CPDs produced by UV light. Although the accessibility of the NER complex to CPDs may be less than that of photolyase, we think that a major reason for the slow repair of CPDs by NER is a limited number of NER complexes for CPD repair in cells (26).

Repeated experiments show that an almost complete repair of either CPDs or 6-4PPs contribute almost equally to the increase in cell survival (Fig. 3, A and C). Supposing that the number of 6-4PPs produced by UV-C light is only about one-third of the number of CPDs (27), then 6-4PPs may be about three times more toxic than CPDs in human cells without NER. This contrasts with NER-defective E. coli in which repair by CPD photolyase increases survival by >10 times more than that by 6-4PP photolyase (12). This difference between human and E. coli cells suggests that the UV light resistance of E. coli cells depends more on the repair of CPDs than is the case for human cells. The photorepair of CPD significantly increases the cell survival of NER-deficient yeast Saccharomyces cerevisiae as well (28), suggesting that the tolerance for UV light-induced damage in the budding yeast S. cerevisiae is similar to that in E. coli. CPD photolyases present in E. coli and yeast play an important role for UV light resistance. It would be interesting to know how the repair of CPDs or 6-4PPs by photolyases increases cell survival in NER-deficient mutants of other organisms and to determine the roles of TLS for UV light-induced damage of the organisms. This may partly explain the distribution of photolyase genes in various organisms.

In vitro data have shown that several DNA polymerases contribute to bypass CPD in human cell (5), pol η can bypass CPD with the same efficiency and accuracy as it bypasses non-damaged template, but pol η cannot bypass 6-4PP and only inserts one deoxynucleotide opposite the 3'-base of 6-4PP (29). In contrast to pol η, pol ζ can bypass CPDs with low efficiency and, although it cannot initiate the bypass of 6-4PP, it can efficiently extend from the base inserted opposite the 3'-base of 6-4PP (30). pol κ cannot insert the nucleotides opposite CPD but can promote efficient lesion bypass by extending from the nucleotides incorporated by other polymerases opposite CPD. However, unlike pol ζ, pol κ cannot extend from the nucleotides incorporated opposite 6-4PP (31). Although pol ι can bypass CPD with a reduced efficiency compared with pol η and can also bypass 6-4PP as well under certain conditions (32), there is no report that pol ι-deficient cells exhibit UV light sensitivity. As discussed above, in the absence of NER the killing effect of 6-4PPs is similar to that of CPDs, suggesting also that a 6-4PP is more difficult to overcome than a CPD. Therefore, it is important to examine whether tolerance to 6-4PPs is present in human cells. Our data showed that the suppression of pol ζ by siRNA increased the UV light sensitivity of NER-deficient cells. This indicates that in NER-defective cells many of the UV light lesions are tolerated by pol ζ. The repair by 6-4PP photolyase, but not by CPD photolyase, compensates the suppression of pol ζ, indicating that pol ζ is indeed involved in the tolerance of 6-4PP. This is the first evidence showing that 6-4PP is bypassed by the pol ζ-dependent translesion synthesis pathway in living cells, including yeast and human cells. Similar analysis using RNA interference to various genes involved in TLS will elucidate their substrates in vivo.

In summary, we established and analyzed NER-deficient human cells expressing photolyase for either CPDs or/and 6-4PPs, and the major findings are as follows. 1) A 6-4PP is severalfold more toxic than a CPD unless it is repaired by NER.

2) The killing effect by the suppression of pol ζ after UV light irradiation is abrogated by repair of 6-4PP photolyase but not with a CPD photolyase. These data showed the importance of NER as well as TLS for the survival of UV-light-irradiated human cells. Our approach will help to extend in vitro data to the analysis of living cells.

Acknowledgments—We thank Drs. Jun-ichi Miyazaki and Kiyoji Tanaka for providing us with the expression vector pCY4B and the XP-A cell line expressing wild-type XPA cDNA, respectively. We thank Dr. Shirley McCready for critical reading of the manuscript.

REFERENCES

1. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) DNA Repair and Mutagenesis, pp. 24 –30, ASM Press, Washington, D. C.

2. Hogejmakers, J. H. (2001) Nature 411, 366–374

3. Mitchell, D. L. (1986) Photochem. Photobiol. 48, 51–57

4. Lindahl, T., and Wood, R. D. (1999) Science 286, 1897–1905

5. Lehmann, A. R. (2002) Mutat. Res. 509, 23–34

6. Masutani, C., Kusumoto, R., Yamada, A., Doihmae, N., Yokoi, M., Yuasa, M., Araki, M., Iwai, S., Takio, K., and Hanaoka, F. (1999) Nature 398, 700–704

7. Johnson, R. E., Kondratiek, C. M., Prakash, S., and Prakash, L. (1999) Science 285, 265–266

8. Yasui, A., and Eker, A. P. M. (1998) in DNA Damage and Repair: DNA Repair in Higher Eukaryotes (Nicoloff, J. A., and Hoeckstra, M. F., eds) Vol. 2, pp. 9–32, Humana Press Inc., Totowa, NJ

9. Wood, R. D., Mitchell, M., Sgouros, J., and Lindahl, T. (2001) Science 291, 1284–1289

10. Satokata, I., Tanaka, K., Miura, N., Narita, M., Mimaki, T., Satoh, Y., Kondo, S., and Okada, Y. (1992) Mutat. Res. 273, 193–202

11. Yasui, Eker, A. P., Yamashita, S., Yajima, H., Kobayashi, T., Takao, M., and Okawa, A. (1994) EMBO J. 13, 6143–6151

12. Nakajima, S., Sugiyama, M., Iwai, S., Hitomi, K., Otsuki, K., Kim, S. T., Jiang, C. Z., Todo, T., Brit, A. B., and Yamamoto, K. (1998) Nucleic Acids Res. 26, 638–644

13. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Gene 108, 193–199

14. Mori, T., Nakane, M., Hattori, T., Matsunaga, T., Ibara, M., and Nikaido, O. (1991) Photochem. Photobiol. 54, 225–232

15. Lin, W., Wu, X., and Wang, Z. (1999) Mutat. Res. 433, 89–98

16. Harm, H. (1980) Biological Effects of Ultraviolet Radiation, pp. 84, Cambridge University Press, Cambridge, United Kingdom

17. Prakash, S., and Prakash, L. (2002) Gene 298, 1872–1883

18. Ashahina, H., Han, Y., Kawanishi, M., Kato, T., Jr., Ayaki, H., Todo, T., Yagi, T., Takebe, K., Ikenaga, M., and Kimura, S. H. (1999) Mutat. Res. 435, 255–262

19. Chiganeas, V., Miyui, E. N., Mustr, A. R., de Fatima Jacysyn, J., Amarante-Mendes, G. P., Yasui, A., and Menck, C. F. (2000) Cancer Res. 60, 2453–2461

20. Niggl, H. J., and Ceruttii, P. A. (1982) Biochem. Biophys. Res. Commun. 105, 1215–1223

21. Mitchell, D. L., Nguyen, T. D., and Cleaver, J. E. (1990) J. Biol. Chem. 265, 5355–5356

22. Mitchell, D. L., Brash, D. E., and Nairn, R. S. (1990) Nucleic Acids Res. 18, 963–971

23. Suter, B., Livingstone-Zatche, M., and Thoma, F. (1997) EMBO J. 16, 2150–2160

24. Thoma, F. (1999) EMBO J. 18, 6585–6588

25. Meijer, M., and Smerdon, M. J. (1999) BioEssays 21, 596–603

26. Dwang, B. J., Ford, J. M., Hanawalt, P. C., and Chu, G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 424–428

27. van Hoffen, A., Venema, J., Meschini, R., van Zeeland, A. A., and Mullenders, L. H. (1995) EMBO J. 14, 360–367

28. Yasui, A., and Laskowski, W. (1975) Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 28, 511–518

29. Johnson, R. E., Harasaca, L., Prakash, S., and Prakash, L. (2001) Mol. Cell. Biol. 21, 3558–3563

30. Johnson, R. E., Washington, M. T., Harasaca, L., Prakash, S., and Prakash, L. (2000) Nature 406, 1015–1019

31. Washington, M. T., Johnson, R. E., Prakash, L., and Prakash, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 1910–1914

32. Tissier, A., Frank, E. G., McDonald, J. P., Iwai, S., Hanaoka, F., and Woodgate, R. (2000) EMBO J. 19, 5259–5266
