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Detrimental Effects of UV-B Radiation in a Xeroderma Pigmentosum-Variant Cell Line

Kimberly N. Herman¹, Shannon Toffton¹, and Scott D. McCulloch^{1,2,*}

¹Department of Biological Sciences, Environmental and Molecular Toxicology Program, North Carolina State University, Raleigh, North Carolina

²Center for Human Health and the Environment, North Carolina State University, Raleigh, North Carolina

Abstract

DNA polymerase η (pol η), of the Y-family, is well known for its in vitro DNA lesion bypass ability. The most well-characterized lesion bypassed by this polymerase is the cyclobutane pyrimidine dimer (CPD) caused by ultraviolet (UV) light. Historically, cellular and whole-animal models for this area of research have been conducted using UV-C ($\lambda = 100\text{--}280\text{ nm}$) owing to its ability to generate large quantities of CPDs and also the more structurally distorting 6-4 photoproduct. Although UV-C is useful as a laboratory tool, exposure to these wavelengths is generally very low owing to being filtered by stratospheric ozone. We are interested in the more environmentally relevant wavelength range of UV-B ($\lambda = 280\text{--}315\text{ nm}$) for its role in causing cytotoxicity and mutagenesis. We evaluated these endpoints in both a normal human fibroblast control line and a Xeroderma pigmentosum variant cell line in which the POLH gene contains a truncating point mutation, leading to a nonfunctional polymerase. We demonstrate that UV-B has similar but less striking effects compared to UV-C in both its cytotoxic and its mutagenic effects. Analysis of the mutation spectra after a single dose of UV-B shows that a majority of mutations can be attributed to mutagenic bypass of dipyrimidine sequences. However, we do note additional types of mutations with UV-B that are not previously reported after UV-C exposure. We speculate that these differences are attributed to a change in the spectra of photoproduct lesions rather than other lesions caused by oxidative stress.

Keywords

DNA damage; DNA polymerase η ; mutagenesis; translesion synthesis; ultraviolet radiation

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*Correspondence to: Department of Biological Sciences, North Carolina State University, 850 Main Campus Drive, Campus Box 7633 Raleigh, NC, USA. scott_mcculloch@ncsu.edu.

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Additional Supporting Information may be found in the online version of this article.

INTRODUCTION

Human exposure to sunlight is a widespread, and often an unavoidable, means of radiation exposure. Solar radiation has a large array of wavelengths, varying from the infrared down to short-wave ultraviolet and beyond. Exposure to ultraviolet radiation has been shown to cause detrimental effects to human skin, including sunburns and skin cancer [Rigel, 2008]. Interestingly, some clinical and epidemiological studies have shown a correlation between sunscreen use and an increase in some forms of skin cancer [Autier et al., 1999]. Although this may seem paradoxical, increased sunblock use tends to correlate with increased sun exposure. For decades, commercially available sunblock has primarily contained ingredients that block both ultraviolet (UV)-C and UV-B radiation although nearly all UV-C is filtered by the stratospheric ozone layer and only ~5% of ultraviolet exposure is coming from UV-B wavelengths [Perdiz et al., 2000]. Despite this fact, UV-C has most often been used for research owing to its potency and ability to generate cyclobutane pyrimidine dimers (CPDs) and other photoproducts [Lima-Bessa et al., 2008]. There is ample evidence that both UV-B and UV-A radiation can also generate CPDs [Sage, 1999; Perdiz et al., 2000]; however, they also make other secondary lesions through their ability to generate reactive oxygen species (ROS) [Kielbassa et al., 1997; Sage, 1999; Brown et al., 2000; Ravanat et al., 2001; Friedberg et al., 2005]. The ability to generate multiple types of lesions adds relevance to the study of UV-B and UV-A radiation with respect to mutagenesis.

The photoproducts caused by UV light include 6-4 photoproducts (6-4 PP) and CPDs. Although both lesions occur between adjacent pyrimidines, available evidence shows that the 6-4 PP is readily recognized and repaired by the nucleotide excision repair (NER) pathway [Lima-Bessa et al., 2008]. CPDs can also be recognized and repaired by the NER system; however, the kinetics of their repair is slower [Lo et al., 2005]. Because of this reason, it is much more likely CPDs will be encountered during replication and therefore will require translesion synthesis (TLS), making them primarily responsible for the mutagenesis that occurs after UV light exposure [You et al., 2001]. There are multiple types of CPD dimers based on the identity of the adjacent bases (i.e., CC, TC, CT, or TT). DNA polymerase η (pol η) is able to readily bypass at least the thymine–thymine dimer (TT dimer) with high efficiency and moderate fidelity [McCulloch et al., 2004]. It is assumed that pol η is able to bypass all of the dimer combinations (and also the corresponding uracil-containing lesions created by deamination of the cytosine base) with similar properties. There is in vitro experimental support for the bypass of a thymine–uracil (TU) dimer [Takasawa et al., 2004; Vaisman et al., 2006] and genetic evidence that in yeast the CC and TC lesions are preferentially processed by pol η [Yu et al., 2001].

The potency of UV-C has led to its popularity as an investigative tool for UV photoproduct mutagenesis. However, UV-C is not the only wavelength spectra that can cause photoproduct lesions. UV-B and UV-A, although less energetic, are much more abundant and environmentally relevant than UV-C [Sage, 1999; Perdiz et al., 2000; Ravanat et al., 2001; Cadet et al., 2005], and they can produce photoproduct lesions as well as generate significant levels of ROS [Douki et al., 1999; Takasawa et al., 2004]. ROS can lead to a wide variety of DNA lesions [Wang et al., 1998; Neeley and Essigmann, 2006], at least one of which, 7-8-dihydro-7,8-oxoguanine (8-oxoG), is also bypassed by pol η [Haracska et al.,

2000; Zhang et al., 2000; McCulloch et al., 2009; Suarez et al., 2013]. Therefore, investigating the types of mutations generated after exposure to UV-B and UV-A radiation may provide clues as to what types of lesions are commonly generated and which of the multiple polymerases available in human cells are involved in their bypass [Bebenek and Kunkel, 2004]. In this study, we sought to understand the role of CPDs compared to other potential lesions caused by environmentally relevant exposures of UV light in relation to their mutagenesis. To begin, we used a moderate dose of UV-B radiation and analyzed its effects on cells both proficient and deficient in the main CPD bypass polymerase, pol η . We have evaluated the cytotoxicity in the presence and absence of caffeine, as well as mutation rates and spectra after single dose of primarily UV-B exposure. Caffeine was used to compare the cytotoxicity of UV-C to that of UV-B; as it has been used previously to increase the cytotoxicity of UV-C by inhibiting the ATM and ATR checkpoint kinases that signal DNA damage [Maher et al., 1976a; Kaufmann et al., 2003]. We compare our results to the previously published study using UV-C and simulated sunlight (SSL) sources of radiation.

MATERIALS AND METHODS

Cell Lines, Growth Conditions, and Treatment Protocols

GM02359-hTERT (Xeroderma pigmentosum variant [XP-V] strain XP115LO) Clone 1B, denoted here as XP-V (containing a nonfunctional DNA polymerase η owing to a truncating mutation at ORF position 1117), has been described previously [Cordeiro-Stone et al., 2002; King et al., 2005]. We use an apparently normal neonatal foreskin fibroblast line as a control (NHF1-hTERT), denoted here as NHF, which has been characterized previously [Bassett et al., 2004; King et al., 2005]. These cell lines were a generous gift from Dr. Marila Cordiero-Stone (University of North Carolina). All media and supplements were obtained from Sigma-Aldrich (St. Louis, MO) or Gibco-Life Technologies (Grand Island, NY). XPV cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 2 mM of L-glutamine, 10% fetal bovine serum (FBS), and 2 $\times$ of MEM nonessential amino acids. NHF cells were maintained in Eagle's MEM with 10% of FBS and 2 mM of L-glutamine. Some expansion cultures for hypoxanthine-guanine phosphoribosyltransferase (HPRT) sequence analysis were maintained with the addition of 100 U penicillin/100 μ g streptomycin. All cultures were maintained in 37°C with 5% CO₂. UV-B treatments were performed by the removal of growth media and addition of Hanks' balanced salt solution (HBSS) to cells. P100 plates were washed with 3 mL of HBSS and 96-well plates were dosed using 100 μ L of HBSS per well. The lamp used has been described previously [Yoon and Smart, 2004]. Exposure to either a 5 or 10 mJ/cm² final dose was performed with no lid and took ~10 and ~20 sec, respectively.

Cell Viability

Cell viability after UV-B treatment was determined using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI). Cells were plated into 96-well, flat-bottom plates (655083; Greiner Bio-one, Austria) at 5,000 cells per well based on the preliminary plating studies, as suggested by the manufacturer. Twenty-four hours after plating, cells were treated with UV-B, UV-B plus caffeine, or given no treatment but

otherwise processed the same. UV-B dosing was as described above. For treatments using caffeine (final concentration, 1 mM), a 1-hr pretreatment was used, followed by its removal immediately prior to UV-B treatment. Caffeine was added back after treatment and left in the media until viability was measured. At 24 and 48 hr, cells were lysed and luminescence was measured using a FLUOstar Omega plate reader (BMG Labtech, Offenburg, Germany) using the protocol recommended by the manufacturer.

Mutation Frequency

The HPRT mutation assay was conducted essentially as described previously [Bassett et al., 2004]. Briefly, cells were preselected for functional HPRT by growth in $1\times$ HAT (hypoxanthine, aminopterin, and thymidine) media (H0262; Sigma-Aldrich, St. Louis, MO) for 10 days. Cells were then expanded and plated at 15–20% confluence and grown for 24 hr, followed by treatment of 10 mJ/cm^2 UV-B, or no treatment. These cells were then maintained in logarithmic growth for 14 days. Selection for loss of HPRT function was performed by replating 4×10^4 cells per P100 plate in growth media + $40\text{ }\mu\text{M}$ 6-thioguanine (6-TG) (A4882; Sigma-Aldrich, St. Louis, MO). Cells were given fresh media/6-TG on day 7 of selective growth day. Mutants were counted and collected on the 14th day of selective growth. Colony-forming efficiency was determined at the time of mutant selection by plating 750 cells per P100 plate in nonselective media (normal growth media). Colony-forming efficiency plates were counted on the same day as mutant collection. Mutation frequencies were calculated as described previously [Bassett et al., 2004]. For colonies not destined for mutation spectra analysis, colonies were fixed with methanol/acetic acid (vol:vol, 3:1) and stained with crystal violet prior to counting. Statistics for HPRT mutation frequencies were conducted using a one-way ANOVA with the Tukey post test, performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA (www.graphpad.com).

Analysis of HPRT Mutation Spectra

Mutation spectra analysis used a modified protocol based on the previously published study [Yang et al., 1989]. Mutant colonies were first identified by inverted light microscopy without crystal violet staining. Colonies were harvested as described previously [Freshney, 2010]. Briefly, the plates were rinsed with PBS or HBSS, an 8-mm sterile plastic cloning ring was placed around them, trypsin solution was added to the ring and incubated at 20 sec at room temperature, and then at 37°C for 15 min after trypsin was removed. Complete media was used to collect the cells and they were replated for continued growth. Expansion of these cells was performed in selective 6-TG-containing media. RNA was collected using the Qiagen RNeasy Mini Kit (Qiagen, Louisville, KY). cDNA was produced using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Life Technologies, Grand Island, NY). After cDNA production, PCR amplification of the HPRT gene was done using LongAmp Taq DNA Polymerase (New England Biolabs, Ipswich, MA), and PCR primers 5'-CTGCTCCGCCACCGCTTCC and 5'-GATAATTTTACTGGCGATGT (primers 1 and 2 from [Yang et al., 1989]) with the cycling parameters: 95°C for 5 min, 35 cycles of 95°C for 1 min, 48°C for 1 min, 65°C for 2 min, with a final extension of 65°C for 2 min. PCR cleanup was performed using a QIAquick PCR Purification Kit (Qiagen, Louisville, KY), followed by a second round of PCR using 1:100 dilution of PCR product,

and primers 5'-CCTGAGCAGTCAGCCCGCGC and 5'-CAATAGGACTCCAGATGTTT (primers 3 and 4 from [Yang et al., 1989]) with the same conditions as the first round of PCR. Amplification products were visualized in a 2% agarose gel. Products were sequenced by Genewiz (Genewiz, Research Triangle Park, NC) using PCR primers 3 and 4. Sequence analysis was performed using Geneious Pro Version 5.4.4 (Biomatters, Auckland, New Zealand). Analysis of the POLH gene in both cell lines was performed by PCR amplification using primers 5'-CCTCACCTCTCCAGACCTGC and 5'-GAGGAGACCATTCTGTCTGGA and the cycling parameters were as follows: 95°C for 1 min, 30 cycles of 95°C for 30 sec, 45°C for 1 min, 68°C for 1.5 min, with a final extension of 68°C for 5 min using Quick-load Taq 2X Master Mix (New England Biolabs, Ipswich, MA). This amplifies a product of ~750 bp that flanks the altered position. Sequencing was performed by Genewiz (Research Triangle Park, NC) using the same primers as the PCR reaction.

RESULTS

Verification of Cell Lines and Dosing

To determine the effect of an environmentally relevant level of UV-B radiation on cells, we dosed a pair of hTERT-immortalized cell lines. The cells were an apparently normal human foreskin fibroblast and a dermal fibroblast line deficient in DNA polymerase η activity in which the deficient cell line contains a point mutation leading in the POLH gene at codon 373, leading to a truncation in the protein that results in a complete loss of DNA polymerase η activity [Cordeiro-Stone et al., 2002; King et al., 2005]. First, we verified the genotypes of both cell lines and found the expected mutation in the XP-V cell line (Fig. 1A). We then exposed exponentially growing cells to doses of biologically relevant UV-B radiation [Yoon and Smart, 2004]. These doses can be achieved by relatively short exposure to direct sunlight (Dr. Jonathon Hall, personal communication) and with the lamp used and a monolayer of cells in culture, required only ~21 sec for the highest dose. Although the peak output of this lamp occurs at 312 nm, it likely does have a minor output in the UV-C range as it emits wavelengths between 280 and 350 nm (according to the manufacturer's literature). Based on the preliminary studies, we chose 5 and 10 mJ/cm² as doses which were not overly cytotoxic to cells (data not shown). The goal of these experiments was to examine if relatively low levels of primarily UV-B radiation were able to affect cells in a manner similar to the potent UV-C exposures that characterize many studies involving pol η [Maher et al., 1976b; King et al., 2005; Wang et al., 2007].

Cell Proliferation and Effects of Caffeine

The CellTiter Glo™ assay was used to measure ATP levels in whole-cell lysates. This correlates with cell number and preliminary studies verified that we were in the linear detection range for total cell number (data not shown). We plotted the luminescence value (i.e., surrogate for cell number) for irradiated cells relative to the same value for nonirradiated cells and defined this ratio as the proliferation index (Fig. 1C; raw luminescence values are shown in Supporting Information Fig. S1). In each experiment, multiple replicate wells were used and the values were averaged. Each value plotted is the average of multiple independent experiments. As shown in Figures 1C and 1D, exposure to

both 5 and 10 mJ/cm² UV-B radiation was not overly cytotoxic to NHF cells in the presence or absence of caffeine (solid lines). At 1 day and 2 days postradiation exposure, cell numbers were at least 90% those of unirradiated cells with no striking difference noted. Supporting Information Figure S1 shows that luminescence values increased with time, indicating that cells were indeed proliferating. This demonstrates that the dose is not excessively cytotoxic. Caffeine does not have any noticeable effect on NHF cells; in contrast, we do see a noticeable effect of adding caffeine to the XP-V cells. At day 1, there is no apparent difference in cell number, but by day 2 we see a decrease in the number of cells exposed to combination treatment of both caffeine and UV-B radiation. The drop in total luminescence over time seen in Supporting Information Figure S1 also supports the conclusion that in XP-V cells, the combination of UV-B light and caffeine caused an increase in cell death. This phenotype is the same as previously described after UV-C radiation and is considered diagnostic of XP-V cells [Maher et al., 1976a; Stary et al., 2003]. It suggests to us that UV-B radiation is capable of causing a replication block in cells lacking pol η that is exacerbated by caffeine, possibly by inducing the G1 cell-cycle block [Kaufmann et al., 2003]. Based on these results, we were interested in investigating whether XP-V cells exposed to UV-B radiation would display increased mutagenesis as they do after UV-C exposure.

Mutagenesis of UV-B

To investigate whether UV-B could cause mutagenesis in these cell lines, we employed the HPRT mutagenesis assay (Fig. 2A). This assay requires the selection of mutants and therefore is not an idle or neutral locus. Hence, although we cannot say definitively that the same mutations would occur in the absence of selection, this assay has been used extensively to look at UV light mutagenesis in multiple cell lines, including those used here [King et al., 2005]. NHF and XP-V cells were dosed with 10 mJ/cm² of UV-B light and then selected for growth in 6-TG to select for loss of HPRT activity. Importantly, we saw no difference in the low-cell density cloning efficiency of the two cell types (12–15%, Table I). We observed that the background mutation frequency for both cell types was similar (0.55×10^{-5} and 0.98×10^{-5} for NHF and XP-V cells, respectively; Fig. 2B and Table I). These results agree with the data from a mouse model in which animals lacking pol η showed no increase in tumors under nonexposed conditions [Lin et al., 2006]. Our data did show a statistically significant increase in the mutation frequency after UV-B exposure (compared to no exposure) in both cell types (~18- and ~25-fold, for NHF and XP-V cells, respectively). Importantly, the observed mutation frequency of NHF and XP-V cells after UV-B exposure was significantly different from each other (Fig. 2B; 8.56×10^{-5} and 25.8×10^{-5} for NHF and XP-V cells, respectively). These observed mutation frequencies after UV-B exposure show a similar trend to the increased mutagenesis after UV-C exposure in XP-V cells. These data are consistent with a lack of pol η -dependent lesion bypass in these cells. These data also demonstrate that even at doses that do not cause excessive cytotoxicity, mutagenesis above background levels occurs even when pol η is present, and that loss of pol η further magnifies this phenotype. We acknowledge that these cell lines are not isogenic and therefore the difference could be owing to factors other than pol η . However, at 1- and 24-hr postirradiation, we saw no evidence of different levels of TT dimer lesions by dot blot

analysis (Supporting Information Fig. S2). This suggests that both initial levels of, and overall repair rates for, this lesion are comparable in these two lines.

Mutation Spectra Evaluation

As UV-B and UV-C radiation can produce multiple different types of lesions, [Ravanat et al., 2001; Cadet et al., 2005], we were interested in how the spectra of mutations caused by UV-B radiation compared to those observed after UV-C exposure. To investigate this, we generated spectra of mutations caused by UV-B light in pol η -proficient and -deficient cells by isolating RNA from 6-TG-resistant clones, PCR amplifying the HPRT open-reading frame from cDNA, and then sequencing these products. Tables II and III and Figure 3 show a marked difference in the types of mutations observed in NHF and XP-V cells. The most obvious difference is the relative levels of base substitutions to insertion/deletions. In NHF cells, insertions/deletions account for 67% of the total mutations, compared to only 26% for XP-V cells. We also observe more variability in the types of insertion/deletion mutations in NHF cells. In NHF cells, we detected three different insertions at two different locations and six different types of deletions ranging from a single base up to more than 100 bases deleted. These occurred at seven separate locations in the gene (Table III). All but one of the insertions/deletions is predicted to cause a truncation of the HPRT protein (Table III). In XP-V cells, no insertions were observed and the deletions were of a much more limited scope (a single one-base deletion and three separate instances of a 77-base deletion) (Table II).

When evaluating the types of changes that did occur, the most striking difference was observed in the numbers and distribution of base substitutions mutations. In NHF cells, all of the base substitutions occur at dipyrimidine sites in one of the two strands (Table III). However, these mutations account for a minority of total changes in the NHF cells. Four out of the six sites are either TT or CC sequences, whereas only one is of a mixed base sequence (the last could be either TT or CT). Of the dipyrimidine-associated mutations, there were three changes each at C and T bases. In XP-V cells, there is a similar trend of most base substitutions occurring at dipyrimidine sites, but we also detected two changes that cannot be attributed to such sequences (Table II). Of the dipyrimidine sites, five are at either CC or TT sites and six are at TC or CT sites, a different distribution than that seen in NHF cells. As observed in NHF cells, the mutations at photoproduct sites also occur equally at T and C residues (five and six changes, respectively). Taken together, the results presented here suggest to us that in NHF cells, functional pol η helps suppress mutagenesis caused by UV-B light, with the majority of changes being more complex insertion/deletions. In XP-V cells lacking functional pol η , an increase in overall mutagenesis is noted as well as a specific increase in base substitutions at dipyrimidine sequences and also mutations at other nondipyrimidine sites. Although we have not determined the genotype of the pol I gene, we assume it to be wild type and therefore expect that in our XP-V cells, the majority of dipyrimidine mutations are caused by low-fidelity bypass of photoproduct-induced lesions by pol I as it has been shown previously [Wang et al., 2007].

DISCUSSION

It is generally believed that most nonmelanoma skin cancers are caused by UV-B and/or UV-A radiation. Most of UV-C radiation is filtered by stratospheric ozone before reaching the ground level, leading to the composition of UV irradiation we are normally exposed to being ~95% UV-A and UV-B [Perdiz et al., 2000; Pfeifer and Besaratinia, 2012]. We were interested in how these less energetic but more environmentally relevant wavelengths of solar radiation affect human cells with respect to cytotoxicity and mutagenesis. A study of yeast strains proficient or deficient for either the *rad30* (pol η) or *rev3* (pol ζ) genes was conducted comparing UV-C to SSL (SSL; UV-B and UV-A in proportions that mimic solar radiation) [Kozmin et al., 2003]. Their results suggested that the lesions created by UV-C are more mutagenic. Interestingly, they saw little to no effect on mutation frequency when pol η was missing using only SSL. Similarly, we found that UV-B did indeed cause an increase in mutagenesis in normal cells; it was less than the reported values for this line using UV-C [King et al., 2005]. This differs somewhat from our results in which our XP-V line showed a further increase in mutation frequency compared to normal cells (Fig. 2). One explanation could be the different types of radiation used. Another explanation could be the lack of pol I in yeast as it is known to be involved in UV light-dependent mutagenesis in the absence of pol η [Wang et al., 2007].

Pol η is considered to be the main polymerase involved in lesion bypass of products caused by UV-C radiation. As we limited ourselves to a comparison of mostly UV-B to UV-C radiation, we reasoned that if the less energetic UV-B were working by a mechanism similar to UV-C, we should observe similar effects. Therefore, we decided to evaluate cytotoxicity both in the presence and in the absence of caffeine as a specific enhancement of cytotoxicity by caffeine is a well-described phenotype of XP-V cells [Maher et al., 1976a; Kaufmann et al., 2003]. We were also interested in investigating whether pol η deficiency would cause an increase in mutations after UV-B radiation and if so, whether the types of mutations found were similar to or different from that reported after UV-C radiation. To this end, we performed the HPRT mutagenesis assay and sequenced the coding sequence of 6-TG-resistant mutants.

Our results show that normal human fibroblasts do not exhibit significant sensitivity to killing by 5 and 10 mJ/cm² UV-B. These doses are low, environmentally relevant, and are able to be absorbed in only a few minutes of direct, mid-day sunlight. However, XP-V cells do exhibit cytotoxicity at these doses but only in the presence of caffeine (Fig. 1C). Based on the previously published reports that used UV-C radiation, we hypothesize that this increased cytotoxicity is the result of collapsed replication forks owing to inefficiency or absence of TLS. In normal cells and in the absence of caffeine, these collapsed forks should trigger the ATR-dependent damage response pathway [Kaufmann et al., 2003]. Caffeine is a known ATM/ATR inhibitor [Yoon and Smart, 2004]. It has been previously shown that in response to UV-C irradiation, XP-V cells show a significant inhibition in proliferation in the presence of caffeine [Maher et al., 1976a; King et al., 2005; Wang et al., 2007]. Those studies used UV-C doses that kill the majority of cells. Our results suggest that even lower doses of radiation that kill only a small percentage of total cells cause the same phenotype. This fact suggests that the response to DNA damage is very sensitive and finely tuned. It

does not require high doses that kill most cells. It also shows that cells respond in the same fashion to both UV-C and UV-B radiation.

We were also interested in understanding how UV-B, specifically, affects mutagenesis, and whether the protective effect of pol η extended to these wavelengths of radiation. We determined mutation frequencies and spectra for UV-B-treated normal and XP-V cells to begin to understand the role that pol η plays in this process. We started this study, assuming that the mutation frequencies would likely be lower than that of UV-C-treated cells, but that there would be a similar trend in terms of mutagenicity and sensitivity in the XP-V line. This indeed was the case. In the previously published study by Bassett et al. [2004], UV-C doses ranging from 4 to 8 J/m² (equivalent to 0.4–0.8 mJ/cm²) on NHF cells gave mutation frequencies ranging from 11×10^{-5} to 19.3×10^{-5} . In XP-V cells, which is the same cell line we used, they used doses of 2–4 J/m² of UV-C (0.2–0.4 mJ/cm²), giving mutation frequencies ranging from 31×10^{-5} to 46.9×10^{-5} . These doses may appear to be relatively small compared to ours (0.2–0.8 mJ/cm² vs. 5–10 mJ/cm² used in our studies), but it is important to keep in mind that UVB radiation is overall less energetic and that the peak wavelength of the emission source we used was 312 nm, significantly higher than the peak absorption maximum of DNA.

As summarized in Table I, NHF cells dosed with 10 mJ/cm² UV-B gave a mutation frequency of 8.56×10^{-5} , compared to XP-V cells with a frequency of 25.8×10^{-5} . These values are significantly different from untreated cells for each cell line, that is UV-B radiation is mutagenic in both cell lines. We also see a statistically significant difference in the mutation frequency of UV-B light when comparing NHF and XP-V cells. The values observed are somewhat lower than those reported for UVC, perhaps owing to the above-noted potency of light with a peak emission of ~254 nm, very close to the maximum absorption wavelength of DNA. Owing to this difference in wavelengths of the light sources used in these various studies, it is possible that a completely different set of DNA lesions are being generated by each exposure [Ravanat et al., 2001; Cadet et al., 2005]. Initially, we assumed that UV-B would cause more mutations at non pyrimidine dimer sites as it is frequently stated that UV-B can cause oxidative stress at relevant levels. We saw only limited evidence for this, as only one of the two nonphotoproduct base substitutions we detected could possibly be attributed to 8-oxoguanine, the most abundant oxidative lesion. This is consistent with Kozmin et al. [2003], who reported that that pol η can accurately bypass 8-oxoguanine after SSL exposure in yeast cells. However, we have not observed an increase in 8-oxoguanine levels in the DNA after this much of UV-B exposure (data not shown), suggesting that the mutagenic effect of the UV-B light used here is largely photoproduct dependent although a minor contribution by oxidative damage cannot be ruled out completely.

Comparison of the mutation spectra of NHF and XP-V cells caused by UV-B light was very interesting. The most notable observation was the striking difference in the number of insertion/deletion mutations versus base substitutions when comparing the two cell lines. In NHF cells, we observed 50% of deletions, 17% of insertions, and 33% of base substitutions. However, in XP-V cells, no insertions were found and only 26% of the changes were deletions, with the remaining 74% of mutations being base substitutions. The most likely

explanation for this difference is that in NHF cells, the presence of functional pol η allows for bypass of CPDs in a more efficient manner and in a way that is more accurate. It is interesting to note, however, that even in NHF cells, all the detected base substitutions were at dipyrimidine photoproduct sites. Possible explanations for this are that TLS of CPDs by pol η is not completely error free as it has been suggested earlier [McCulloch et al., 2004], or that other polymerases (likely pol I) also play a role in normal TLS of UV light-generated lesions [Wang et al., 2007]. We do note that a published report (compiling both previous study and new data) [Wang et al., 2007] showed that in “wild-type” cells (i.e., both pols η and I present) the majority of base substitutions caused by UV-C were transitions (87%; T→C or C→T), whereas our results show that only ~33% of base substitutions were transitions (Table II). It is entirely possible that this is caused by the distribution of photoproduct lesions after UV-C versus UV-B radiation [Mitchell et al., 1992; Sage, 1999].

Breaking down the base substitutions observed in XPV cells, we see a similar distribution of changes at photoproduct sites when compared to the previously published reports [Wang et al., 2007] (Table II and Fig. 3). Specifically, roughly 63% of the changes were transversions, either T→A or C→A changes. This matches 55–70% of the value reported by Wang et al. for two different XPV cell lines that were dosed with UV-C light (Tables I and II in Wang et al., [2007]). Results from the study of Kozmin et al. [2003] also confirm that UV-C causes predominantly transversions, but they report that SSL caused only 50% of transitions and 28% of transversions. We speculate that for our results, the absence of pol η , even with the less cytotoxic UV-B, the same replication blocking lesions are produced as in UV-C, albeit possibly in lower overall levels. When these lesions are bypassed in a pol I-dependent manner, they cause the same types of errors. More evidence that the mutagenic response to UV-B light is similar to that of UV-C is the fact that we see roughly equal mutations on the transcribed and nontranscribed strands of HPRT (45%/55% split) in XP-V cells, similar to the 44%/56% split observed in XPB4E and XP115LO cells from the study of Wang et al. [2007] using UV-C radiation. Although no data were presented in their report regarding strand specificity in wild-type cells, we also observed no difference in our NHF cells (50%/50% transcribed and nontranscribed strand, respectively).

Our data show that UV-B acts in a very similar fashion to UV-C, albeit with somewhat lesser effects with regards to cytotoxicity. The fact that it can induce mutagenesis even in the presence of pol η lends support to the notion that after UV-B exposure is a risk factor for skin cancer. Our data suggest that the majority of the mutations are associated with photoproduct sites. This mutation frequency increase in the absence of pol η suggests that CPDs are the main culprit of the mutagenesis. As UV-B is the minor component of the UV radiation, we are exposed to, it will be important to extend these studies to the UV-A range of wavelengths, and also to study the combined effects of various wavelengths (i.e., using SSL lamps). Some of these studies have been performed in yeast cells [Kozmin et al., 2003], but the relevance to human health cannot be determined, given the differences in TLS polymerases and repair systems available to each organism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A: Results of sequencing a portion of the POLH cDNA in NHF and XP-V lines, confirming C1117T change in the latter. **B:** Schematic representation of cell viability assay as determined by CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega). NHF and XP-V cells were seeded at 5,000 cells/well. Caffeine was added to a final concentration of 1 mM 23-hr postplating to appropriate wells. Twenty-four-hour postplating, media was removed and replaced with HBSS, and cells were treated with UVB or no treatment. HBSS was removed and fresh media was then added (caffeine, ± 1 mM). Viability assay was performed at 24- and 48-hr post-treatment. **C:** Graph of relative cell viability (compared to no UV-B treatment) as measured at 24-hr post-treatment. Cell number is proportional to luminescence as measured by the amount of ATP present in cell lysates. **D:** Graph of relative cell viability as measured at 48-hr post-UVB treatment.

Fig. 2.

A: Schematic diagram of HPRT mutation assay. Cells were plated at 15–20% confluence, grown for 24 hr, and then treated with UVB (or no treatment). Cells were kept in exponential growth for 14 days and then plated at 40,000 cells per plate for selection plates or 750 cells per plate to determine the plating efficiency. After 14 days in selective media (DMEM or EMEM + 40 μ M 6-TG), plates were stained with crystal violet and visually examined by microscope to count colonies. For plates that were used to expand cultures for cDNA analysis, colonies were counted without the aid of crystal violet staining. **B:** Mutation frequencies were calculated as described by Bassett et al.; mutation frequency = (number of colonies/number of cells plated)/(CFE). Mutation frequencies were calculated for each separate experiment, with a minimum of three experiments for each treatment. The averages of multiple trials are shown on the graph $\pm$ SEM. The graph was generated using GraphPad Prism 5.0. Statistics used an ANOVA with Tukey's Multiple Comparisons test (* P 0.05, ** P 0.01, *** P 0.001, and **** P 0.0001). Results significance between no treatment and treatment in both the NHF and the XP-V cells, as well as between the treated XP-V and the NHF cells.

Fig. 3.

6-TG-resistant colonies were collected by ring cloning, expanded and mRNA harvested to produce HPRT cDNA. Sequencing of nested PCR products was performed and compared to the wild-type HPRT cDNA sequence. Analysis of sequences was performed using Geneious Pro Version 5.4.4 software. All changes were recorded and categorized by type of mutation (base substitution, insertion, and deletion). A: Breakdown of the types of changes observed in NHF and XP-V cells. B: Breakdown of the types of base substitutions assumed to be photoproduct errors in NHF and XP-V cells.

TABLE I

Average Mutation Frequency at the HPRT Gene after a Single Dose of UV-B Radiation on NHF and XP-V Cells with at Least three Replicate Experiments^a

	NHF		XP-V	
	No treatment	10 mJ/cm ² UVB	No treatment	10 mJ/cm ² UVB
Mutant colonies	4	97	7	177
Colony-forming efficiency	0.13	0.12	0.12	0.15
Mutation frequency	0.55×10^{-5}	8.56×10^{-5}	0.98×10^{-5}	25.8×10^{-5}
Number sequenced	0	18	0	19

^a Mutant colonies were counted by inverted microscope after 14 days of 6-TG selection. Mutation frequency was calculated as (number of resistant colonies/number of cells plated for selection)/(CFE). Colony-forming efficiency is the number of colonies on the unselected low-density plates/total cells plated for plating efficiency.

TABLE II

Mutations Observed in the HPRT Gene of UV-B-irradiated XP-V Cells after 6-TG Selection^a

Base substitutions					
Position	Mutation	Photoproduct	Strand	Sequence ^b	Effect
2	A→T or T→A	No		GCCATAA	No start codon
617	C→A or G→T	No		ACACAAA	C206F
39,40 ^c	C→A; A→T	Yes	T	GTTCATCA	14–218
40	C→A	Yes	T	GTTCATC	14–218
62	T→A	Yes	NT	ATTIATT	21–218
222	C→A	Yes	NT	ATTCTTT	F74L
222	C→A	Yes	NT	ATTCTTT	F74L
259	T→A	Yes	T	TTCTATT	87–218
464	C→T	Yes	NT	ATCCAAA	P155L
606	C→A	Yes	T	ATTCAAA	L202F
616	T→C	Yes	NT	GTTTGTG	C206R
616	T→C	Yes	NT	GTTTGTG	C206R
616	T→C	Yes	T	GTTTGTG	C206R
Insertions/deletions					
Position	Size (bp)	Sequence			Effect
18	–1	GCC(A)GGG			9–218
531	–77	CAT(GATTCAAATC...AAATCCAACA)AAG			183–218
532	–77	ATG(ATTCAAATCC...AATCCAACAA)AGT			183–218
532	–77	ATG(ATTCAAATCC...AATCCAACAA)AGT			183–218

^aListed mutations for likely CPD sites are given by assuming the dimer was the source of the mutation. The multiple occurrences of mutations at the same position (222, 532, and 616) were observed in different experiments, demonstrating that they are independent events.

^bGiven 5'→3' of the relevant strand for photoproducts with the altered base in bold font and the photoproduct site underlined. For nonphotoproduct site, the sequence of the transcribed strand is given. Sequences of deleted bases are shown in bold font within parentheses, with the position listed being the last present prior to the deleted region.

^cTandem base substitution in which only one is within a photoproduct site.

TABLE III

Mutations Observed in the HPRT Gene of UV-B-irradiated NHF Cells after 6-TG Selection^a

Base substitutions					
Position	Mutation	Photoproduct	Strand	Sequence ^b	Effect
34, 36 ^c	A→T, C→A	Yes	T	ATCAT <u>C</u> ACT	D12Stop
62	T→A	Yes	NT	AT <u>T</u> TATT	L21Stop
208	C→T	Yes	T	CCCC <u>C</u> TT	G70R
209	C→T	Yes	T	CCCC <u>C</u> CA	G70E
223	T→A	Yes	NT	TT <u>C</u> TTG	F75I
623	T→A	Yes	NT	TCAT <u>T</u> AG	V208N
Insertions/deletions					
Position	Size (bp)	Sequence			Effect ^d
453	+1	CTG(A)CCT			154–218
609	+14	ATG(CTATAAAAAAAAAAT)ATT			<i>e</i>
609	+15	ATG(CTATAAAAAAAAAAT)ATT			208–218
18	–1	GCC(A)GGG			9–218
18	–1	GCC(A)GGG			9–218
27	–107	GTC(CTGTCCATAA...CATCACTAAT)CAC			10–218
384	–18	ATC(TTCCACAATCAAGACATT)CTT			129–134
399	–4	TAT(CTTC)CAC			135–218
403	–1	TAT(C)TTC			136–218
485	–47	CAA(AGTCTGGCTT...CACCAGCAAG)CTT			166–218
532	–77	ATG(ATTCAAATCC...AATCCAACAA)AGT			183–218
535	–77	AAC(ATGATTCAAA...TCAAATCCAA)CAA			182–218

^aListed mutations for likely CPD sites are given by assuming the dimer was the source of the mutation. The multiple occurrences of mutations at the same position (18, 609) were observed in different experiments, demonstrating that they are independent events.

^bGiven 5'→3' of the relevant strand for photoproducts with the altered base in bold font and the photoproduct site underlined. For nonphotoproduct site, the sequence of the transcribed strand is given. Sequences of inserted or deleted bases are given in bold font inside parentheses, with the position listed being the base prior to the inserted or deleted position.

^cThis sample contained two base substitutions separated by a single base. Only one of the changes is in a potential photoproduct site.

^dThe numbers given indicate the size of the truncated protein (i.e., 182–218 indicates the mutated protein was 181 amino in length). Most of the deletions also contained changes in amino acids at the C-terminal end of the truncated protein compared to wild-type sequence.

^eThis mutation changes amino acids starting from residue 204 and takes the normal stop codon out of frame, giving a new reading frame of at least 223 amino acids.