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Cyclobutane Pyrimidine Dimer Density as a Predictive Biomarker of the Biological Effects of Ultraviolet Radiation in Normal Human Fibroblast

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Abstract

This study compared biological responses of normal human fibroblasts (NHf1) to three sources of ultraviolet radiation (UVR), emitting UVC wavelengths, UVB wavelengths, or a combination of UVA and UVB (solar simulator; emission spectrum, 94.3% UVA and 5.7% UVB). The endpoints measured were cytotoxicity, intra-S checkpoint activation, inhibition of DNA replication and mutagenicity. Results show that the magnitude of each response to the indicated radiation sources

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SUPPORTING INFORMATION

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

Figure S1. Activation of checkpoint kinases by UVR-induced DNA damage in normal human melanocytes. Lightly pigmented NHM4 + hTERT and heavily pigmented NHM28 + hTERT melanocytes were sham treated or irradiated with UVC, UVB (a broad-band source emitting 280–370 nm, peak emission at 316 nm) or a solar simulator source emitting 94.3% UVA and 5.7% UVB (UVA-UVB) using fluences predetermined to induce equal CPD densities in human fibroblasts. After 30-min incubation, cells were harvested for immunoblot analysis of the phosphorylation status of ATM (P-ATM, Cell Signaling 4526; ATM, Santa Cruz SC-7230) and CHK1. P-ATM/ATM and PCHK1/CHK1 ratios were normalized to the sham-treated control. The lower ratios determined for the NHM28 + hTERT melanocytes are expected to be due to an attenuation of the DNA damage burden in these heavily pigmented cells. Experiment performed by Dennis Simpson, Ph.D.

Figure S2. Repair of CPD or 6-4PP in NHf1 and NHM4. Cultures of NHf1 or NHM4 were irradiated with 12 J m⁻² UVC and harvested 0, 3, 6, 12, or 24 h post irradiation (experiment performed in the laboratory of Dr. William Kaufmann). DNA was purified and CPD and 6-4PP were quantified by RIA in the laboratory of Dr. David Mitchell.

Table S1. Quantification of 8-oxo-dG in NHf1 cells exposed to UVA-UVB or UVC. LC/MS/MS was used to quantify 8-oxo-dG levels in NHf1 cells exposed to UVA-UVB (0, 14.7, 29.2, or 43.7 kJ m⁻²) or UVC (0, 4, 8, 10, or 12 J m⁻²). Fluences were chosen to produce equal CPD densities. DNA was isolated in the presence of 2,2,6,6-tetramethyl-piperidinoxyl (TEMPO, 20 mM final concentration) to minimize artifacts due to oxidation during DNA purification. Assay was performed by the Biomarker Mass Spectrometry Core Facility, Center for Environmental Health and Susceptibility (CEHS), University of North Carolina, Chapel Hill as described in Boysen, G. *et al.* (2010). *J. Chromatogr. B. Analyt Technol. Biomed. Life. Sci.* 878, 375–380.

was best predicted by the density of DNA cyclobutane pyrimidine dimers (CPD). The density of 6-4 pyrimidine–pyrimidone photoproducts was highest in DNA from UVC-irradiated cells (14% of CPD) as compared to those exposed to UVB (11%) or UVA–UVB (7%). The solar simulator source, under the experimental conditions described here, did not induce the formation of 8-oxo-7,8-dihydroguanine in NHF1 above background levels. Taken together, these results suggest that CPD play a dominant role in DNA damage responses and highlight the importance of using endogenous biomarkers to compare and report biological effects induced by different sources of UVR.

INTRODUCTION

Ultraviolet radiation (UVR) is an established environmental carcinogen that induces DNA damage in basal keratinocytes and melanocytes, potentially leading to cancer development (1,2). The UVR spectrum is divided into three wavelength ranges: UVC (100–280 nm), UVB (280–315 nm) and UVA (315–400 nm). Wavelengths in the UVC range are absorbed by the Earth's atmosphere; hence, UVR reaching the surface of the planet consists of approximately 5%–10% UVB and 90%–95% UVA. Although the genotoxicity of UVB can be attributed to direct absorption of photons by DNA, the mechanism by which UVA causes genotoxicity is less clear. The predominant view has been that DNA does not readily absorb the energy from UVA and that endogenous photosensitizers are required to transfer the energy of UVA to DNA (3,4). However, experimental evidence that UVA can damage DNA directly has emerged (5–7). Although the mechanism is in question, it is widely accepted that wavelengths above 300 nm are capable of inducing the most common forms of DNA photodamage, including the cyclobutane pyrimidine dimers (CPD) (8,9), and 6-4 pyrimidine–pyrimidone photoproducts (6-4PP) (10,11). In addition, there is evidence that long wavelengths of UVR induce oxidative damage to DNA, possibly through the formation of singlet oxygen or other reactive oxygen species, resulting in oxidized bases, such as 8-oxo-7,8-dihydroguanine (8-oxo-dG) (3,4).

Much of the research examining the biological effects of UVR have utilized UVC, whereas comparatively fewer studies have been conducted with wavelengths that model the more naturally occurring exposures to UVA and UVB. The common use of UVC lamps can be attributed to their availability and low cost, as well as the convenience of using a highly energetic source that allows short irradiation times for the induction of DNA photoproducts and the triggering of UVR-induced responses. Indeed, there is evidence that even wavelengths in the UVA range of the UVR spectrum are mutagenic (10,12) and induce the most abundant of the photoproducts (CPD) in the nuclear DNA of cultured cells (4,9,10,13), albeit with lower efficiency and admixed with oxidative lesions. Although there is little doubt that the CPD is the primary mutagenic lesion following exposure to UVC or UVB, the role of the CPD in UVA-induced mutagenesis is less clear (reviewed in [14]), but cannot be neglected (5–7).

Ultraviolet radiation damage to DNA has been shown to trigger various DNA damage responses, including repair, apoptosis, translesion synthesis (TLS) and the activation of cell cycle checkpoints. In most of these studies, UVC was used to characterize these responses,

including activation of the intra-S checkpoint (15,16). This checkpoint has been described as an active signaling system that recognizes UV-induced DNA damage and slows progression through S-phase, thereby reducing the probability that damaged DNA will be replicated, potentially lowering the risk for induced mutations (reviewed in [17]). The intra-S checkpoint response to UVC was found to require the activity of checkpoint proteins ataxia telangiectasia and Rad3-related (ATR) kinase, and its substrate checkpoint kinase 1 (CHK1), to act on downstream targets to inhibit the firing of new origins of replication (18–20) and to slow rates of DNA chain elongation (21–23). Comparatively less research has examined the intra-S checkpoint response to UVA and UVB, despite observations that the spectra of DNA damage induced by these wavelengths are not identical. At present, it remains unclear which type of DNA damage is primarily responsible for UV activation of the intra-S checkpoint, although there is evidence that supports CPD, the most abundant, and 6-4PP as the UVC-induced lesions responsible for stalling of replication forks (17).

The main objective of the experiments reported here was to assess the biological outcomes of irradiating human dermal fibroblasts with three sources of UVR, each emitting different spectra of wavelengths. CPD and 6-4PP densities were quantified and used as biomarkers of exposure following irradiation with a source emitting UVA–UVB (modeling UV present in sunlight), a source emitting a narrow range of UVB or a lamp-emitting UVC wavelengths. Comparing irradiation conditions that produced similar CPD densities, we then assessed UV-induced cytotoxicity for these three sources. Using the shortest (UVC) and the longest wavelength range of UVR (UVA–UVB), we also characterized the activation of the intra-S checkpoint and compared their mutagenic potential. Our results suggest that in our model system the CPD burden efficiently predicts the cytotoxicity, activation of the intra-S checkpoint and mutagenicity induced by these sources of UVR.

MATERIALS AND METHODS

Cell culture

The normal human fibroblast cell line NHF1-hTERT was derived from neonatal foreskin fibroblasts (24) and immortalized by ectopic expression of the catalytic subunit of human telomerase (18). The xeroderma pigmentosum variant (XPV) cell line GM02359-hTERT (XP115L0) was derived in the laboratory of Dr. Roger A. Shultz (University of Texas Southwestern Medical Center, Dallas, TX; [25]) and clone 1B (26) was compared with NHF1 in the studies reported here. Cells were cultured in minimum essential medium (MEM) supplemented with 2 mM L-glutamine and 10% fetal calf serum. GM02359-hTERT cells were additionally supplemented with 1× MEM nonessential amino acids (Gibco). All cell cultures were maintained at 37°C in humidified 95% air and 5% CO₂.

Irradiation

Cells were rinsed in phosphate-buffered saline (PBS) prior to irradiation and all cell culture plates were irradiated with lids removed. UVC irradiations were performed with a germicidal fluorescent lamp (Sylvania G8T5, 90% emission at 254 nm). UVB irradiations were performed with two Philips TL20W/01 NB-UVB fluorescent lamps emitting between 300 and 315 nm, with a maximum emission at 312 nm. Sunlight simulating UVA–UVB

irradiations were performed with four Houvalite F20T12BL/HO PUVA lamps; this source emits wavelengths between 300 and 400 nm (94.3% UVA and 5.7% UVB), with a peak at 350 nm; hence, it is identified in the text as either the UVA–UVB or the solar simulator source. UVC irradiations were performed with no liquid in the plates, whereas PBS was used to cover the cultures during UVB and UVA–UVB exposures (as detailed below). Sham-irradiated plates were handled the same way minus the exposure to UVR. These control plates were covered with aluminum foil and placed in the irradiation chamber along with those exposed to the highest fluence used in the experiment. Lamp irradiance was measured using a UVX Digital Radiometer (UVP, LLC) and the following sensors: UVX-25 (250–290 nm range, calibrated at 254 nm), UVX-31 (280–340 nm range, calibrated at 310 nm) and UVX-36 (335–380 nm range, calibrated at 365 nm).

Cytotoxicity

UVR-induced inhibition of [³H]-thymidine incorporation was measured as an index of cytotoxicity, as described previously (27,28). This short-term assay for inhibition of cell proliferation yields results in close agreement with those based on reduction in efficiency of colony formation (27). Logarithmically growing cells were seeded in 6-well plates at 1000 cells per well. The following day, cells were rinsed with PBS and irradiated with increasing fluences of the specified UVR sources; 500 μ L or 1 mL PBS was used to cover cells in each well during irradiation with UVB or UVA–UVB, respectively. Approximately 72 h post irradiation, cells were pulse labeled for 60 min at 37°C in medium containing 2 μ Ci mL⁻¹ [³H]-thymidine. Afterward, the plates were placed on ice and the wells rinsed 2 $\times$ with cold PBS. Cells were then fixed in 5% trichloroacetic acid (TCA) for 30 min and rinsed 2 $\times$ with 5% TCA and 2 $\times$ with 95% ethanol. Fixed cells were allowed to dry overnight and then dissolved for 1 h in 1 mL of 0.3 M sodium hydroxide. Equal volumes of solubilized cells were analyzed by liquid scintillation counting.

Immuno-slot-blot quantification of CPD

Cell cultures were irradiated in 10 cm dishes after rinsing and removing the liquid from the plates (UVC), or while covered with 2 mL (UVB) or 6 mL (UVA–UVB) PBS. DNA from irradiated cells was purified using a Qiagen DNeasy Blood and Tissue kit, using RNase A to remove RNA in the samples, and CPD quantified as published (29). After denaturation at 100°C for 10 min, 200 ng of DNA was combined with an equal volume of 2 M ammonium acetate and placed on ice. Each DNA sample was spotted onto a nitrocellulose membrane (equilibrated in 1 M ammonium acetate) in triplicate, using a Minifold slot-blot manifold (Schleicher & Shuell). Membranes were then washed in 5 $\times$ saline sodium citrate buffer for 15 min in a 37°C water bath and dried in a vacuum oven for 30 min at 80°C. After blocking in 5% powdered milk in TBST (0.5% Tween-20, 10 mM Tris-HCl, 10 mM NaCl, pH 7.4), membranes were probed with mouse anti-CPD antibody (Cosmo-Bio, NMDND001). Following secondary antibody application (HRP-conjugated, GE Healthcare), enhanced chemiluminescence (ECL) was used to detect on film the antibody-dependent signal from each DNA band. Densitometry of film bands was performed using an Alpha Innotech Fluor-Chem HD2. Each membrane contained a standard curve prepared from UVC-irradiated calf-thymus DNA, in which the CPD density had previously been determined by radioimmunoassay (RIA).

Radioimmunoassay

Radioimmunoassay was used to quantify CPD and 6-4PP. RIA is a competitive binding assay between radiolabeled DNA and sample DNA for antisera raised against UV-irradiated DNA. DNA damage frequencies in samples used for the standard curve were determined using HPLC tandem mass spectrometry (Thierry Douki, CEA, Grenoble). These details, as well as those concerning the specificities of the RIAs and standards used for quantification, are described in (30–32).

Western blotting and antibodies

Cells were harvested by trypsinization 1 h after irradiation, pelleted and flash frozen in liquid nitrogen. Cell pellets were resuspended in urea lysis buffer (8 M urea, 5 M NaH₂PO₄, 1 M Tris-HCl pH 8) for 30 min on ice and then clarified by centrifugation at 16 000 × *g* and 4°C. Protein concentrations were determined using the Bio-Rad Protein Assay kit. Protein lysates were combined with equal volumes of loading buffer (125 mM Tris pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 4% sodium dodecyl sulfate, 0.05% bromo-phenol blue) and equal amounts of protein were loaded onto BioRad Criterion-TGX 4%–15% gradient gels (run at approximately 150–200 V for 2–4 h). Size-separated proteins were transferred (100 mA, overnight) onto a nitrocellulose membrane, and blocked in 5% powdered milk in TBST. The following antibodies were used: rabbit anti-P-CBK1 S345 (2348s Cell Signaling), mouse anti-CBK1 (SC-8408, Santa Cruz) and goat anti-ACTIN (SC-1616, Santa Cruz). Following incubation with secondary antibody (HRP-conjugated, GE Healthcare), bands were visualized on film by ECL. Densitometry of film bands was performed using an Alpha Innotech Fluor-Chem HD2.

Velocity sedimentation

The steady-state size distribution of nascent DNA was determined 45 min after UV irradiation by centrifugation in an alkaline sucrose density gradient as described previously (15,33). Cells were plated onto 60 mm culture dishes and their DNA uniformly prelabeled by incubation with 5–10 nCi mL⁻¹ [¹⁴C]-thymidine for at least 36 h during logarithmic growth. Medium containing [¹⁴C]-thymidine was removed and the cultures incubated overnight in fresh, label-free medium. The following morning, this medium was collected and reserved for use during the post irradiation incubation. Cells were covered with 2 mL PBS during exposure to the solar simulator source. After UV irradiation, cells were incubated for 30 min in the reserved medium and then pulse labeled with 25 μCi mL⁻¹ [³H]-thymidine for 15 min. Cells were washed, then scraped on ice into 0.1 M NaCl, 0.01 M EDTA (pH 8) and 0.5 mL added to an equal volume of lysis buffer (1 M NaOH, 0.02 M EDTA) on top of an alkaline sucrose gradient (5%–20% sucrose in 0.4 M NaOH, 2 M NaCl, 0.01 M EDTA). These gradients were left under fluorescent lighting for 45 min and then centrifuged in a Beckman SW32Ti rotor at 25 000 rpm (46 800–106 800 *g*) and 20°C for 5 h. Gradients were fractionated into equal volumes through a hole punctured in the bottom of the centrifuge tube and acid-precipitable material filtered onto glass microfiber filters (Whatman GF/C 24 mm), which were then analyzed by liquid scintillation counting. All experiments included cells prelabeled with [¹⁴C]-thymidine, but not pulse labeled with [³H]-thymidine, which were used to measure the ¹⁴C CPM values detected in the ³H channel

(spillover) during liquid scintillation counting. Normalized ^3H CPM were the counts in each fraction detected in the ^3H channel, corrected for the ^{14}C spillover, and divided by the total ^{14}C in the gradient (proportional to the number of cells analyzed).

HAT selection

Cells used for mutagenesis experiments were preselected for functional HPRT by expanding cultures for 10–14 days in medium supplemented with $1\times$ HAT (34). The $100\times$ lyophilized HAT solution contains 10 mM sodium hypoxanthine, 40 M aminopterin and 1.6 mM thymidine. Aliquots of HAT selected cells were stored frozen in liquid nitrogen. A new aliquot was used for every *HPRT* mutagenesis experiment; cells were replated directly in culture medium without HAT and expanded for 10–14 days prior to mutagenesis experiments.

Mutagenesis

Cells were plated at 5×10^5 per 10 cm dish (2 dishes per treatment) and irradiated the following day. Cell cultures were irradiated after rinsing and removing the liquid from the plates (UVC), or while covered with 6 mL PBS (UVA–UVB). These cultures were maintained in logarithmic growth for 4–6 population doublings prior to selection. *HPRT* mutants were selected by plating 4×10^4 cells per 10 cm dish (55 dishes per treatment) in medium containing 40 μM 6-thioguanine. Colony-forming efficiency was determined at the time of mutant selection by plating 400 cells per dish in medium lacking 6-thioguanine. After 2 weeks, colonies were fixed in 3:1 methanol:acetic acid and stained with 0.05% crystal violet. Colonies with more than 50 cells were counted. Mutation frequencies were calculated from the number of plates without any mutant colonies, using the Poisson distribution as follows: $(-\ln [\text{number of plates without 6-thioguanine resistant colonies}/\text{total number of plates}]) / ([\text{number of cells plated for selection}] \times [\text{colony-forming efficiency at time of selection}])$.

Statistical methods

Statistical comparisons were performed to determine whether 6-4PP density, cytotoxicity, CHK1 phosphorylation, inhibition of replicon initiation, inhibition of DNA strand growth and mutagenicity varied significantly between different sources of UVR. The linear regression model was used to carry out the data analysis for estimating various parameters of interest with appropriate 95% confidence intervals, and hypothesis testing. Specifically, the linear model was used to model the density of 6-4PP and the inhibition of DNA strand growth as response variables with the density of CPD, the radiation source and their interactions as covariates. Wald statistics were used to determine the statistical significance of the comparisons. The Wilcoxon rank-sum test was used to do the group-wise comparisons at different CPD levels and sources of UVR for cell survival percentage, normalized ratios of PCHK1 to total CHK1, inhibition of replicon initiation and the mutation frequencies. Exact test statistics were used to determine the statistical significance of the comparisons. All statistical analyses were performed using SAS 9.3 (SAS Institute Inc., Cary, NC).

RESULTS

Dosimetry curves

Immunoblotting was first used to assess the induction of CPD in NHF1 cells exposed to UVA–UVB, UVB, or UVC (Fig. 1). As expected, UVC was the most efficient UVR source, causing the formation of approximately 34 CPD per Mb of DNA for each 1 J m^{-2} increase in incident fluence (slope of the fluence-response curve). The narrowband UVB and the solar simulator sources also produced CPD in a fluence-dependent manner, although at much lower efficiencies (121 and $9.4 \text{ CPD/Mb per kJ m}^{-2}$, respectively). These dosimetry curves were used in all subsequent experiments to select exposures to different UVR sources to compare their biological effects on an equal CPD density basis.

Later experiments used DNA from independently irradiated NHF1 cultures and RIA to determine the UVR induction of both CPD and 6-4PP in the same DNA samples (Fig. 2). The observed relative densities of 6-4PP per 100 CPD were 14, 11 and 7 for the UVC, UVB and solar simulator sources, respectively. UVC was marginally more efficient at producing 6-4PP than UVB, but the difference did not reach statistical significance ($P = 0.09$). UVC was significantly more efficient at producing 6-4PP than irradiation with the solar simulator ($P = 0.04$). DNA from irradiated cells was also analyzed for the presence of 8-oxo-dG, the most common oxidative DNA lesion; 8-oxo-dG was not formed in a fluence responsive manner and was not produced in levels appreciably higher than background (see Supplemental Material Table S1).

Cytotoxicity

Figure 3a shows the cytotoxicity curves for UVA–UVB, UVB and UVC, expressed as the percent survival relative to CPD burden. As expected, irradiation with each source of UVR produced a dose-dependent decrease in survival. The increase in fluences required to decrease the surviving fraction from 100% to 37% (D_0) was 16.8 kJ m^{-2} , 2.0 kJ m^{-2} and 5.5 J m^{-2} , or 159, 236 and 190 CPD/Mb , for UVA–UVB, UVB and UVC, respectively. When expressed in terms of the CPD density, UVB and UVC caused similar toxicity profiles ($P = 0.41$ for the comparison of D_0 values); whereas the UVA–UVB cytotoxicity curve did not overlap with those for UVB and UVC ($P < 0.005$ for both UVA–UVB vs UVB and UVA–UVB vs UVC, linear regression analysis), the differences in the estimated D_0 did not reach statistical significance (UVA–UVB vs UVB $P = 0.17$, UVA–UVB vs UVC $P = 0.23$). When cytotoxicity is expressed as the percent survival relative to the 6-4PP burden (Fig. 3b), the D_0 value for the solar simulator (3.0 6-4PP/Mb) was significantly different from those produced by the UVB (7.6 6-4PP/Mb , $P = 0.007$) or UVC (8.6 6-4PP/Mb , $P < 0.0001$). Similarly, the cytotoxicity curve produced by the solar simulator did not overlap with those produced by the UVB or UVC sources when compared on the basis of equal 6-4PP densities (both $P < 0.0001$, linear regression analysis). CPD density and 6-4PP density described the percent survival induced by all three sources with a correlation coefficient (r^2) of 0.80 and 0.45, respectively.

Activation of the intra-S phase checkpoint

The activation of the intra-S phase checkpoint was examined in NHF1 cells irradiated with either the solar simulator or the UVC lamps. Should there be significant differences in the biological responses to DNA damage induced by different wavelengths of UVR, they would most likely be observed between these two irradiation sources with distinct UV spectra. Phosphorylation of CHK1 at serine 345 is commonly used as an index of DNA damage-induced activation of the intra-S checkpoint. Based on the dosimetry curves illustrated in Fig. 1, NHF1 cells were irradiated with fluences of UVA–UVB or UVC calculated to produce equal CPD densities. Cells were harvested 45 min post irradiation and protein extracts were probed for P-CHK1 (S345), total CHK1 and ACTIN (Fig. 4a). Figure 4b summarizes the quantification by densitometry of immunoblot signals from three independent experiments. The ratios of P-CHK1 to total CHK1 were normalized to the same ratio determined for the sample with the highest signal in each experiment and plotted against CPD/Mb. Irradiation with either the solar simulator or UVC induced CHK1 phosphorylation at S345 as the CPD burden increased, but no statistically significant differences were observed in the magnitude of the responses induced by the two UVR sources.

Intra-S checkpoint activation was also functionally assessed by velocity sedimentation analyses of nascent DNA pulse labeled with [³H]-thymidine. This technique was used to determine the size distribution of newly replicated DNA and to examine how exposures to UVA–UVB or UVC affected that distribution. First, low fluences of UVR were used to examine the selective inhibition of synthesis of low molecular weight (MW), origin-proximal nascent DNA. NHF1 cells were exposed to 3.8 kJ m⁻² UVA–UVB or 1 J m⁻² UVC, fluences shown to result in similar CPD burdens Fig. 1 and cause minimal cytotoxicity (Fig. 3, 86% and 78% survival, respectively). After irradiation, cells were incubated for 30 min, pulse labeled with [³H]-thymidine for 15 min, harvested and lysed on top of alkaline sucrose gradients (Fig. 5). Low fluences of UVC have been previously shown to selectively inhibit the production of small MW DNA intermediates that initiated DNA synthesis during the 30-min incubation after irradiation or within the 15 min pulse labeling with [³H]-thymidine (15,16) and that this inhibition is dependent on signaling by the checkpoint kinases ATR and CHK1 (18,20). As a functional biomarker for the inhibition of replicon initiation, reduction in labeling of small MW nascent DNA was used to assess activation of the intra-S phase checkpoint. The observed inhibition was 34% ± 6% after a low fluence of UVA–UVB (3.8 kJ m⁻²), by comparison to the matched sham control, and 40% ± 7% after 1 J m⁻² UVC.

Velocity sedimentation can also be used to assess the effect of irradiation on DNA strand growth by examining the abundance of labeled DNA intermediates of high MW. Fluences of UVR higher than those used to document the inhibition of replicon initiation also inhibit the production of multireplicon-sized DNA intermediates in a dose-dependent manner. Once again, fluences were chosen to cause similar CPD densities in cells irradiated with UVA–UVB (Fig. 6a) or UVC (Fig. 6b). Figure 6c summarizes the data from several experiments and shows that DNA strand growth was inhibited as the CPD burden increased, regardless of the source used to irradiate the fibroblasts.

Mutagenesis

UVR-induced mutation frequencies at the *HPRT* locus were measured in NHF1 cells exposed to either UVA–UVB or UVC fluences inducing similar densities of CPD. Colonies resistant to 6-thioguanine were counted as mutants lacking functional HPRT and the mutation frequency expressed per 10^6 surviving cells (Fig. 7). Mutagenicity results from one experiment with XPV fibroblasts exposed to UVC were included as a positive control, as these cells lack the TLS DNA polymerase eta and are known to be hypermutable in response to UVR (34). In NHF1, both the lower (4 J m^{-2}) and the higher (7.5 J m^{-2}) fluences of UVC induced mutation frequencies above the matched sham-irradiated controls ($P = 0.03$ and 0.01 respectively). Exposure to the solar simulator source (UVA–UVB) at incident fluences inducing similar CPD densities in nuclear DNA (14.7 and 27.4 kJ m^{-2} , respectively) resulted in a mutation frequency higher than the matched control only at the highest fluence ($P = 0.059$). Nonetheless, after expressing the mutation frequency against CPD burden, the overall dataset did not disclose any significant differences in the frequencies of *HPRT* mutations induced in NHF1 exposed to UVA–UVB or UVC.

DISCUSSION

The health effects of prolonged skin exposures to the sun have been connected mechanistically to the cellular DNA damage induced by UVR. However, there is still much debate over the relative contribution of UVA toward solar radiation-induced pathology and whether or not exposure primarily to UVA (e.g. through tanning beds) carries the same risks as sunlight, which also includes wavelengths within the UVB range. The properties of different wavelengths of UVR, in particular skin penetration and efficiency of inducing different types of cellular damage (including those triggering inflammation) have made the detailed investigation of this complex issue quite difficult.

This study focused specifically on DNA damage caused by UVR in cultured human fibroblasts and the attendant biological responses, both shortly after irradiation (activation of the intra-S checkpoint, inhibition of DNA synthesis), as well as the resulting cytotoxicity and frequency of *HPRT* mutations in the surviving populations. Biological effects produced by UVR cannot be compared from one study to another on the basis of the incident fluence alone, which are often measured at a single wavelength, while the source spectral profiles can vary widely. By the same token, to characterize and compare biological responses to the UVR sources used in this study, DNA damage dosimetry curves for CPD (Fig. 1) and 6-4PP (Fig. 2) were produced. CPD densities per J m^{-2} indicated that UVC was 284 times more efficient than UVB and 3654 times more efficient than UVA–UVB, in producing this type of DNA lesion. It is well established that short wavelength UVR produces CPD in either irradiated cells or purified DNA in solution more efficiently than long wavelength UVR (5,12,35,36). The relative amounts of CPD induced by the solar simulator used here are higher than values reported for UVA; this is not surprising given that the lamp used emits ~5.7% of its radiant energy in the UVB range. UVC was also more efficient in producing 6-4PP; after normalization to the CPD density in the same sample, the density of 6-4 PP per CPD was 1.3 and 2 times higher in DNA from cells exposed to UVC than in those exposed to the UVB and UVA–UVB sources, respectively (Fig. 2). This relative 6-4PP production is

similar to values reported in human dermal fibroblasts, where UVC induced 1.4 times more 6-4PP per CPD than did irradiation with a narrowband UVB source, such as the one used here (35); but, it is lower than observed in DNA from mouse embryonic fibroblasts, where UVC produced 5.4 times more 6-4PP per CPD than did a monochromatic source of UVB (305 nm) (10). It is also expected that the 6-4PP density measured in NHF1 exposed to the UVA–UVB lamp was produced by the UVB wavelengths emitted by this source, as 6-4PP are not expected to be produced by wavelengths greater than 310 nm (10,37,38). Based on this premise, we can calculate from the fluence-dependent induction of CPD and 6-4PP, by either the narrowband UVB source or the solar simulator, that the shorter wavelengths contained in the latter were responsible for the formation of about 75% of the CPD in DNA of fibroblasts exposed to the solar simulator.

Survival curves generated using the solar simulator, UVB and UVC lamps and constructed against CPD densities suggest that this type of DNA damage was primarily responsible for the cytotoxicity induced by these sources of UVR (Fig. 3a). Although the estimated D_0 values were not statistically different between the three sources of UVR, the solar simulator cytotoxicity curve did not overlap with those induced by UVB or UVC (linear regression analysis), suggesting that other types of cellular damage induced by the solar simulator may have contributed to the cytotoxicity. However, regardless of the source used, CPD densities ($r^2 = 0.80$) correlated better with the overall survival than the 6-4PP densities ($r^2 = 0.45$) (Fig. 3b). Previous research reported a wavelength-dependent increase in cytotoxicity, with longer wavelengths causing higher inactivation of colony formation, when compared on the basis of equivalent T4 endonuclease sensitive sites (12), even when a narrowband UVB source was compared with 254 nm radiation. In contrast, the results reported here, utilizing the same narrowband UVB lamp and the same cell type, suggest that cytotoxicity was equivalent between the UVC and UVB sources when normalized on the basis of CPD density. It has also been reported that cytotoxicity in cultured cells irradiated with UVC or UVB (narrow band or broad band) correlated best with the 6-4PP density (35). The results reported here, utilizing the same narrow band UVB lamp, suggest that CPD density, and not 6-4PP density, adequately described the cytotoxicity of NHF1 exposed to UVC or UVB radiation. The results reported here are in agreement with other research supporting that CPD density is a good biomarker for UV-induced cytotoxicity induced by multiple sources of UVR (36).

Cyclobutane pyrimidine dimers density was well correlated with the activation of the intra-S checkpoint in fibroblasts exposed to UVA–UVB and UVC. Phosphorylation of CHK1 at S345 (Fig. 4), inhibition of replicon initiation (Fig. 5) and inhibition of DNA strand growth (Fig. 6) were all adequately described by CPD density. The observation that CPD density, and not the 6-4PP density, correlated best with these endpoints is consistent with a model that the CPD, as the most abundant lesion induced by UVR, is the primary UV-induced DNA damage encountered by DNA replication forks, thus initially creating a majority of genome sites where the activation of ATR/CHK1 takes place to trigger the intra-S checkpoint responses.

The time course of inhibition of DNA synthesis and the lesion repair kinetics in human fibroblasts have been used to suggest that CPD were not likely to be predominantly

responsible for intra-S phase checkpoint activation. The maximal CHK1 phosphorylation signal and inhibition of DNA synthesis are both reported to occur 45–120 min following UVC irradiation and to recover to basal levels within 6 h post irradiation (16,39). While significant numbers of CPD remain after DNA synthesis has recovered, the kinetics of 6-4PP repair closely matches this time course, with more than 80% of the 6-4PP being removed within 6 h post irradiation (40). The data presented here, however, are more consistent with the CPD, as the most abundant lesion, being primarily responsible for checkpoint activation, and that recovery of DNA synthesis occurs even in the presence of persisting CPD. This interpretation is supported by data showing that UV-induced γ -H2AX foci, also a marker of ATR activation, are formed within the first hour post irradiation, continue to accumulate 48 h post irradiation and are reduced by 50% upon CPD photoreactivation (41,42). Thus, it is plausible that the decay in checkpoint signaling is related not to repair of the less frequent 6-4PP (although it would be a contributing factor), but to the efficiency of TLS across UV-induced CPD; indeed, TLS-defective XP-V fibroblasts display delayed intra-S phase checkpoint recovery following UV irradiation (43) despite having normal rates of nucleotide excision repair (44,45).

Irradiation of SV40-transformed normal human fibroblasts and AT-fibroblasts showed that both displayed inhibition of DNA synthesis following irradiation with UVA or UVC (46). Treatment with 5 mM caffeine (an ATM and ATR inhibitor) abrogated this inhibition in both cell types following irradiation with UVC but not with UVA, suggesting that activation of the ATR/CHK1 pathway was not sufficient to explain UVA-induced inhibition of DNA synthesis. These studies also reported a relatively low production of CPD (less than 2 TT CPD/Mb) at UVA fluences that caused significant cytotoxicity, leading the authors to conclude that DNA damage (TT CPD, 8-oxo-dG, or strand breaks) was not the main cause of the observed inhibition of DNA synthesis. Instead, they suggested that the inhibition of DNA synthesis was more likely due to UVA-induced ROS causing oxidation of replication proteins. These findings are pertinent to experimental conditions utilizing an irradiation source emitting only UVA, which is relatively inefficient at producing CPD, but still capable of producing cellular ROS. Such experimental conditions are very different from those used here, which support the interpretation that the inhibition of DNA synthesis caused by the solar simulator was dominated by the UVB-induced production of CPD and 6-4PP, and that the UVA portion of this source produced significantly lower levels of UVA-induced ROS, as suggested by the absence of detectable 8-oxo-dG (see Supplemental Material, Table S1).

No differences in mutation frequencies at the *HPRT* locus were observed in NHF1 cells irradiated with the solar simulator or UVC when compared on an equal CPD basis Fig. 7. These data suggest that in this model system, the CPD is the photolesion predominantly contributing to mutagenesis under the irradiation conditions used in this study. UVA radiation emitted by the solar simulator did not contribute significantly to the observed mutation frequency, except for the induction of a fraction of the total CPD. Other research examining mutagenesis in response to different sources of UV has produced varying results. Human fibroblasts irradiated with longer wavelengths of UVR displayed higher mutation frequencies than those irradiated with short wavelength UVR when compared on the basis of equal T4 endonuclease sensitive sites, a common marker for CPD (12). In contrast, approximately two-fold higher mutation frequencies were observed in a shuttle vector

carried by human embryonic kidney cells when cultures were irradiated with equitoxic fluences of UVA as compared to UVB, but only when using fluences inducing ~1% cell survival (47); mutation frequencies were equal at equitoxic fluences of UVA and UVB resulting in 10% cell survival. Mutation frequency in primary neonatal human fibroblasts was also higher in cells irradiated with UVA as compared to roughly equitoxic fluences of UVB (48), but quantitative toxicity and dosimetry data were not provided. Each of these studies utilized UVR sources emitting exclusively in the UVA range. In contrast, the data presented here support the conclusion that the mutation frequency induced by our solar simulator was predominantly a result of UVB-induced photolesions, and that its UVA component did not significantly contribute to the observed mutation frequencies.

The observation of similar mutation frequencies, after normalization to CPD density, in NHF1 irradiated with UVA–UVB and UVC, is consistent with other research examining the mutation spectrum induced by different wavelengths of UVR. The C:G→T:A transition and the CC:GG→TT:AA tandem mutations are considered UV signatures arising from replication past CPD and 6-4PP (49). Sequencing of the *HPRT* gene in primary neonatal fibroblasts showed that C:G→T:A transitions were by far the most abundant UV-induced mutation induced by either UVA or UVB, and that these tended to occur in pyrimidine rich hotspots (48). In addition, most of these mutational hotspots were shared by cells irradiated with UVA or UVB, suggesting that these mutations were formed by a common mechanism. Similarly, C:G→T:A transitions at dipyrimidine sites were also the most common base pair change found in mutants isolated from the epidermis of transgenic mice harboring λ -phage-based *lacZ* mutational reporter genes after irradiation with UVA or UVB (50). These findings support the conclusion that the CPD is the primary lesion associated with solar UVR-induced mutagenesis.

One limitation of the current analysis is the use of dermal fibroblasts that are not the target of UVR carcinogenesis in skin. We have compared the effects of UVC, UVB and UVA–UVB on human dermal melanocytes. We found that melanocytes displayed levels of phosphorylation of checkpoint kinases in response to UVR similar to fibroblasts (see Supplemental Material, Fig. S1). Normal human melanocytes were also found to repair CPD and 6-4PP with kinetics similar to fibroblasts (see Supplemental Material, Fig. S2 and [51]). Thus, the radiobiological responses reported here for fibroblasts are likely to hold true for melanocytes.

Taken together, the results described in this study constitute strong evidence that CPD density is the most predictive biomarker for the biological effects derived from exposure to solar radiation. The broad-spectrum solar simulator lamp used in this study emits ~5.7% UVB wavelengths (based on manufacturer's spectral output curves) and this percentage is similar to that present in natural sunlight. This UVB representation is expected to have contributed to the abundance and relative proportions of CPD and 6-4PP, and thus also to the biological responses measured in the fibroblasts irradiated with the solar simulator. Therefore, the importance of including quantitative DNA damage dosimetry to studies examining UVR-induced biological responses cannot be emphasized enough.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cyclobutane pyrimidine dimers (CPD) dosimetry curves. NHF1 were exposed to three different sources of ultraviolet radiation: a) a UVC germicidal lamp (open diamonds) and b) a narrowband UVB lamp (open squares) or a solar simulator emitting wavelengths in both the UVB (5.7%) and UVA ranges (94.3%) (closed circles). CPD densities were determined by immunoblotting, using standard curves prepared with irradiated calf-thymus DNA with known CPD densities, which were independently determined by radioimmunoassay (each point represents the mean of 2–4 measurements; error bars indicate SEM).

Figure 2. 6-4PP dosimetry curves. Dose–response curves for the formation of 6-4 pyrimidine-pyrimidone photoproduct in NHF1 exposed to a solar simulator (UVA–UVB, closed circles), UVB only (open squares) or UVC (open diamonds) as a function of cyclobutane pyrimidine dimers density. The 6-4PP density produced by UVC was statistically different from that produced by irradiation with a solar simulator ($P = 0.04$ by linear regression analysis). Both lesions were quantified in each sample by radioimmunoassay ($n = 3–4$, mean $\pm$ SEM).

Figure 3. Cytotoxicity as a function of a) CPD density or b) 6-4PP density, for NHF1 exposed to a solar simulator (UVA+UVB, closed circles), UVB only (open squares), or UVC (open diamonds). Percent survival measured by [^3H]-thymidine incorporation 72 h post irradiation as compared to the unirradiated sham ($n = 2-4$, mean $\pm$ SEM). There was no statistical difference between the D_0 values expressed in terms of CPD density (Wald test p-values were 0.17 for UVA+UVB vs UVB, 0.23 for UVA+UVB vs UVC, and 0.41 for UVB vs UVC). Regression analysis showed that the UVA+UVB survival curve was distinct from those for UVB and UVC ($P < 0.005$). When expressed in terms of 6-4PP density, the D_0 value for the solar simulator was statistically different from the UVB or UVC source ($P = 0.007$ and $P < 0.0001$, respectively). Regression analysis also showed that the solar simulator survival curve was statistically distinct from the UVB and UVC sources when compared on the basis of 6-4PP density ($P < 0.0001$ for both).

Figure 4. Checkpoint kinase 1 (CHK1) phosphorylation in NHF1 exposed to UVA–UVB or UVC. Fluences of UVA–UVB or UVC were chosen so as to produce equivalent CPD burdens. a) Immunoblot showing P-CHK1 (S345), total CHK1 and ACTIN. b) Quantification of immunoblots ($n = 3$) by densitometry $\pm$ SEM. Densitometry values were normalized to the highest ratio of P-CHK1:total CHK1 in each experiment. The normalized ratios of P-CHK1 to total CHK1 produced by the UVA–UVB source (open bars) were not statistically different (Wilcoxon rank-sum test) from those produced by UVC (filled bars) at equal CPD densities.

Figure 5.

Inhibition of replicon initiation by UVC or UVA–UVB. Velocity sedimentation analysis of nascent DNA from NHF1 cells exposed to low fluences of a) UVC (1 J m^{-2} open squares; sham control-closed diamonds) or b) UVA–UVB (3.8 kJ m^{-2} open squares; sham control-closed diamonds). Fluences were chosen so as to induce similar CPD densities ($\sim 19 \text{ CPD/Mb}$). Inhibition of small MW DNA, measured from the area under the curve between the two arrows, was $34\% \pm 6\%$ (UVA–UVB) and $40\% \pm 7\%$ (UVC), $n = 3$ (mean $\pm$ SEM), and these values were not statistically different ($P = 0.4$, Wilcoxon rank-sum test).

Figure 6.

Inhibition of DNA strand growth by UVC or UVA-UVB. Representative velocity sedimentation analysis of nascent DNA from NHF1 cells exposed to high fluences of **a)** UVC (0, 5, 8, or 12 J m⁻²) or **b)** UVA-UVB (0, 18.3, 29.2, or 41.6 kJ m⁻²). Fluences were chosen to induce equal cyclobutane pyrimidine dimers densities (0, 156, 259, or 397 CPD/Mb, respectively). **c)** Inhibition of DNA strand growth (calculated from the area under curve between the two arrows as compared to sham irradiated) is shown as a function of CPD burden for NHF1 exposed to UVA-UVB (closed circles) or UVC (open diamonds). Figure represents the combination of five different experiments examining increasing irradiation fluences from the UVC or the solar simulator sources. There was no statistical difference in the percent inhibition of DNA strand growth by UVA-UVB or UVC (linear regression analysis, $P = 0.59$).

Figure 7. UVA–UVB or UVC-induced mutation frequency at the *HPRT* locus. *HPRT* mutation frequency in xeroderma pigmentosum variant cells exposed to UVC (closed triangles, $n = 1$) and NHH1 exposed to a solar simulator (closed circles, $n = 4$) or UVC (open diamonds, $n = 4$) expressed as mutants per 10⁶ surviving cells $\pm$ SEM. The mutation frequency induced by UVA–UVB was not statistically different from that induced by UVC ($P = 0.2$ and 0.69 for 122 or 242 CPD/Mb, respectively, Wilcoxon rank-sum test).