Short- and Long-Wave UV Light (UVB and UVA) Induce Similar Mutations in Human Skin Cells

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While the mutagenic and carcinogenic properties of longwave UV light (UVA) are well established, mechanisms of UVA mutagenesis remain a matter of debate. To elucidate the mechanisms of mutation formation with UVA in human skin, we determined the spectra of UVA- and UVB-induced mutations in primary human fibroblasts. As with UVB, we found the majority of mutations to be C-to-T transitions also with UVA. For both UVA and UVB, these transitions were found within runs of pyrimidines, at identical hotspots, and with the same predilection for the nontranscribed strand. They also included CC-to-TT tandem mutations. Therefore, these mutations point to a major role of pyrimidine dimers not only in UVB but also in UVA mutagenesis. While some differences were noted, the similarity between the spectra of UVA- and UVB-induced mutations further supports similar mechanisms of mutation formation. A non-dimer type of DNA damage does not appear to play a major role in either UVA or UVB mutagenesis. Therefore, the previously reported increasing mutagenicity per dimer with increasing wavelengths cannot be due to non-dimer DNA damage. Differences in the cellular response to UVA and UVB, such as the less prominent activation of p53 by UVA, might determine a different mutagenic outcome of UVA- and UVB-induced dimers.

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INTRODUCTION

The mutagenic and carcinogenic properties of sunlight have been attributed to the short-wavelength range (UVB, 290–320 nm) of the solar UV spectrum. Despite being well established that UVA (320–400 nm) can also damage DNA and that it has mutagenic and carcinogenic properties (Stary and Sarasin, 2000), the relevance of these effects for solar mutagenesis and skin carcinogenesis remains unproven. There are several indications that UVA in particular might play an important role in the pathogenesis of cutaneous malignant melanoma, the deadliest type of skin cancer (Moan *et al.*, 1999; Rünger, 1999; Wang *et al.*, 2001). However, this has recently been questioned, as only UVB, but not UVA, induced melanoma in a transgenic mouse model (De Fabo *et al.*, 2004).

The exact mechanisms of UVA mutagenesis are still a matter of debate (Douki *et al.*, 1999). It is generally accepted that pyrimidine dimers, namely cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6,4) pyrimidone photoproducts, are the major mutagenic lesions for UVB. Several lines of evidence indicate that pyrimidine dimers are not the only or major mutagenic lesions with UVA: Enninga *et al.* (1986) reported that mutagenicity per dimer increased with increasing wavelength, in particular within the UVA range. Further evidences include differences in the spectrum of UVA- and UVB-induced mutations (Drobetsky *et al.*, 1995; Robert *et al.*, 1996; Besaratinia *et al.*, 2004), a lower incidence of p53 mutations in UVA- as compared to UVB-induced skin tumors in mice (van Kranen *et al.*, 1997), and a second peak of tumor formation in the UVA range of the squamous cell carcinoma action spectrum in mice, without a second peak of dimer formation in that wavelength range (de Gruijl *et al.*, 1993). However, to our knowledge, epigenetic differences in the cellular responses to UVA and UVB have as yet not been discussed to account for the different mutagenic properties of UVA and UVB.

UVA mutagenesis has been suggested to result from oxidative DNA base modifications (Darr and Fridovich, 1994; Stary and Sarasin, 2000), such as 7,8-dihydro-8-oxoguanine (8-oxoG). This mutagenic lesion is a likely candidate for UVA mutagenesis, as one peak of its formation has been shown for the UVA range (Kielbassa *et al.*, 1997). If this is true, one would expect a high proportion of mutations typically induced by 8-oxoG in the spectrum of UVA-induced mutations (G→T transversions, generated by mispairing of the template 8-oxoG with adenine, or A→C transversions, generated by misincorporation of 8-oxoG as the substrate opposite adenine (Epe, 1991; Cheng *et al.*, 1992)). Indeed, Besaratinia *et al.* (2004) found 25% of

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Abbreviations: cDNA, complementary DNA; CHO, Chinese hamster ovary; CPD, cyclobutane pyrimidine dimers; 8-oxoG, 7,8-dihydro-8-oxoguanine

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UVA-induced mutations in rodent cells to be G→T transversions, and Persson *et al.* (2002) found three G→T transversions in the *p53* gene of single keratinocytes derived from UVA-irradiated human skin. Drobetsky *et al.* (1995) did find a large proportion of A→C transversions (but not G→T transversions) in the spectrum of UVA-induced mutations in hamster cells, but not with UVB, and suggested that these mutations are fingerprints for exposure to UVA. Description of this mutation in human squamous skin tumors has been interpreted to indicate a role for UVA in human skin carcinogenesis (Agar *et al.*, 2004). Other oxidative DNA lesions or oxygen-radical-induced DNA strand breaks might also mediate mutation formation following UVA-induced oxidative stress (Peak *et al.*, 1987; Kuluncsics *et al.*, 1999). If, for example, thymine glycol, an oxidized pyrimidine lesion, contributed to UVA mutagenesis, T→C transition mutations would be expected in the spectrum of UVA-induced mutations, as thymine glycol has been reported to induce such lesions (Essigmann *et al.*, 1989). Based on the assumption that oxidative base damage contributes to solar mutagenesis, the use of antioxidants in sunscreens has been widely advertised for the prevention of photocarcinogenesis.

Despite its relatively weak ability to induce pyrimidine dimers through direct excitation of the DNA molecule, such lesions have nevertheless been suggested to contribute at least in part also to UVA mutagenesis. If this is true, C→T transitions and CC→TT tandem mutations would be expected in the spectrum of UVA-induced mutations. Recently, it was suggested that UVA generates CPDs via a photosensitized triplet energy transfer (Douki *et al.*, 2003; Rochette *et al.*, 2003), in contrast to formation via direct excitation of DNA by UVB.

Currently available investigations of UVA-induced mutations produced conflicting results and used model systems that might not reflect well the situation in humans (Drobetsky *et al.*, 1995; Robert *et al.*, 1996). So far, a detailed spectrum of UVA-induced mutation in primary human skin cells has not been available and an understanding of the exact mechanism of UVA mutagenesis in humans remains elusive today. In order to study in detail the mechanisms of mutation formation following UVA exposure, we generated, sequenced, and compared mutations in the hypoxanthine-phosphoribosyl-transferase (*hprt*) gene generated by exposure to UVA or UVB in primary human skin fibroblasts.

RESULTS

Mutation formation and cytotoxicity by UVA and UVB

The frequency of spontaneous mutation formation in sham-irradiated cells was low, with 0.3±0.6 mutants/one million colony-forming cells (mean±standard deviation; $n=5$). Increasing mutation frequencies were observed with increasing doses of UVA or UVB (Figure 1). With 200 kJ/m^2 UVA, the induced mutation frequency was 235-fold higher than the spontaneous mutation rate. Samples that were UVA-irradiated through a 320 nm cutoff filter did not exhibit a lower mutation frequency ($n=2$), as compared to samples from the same donor irradiated without the cutoff filter ($n=2$). The frequency of mutations induced by 200 J/m^2 UVB was 68-fold

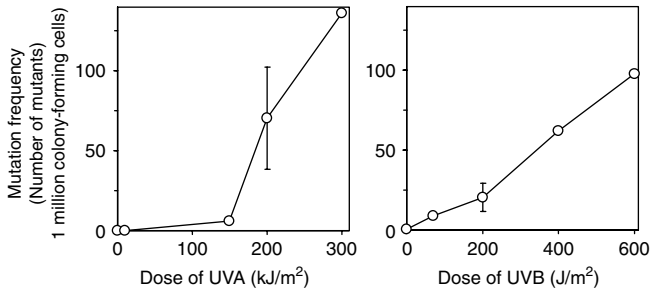

Figure 1. Dose-response of *hprt*-mutation formation in UVA- or UVB-irradiated primary neonatal human fibroblasts. ($n=4$–5 for sham-irradiated samples, and those irradiated with 200 kJ/m^2 UVA or 200 J/m^2 UVB. $n=1$ for all other samples. Error bars show standard deviation).

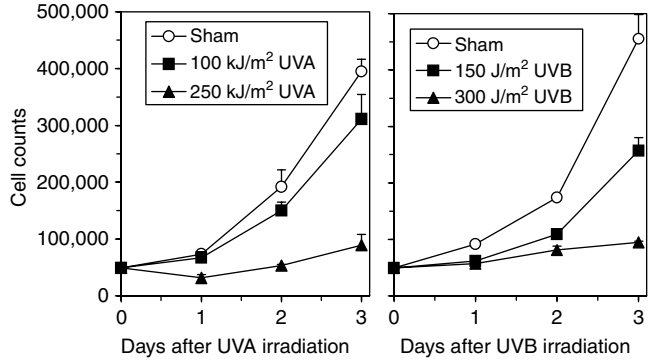

Figure 2. Growth curves of primary neonatal human fibroblasts after irradiation with UVA or UVB. The means±standard deviation of triplicate samples are shown.

higher than the spontaneous mutation frequency. In these dose ranges, cells exhibited a similar, mild to moderate cytotoxicity, as shown in the growth curves (Figure 2). Comparing roughly equitoxic doses (200 kJ/m^2 UVA or 200 J/m^2 UVB), our UVB source yields approximately 300-fold more mutations per J/m^2 than the UVA source. This is well within the range of published factors or factors calculated from published data. For human cells, these factors range from 150 to 1,100 (Wells and Han, 1984; Enninga *et al.*, 1986; Jones *et al.*, 1987) and for rodent cells from 700 to 50,000 (Drobetsky *et al.*, 1995; Dahle and Kvam, 2003).

Characterization of UVA- and UVB-induced mutants

As the frequency of UV-induced mutations was that much higher than the background of spontaneous mutation formation, we are convinced that the vast majority of sequenced mutants in the UVA- or UVB-irradiated samples were indeed induced by UV. The *hprt*-messenger RNA of 55 independent UVA-induced mutants (derived from 14 independent samples and cells from 11 different donors) and 58 independent UVB-induced mutants (derived from 15 independent samples and cells from nine different donors) was sequenced and the result confirmed by sequencing in opposite direction. In all, 16% of UVA- and 10% of UVB-induced mutations (difference not significant) were simple exon deletions, consistent with mutations at splice-acceptor or splice-donor sites. These

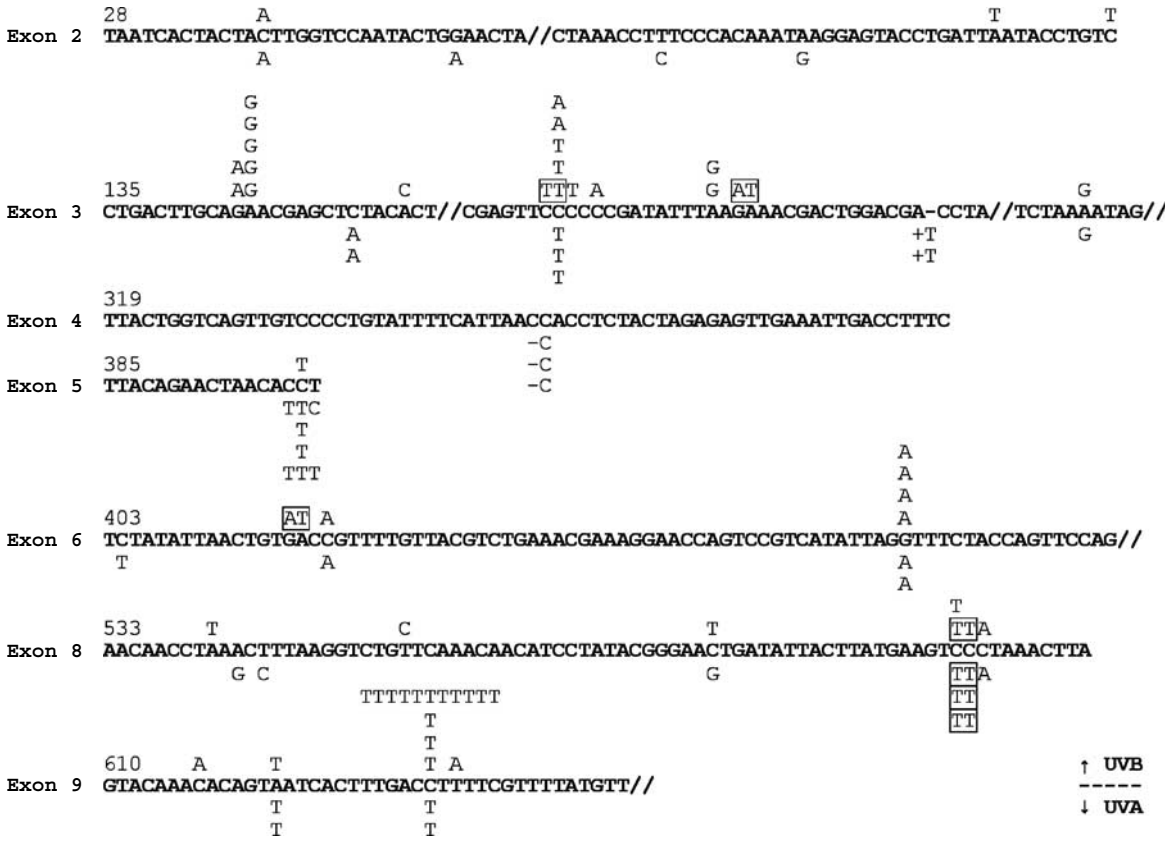

Figure 3. Localizations and types of *hprt* single-base mutations induced by UVB or UVA in primary neonatal human fibroblasts. Parts of the sequence of the nontranscribed strand in which single-base mutations were found are shown in bold, and UVB-induced mutation above and UVA-induced mutation below the cDNA sequence. The first base of the start codon is designated as position 1. A double backlash indicates a break in the sequence. Tandem mutations are boxed.

frequencies are consistent with a published rate of 12.5% of human *hprt* mutations, resulting in alterations in RNA splicing (O'Neill *et al.*, 1998). Figure 3 shows parts of the *hprt*-complementary DNA (cDNA) sequence in which we found mutations and the type of single-base mutations induced by UVB (shown above the cDNA sequence) and UVA (shown below the cDNA sequence). Six hotspots for C:G→T:A transitions and CC:GG→TT:AA tandem mutations were found, most of which were located within long runs of pyrimidines (exon 3, positions 145 and 208; exon 5, position 400; exon 6, position 465; exon 8, position 599/600; exon 9, position 635). Four of these hotspots were identical for UVA and UVB. One weak hotspot (exon 3, position 146) was only observed with UVB. At one hotspot with UVA (exon 5, position 400) only a single mutation event was observed with UVB. When including the UVA hotspot in exon 5, the frequency of C:G→T:A transitions in the four/five common hotspots per mutant is very similar for UVA and UVB (41 and 46%, respectively; difference not significant). In addition, CC:GG→TT:AA tandem mutations also occurred at these hotspots, again with both UVB and UVA. Three more hotspots were observed, two for A:T→G:C transitions in exon 3 (UVB only) and one for A:T→T:A transversions in exon 9 (hotspot for UVA, single occurrence with UVB). Single base-pair insertions ($n=2$) and single base-pair deletions ($n=3$) were induced by UVA, but not by UVB.

Figure 4 compares the frequency of each type of single and tandem base mutations with UVA and UVB. For both wavelengths, the C:G→T:A transition mutation is by far the most common type of mutation, at least twice as frequent as any of the other types of mutations, and their frequency (including tandem mutations) is not significantly different between UVA and UVB (46 and 54%, respectively). Except for the A:T→T:A transversion, which was almost twice as frequent with UVA than UVB (15 *vs* 8%; $\chi^2=3.94$, $P=0.047$), all other types of single base exchange mutations showed very similar frequencies in the UVA and UVB spectrum, or were too rare to allow meaningful comparison. Overall, there is a marked similarity between the UVA- and UVB-induced mutation spectra.

p53 activation following exposure to UVA and UVB

Following exposure to the same dose range of UVA and UVB as used for the mutagenesis studies, primary human neonatal fibroblasts showed a dose-dependent activation of p53, as demonstrated by phosphorylation at serine 15 (Figure 5). However, with UVA, the p53 activation is less pronounced and more short-lived than with UVB.

DISCUSSION

We describe a detailed spectrum of mutations induced by UVA and UVB, for the first time in nontransformed, human

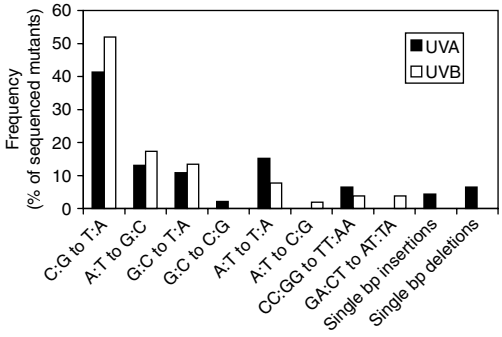

Figure 4. Distribution of mutations induced by UVA or UVB in the *hprt* gene of primary neonatal human fibroblasts.

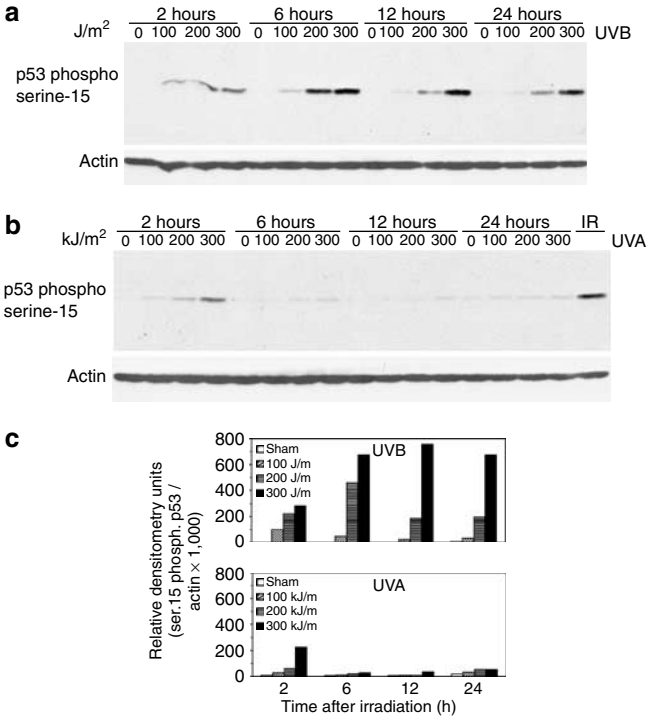

Figure 5. Stronger and longer-lasting activation of p53 in primary human neonatal skin fibroblasts after exposure to increasing doses of UVB, as compared to increasing doses of UVA. Densitometric analysis of serine-15 phosphorylated p53, derived from the UVB- and UVA-irradiated samples (shown in (**a**) and (**b**), respectively), is shown in (**c**). IR: positive control, 2 hours after 10 Gy of ionizing radiation.

primary skin cells. The C:G→T:A transition was by far the most common mutation for both UVA and UVB (41 and 52% of all mutants, respectively). Such mutations are considered signature mutations for UV light, as these are only rarely found after exposure to non-UV carcinogens (Ziegler *et al.*, 1993; Wikondahl and Brash, 1999). This is even more true for CC:GG→TT:AA tandem mutations, which we also found after exposure to UVA and UVB (7 and 4% of all mutations, respectively). Since pyrimidine dimers (CPDs and (6,4) pyrimidone photoproducts) are generally accepted to cause C:G→T:A and CC:GG→TT:AA mutations, our data suggest that pyrimidine dimers are not only the major contributors to UVB mutagenesis but also to UVA mutagenesis. This is further supported by the location of most these mutations within runs of pyrimidines, as pyrimidine dimer-induced mutations have been described previously to occur most commonly in such areas (Brash *et al.*, 1987). In addition, the fact that most of the hotspots of C:G→T:A transitions were identical with UVA and UVB also suggests that the UVA- and UVB-induced C:G→T:A transitions were formed through the same mechanisms, namely pyrimidine dimers. It remains unclear if smaller differences reflect experimental variability or mechanistic differences in mutation formation.

Dimer-induced C:G→T:A transitions have been described to be more common on the nontranscribed strand, presumably due to faster repair of dimers on the transcribed strand (McGregor *et al.*, 1991). After irradiation of cells in G1 phase, 80% of UV-induced C:G→T:A transitions were reported to be located on the nontranscribed strand of *hprt* of human fibroblasts. This is consistent with our data which show most of the C:G→T:A transitions also located on the nontranscribed strand (85% for UVA; 78% for UVB), including four of the five C:G→T:A hotspots. This further substantiates the interpretation that our C:G→T:A transitions have indeed been generated by pyrimidine dimers.

The possibility that an undetected contamination of our UVA source with UVB could have been responsible for dimer formation and subsequent C:G→T:A transition formation was excluded by showing that the use of a 320 nm cutoff filter did not reduce the frequency of UVA mutations.

Although the ability of UV light to induce DNA photoproducts decreases exponentially with increasing wavelengths, UVA is still able to generate such damage, *in vitro* and *in vivo* (Freeman *et al.*, 1987; Matsunaga *et al.*, 1991; Kielbassa *et al.*, 1997; Ley and Fourtanier, 2000). Douki *et al.* (2003) reported that single-strand breaks, oxidized pyrimidines, oxidized purines (mainly 8-oxoG), and CPDs are formed in a 1:1:3:10 ratio with UVA, using a very similar UVA source as we did in this study. Considering that we have used a 1,000-fold higher dose of UVA than UVB, it may not be surprising to find pyrimidine dimer-induced C:G→T:A transitions after exposure to UVA. Douki *et al.* (2003) also reported the distribution of bipyrimidine photoproducts at the four possible dipyrimidine sites (TT, CC, TC, and CT) within the DNA of Chinese hamster ovary (CHO) cells after irradiation with UVA or UVB. While the TT-CPD was the most common type for both wavelengths, UVB induced several other types with almost the same frequency, while many types were not detected after UVA (most notably (6–4) adducts and CC-CPDs), and TC- and CT-CPDs were detected only with a much lower frequency than TT-CPDs. As similar results were recently reported for UVA and primary human keratinocytes and fibroblasts (Courdavault *et al.*, 2004), our data suggest, consistent with published data (sometimes referred to as the A-rule; Strauss, 1991), that TT-CPDs are not very mutagenic, and that CPDs at cytosine-dipyrimidine sites contribute most to UVA and UVB mutagenesis, despite their less frequent, and in the case of UVA, much less frequent

formation. This has been suggested to be due to the mutation-prevention effects of DNA polymerase η or other translesional DNA polymerases (Taylor, 2002; Choi and Pfeifer, 2005; Lehmann, 2005). Our data, showing that most of our UVA-induced C:G→T:A transitions are located at CT sites, and less at TT sites is consistent with this explanation. A notable exception is the hotspot in exon 3, which is located within a run of cytosines, presumably caused by a CC dimer, a type that was not found after UVA exposure by Douki *et al.* (2003) and Courdavault *et al.* (2004).

Unlike UVC, which is considered to induce mostly, if not exclusively, pyrimidine dimer-type DNA damage, both UVB and UVA have been reported to generate a variety of different types of DNA damage besides pyrimidine dimers, including various types of oxidative DNA damage, DNA strand breaks or other ''minor'' photoproducts (Peak and Peak, 1990; Kielbassa *et al.*, 1997). It is therefore difficult to extrapolate from a given mutation to the inducing DNA lesion. For example, the A:T→G:C can be generated at a pyrimidine dimer, or at the oxidative thymine base modification thymine glycol (Basu *et al.*, 1989). In our mutation spectra, all of these mutations were found to be located at possible pyrimidine dimer sites. They do not, however, show the bias for the nontranscribed strand, as seen for the C:G→T:A transitions. Two hotspots for the formation of A:T→G:C mutations were observed with UVB on exon 3, but none with UVA. While the overall frequency of this type of mutation was not significantly different between UVA and UVB (13 and 17%, respectively), the difference in hotspot formation might indicate that UVA and UVB generate them through a different mechanism. Similarly, the difference in the frequencies and hotspot formation with the A:T→T:A mutation might also point to differences in mutation formation. However, as the frequencies of these two types of mutations are low overall, these differences can only be considered minor. The G:C→T:A and G:C→C:G transversions have been described to be typical mutations at sites of 8-oxoG, and A:T→C:G transversions to be caused by misincorporation of 8-oxoG as the substrate opposite of adenine (Epe, 1991; Cheng *et al.*, 1992). In our data, such mutations occurred at similar frequencies after irradiation with UVA (13%) and UVB (15%). This is consistent with both UVA and UVB being able to induce 8-oxoG (Kielbassa *et al.*, 1997). The fact that two UVB-induced G:C→T:A transversions occurred at the same site as the hotspot for G:C to A:T transitions in exon 3 (Figure 3) might indicate that even this type of mutation was not formed by an oxidized guanine, but at a pyrimidine dimer on the other strand. This possibility is supported by the description of G:C→T:A transversions with UVC (254 nm), a wavelength considered to generate almost exclusively pyrimidine dimers (McGregor *et al.*, 1991). Even if all transversions were induced by 8-oxoG, our data clearly indicate that, in comparison to pyrimidine dimers, 8-oxoG could only be a minor contributor to either UVA or UVB mutagenesis. As a practical consequence, the current advocating of antioxidants for skin cancer protection might need to be questioned. The recently reported somewhat higher frequency of G:C→T:A transversions (25%) with UVA in rodent cells (Besaratinia *et al.*, 2004) might be attributable to the difference between rodent and human cells. A rate of this type of mutations with UVB was not reported in that study.

Single-base insertions and single-base deletions require formation of a single- or double-strand break, either as initial lesion, or as a repair intermediate. As such mutations were only found with UVA, but not with UVB, this might point to a contribution, albeit a small one, of DNA strand breaks to UVA mutagenesis in particular. UV-induced −1 frameshift mutations have also been described following dimerization of nonadjacent pyrimidines with a single intervening nucleotide (Nguyen and Minton, 1988; Lingbeck and Taylor, 1999). However, at the site of our −1 deletion, such dimers could not be formed, because the deleted base is flanked by one pyrimidine and one purine.

Drobetsky *et al.* (1995) sequenced UVA- and UVB-induced mutations using an immortalized strain of CHO cells. With UVB, they also found predominantly C:G→T:A transitions, but the spectrum of the less common mutations differs considerably from ours. In contrast to our data with a marked similarity between the UVB- and the UVA-induced mutation spectra, they found large differences between UVB and UVA, with a lower frequency of C:G→T:A transitions (albeit the same frequency of CC:GG→TT:AA tandem mutations), and a much higher frequency of A:T→C:G transversions with UVA. The latter was suggested to constitute a molecular fingerprint for mutations induced by UVA in rodent cells and for that reason has been used to attribute mutations found in human cutaneous squamous cell carcinomas and actinic keratoses to UVA (Agar *et al.*, 2004). Our data do not confirm this UVA fingerprint mutation for human cells. While Drobetsky *et al.* (1995) acknowledge a possible contribution of pyrimidine dimers to UVA mutagenesis, our data point to a much more prominent role of these lesions in UVA mutagenesis. The type of cells used might explain these differences: While we used primary human skin cells, they studied CHO cells. These cells, as rodent cells in general, are well known to be relatively deficient in nucleotide excision repair, as compared to human cells (Hanawalt, 2001). In addition, immortalization is known to often alter the way how cells respond to DNA damage. For example, p53, a pivotal, UV-activated regulator of apoptosis, cell cycle, and DNA repair, is commonly inactivated in immortalized cells (Finlay, 1992; Tiemann and Deppert, 1994). As the spontaneous mutation frequency with these cells was high, irradiation with high doses of UVA for 2 hours was needed to observe a UVA-induced mutation frequency three- to five-fold above the spontaneous background. This means that 20–33% of the mutations observed after UVA irradiation were actually not UVA-induced, but represent spontaneous mutations. In our experiments, the frequency of UVA-induced mutations was 68- to 235-fold higher than the spontaneous background, indicating that only 0.4–1.5% of our mutations observed after UVA irradiation represent spontaneous mutations. Finally, differences might also be attributable to the different emission spectra of UVA lamps used and the much weaker output of their fluorescent UVA lamp, which required irradiation for 2 hours, while our

stronger UVA emitter required irradiation times of only less than 15 minutes.

Robert *et al*. (1996) studied UVA- and UVB-induced mutations in adenovirus-transformed human embryonic kidney cells, stably transfected with an extrachromosomal shuttle vector harboring the *lacZ* bacterial gene as mutagenesis target. Consistent with our data, they also found a majority of point mutations to be C:G→T:A transitions with both UVB and UVA (68% out of 34 point mutations and 54% out of 22 point mutations, respectively). The spectra of the remaining mutations were also similar to our results. While almost all mutations were located at dipyrimidine sites, the distribution of UV-induced mutations in the mutagenesis target gene was significantly different for UVB and UVA. This is in contrast to our data, where we found the same hotspots for C:G→T:A transitions with UVA and UVB. These differences might again be attributable to the different model systems (their extra-chromosomal, plasmid-encoded bacterial gene *versus* our human chromosomal gene; their transformed kidney cell line *versus* our primary skin cells).

Rochette *et al*. (2003) quantified CPD formation at nucleotide resolution along one exon of *hprt* in CHO cells following exposure to UVA, UVB, UVC, and solar simulated light, and aligned the resulting DNA damage spectrum with the corresponding mutational spectrum previously reported, as mentioned above, by Drobetsky *et al*. (1995). In contrast to UVC, UVB, and solar simulated light, where CPD damage hotspots corresponded to C:G→T:A mutation hotspots, UVA generated CPDs more frequently at TT sites, correlating well with the high proportion of mutations recovered opposite of TT dipyrimidines in UVA-irradiated CHO cells. They conclude that UVA-induced CPDs are formed through means other than (or in addition to) direct absorption by DNA, and suggest triplet photosensitization to be responsible for these UVA-induced CPDs at TT sites. While their conclusion that CPDs are a major promutagenic DNA photoproduct in UVA mutagenesis is consistent with our data, we did not find any indications that UVA-induced mutations are generated at CPDs formed through a mechanism different from those responsible for UVB-induced mutations, as the mutation spectra, including hotspots, were very similar for UVA and UVB, and no particular predominance of mutations at TT sites was noted. These differences might again be due to the different UVA sources, cell types, and experimental designs used by Drobetsky *et al*. (1995), as discussed above, as the conclusions of Rochette *et al*. (2003) are based on the mutation spectra from Drobetsky *et al*. (1995). In addition, their findings might have been, at least in part, influenced by the unphysiologically high doses of UVA, as they used up to more than 20-fold higher UVA doses than we did in our mutagenesis studies. Alternatively, dimers might be formed preferentially at TT sites, but not contribute to mutation formation in human cells, as discussed above.

Pyrimidine dimer-induced C:G→T:A and CC:GG→TT:AA mutations are regarded as UV-signature mutations. Here we provide strong evidence that these mutations are signatures not only for an exposure to UVB but also to UVA. Our results with the striking similarity between the UVA- and UVB-induced mutation spectra do not support a major role or an increasing role of non-dimer DNA damage with increasing wavelength. The findings of increasing mutagenicity per dimer with increasing wavelength (Enninga *et al*., 1986) and the second peak in the UVA range of the carcinogenesis action spectrum without a second peak of dimer formation within that range of wavelengths (de Gruijl *et al*., 1993) might therefore need to be explained by epigenetic differences in the cellular responses to UVB and UVA. We would like to speculate that a less protective cellular DNA damage response to UVA, as compared to UVB, could reconcile our data with previously published ones. Just as one example of a profoundly different DNA damage response, we would like to suggest that the less prominent and less long-lasting activation of p53 after exposure to UVA (Figure 5) might be contributing to the different mutagenic outcomes of UVB- or UVA-induced pyrimidine dimers. While the strong activation of p53 after UVB assures its function as ''guardian of the genome'', the weaker activation of p53 after UVA increases the chance that a pyrimidine dimer leads to mutation formation, due to less cell cycle arrest and a subsequent higher chance of replication of a damaged template, and due to less p53-mediated induction of DNA repair. Due to less p53-mediated apoptosis, it might also entail a higher chance that a cell with DNA damage or mutations will survive, and potentially go on to form skin cancer. If this speculation is true, that an UVA-induced dimer is more mutagenic due to a less pronounced protective DNA damage response, one would have to conclude that exposure to a given UVA dose from a pure UVA source, such as those often used in tanning parlors or in medical devices for high-dose UVA1 therapy, might be more harmful than receiving the same UVA dose from a mixed UVA–UVB source, such as natural sunlight, as the UVB will induce a better protective cellular response and reduce the mutability of UVA-induced DNA damage. More work is required to confirm the impact of different DNA damage responses to UVA and UVB and of interactions between UVA and UVB on mutagenic outcomes.

MATERIALS AND METHODS

Cell lines and culture conditions

Primary human fibroblasts were derived from dermal explants of the neonatal foreskin without identifiable patient data as described previously (Stanulis-Praeger and Gilchrest, 1989) and cultured in Dulbecco's modified Eagle's medium (Gibco/BRL, Rockland, MA) supplemented with 10% calf serum at 37°C and 5% CO_2. Only low passage number cells (maximum passage 3) were used. Cells from a different donor were used for each experiment. Limiting the source to neonatal cells excludes the possibility of variability due to donor age and age-dependent decline in DNA repair capacity. All experiments were performed with institutional approval.

HPRT-mutagenesis assay

The HPRT-mutagenesis assay was used as described previously to detect and characterize UV-induced *hprt* mutations (McCormick and Maher, 1988; Zdzienicka, 1999). Exponentially growing cells were irradiated with UVA, UVB, or sham (see below) at approximately 40% confluence. After irradiation, cells were propagated for 3.5–4

population doublings (expression period), as verified by cell counts in parallel dishes, to allow for expression of mutated *hprt*. After 5–12 days, cells were transferred into 10–20 tissue culture dishes, at a density of 5,100 cells/cm^2, with a selective medium containing 7 μg/ml 6-thioguanine (Sigma, St. Louis, MO). In this selective medium, only cells with HPRT-inactivating mutations can grow and form colonies, as they do not metabolize 6-thioguanine to a toxic agent. After a selection period of 4–6 weeks, *hprt*-mutant, 6-thioguanine-resistant cell colonies were either stained with 0.2% methylene blue and counted for mutation frequency, or harvested for *hprt* sequencing. The mutation frequency was calculated as the number of mutants/number of plated cells × plating efficiency. The latter was determined by plating 100 cells/dish separately in nonselective medium at the end of the expression period. The mean plating efficiency was 50 ± 8%.

UV-irradiation protocol

After washing twice with phosphate-buffered saline, cells were irradiated with a thin cover of phosphate-buffered saline (2 ml in p100 dishes) to avoid drying, and on a water bath at room temperature to avoid overheating during irradiation. Irradiation was performed through the lid of the tissue culture dish (Falcon, Becton Dickinson, Franklin Lakes, NJ) to ensure sterility of the long-term cultures.

For UVA1 irradiation (with only small amounts of contaminating UVA2 and no detectable UVB with a minimum detection sensitivity of 1×10^{-6} W/cm^2), we used a 2 kW metal halogenide UVA lamp (SELLAS Sunlight, Germany) with an emission spectrum ranging from 335 to 440 nm and an emission maximum at 375 nm (Figure 6). In all, 99.954% of the emitted UVA is UVA1 (340–400 nm). The spectral irradiance of the lamp was measured with a doublemonochromator spectroradiometer Bentham DM150 (Bentham Instruments Ltd, Reading, UK), which is designed for optimized dynamic range and straylight reduction in the UV wavelength range. The detector was a photomultiplier tube, the input optics consisted of a 2.5-cm-diameter teflon diffusor with cosine response characteristic. The calibration was performed with a quarz tungsten halogen calibration lamp traceable to Physikalisch Technische Bundesanstalt. The stepwidth and bandwidth was 1 nm.

After warm-up, an irradiance value (not spectrally weighted) of approximately 30 mW/cm^2 at 35 cm distance allowed irradiation with, for example, 200 kJ/m^2 in less than 12 minutes. The lamp also contained an infrared filter that prevented excessive warming of irradiated samples. For most experiments, we used a UVA dose of 200 kJ/m^2. This is a relevant and solar available dose, since it can be acquired during about 1 hour summer sun exposure at noon time (Jeanmougin and Civatte, 1987). Tanning beds use even higher doses, up to 600 kJ/m^2. For one experiment, two tissue culture dishes were irradiated with 200 kJ/m^2 through a 320 nm cutoff filter (1 mm; Schott, Duryea, PA), parallel to two samples irradiated with the same dose without this filter. Irradiation time was adjusted for equal dosing and was 14.7% higher for the samples irradiated through the filter.

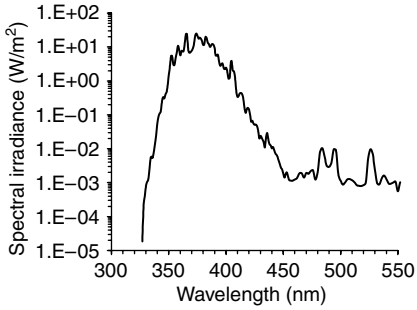

Figure 6. Emission spectrum of the UVA lamp. Note the log scale for the ordinate.

A solar simulator (LH 153, Kratos Analytical, Ramsey, NJ) was used to expose cells to broad-spectrum UVB. The tissue culture lid also removes contaminating UVC and only slightly reduces irradiance of the shorter wavelengths of the UVB range. The resulting emission spectrum was published by Werninghaus *et al.* (1991). After warm-up, an irradiance level of approximately 0.06 mW/cm^2 allowed irradiation with, for example, 200 J/m^2 UVB in less than 6 minutes. The UVA emitted by this source at the maximum UVB doses used was less than 2.5 kJ/m^2, consistent with previous reports that solar simulators often emit insufficient long-wave UVA to adequately simulate natural sunlight (Sayre *et al.*, 1990; Stary and Sarasin, 2000). This low UVA dose did not affect cell survival or proliferation. With a four-fold higher dose of UVA (10 kJ/m^2), we did not detect any mutation formation above background (Figure 1), indicating that the UVA contained in the solar simulator did not contribute to mutation formation. For most experiments, we used a UVB dose of 200 J/m^2, also a solar available dose, and easily accumulated during 10–15 minutes of midsummer mid-day sun exposure.

Radiometric measurements were performed for each experiment. For dosimetry, an IL-1700 Research Radiometer (International Light, Newburyport, MA) was used, equipped with a UVA sensor (SEF 015) or a UVB sensor (SED 240).

Immediately after irradiation, the phosphate-buffered saline cover was removed, and cells were fed with fresh prewarmed complete medium.

Sequencing of hprt-mutant cells

HPRT-cDNA was amplified after isolation of messenger RNA from single mutant colonies by nested RT-PCR using two published primer pairs (Yang *et al.*, 1989) and a high-fidelity *Taq* polymerase (Invitrogen, Carlsbad, CA). HPRT was sequenced using automated DNA sequencing, using PCR primers, and aligned with the published human *hprt* sequence (Jolly *et al.*, 1983) using Jellyfish software (Version 3.0, LabVelocity, Los Angeles, CA). All mutations found were confirmed by sequencing in reverse direction. Identical mutations were only counted if the mutant colonies were derived from separate dishes. While this might underestimate mutation hotspots, it ensures that mutant colonies derived from the same mutation event are not counted more than once. The χ^2 test was used to compare counts of different types of UVA- and UVB-induced mutations.

Immunoblotting

At 2 days after plating, subconfluent, exponentially growing cultures of primary neonatal fibroblasts from one donor were irradiated with various doses of UVA or UVB as described above in p60 tissue culture dishes. At 2, 6, 12, or 24 hours after irradiation, whole-cell

extracts were prepared and Western blotting was carried out using standard procedures with 20 μg of protein. UVA- and UVB-irradiated samples were run in parallel on two gels (10% acrylamide) in the same electrophoresis apparatus at the same time. The primary antibodies utilized were directed against serine-15 phosphorylated p53 (Cell Signaling, Beverly, MA) and actin (I-19) horseradish peroxidase conjugate (Santa Cruz, Santa Cruz, CA). The secondary antibody for the detection of serine-15 phosphorylated p53 was enhanced chemiluminescent-anti-rabbit peroxidase-linked species-specific antibody from donkey (Amersham Biosciences, Piscataway, NJ). Autoradiography of the two membranes was performed simultaneously onto a single sheet of film. Densitometry of the single autoradiogram (with both the UVA- and the UVB-irradiated samples) was performed using Quantity One software (Bio-Rad, Hercules, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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