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# UVA1 Genotoxicity Is Mediated Not by Oxidative Damage but by Cyclobutane Pyrimidine Dimers in Normal Mouse Skin

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UVA1 induces the formation of 8-hydroxy-2′-deoxyguanosines (8-OH-dGs) and cyclobutane pyrimidine dimers (CPDs) in the cellular genome. However, the relative contribution of each type of damage to the *in vivo* genotoxicity of UVA1 has not been clarified. We irradiated living mouse skin with 364-nm UVA1 laser light and analyzed the DNA damage formation and mutation induction in the epidermis and dermis. Although dose-dependent increases were observed for both 8-OH-dG and CPD, the mutation induction in the skin was found to result specifically from the CPD formation, based on the induced mutation spectra in the skin genome: the dominance of C→T transition at a dipyrimidine site. Moreover, these UV-specific mutations occurred preferentially at the 5′-TCG-3′ sequence, suggesting that CpG methylation and photosensitization-mediated triplet energy transfer to thymine contribute to the CPD-mediated UVA1 genotoxicity. Thus, it is the CPD formation, not the oxidative stress, that effectively brings about the genotoxicity in normal skin after UVA1 exposure. We also found differences in the responses to the UVA1 genotoxicity between the epidermis and the dermis: the mutation induction after UVA1 irradiation was suppressed in the dermis at all levels of irradiance examined, whereas it leveled off from a certain high irradiance in the epidermis.

## INTRODUCTION

Sunlight reaching the earth's surface contains UV radiation consisting of UVB (290–320 nm) and UVA (320–400 nm). Although the UVB component is known to include the most carcinogenic wavelengths for mammalian skin (De Gruijl and Van der Leun, 1994), the UVA component is also suggested to play an important role in the skin genotoxicity of solar UV (Setlow *et al.*, 1993). The genotoxicity of UVB derives from its potent mutagenicity through the photochemical formation of

specific types of DNA base damage such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6-4)pyrimidone photoproducts (64PPs) after direct absorption of the photon energy to DNA molecules (Friedberg *et al.*, 2005). UVA genotoxicity for mammalian cells depends on the wavelength: at a shorter wavelength range (320–340 nm), the UVA2 region, the effect is exerted mainly through the same direct photochemical reaction with DNA as that by UVB, although its efficiency is very low (Kielbassa and Epe, 2000; Ikehata and Ono, 2007), whereas indirect genotoxic effects also occur through the production of reactive oxygen species (ROS) by photosensitization of biological molecules other than DNA, especially in the longer wavelength range of UVA1 (340–400 nm) (Kvam and Tyrrell, 1997; Kielbassa and Epe, 2000; Tyrrell, 2000).

The significance of the indirect oxidative damage-mediated genotoxicity of UVA for the mammalian genome, however, has been controversial. Although several studies supported the ROS-mediated genotoxicity of UVA (Besaratinia *et al.*, 2004, 2007; Wood *et al.*, 2006), studies showing a major contribution of CPD, which can be induced in cellular DNA directly or indirectly by UVA (Courdavault *et al.*, 2004), have also been reported (Ikehata *et al.*, 2003a), even within the UVA1 range (Van Kranen *et al.*, 1997; Kappes *et al.*, 2006). In addition, another DNA modification by UVA was also suggested in previous studies (Drobetsky *et al.*, 1995;

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Agar *et al.*, 2004), and this modification specifically induced a rare type of transversion mutation, T→G, which was referred to as the UVA fingerprint in those studies, although the DNA lesions or mechanisms causing the mutation have not been established. Discrepancies in the types of UVA genotoxicity among those studies may have resulted in part from differences in the UVA source used in each study: the inclusion of a shorter wavelength component in the emitted UVA could, even if it is very small, modify or overwhelm the resultant mutation spectrum with a UVB-like profile. However, attention should be given to the experimental conditions during the cellular exposure to UVA, because ROS production by irradiation can be influenced profoundly by the cellular environment such as the ingredients in the cultured media and oxygen concentration, which could mediate the indirect genotoxicity of UVA as photosensitizers or effectors (Besaratinia *et al.*, 2007). As long as cells are cultured in artificial conditions, such ambiguities concerning the causes of the UVA genotoxicity cannot be resolved.

To exclude such problems that are intrinsic to cultured cell systems, we employed *in vivo* analysis of UVA genotoxicity, in which transgenic mice developed for mutation research were irradiated and the induced mutations were evaluated directly in the exposed skin (Ikehata *et al.*, 2003a). The induced mutation spectrum observed in the epidermis was rather similar to that induced by UVB, which we determined previously (Ikehata *et al.*, 2003b), although much more remarkable hot spots for UV-specific C→T transitions appeared at methylated CpG-associated dipyrimidine sites after UVA irradiation (Ikehata *et al.*, 2003a). However, the UVA source used in that study emitted not only UVA1 but also UVA2 and even a small amount of the boundary wavelengths of UVB, which might have affected the results. In this study, we used a laser emitting a purely monochromatic 364-nm UV light to avoid the problem of contamination by shorter wavelengths, and analyzed the mutation spectrum as well as the DNA lesions induced in the exposed mouse skin.

## RESULTS

### Induction of DNA damage in skin

A significant dose-dependent increase in the amount of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) in genomic DNA was observed in both the epidermis and dermis of mouse skin ($P < 0.0001$, two-way analysis of variance) after exposure to $0.85-3\ MJ\ m^{-2}$ of the 364-nm UVA1 light (Figure 1a), confirming a genotoxic effect on the skin through the UVA1-mediated production of ROS, which should then oxidize the cellular DNA to produce oxidative damage represented by 8-OH-dG (Kvam and Tyrrell, 1997; Kielbassa and Epe, 2000; Tyrrell, 2000). Simultaneously, a significant linear increase in the amount of CPD along with the increase in UVA1 irradiance was also observed in both the tissues ($P < 0.0001$, two-way analysis of variance), whereas no significant induction of 64PP was detected in either tissue at any level of irradiance examined (Figure 1b). These amounts of CPD produced after the UVA1 irradiation were quite comparable to those of the photolesions induced with
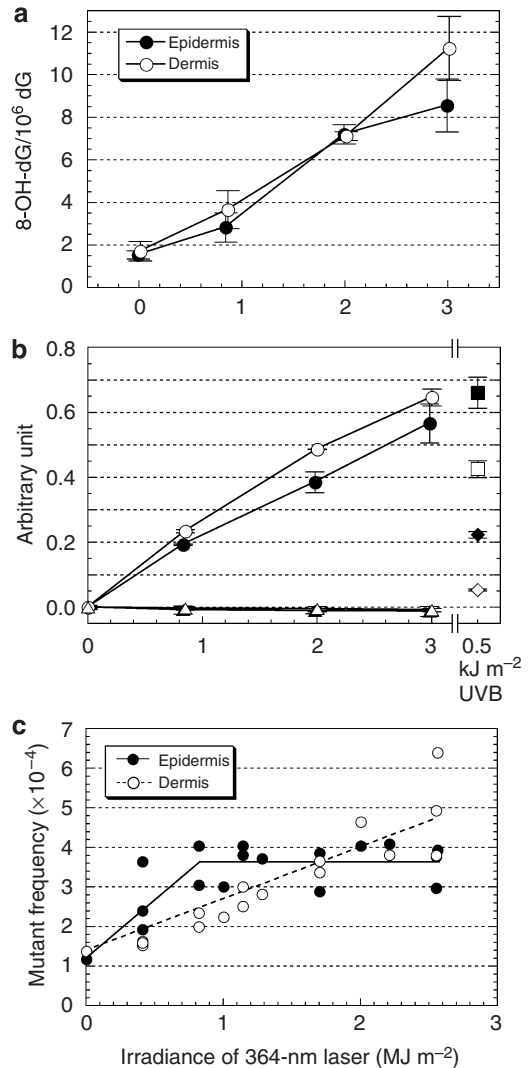


**Figure 1**. **Genotoxic effects of 364-nm UVA1 on mouse epidermis and dermis.** (**a**) Dose-dependent production kinetics of 8-OH-dG in the epidermis (closed circles) and dermis (open circles) after UVA1 irradiation. The amounts of 8-OH-dG in the genomic DNA from each tissue were quantified with an HPLC/electrochemical detector system. Data points were obtained from two to three mice for each dose. (**b**) Dose-dependent induction kinetics of CPD (circles) and 64PP (triangles) in the epidermis (closed symbols) and dermis (open symbols) after UVA1 irradiation. For comparison, the amounts of CPD (squares) and 64PP (diamonds) induced after $0.5\ kJ\ m^{-2}$ UVB are given on the right side. The amounts of the photolesions were evaluated with an ELISA using specific mAbs. Each set of data points was obtained from two mice. (**c**) Dose-dependent induction kinetics of MF of the *lacZ* transgene in the epidermis (closed circles) and dermis (open circles) after UVA1 irradiation. Each data point was from a single animal. Regression lines are given for the epidermal data points less than $1\ MJ\ m^{-2}$ (solid line, $R^2 = 0.934$, $P < 0.001$) and for all the dermal points (dotted line, $R^2 = 0.932$, $P < 0.0001$) under the presumption that the *y*-intercept equals the background MF. For the data points of epidermis over $0.8\ MJ\ m^{-2}$, a horizontal solid line is drawn at the mean of those data ($3.64 \pm 0.47 \times 10^{-4}$), which is significantly different from the background MF ($P < 0.0001$), to show the suppression of MF induction.

$0.5\ kJ\ m^{-2}$ of UVB (Figure 1b), a dose sufficient to induce mutations at more than 10-fold the background level and the minimal dose provoking inflammation in mouse skin (Ikehata and Ono, 2002), indicating that the amounts of CPD

produced at these levels of UVA1 irradiance would be enough to exert genotoxicity in the skin. In contrast to UVB, UVA1 induced similar amounts of CPDs in the epidermis and dermis (Figure 1b), and a similar situation was also observed for the amounts of 8-OH-dG (Figure 1a), probably reflecting the high penetration of the epidermis by wavelengths >300-nm UV (Bruls *et al.*, 1984) and the thinness of the mouse epidermis.

## Mutation induction in skin

Sixteen mice were exposed to the 364-nm UVA1 light at irradiances of 0.41–2.56 MJ m$^{-2}$, and the frequencies of the mutations induced in the epidermis and dermis were evaluated 4 weeks later using the *lacZ* transgene as a mutational reporter. The observed mutant frequencies (MFs) are plotted in Figure 1c along with the background MFs ($1.18 \times 10^{-4}$ for the epidermis and $1.38 \times 10^{-4}$ for the dermis; Ikehata and Ono, 2002). A significant dose-dependent linear increase in MF was observed in the dermis at the entire dose range examined (regression analysis: slope $= 1.32 \times 10^{-10}$ per Jm$^{-2}$, $R^2 = 0.932$, $P < 0.0001$), and in the epidermis at doses up to 0.82 MJ m$^{-2}$ (slope $= 2.97 \times 10^{-10}$ per Jm$^{-2}$, $R^2 = 0.934$, $P < 0.001$). The slopes were significantly different between the two tissues ($P < 0.001$): 2.3-fold steeper in the epidermis. However, at doses more than 0.82 MJ m$^{-2}$, the MF increase in the epidermis was suppressed almost completely to a constant level of MF: mean $= 3.64(\pm 0.47) \times 10^{-4}$ ($n = 13$), which was still significantly higher than the background MF (3.1-fold, $P < 0.0001$). During the experiments, we noticed inflammation in the exposed skin area in all the mice 2 days after the irradiation, even at the smallest irradiance examined (0.41 MJ m$^{-2}$).

## UVA1-induced mutation spectrum in the epidermis

In total, 147 *lacZ* mutants were isolated from the epidermis of six mice exposed to 1.00–2.56 MJ m$^{-2}$ of 364-nm UVA1. The entire coding region of the *lacZ* gene of these mutants was sequenced, and mutations were detected for all the mutants (Table S1). We found that 92% ($n = 134$) of these mutants had a single-base substitution. The others were five mutants with a frameshift, two with a complex mutation, which is a frameshift associated with base changes, four with a deletion, and two with an identical 1.3-kb insertion. The last two mutants were excluded from further analysis because their insertion included a bacterial transposable element and most likely resulted from an *ex vivo* mutation that occurred during recovery of the transgene. The same insertion mutation was recovered and characterized in our previous study (Ikehata *et al.*, 2007b). The obtained mutation spectrum for the UVA1-exposed epidermis is summarized in Table 1, and compared with the UVB-induced and background spectra, which we reported before (Ikehata *et al.*, 2003b).

Among the 134 mutants with a single-base substitution recovered in the irradiated epidermis, 94 (65% of total mutants) were C→T transition, 90% of which ($n = 85$) occurred at dipyrimidine sites, where UV photolesions are preferentially produced. In contrast to the high recovery of these UV-specific mutations of C→T at dipyrimidine sites,

### Table 1. Summary of mutation spectra in mouse skin epidermis

| | 364-nm UVA1 | | UVB[1] | | Background[1] | |
|---|---|---|---|---|---|---|
| | Number (%) | % (Py-Py)[2] | Number (%) | % (Py-Py)[2] | Number (%) | % (Py-Py)[2] |
| Base substitution | 134 (92) | | 75 (97) | | 42 (95) | |
| Transition | | | | | | |
| C→T (CpG) | 80 (55) | 93 | 26 (34) | 96 | 24 (55) | 63 |
| C→T (non-CpG) | 14 (10) | 79 | 39 (51) | 100 | 2 (5) | 50 |
| T→C | 25 (17) | 0 | 1 (1) | 100 | 6 (14) | 0 |
| Transversion | | | | | | |
| G→C | 1 (1) | 100 | 2 (3) | 100 | 1 (2) | 0 |
| G→T | 11 (8) | 55 | 1 (1) | 100 | 5 (11) | 80 |
| T→G | | | | | 1 (2) | 0 |
| T→A | 3 (2) | 100 | 4 (5) | 100 | 2 (5) | 100 |
| Tandem | | | 2 (3) | 100 | 1 (2) | 100 |
| Frameshift | 5 (3) | 60 | 2 (3) | 50 | 1 (2) | 0 |
| Complex[3] | 2 (1) | 75* | | | | |
| Duplication | | | | | 1 (2) | |
| Deletion | 4 (3) | | | | | |
| Total | 145 | | 77 | | 44 | |

Py, Pyrimidine.
[1]Ikehata *et al.* (2003b).
[2]Percentage of the fraction of the mutations that occurred at dipyrimidine sites.
[3]Frameshift mutations associated with base changes.
*For one of the two mutations, only one nucleotide of the affected dinucleotide resided in a dipyrimidine site.

G→T transversion, which is known to be one of the most representative ROS-induced mutations and to result from 8-OH-dG (Grollman and Moriya, 1993), was recovered as a minor fraction ($n = 11$, 8%), and no T→G base substitution, the UVA fingerprint mutation (Drobetsky *et al.*, 1995), was detected (Table 1). We also analyzed the DNA sequence changes in the *lacZ* mutants recovered from the dermis of the UVA1-exposed skin, and found a mutation spectrum similar to that in the epidermis: a dominance of the UV-specific mutation and small numbers of the ROS-specific and UVA fingerprint mutations (data not shown).

The mutation spectrum observed in the UVA1-exposed epidermis was significantly different from that in the UVB-exposed epidermis (Table 1; $P < 0.001$, Adams–Skopek test). The difference seems to result mainly from the larger ratio of C→T transitions in the UVB spectrum than in the UVA1 spectrum (85 vs 65%) and especially from the more frequent occurrences of those C→T mutations at non-CpG sites in the UVB spectrum (39 at non-CpG/65 total for UVB vs 14/94 for
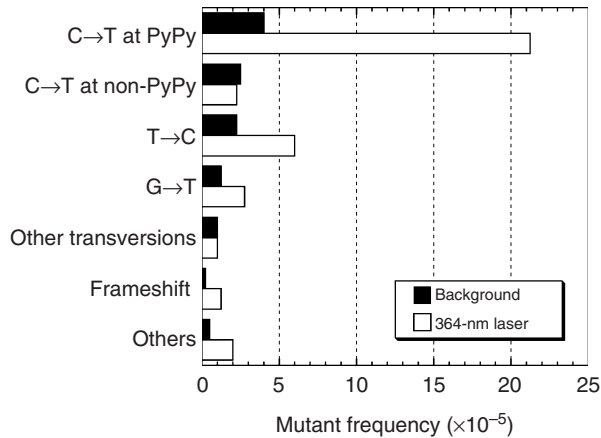
**Figure 2**. **Comparison of the frequencies of mutants with each class of mutations in the epidermis between unirradiated (background) and UVA1-irradiated (364-nm laser) mice.** The MF of each class was calculated by multiplying the observed overall MF ($1.18 \times 10^{-4}$ for the background and $3.64 \times 10^{-4}$ for the irradiated) with the fraction of the class in overall mutations. PyPy, a dipyrimidine site.

UVA1; Table 1). On the contrary, the UVA1 spectrum was not significantly different from that in the unirradiated epidermis (the background shown in Table 1) for both overall and base substitution mutations (Adams–Skopek test), probably reflecting in part the low level of MF induction by UVA1. However, a significant difference was detected when the numbers of UV-specific and non-UV-specific C→T transitions, which differ in association with dipyrimidine sites, were compared between the unirradiated (16 and 10) and the irradiated (85 and 9) epidermis ($P < 0.005$, Fisher's exact probability test), reflecting the more frequent occurrence of C→T mutations at dipyrimidine sites in the exposed epidermis (90%) than in the unirradiated epidermis (62%) (see also Table 1). Furthermore, the frequency of mutants with each class of mutations was calculated from the overall MFs and its proportion in the mutation spectra, and was compared between the background and the induced mutations, as shown in Figure 2, which clearly demonstrated that the observed MF increase in the epidermis after 364-nm UVA1 irradiation mainly resulted from the induction of UV-specific C→T transitions but not 8-OH-dG-mediated G→T or other transversion mutations. Although we detected both CPD and 8-OH-dG induction in mouse skin by UVA1, these results indicate that only CPD, which is known to induce UV-specific mutations (Tessman *et al.*, 1992), can effectively bring about genotoxicity in the skin after UVA1 irradiation and that the amount of 8-OH-dG produced simultaneously would not appear to be enough to harm the skin genome, at least in normal mice whose response to ROS-induced DNA lesions should not be compromised.

### Characterization of UVA1-induced mutations

To investigate the preferred DNA sequence contexts for the UV-specific C→T transitions induced in UVA1-exposed mouse epidermis, all the cytosine residues in dipyrimidine sites on both strands of the *lacZ*-coding region were

categorized into 12 groups based on their surrounding sequence context as shown in Table 2, in which the cytosine subjected to the mutation was located at the middle position of triplet sequences that include dipyrimidines. Sites for the UVA1-induced UV-specific mutation were found most frequently in the 5′-TCG-3′ context (see the mutabilities in context in Table 2), and an outstanding number of mutants with the mutation were also recovered in the same sequence context ($n = 70$; see the number of mutants in Table 2). Moreover, the mutation-detected sites with the 5′-TCG-3′ context seemed to produce the UV-specific mutations much more frequently than those with other contexts, as evidenced by the average recurrences in Table 2 (7.78 vs 1.00–2.00). These estimations indicate that the UV-specific C→T transition induced by UVA1 occurs most preferably in the 5′-TCG-3′ sequences.

This association of UVA1-induced mutations with the 5′-TCG-3′ sequence context suggests the importance of cytosine methylation at CpG sites (Grünwald and Pfeifer, 1989) for UVA1 mutagenesis in the mammalian genome, as it has been shown that transgenes of the mice used here are fully methylated in the skin (Ikehata *et al.*, 2003b). When examining the distribution in the *lacZ* coding sequence of mutations recovered from the UVA1-exposed epidermis (Figure 3), almost all of the C→T mutations at 5′-TCG-3′ sites (69/70) were found to occur repeatedly at several certain positions in the gene, some of which showed hot spots for this mutation type (positions 928, 1,187, 1,627, and 2,392). This observation suggests that UVA1-mediated genotoxicity in mammalian skin depends on CpG methylation. In the distribution, another hot spot, where T→C transitions occurred exclusively, was noticed at position 625 (Figure 3). The same outstanding hot spot was also observed in the distribution of the background mutations, as shown before (Ikehata *et al.*, 2003b), indicating that these hot spot mutations would occur spontaneously, not by UVA exposure.

### DISCUSSION

After 364-nm UVA1 irradiation, we observed the induction of 8-OH-dG and CPD but not of 64PP in both the epidermis and dermis of living mice. The simultaneous formation of 8-OH-dG and CPD without 64PP induction by UVA1 was also reported in cultured cells (Kielbassa and Epe, 2000; Courdavault *et al.*, 2004; Besaratinia *et al.*, 2005) and skin specimens (Mouret *et al.*, 2006). The 8-OH-dG formation would result from DNA oxidation by ROS produced through UVA-mediated photosensitization of intrinsic biomolecules such as riboflavin and porphyrin (Tyrrell, 2000; Besaratinia *et al.*, 2007). For the CPD production, there are two possible pathways: one is a photochemical reaction through the direct absorption of UVA1 energy to DNA, and the other is an indirect triplet energy transfer mediated by some photosensitized molecules (Lamola, 1970), although the identity of those mediators is currently not known. The absence of 64PP induction favors the latter possibility because direct energy transfer by the former mechanism should have produced 64PPs equally, although the photochemical conversion to Dewar valence isomers

**Table 2. Influence of adjacent bases on UV-specific C→T mutations induced by 364-nm UVA1**

| Sequence context[1] | Number of sites in the *lacZ* coding | | | Number of mutants | Mutability in context[4] | Average recurrence[5] |
|---|---|---|---|---|---|---|
| | All | Mutable[2] | Detected[3] | | | |
| 5′-TCA-3′ | 112 | 71 | 1 | 1 | 0.01 | 1.00 |
| 5′-CCA-3′ | 139 | 100 | 2 | 2 | 0.02 | 1.00 |
| 5′-TCG-3′ | 113 | 74 | 9 | 70 | 0.12 | 7.78 |
| 5′-CCG-3′ | 152 | 99 | 3 | 4 | 0.03 | 1.33 |
| 5′-ACT-3′ | 64 | 24 | 0 | 0 | <0.04 | — |
| 5′-ACC-3′ | 112 | 88 | 1 | 2 | 0.01 | 2.00 |
| 5′-GCT-3′ | 121 | 53 | 0 | 0 | <0.02 | — |
| 5′-GCC-3′ | 148 | 119 | 0 | 0 | <0.01 | — |
| 5′-TCT-3′ | 52 | 20 | 1 | 2 | 0.05 | 2.00 |
| 5′-TCC-3′ | 91 | 62 | 2 | 3 | 0.03 | 1.50 |
| 5′-CCT-3′ | 60 | 24 | 1 | 1 | 0.04 | 1.00 |
| 5′-CCC-3′ | 71 | 57 | 0 | 0 | <0.02 | — |

[1]Either base adjacent to the 5′ or 3′ side of the cytosine that should be subject to mutation to thymine is shown. Tandem pyrimidines are underlined.
[2]Sites where an amino-acid change or termination is expected if a C→T transition occurs.
[3]Sites where C→T transitions were detected for 364-nm UVA1-irradiated skin epidermis.
[4]Numbers of detected sites were divided by numbers of the mutable sites of the same sequence context.
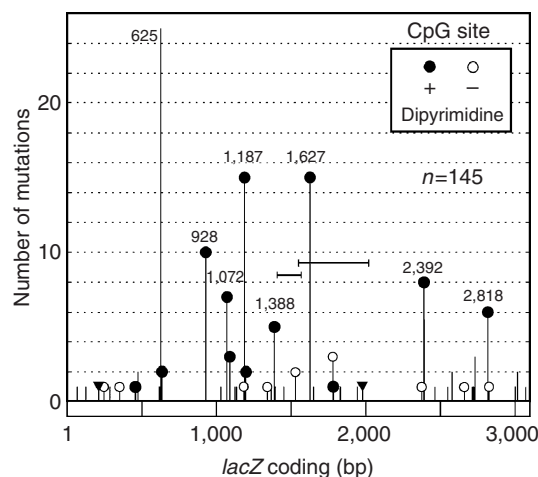[5]Numbers of mutants were divided by numbers of the detected sites of the same sequence context.



**Figure 3**. **Distribution in the *lacZ* transgene of mutations detected in 364-nm UVA1-exposed mouse skin epidermis.** The horizontal open bar indicates positions in the *lacZ* coding sequence. The number of mutations at each position is shown by vertical lines above the horizontal bar. The positions at which cytosine mutations were detected within Py-CpG and Pu-CpG sequences are shown by closed and open lollipops, respectively (Py, pyrimidine; Pu, purine). The sites for small (<20 bp) and large (>100 bp) deletions are indicated by reverse triangles and horizontal lines with short vertical lines on their ends, respectively. Position numbers are given for recurrent sites at which more than four identical mutations were detected.

might have precluded the detection of such 64PPs. A study with HPLC coupled with tandem mass spectrometry, however, showed no formation of 64PPs and Dewar isomers in cellular DNA after UVA1 irradiation (Courdavault *et al.*, 2004), and reported a preferential CPD formation at TT

dipyrimidines (Courdavault *et al.*, 2004; Mouret *et al.*, 2006), which also supports the indirect triplet energy transfer mechanism because the energy level of the excited triplet state of thymine is the lowest among DNA bases. In addition, we observed the induction of similar but significantly larger amounts of CPD in the dermis compared to the epidermis after UVA1 irradiation ($P<0.05$, two-way analysis of variance; Figure 1b), which might suggest a biased distribution of some unidentified photosensitizers toward the dermis because, if the direct energy absorption reaction were relevant, the amounts of CPD should be larger in the epidermis, even if the difference is very small due to the high penetration of UVA1 into the skin. Moreover, an action spectrum of CPD formation in naked and cellular DNA previously reported showed a shoulder at the UVA1 wavelength range (Matsunaga *et al.*, 1991; Kielbassa and Epe, 2000), suggesting some photochemical reaction producing CPDs that was different from the direct energy transfer mechanism that peaks at 260 nm. These observations favor the photosensitized triplet energy transfer pathway for the CPD formation mechanism by UVA1.

Although we observed 8-OH-dG formation in the skin genome with UVA1 irradiation, there was no evidence that it significantly contributed to the genotoxicity in the skin in the following mutation studies (Table 1 and Figure 2). This result may suggest that the ability of normal mouse skin to repair ROS-mediated DNA damage is high enough to suppress the mutagenicity of such DNA lesions, which is consistent with previous studies reporting fast and efficient repair of 8-OH-dG in cultured normal keratinocytes and fibroblasts (Klungland *et al.*, 1999; Orimo *et al.*, 2006). In those studies, 50% of 8-OH-dGs were removed from the cellular genome

within 6 hours, a time length in which no significant repair of CPD was observed in cells and skin tissues of mice (Mizuno *et al.*, 1991; Ikehata *et al.*, 2007a). Apoptotic responses to ROS-induced DNA damage or to ROS itself might also be related to the suppression of genotoxicity. Nevertheless, because we used only a single wavelength within the UVA1 range in this study, it could be suggested that the amount of 8-OH-dG, in other words the abundance of ROS, induced here was not large enough to reflect the real situation under actual solar UVA. This would not be the case, however, as the observed amounts of 8-OH-dG induced after the 364-nm UVA1 (4–10 molecules per $10^6$ deoxyguanosines; Figure 1a) were nearly equal to those observed in studies with broadband UVA1 sources (Besaratinia *et al.*, 2005; Mouret *et al.*, 2006). Thus, the ROS induced after UVA1 irradiation would not seem to be potent enough to cause genotoxicity in normal cells or skin, even if the irradiance is above the maximal physiological level of daily UVA from natural sunlight ($\leqslant 1.5\,MJ\,m^{-2}$).

The sequence analysis of mutants in this study clearly showed that the mutation induction by UVA1 exposure was mediated mainly by CPD formation in both the epidermis and dermis. However, the initial increments in MF were 2.3-fold larger in the epidermis than in the dermis, although similar amounts of CPD were produced in both tissues at each level of irradiance examined (Figure 1b and c). This apparent inconsistency may suggest some differences in the response to UVA1 between the epidermis and the dermis. One possibility is that DNA repair for CPDs is more active or efficient in the dermis than in the epidermis. Although it has been reported that keratinocytes *in vitro* show a higher level of CPD removal than skin fibroblasts after UVB irradiation (D'Errico *et al.*, 2003), the difference in cell proliferation rates *in vivo* between the two tissues could contribute to the situation: if fibroblasts proliferate in skin tissue after irradiation much more slowly than keratinocytes, more repair time would be available to the fibroblasts, resulting in more efficient DNA repair in the dermis. Another possibility is that the fibroblasts in the dermis are more sensitive to UVA1 genotoxicity than the keratinocytes in the epidermis because of some cell-exclusion responses such as apoptosis, which could lead to more efficient exclusion of damaged cells from the dermis than from the epidermis, with the result that the remaining cells in the dermis show fewer mutations. Consistent with this idea, higher sensitivities to UVA1-mediated cell killing have been demonstrated for dermal fibroblasts than for keratinocytes (Leccia *et al.*, 1998; Courdavault *et al.*, 2004).

Furthermore, at a higher dose range than $0.8\,MJ\,m^{-2}$, the mutation induction in the epidermis seemed to be repressed completely, although the amount of CPD in the tissue continued to increase lineally with the increase in UVA1 irradiance (Figure 1b and c). This observation might suggest some antigenotoxic response specific to the epidermis, which should be different from the mutation-suppressing response observed in the dermis mentioned above, because the mutation suppression kinetics were quite different between the two tissues: the slope was suppressed at all irradiances for the dermis, whereas it leveled off from a certain high-dose point in the epidermis (Figure 1c). A level-off kinetics of mutation induction was also reported previously for the epidermis of UVB-exposed mice (Ikehata and Ono, 2002), which may indicate that this level-off response to mutation induction is epidermis specific and acts in response to multiple environmental genotoxic agents, including UVB and UVA1, although the mechanism of this response remains unknown. Interestingly, the level-off response appears at a lower MF for UVA1 ($3.64 \times 10^{-4}$) than for UVB ($18.10 \times 10^{-4}$) (Ikehata and Ono, 2002). This difference might explain the observation in a skin carcinogenesis study with hairless mice that UVA1 carcinogenesis was less dose dependent than UVB carcinogenesis (De Laat *et al.*, 1997), because, irrespective of the given doses, the genotoxic effect at a single exposure would be smaller with UVA1 than with UVB.

We found that UVA1 caused UV-specific C→T transitions most frequently and that these mutations occurred preferentially at 5′-TCG-3′ sequences (Tables 1 and 2) forming hot spots at several sites in the reporter gene (Figure 3). These observations suggest that the CpG methylation in the mammalian genome (Grünwald and Pfeifer, 1989) may contribute to the UVA1-induced mutation. The cytosine methylation in DNA is known to promote UVB/sunlight-induced CPD formation at cytosine-containing dipyrimidine sites (Drouin and Therrien, 1997; Tommasi *et al.*, 1997), and UVB, UVA2, and sunlight have been shown to induce C→T mutations frequently at those dipyrimidine sites associated with a methylated CpG (Pfeifer *et al.*, 2005; Ikehata and Ono, 2007). The results in this study might expand this concept of the effect of CpG methylation on UV genotoxicity from the UVB–UVA2 wavelength range to that of the UVA1. However, UVA1 did not induce mutations at the 5′-CCG-3′ sequence, which is the other triplet including both dipyrimidine and CpG motifs (Table 2). This discrepancy could be explained by the previous observation that UVA1 produced CPDs at 5′-TC-3′ and 5′-CT-3′ dipyrimidines but not at CC dipyrimidines in mammalian cellular and skin DNA, although the highest yield was observed at TT dipyrimidines (Courdavault *et al.*, 2004; Mouret *et al.*, 2006). If the photosensitization-mediated triplet energy transfer occurs specifically to thymines and produces CPDs selectively at thymine-containing dipyrimidines, and if cytosine methylation can promote this process, the preferential recovery of the UV-specific C→T mutation at the 5′-TCG-3′ would be explainable. Interestingly, the biased occurrence of the CpG-associated UV-specific mutation toward 5′-TCG-3′ against 5′-CCG-3′ is also noticed for the mutations in UVB-, sunlight-, and UVA2-exposed epidermis, which were reported in our previous studies (Ikehata *et al.*, 2003a, b, 2004), although the degrees of the bias are smaller than UVA1: numbers of the C→T mutation at 5′-TCG-3′ and 5′-CCG-3′ sites are 23 and 2 for UVB, 27 and 7 for sunlight, 49 and 4 for UVA2, and 70 and 4 for UVA1, respectively. In addition, most of the 5′-TCG-3′-associated C→T mutations detected in those previous studies occurred at the same specific hot spots as observed in this study, producing similar patterns of the hot spot distribution as that with UVA1 (Figure 3), although hot spots were less prominent in the UVB and sunlight patterns (Ikehata and Ono, 2007). This might

suggest that UV wavelengths shorter than UVA1 can also induce CPDs through the photosensitization-mediated triplet energy transfer mechanism, even though less efficient than UVA1. However, this presumption could not be accepted completely because the UV sources used in those studies also emit UVA1 as a part of their output (Ikehata *et al.*, 2004). Actually, the majority of the UV-specific C→T mutations detected in our UVB and sunlight studies did not occur at CpG sites (Table 1; Ikehata *et al.*, 2004), indicating that main pathways for the mutagenesis with those UV sources should be different from the CPD-mediated mechanism by UVA1 discussed above. A similar bias of C→T mutations toward 5′-TCG-3′ sites was also observed for the background mutation (10 mutations at 5′-TCG-3′ vs 5 mutations at 5′-CCG-3′). However, the extent of the bias was much smaller than any of those observed for the UV-induced mutations.

In this and previous studies (Ikehata *et al.*, 2003a), we demonstrated that UVA genotoxicity occurs mainly through CPD formation, not through ROS generation, in healthy skin. However, we should be cautious in using the results obtained from this study to predict genotoxic effects of UVA on human skin because human epidermis is much thicker than mouse epidermis, which might make a difference in responses of the dermal tissue to the UVA genotoxicity between humans and mice. Moreover, our studies indicate that, within the dose range of daily irradiance that we are exposed to under natural sunlight in the middle latitudes in midsummer, the skin genotoxicity by UVA1 is extremely small compared to that by UVB (Ikehata and Ono, 2002) or natural sunlight (Ikehata *et al.*, 2004): the UVA1 component in sunlight could induce MF in the epidermis only threefold at most above the background level even after a whole day of exposure on a clear day ($\leqslant 1\,\mathrm{MJ\,m^{-2}}$), whereas the UVB component and total sunlight would cause increases of 15-fold and 35-fold, respectively, after just an hour of exposure at noon. Thus, the harmfulness of the UVA1 component of natural sunlight would appear to be negligible compared to the UVB and, probably, UVA2 components (Ikehata *et al.*, 2003a). However, we studied only the effect of a single exposure of UVA1. Repetitive irradiation might affect the situation. In fact, we showed previously that the delivery of multiple doses of UVB at a certain interval cancels the level-off suppression of the mutation induction observed in the epidermis and causes the reappearance of a dose-dependent MF increase (Ikehata and Ono, 2002). Accordingly, studies of UVA1 genotoxicity in this aspect should be conducted in the future.

## MATERIALS AND METHODS

### Mice and laser irradiation
All experimental procedures, including animal husbandry, were conducted according to the Guidelines for Animal Welfare and Experimentation at Tohoku University and the National Institutes of Natural Sciences. A transgenic mouse strain harboring λ-phage-based *lacZ* mutational reporter genes (Gossen *et al.*, 1989) was used. The UVA1 source used was a continuous-wave 364-nm Ar laser maintained by the National Institute for Basic Biology (Okazaki, Japan). The beam was expanded through a set of lenses to irradiate a $60 \times 60\,\mathrm{mm}$ flat field at a homogeneous intensity of $300–400\,\mathrm{W\,m^{-2}}$.

Irradiation to the mouse skin was performed as described (Ikehata *et al.*, 2003a). Briefly, the depilated dorsal skin of anesthetized 8- to 12-week-old mice was irradiated in a black box and monitored with an infrared camera under lighting with 945-nm photodiodes. During the irradiation, no temperature increase was observed.

### DNA damage assay
Mice exposed to 0, 0.85, 2, or $3\,\mathrm{MJ\,m^{-2}}$ of the 364-nm laser light were killed immediately after irradiation, and the epidermal and dermal genomic DNA was isolated separately from the exposed skin area using a phenol–chloroform-based extraction method (performed at room temperature), and assayed for the quantification of CPD and 64PP as described (Ikehata *et al.*, 2007a) using mAbs specific to each photolesion, and analyzed for the quantification of 8-OH-dG using an HPLC equipped with an electrochemical detector as described (Kawai *et al.*, 2007). In the processes for 8-OH-dG quantification, DNA samples were stored at $-80\,^{\circ}\mathrm{C}$ at every step waiting for next treatments to avoid oxidation of the samples. For comparison of the efficiencies in the induction of the UV photolesions, UVB light was also irradiated with broadband UVB fluorescent lamps (peak emission 313 nm, FL20S.E; Toshiba, Tokyo, Japan).

### Mutation analyses
Four weeks after irradiation, mice were killed. The epidermal and dermal genomic DNA isolated from the exposed skin was used for the detection of *lacZ* mutants, evaluation of the MFs, and analysis of the DNA sequence changes by the mutations (Wang *et al.*, 2006).

### Statistical analyses
Differences in the dose-dependent induction kinetics of 8-OH-dG and UV photolesions were evaluated by two-way analysis of variance and a *post hoc* Bonferroni/Dunn's test. Irradiance (0, 0.85, 2, and $3\,\mathrm{MJ\,m^{-2}}$) and tissue (epidermis and dermis) were set as the independent variables and the lesion amount as the dependent variable. For evaluation of the irradiance-dependent MF induction, linear regression analysis was performed under the presumption that the *y*-intercept equals the background MF. Differences between mutation spectra were estimated with the Adams–Skopek test (Cariello *et al.*, 1994) and Fisher's exact probability test.

### SUPPLEMENTARY MATERIAL
**Table S1.** *lacZ* mutations in 364-nm UVA1-exposed mouse skin epidermis.

### REFERENCES
Agar NS, Halliday GM, StC Barnetson R, Ananthaswamy HN, Wheeler M, Jones AM (2004) The basal layer in human squamous tumors harbors more UVA than UVB fingerprint mutations: a role for UVA in human skin carcinogenesis. *Proc Natl Acad Sci USA* 101:4954–9

Besaratinia A, Kim S, Bates SE, Pfeifer GP (2007) Riboflavin activated by ultraviolet A1 irradiation induces oxidative DNA damage-mediated mutations inhibited by vitamin C. *Proc Natl Acad Sci USA* 104:5953–8

Besaratinia A, Synold TW, Chen H, Chang C, Xi B, Riggs AD *et al.* (2005) DNA lesions induced by UV A1 and B radiation in human cells: comparative analyses in the overall genome and in the *p53* tumor suppressor gene. *Proc Natl Acad Sci USA* 102:10058–63

Besaratinia A, Synold TW, Xi B, Pfeifer GP (2004) G-to-T transversions and small tandem base deletions are the hallmark of mutations induced by ultraviolet A radiation in mammalian cells. *Biochemistry* 43:8169–77

Bruls WAG, Slaper H, Van der Leun JC, Berrens L (1984) Transmission of human epidermis and stratum corneum as a function of thickness in the ultraviolet and visible wavelengths. *Photochem Photobiol* 40:485–94

Cariello NF, Piegorsch WW, Adams WT, Skopek TR (1994) Computer program for the analysis of mutational spectra: application to *p53* mutations. *Carcinogenesis* 15:2281–5

Courdavault S, Baudouin C, Charveron M, Favier A, Cadet J, Douki T (2004) Larger yield of cyclobutane dimers than 8-oxo-7,8-dihydroguanine in the DNA of UVA-irradiated human skin cells. *Mutat Res* 556:135–42

D'Errico M, Teson M, Calcagnile A, De Santis LP, Nikaido O, Botta E *et al.* (2003) Apoptosis and efficient repair of DNA damage protect human keratinocytes against UVB. *Cell Death Differ* 10:754–6

De Gruijl FR, Van der Leun JC (1994) Estimate of the wavelength dependency of ultraviolet carcinogenesis in humans and its relevance to the risk assessment of a stratospheric ozone depletion. *Health Phys* 67:319–25

De Laat A, Van der Leun JC, De Gruijl FR (1997) Carcinogenesis induced by UVA (365-nm) radiation: the dose-time dependence of tumor formation in hairless mice. *Carcinogenesis* 18:1013–20

Drobetsky EA, Turcotte J, Châteauneuf A (1995) A role for ultraviolet A in solar mutagenesis. *Proc Natl Acad Sci USA* 92:2350–4

Drouin R, Therrien J-P (1997) UVB-induced cyclobutane pyrimidine dimer frequency correlates with skin cancer mutational hot spots in *p53*. *Photochem Photobiol* 66:719–26

Friedberg EC, Walker GC, Siede W, Wood RD, Schultz RA, Ellenberger T (eds) (2005) *DNA repair and mutagenesis*. 2nd ed. Washington, DC: ASM Press

Gossen JA, De Leeuw WJF, Tan CHT, Zwarthoff EC, Berends F, Lohman PHM *et al.* (1989) Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying mutations *in vivo*. *Proc Natl Acad Sci USA* 86:7971–5

Grollman AP, Moriya M (1993) Mutagenesis by 8-oxoguanine: an enemy within. *Trends Genet* 9:246–9

Grünwald S, Pfeifer GP (1989) Enzymatic DNA methylation. *Prog Clin Biochem Med* 9:61–103

Ikehata H, Kudo H, Masuda T, Ono T (2003a) UVA induces C→T transitions at methyl CpG-associated dipyrimidine sites in mouse skin epidermis more frequently than UVB. *Mutagenesis* 18:511–9

Ikehata H, Masuda T, Sakata H, Ono T (2003b) Analysis of mutation spectra in UVB-exposed mouse skin epidermis and dermis: frequent occurrence of C→T transition at methylated CpG-associated dipyrimidine sites. *Environ Mol Mutagen* 41:280–92

Ikehata H, Nakamura S, Asamura T, Ono T (2004) Mutation spectrum in sunlight-exposed skin epidermis: small but appreciable contribution of oxidative stress-induced mutagenesis. *Mutat Res* 556:11–24

Ikehata H, Ono T (2002) Mutation induction with UVB in mouse skin epidermis is suppressed in acute high-dose exposure. *Mutat Res* 508:41–7

Ikehata H, Ono T (2007) Significance of CpG methylation for solar UV-induced mutagenesis and carcinogenesis in skin. *Photochem Photobiol* 83:196–204

Ikehata H, Saito Y, Yanase F, Mori T, Nikaido O, Ono T (2007a) Frequent recovery of triplet mutations in UVB-exposed skin epidermis of *Xpc*-knockout mice. *DNA Repair* 6:82–93

Ikehata H, Yanase F, Mori T, Nikaido O, Tanaka K, Ono T (2007b) Mutation spectrum in UVB-induced skin epidermis of *Xpa*-knockout mice: frequent recovery of triplet mutations. *Environ Mol Mutagen* 48:1–13

Kappes UP, Luo D, Potter M, Schulmeister K, Rünger TM (2006) Short- and long-wave UV light (UVB and UVA) induce similar mutations in human skin cells. *J Invest Dermatol* 126:667–75

Kawai K, Li Y, Kasai H (2007) Accurate measurement of 8-OH-dG and 8-OH-Gua in mouse DNA, urine and serum: effects of X-ray irradiation. *Genes Environ* 29:107–14

Kielbassa C, Epe B (2000) DNA damage induced by ultraviolet and visible light and its wavelength dependence. *Methods Enzymol* 319:436–45

Klungland A, Rosewell I, Hollenbach S, Larsen E, Daly G, Epe B *et al.* (1999) Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. *Proc Natl Acad Sci USA* 96:13300–5

Kvam E, Tyrrell RM (1997) Induction of oxidative DNA base damage in human skin cells by UV and near visible radiation. *Carcinogenesis* 18:2379–84

Lamola AA (1970) Triplet photosensitization and the photobiology of thymine dimers in DNA. *Pure Appl Chem* 24:599–610

Leccia MT, Richard MJ, Joanny-Crisci F, Beani JC (1998) UV-A1 cytotoxicity and antioxidant defence in keratinocytes and fibroblasts. *Eur J Dermatol* 8:478–82

Matsunaga T, Hieda K, Nikaido O (1991) Wavelength dependent formation of thymine dimers and (6-4)photoproducts in DNA by monochromatic ultraviolet light ranging from 150 to 365 nm. *Photochem Photobiol* 54:403–10

Mizuno T, Matsunaga T, Ihara M, Nikaido O (1991) Establishment of a monoclonal antibody recognizing cyclobutane-type thymine dimers in DNA: a comparative study with 64M-1 antibody specific for (6-4)photoproducts. *Mutat Res* 254:175–84

Mouret S, Baudouin C, Charveron M, Favier A, Cadet J, Douki T (2006) Cyclobutane pyrimidine dimers are predominant DNA lesions in whole human skin exposed to UVA radiation. *Proc Natl Acad Sci USA* 103:13765–70

Orimo H, Tokura Y, Hino R, Kasai H (2006) Formation of 8-hydroxy-2′-deoxyguanosine in the DNA of cultured human keratinocytes by clinically used doses of narrowband and broadband ultraviolet B and psoralen plus ultraviolet A. *Cancer Sci* 97:99–105

Pfeifer GP, You Y, Besaratinia A (2005) Mutations induced by ultraviolet light. *Mutat Res* 571:19–31

Setlow RB, Grist E, Thompson K, Woodhead AD (1993) Wavelengths effective in induction of malignant melanoma. *Proc Natl Acad Sci USA* 90:6666–70

Tessman I, Liu S-K, Kennedy MA (1992) Mechanism of SOS mutagenesis of UV-irradiated DNA: mostly error-free processing of deaminated cytosine. *Proc Natl Acad Sci USA* 89:1159–63

Tommasi S, Denissenko MF, Pfeifer GP (1997) Sunlight induces pyrimidine dimers preferentially at 5-methylcytosine bases. *Cancer Res* 57:4727–30

Tyrrell RM (2000) Role for singlet oxygen in biological effects of ultraviolet A radiation. *Methods Enzymol* 319:290–6

Van Kranen HJ, De Laat A, Van de Ven J, Wester PW, De Vries A, Berg RJW *et al.* (1997) Low incidence of *p53* mutations in UVA (365-nm)-induced skin tumors in hairless mice. *Cancer Res* 57:1238–40

Wang F, Saito Y, Shiomi T, Yamada S, Ono T, Ikehata H (2006) Mutation spectrum in UVB-exposed skin epidermis of a mildly-affected *Xpg*-deficient mouse. *Environ Mol Mutagen* 47:107–16

Wood SR, Berwick M, Ley RD, Walter RB, Setlow RB, Timmins GS (2006) UV causation of melanoma in *Xiphophorus* is dominated by melanin photosensitized oxidant production. *Proc Natl Acad Sci USA* 103:4111–4115