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Quantitative analysis of UV photolesions suggests that cyclobutane pyrimidine dimers produced by UVB are more mutagenic than those produced by UVC

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Abstract

The amount of photolesions produced in DNA after exposure to physiological doses of ultraviolet radiation (UVR) can be estimated with high sensitivity and at low cost through an immunological assay, ELISA, which, however, provides only a relative estimate that cannot be used for comparisons between different photolesions such as cyclobutane pyrimidine dimer (CPD) and pyrimidine(6-4)pyrimidone photoproduct (64PP) or for analysis of the genotoxicity of photolesions on a molecular basis. To solve this drawback of ELISA, we introduced a set of UVR-exposed, calibration DNA whose photolesion amounts were predetermined and estimated the absolute molecular amounts of CPDs and 64PPs produced in mouse skin exposed to UVC and UVB. We confirmed previously reported observations that UVC induced more photolesions in the skin than UVB at the same dose, and that both types of UVR produced more CPDs than 64PPs. The UVR protection abilities of the cornified and epidermal layers for the lower tissues were also evaluated quantitatively. We noticed that the values of absorbance obtained in ELISA were not always proportional to the molecular amounts of the lesion, especially for CPD, cautioning against the direct use of ELISA absorbance data for estimation of the photolesion amounts. We further estimated the mutagenicity of a CPD produced by UVC and UVB in the epidermis and dermis using the mutation data from our previous studies with mouse skin and found that CPDs produced in the epidermis by UVB were more than two-fold mutagenic than those by UVC, which suggests that the properties of CPDs produced by UVC and UVB might be different. The difference may originate from the wavelength-dependent methyl CpG preference of CPD formation. In addition, the mutagenicity of CPDs in the dermis was lower than that in the epidermis irrespective of the UVR source, suggesting a higher efficiency in the dermis to reduce

the genotoxicity of CPDs produced within it. We also estimated the minimum amount of photolesions required to induce the mutation induction suppression (MIS) response in the epidermis to be around 15 64PPs or 100 CPDs per million bases in DNA as the mean estimate from UVC and UVB-induced MIS.

Introduction

Ultraviolet radiation (UVR) induces mutations in the cellular genome, which can lead to carcinogenesis in repetitively UVR-exposed tissues, as evidenced in sun-exposed human skin.[1–4] UVR produces base photolesions such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6-4)pyrimidone photoproducts (64PPs) at dipyrimidine sites in DNA, where two pyrimidine nucleotides are juxtaposed tandemly.[5–7] Among these ultraviolet (UV) photolesions, cytosine-containing CPDs are known as the main mutagenic DNA damage, specifically inducing cytosine (C) → thymine (T) base substitution mutations at dipyrimidine sites.[8] However, studies on the quantitative relationship between the amount of CPD and the occurrence of mutation have been lacking so far.

Evaluation of the amounts of UV photolesions has been performed through several methods such as chromatographic analyses, DNA breakage assays with enzymatic or chemical strand scission specific to UV photolesions, immunological assays, and HPLC with electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) detection.[6,9–13] The latter two methods are sensitive enough to detect physiologically significant, low amounts of UV photolesions, and one of the immunological assays, enzyme-linked immunosorbent assay (ELISA), is widely used because of its well established, conventional protocols and the general availability of monoclonal

antibodies highly specific and sensitive to CPDs and 64PPs.[14,15] However, the quantification with immunological methods is not absolute but relative, so that it is difficult to compare between different photolesions and to determine the molecular amounts of the lesions with this approach alone. Although a pioneer work quantifying the absolute amounts of UV photolesions with an immunological assay was performed previously using calibrated DNA whose UV photolesion amounts were predetermined, the determination of the photolesion amounts in the calibrated DNA depended on indirect enzymatic assays whose sensitivities were relatively low.[9] HPLC-ESI-MS/MS technology has solved these problems by enabling the direct determination of the absolute amounts of UV photolesions with high sensitivity, but this approach is limited by the expense of equipment specifically adjusted for the quantification of UV photolesions.[6]

In the present study, we introduced to a conventional ELISA assay a set of UVR-exposed DNA samples, for which the amounts of UV photolesions had been determined in advance with the HPLC-ESI-MS/MS method as a calibration standard for the estimation of the molecular amounts of CPDs and 64PPs in DNA. For the ELISA, TDM-2 and 64M-2 monoclonal antibodies were utilized because they can detect with high sensitivity CPDs and 64PPs formed at all kinds of dipyrimidine sequences, respectively, as shown previously,[16] although the sensitivities may be not always equal among those dipyrimidine sequences. With this calibrated ELISA assay, we evaluated quantitatively, based on the molecular amounts of photolesions, the effects of these UV photolesions on the mutation induction in mouse skin exposed to UVC and UVB.

Materials and Methods

Standard UVR-exposed DNA

Calf thymus DNA (D1501, Sigma-Aldrich) dissolved in water at 400–500 μg/ml was exposed to 50, 100, 200, 300, 400 and 566 Jm^{-2} of UVB emitted from broadband UVB lamps (313 nm peak; FL20S.E, Toshiba, Japan; Fig. 1). The molecular amounts of UV photolesions produced in the DNA were determined with HPLC-ESI-MS/MS as described previously.[11,12]

Mice, UVR irradiation and skin DNA preparation

All procedures for the animal experiments including the husbandry, which were approved by the Institutional Animal Care and Use Committee of Tohoku University, were conducted according to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Regulations for Animal Experiments and Related Activities at Tohoku University. The mice used were a transgenic mouse strain harboring λ-phage-based *lacZ* mutational reporter genes.[17] The UVB source used was the same as that used for the standard DNA. The UVC source was GL15 germicidal lamps (peak emission 254 nm; Hitachi, Japan; Fig. 1). UVC and UVB dosimetry was performed with a UVX radiometer equipped with UVX-25 and UVX-31 sensors, respectively (UVP, San Gabriel, CA). For DNA damage analysis, two or four mice were exposed to UVC and UVB at each of several dose points in ranges of 25-300 and 100-3000 Jm^{-2}, respectively. UVR irradiation and DNA preparations from the exposed mouse skin were performed as described previously.[18] Briefly, the depilated dorsal skin of 8–12-week old mice was irradiated under anesthesia, excised immediately after euthanasia, and

separated into the epidermis and dermis with thermolysin after incubation at 55°C for 5 min to inactivate endogenous DNA repair activities. Genomic DNA was extracted from each tissue and assayed for UV photolesions with ELISA.

ELISA

ELISA was performed with monoclonal antibodies TDM-2 and 64M-2 (prepared on our own; also available from Cosmo Bio Co., Ltd, Japan), which are specific respectively to CPDs and 64PPs,[16] using the protocols described in the provider's instructions (NMDND001 and 002, Cosmo Bio Co.) except for the use of biotin-goat anti-mouse IgG (H+L) (62-6540, Invitrogen) for the second antibody. The color development reactions of *o*-phenylene diamine by streptavidin-conjugated peroxidase (43-4323, Invitrogen) at 37°C were monitored without adding the stop solution of 2M H_2SO_4, by measuring continually absorbance at 450 nm (A_{450}) in 2-min intervals up to 30 min using a microplate reader SUNRISE (TECAN, Austria).

UV photolesion quantification

In ELISA, each DNA sample derived from the UVR-exposed mouse skin was assayed quadruply along with the UVB-exposed standard DNA in the same plate. Based on the relationship between A_{450} and CPD or 64PP amounts, which was determined based on the data from the standard DNA by regression analysis (see Fig. 2A, C), the amounts of UV photolesions in the epidermal or dermal DNA from UVR-exposed mouse skin were estimated.

Data on mutation induction in the UVR-exposed mouse skin

We analyzed previously the mutation induction kinetics in UVC- and UVB-exposed mouse skin by determining the mutant frequencies (MFs) of the *lacZ* transgene in the epidermis and dermis of UVR-exposed skin using the transgenic mice mentioned above.[18–20] In the present study, those data were utilized to quantitatively evaluate the effects of photolesions on the modulation of mutation induction.

Statistical Analyses

Regression analysis was performed with computer software JMP Pro (SAS Institute Japan) and KaleidaGraph (Synergy Software, USA).

Results and Discussion

Preparation of calibration DNA for the absolute quantification of UV photolesions with ELISA

Commercially available calf thymus DNA was dissolved in water and divided in aliquots, which were exposed to a series of UVB doses from broadband UVB lamps to prepare a set of standard DNA samples as calibrations for the quantification of the absolute amounts of UV photolesions. The molecular amounts of CPDs and 64PPs in each standard sample were determined with HPLC-ESI-MS/MS (Fig. 2A). These standard samples were also assayed with ELISA for the detection and quantification of CPDs and 64PPs, and A_{450} values were measured continually in the time course of the color development reaction of ELISA and showed the saturation of the reactions by 30 min both for CPDs and 64PPs (Fig. 2B), indicating no need of further incubation or reaction-stopping agents to obtain stable chromatic values of absorbance. For the obtained UVB dose-A_{450} plots after 30 min incubation, regression curves were

determined (Fig. 2C). For CPDs, at any time point, even at the time points immediately after starting the reaction, the plot curve was not linear, but rather extended upwards with an upwardly convex curve (Fig. 2B, left), so that an exponential rise curve (y = a{1-exp(-bx)}; a, b: positive constant coefficients determined by the regression analysis) was adopted for the regression analysis because the A_{450} values seemed to approach a horizontal line asymptotically with the UVB dose increasing (Fig. 2C). On the other hand, plot curves for 64PPs showed linear increases at every time point except for the dose range greater than 400 Jm^{-2}, at which the A_{450} values began to saturate (Fig. 2B, right), so that a linear regression analysis was performed for the 64PP plots using the A_{450} values up to 400 Jm^{-2} (Fig. 2C). Hence, the estimation would be valid up to the amount of 64PPs produced in DNA exposed to 400 Jm^{-2} of UVB, which is roughly 50 64PPs per million DNA bases, judging from Fig. 2A.

Thus, regression analyses of the relationships between the UVB dose and A_{450} for CPDs and 64PPs were performed according to the following formulae: A = a{1-exp(-bD)} and A = cD (A: A_{450}, D: UVB dose), respectively, providing the values of the constant coefficients as a = 1.0375, b = 0.0065031 and c = 0.00099223, as shown in Fig. 2C. Since, in Fig. 2A, the relationships between the UVB dose and photolesion amount (molecules/10^6 bases) for CPDs and 64PPs were obtained by regression analyses as L = 1.3259D and L = 0.08914D, respectively (L: photolesion amount), the relationships between A_{450} and the photolesion amount for CPDs and 64PPs were expressed by the following formulae: L = -1.3259/b*ln(1-A/a) and L = 0.08914/c*A, respectively, which convert the A_{450} values to the absolute molecular amounts of photolesions independently of the UVR dose. Using the values of the constant coefficients obtained by the regression analyses shown in Fig. 2, the calibration

formulae for the estimation of the CPD and 64PP molecular amounts were derived as $L = -203.9*\ln(1-A/1.0375)$ and $L = 89.84A$, respectively. Thus, the calibration formulae for the estimation of the absolute photolesion amounts from the relative values evaluated in ELISA can be determined. However, the constant coefficients would vary assay by assay in ELISA, so that they should be determined in every ELISA assay by including the calibration DNA on the same ELISA plate with the samples under analysis.

It should be noticed that the A_{450} values did not linearly increase with the UVB dose obviously for CPDs and, as seen at the highest dose, probably even for 64PPs (Fig. 2B), although the molecular amounts of both photolesions increased proportionally with the UVB dose in the dose range examined here as determined with HPLC-ESI-MS/MS (Fig. 2A), confirming the previous studies.[11,12] This observation would suggest that high densities of photolesions in DNA reduce the efficiency of antibody binding to the target moiety of photolesions, probably because of some steric hindrance by the formation of a lattice structure between antibodies and antigenic photolesions. The higher molecular densities in DNA of CPDs compared to 64PPs at the same dose (Fig. 2A) would have made the non-linear kinetics of dose-A_{450} curves more remarkable in the ELISA for CPDs than that for 64PPs (Fig. 2B). Thus, direct use of the values of absorbance from ELISA could be misleading in the estimation of the UV photolesion amounts.

Quantification of UV photolesions in mouse skin exposed to UVC and UVB

Mice were exposed on their hair-removed dorsal areas to UVR from UVC and UVB lamps up to 300 and 3000 Jm^{-2}, respectively, and the amounts of CPDs and 64PPs produced in the skin epidermis and dermis were evaluated with ELISA (Fig. 3A). From

the obtained A_{450} values for the skin DNA samples, the absolute amounts of the UV photolesions were determined by converting the A_{450} values to molecular amounts of photolesions as mentioned above (see Fig. 2A and C), using the regression curves estimated from the standard UVB-exposed DNA samples which were assayed simultaneously in the same ELISA reaction plate (Fig. 3B). For the evaluation of the efficiencies of photolesion formation, the amounts of CPDs and 64PPs formed by a unit dose of UVR from UVC or UVB lamps were also estimated from the slopes of regression lines for the data points given in the graphs (Fig. 3B). For the regression, data points that deviated largely from the regression lines taken for the data points at lower doses were excluded from the analysis. The deviation would result from photoreversion of CPDs by shorter wavelength components[21,22] and a limit of the calibration by the standard UVB-exposed DNA, which could cover reliably a certain range of the amount of each photolesion judging from Fig. 2A (ranges $\leq$500 CPDs/10^6 bases and $\leq$50 64PPs/10^6 bases).

The estimated amounts of the unit-dose CPD or 64PP formation in mouse skin exposed to UVR from the UVC or UVB lamps are given in Table 1 and Fig. 3C, along with those for the standard UVB-exposed DNA. The differences in the epidermis between the UVR sources indicate that the efficiencies of CPD and 64PP formation were about 10 times higher for UVC than for UVB, which is consistent with a previous report on naked DNA[12] and the fact that the peak wavelength of the energy output of the UVC lamp (254 nm) is much closer to the peak absorption wavelength of DNA (260 nm) than that of the UVB lamp (313 nm), although the UVR from UVB lamps was not purified to exclude the small amounts of UVC and UVA wavelengths by filtration (see Fig.1). The differences between the epidermis and dermis for the same UVR sources

should reflect the capacity of the epidermis to prevent UVR penetration to the dermis. Compared to the values for the epidermis, CPD and 64PP formations were reduced in the dermis to 14% and 8%, respectively, against UVC, and to 66% and 38%, respectively, against UVB (Fig. 3C and Table 1), indicating a higher protection ability of the epidermis for shorter UV wavelengths. These results are consistent with our previous observations of lower inductions of mutation in the dermis than in the epidermis of mouse skin exposed to UVC and UVB.[18,19] In addition, the differences between the epidermis and naked DNA also shows the ability of the epidermal cornified layers to protect against UVB, which reduced CPD and 64PP formations to less than a fourth and a half, respectively (Fig. 3C and Table 1). Similar results were also reported previously for human skin and reconstituted skin.[23,24]

We also noticed differences in the amount ratios of 64PPs to CPDs between the UVR sources or the skin tissues (Fig. 3D and Table 1). In all cases, 64PPs were produced less than CPDs at a molar basis and produced a fifth to a fifteenth as much as CPDs, consistent with previous studies using naked, cellular and skin DNA.[9,11,12,25,26] Moreover, UVC produced 64PPs at higher molar ratios of 64PP/CPD for both the epidermis and dermis than UVB, suggesting that 64PPs are formed preferably by shorter UV wavelengths compared to CPDs, which would be consistent with the lower 64PP/CPD ratios in the dermis than in the epidermis observed in both UVC and UVB-exposed skin (Fig. 3D, Table 1), because the epidermal layer should attenuate the shorter UV wavelengths more efficiently, as shown in Fig. 3C. The lower yield of 64PPs at longer wavelengths may reflect the different photochemical mechanisms recently proposed for the two photolesions,[27] in which 64PP formation should drop more remarkably as the wavelength increases than CPD formation. Another explanation

for the lower 64PP/CPD ratios would be the accelerated decrease in the amount of 64PPs by their isomerization to Dewar isomers by UVB, which emits the wavelengths around 320 nm most effective for the photochemical isomerization reaction. However, in the present study, the estimation of the 64PP/CPD ratio was performed using the unit-dose amounts of photolesions determined with the data in the initial low dose range, which would not induce the isomerization sufficient to affect the 64PP/CPD ratio largely, as reported previously.[28]

Interestingly, the 64PP/CPD molar ratios were remarkably different between the epidermis and naked DNA exposed to UVB (Fig. 3D, Table 1). The lower 64PP formation efficiency for naked DNA would reflect the influence of chromatin structure or other cellular environments in the epidermis on the formation of UV photolesions, which seems to enhance 64PP formation or to suppress CPD formation in the epidermis. Actually, it has been reported that the formation of CPDs in chromatin is restricted by the rotational position of dipyrimidines in DNA to the nucleosome surface, whereas 64PP formation seems to be uniform within a nucleosome and rather prefers linker DNA regions,[29] which might be responsible for the difference in the 64PP/CPD ratio between naked and epidermal DNA observed in Fig. 3D.

Quantitative evaluation of mutation induction by photolesions in UVR-exposed mouse skin

To analyze the relationship between the DNA damage amounts and mutation induction, data for the mutation induction kinetics in mouse skin exposed to UVC and UVB, which we have already reported previously,[18–20] were reevaluated as shown in Fig. 4A. The efficiency of mutation induction, which is defined as mutagenicity here, was

estimated as a unit-dose increase of the mutant frequency (MF), namely, the slope of the MF increase, which was calculated by regression analysis for each MF increase curve shown in Fig. 4A (also given in Table 2 as UVR mutagenicity) although, for the epidermis, only the MF data points before the MF started to plateau were used for the analysis. In the epidermis, the response of mutation induction suppression (MIS) was evident after exposure to UVC or UVB more than a certain dose (Fig. 4A). MIS is an important tissue response that is characteristic of mutation induction in UVR-exposed skin, as we reported previously.[19,30,31] As the quantifiable value for the MIS response, the minimum dose for MIS induction, namely the minimum MIS dose (MMISD), was used,[30] and estimated to be 25 Jm^{-2} for UVC and 500 Jm^{-2} for UVB from the MF induction kinetics in the epidermis for each type of UVR (Fig. 4A).

Because CPDs are the major UVR-specific DNA damage and are known to cause most of the mutations by UVR, at least, in DNA repair-proficient cells and skin,[8,32] we focused on the mutagenicity of CPDs produced by each UVR source. To evaluate the CPD mutagenicity, the amounts of CPDs formed in a *lacZ* transgene by a unit dose of UVC and UVB were calculated as shown in Table 2, and the unit-dose mutagenicity of each UVR (UVR mutagenicity, Table 2) estimated from the slope of the regression lines in Fig. 4A was divided by the calculated value to determine the mutagenicity of a CPD produced by UVC and UVB in the epidermis and dermis (CPD mutagenicity, Table 2). The resultant CPD mutagenicities were significantly different between UVC and UVB in both skin tissues (Fig. 4B and Table 2). CPDs produced by UVB were more than 2-fold mutagenic than those by UVC in the epidermis and this difference was larger in the dermis, which suggests that the properties of the CPDs produced by UVC and UVB might be different. In other words, UVB might produce more mutagenic CPDs than

UVC, although some differences in the cellular responses to these UVRs, such as the induction of DNA repair processes and/or proliferative/senescent/apoptotic responses, might have influenced the differences in CPD mutagenicity.[33] It should also be mentioned that the CPD mutagenicity is determined based on the initial slope of mutation induction, that is, deduced from mutations induced in the low dose range of UVR, which means that the CPD mutagenicity is unlikely to reflect the cellular responses induced by higher doses of UVR.

It was also noticed that CPDs in the dermis were less mutagenic than those in the epidermis, although they were induced by the same UVR sources (Fig. 4B and Table 2). This observation suggests a more efficient elimination of CPDs in the dermis by some biological processes such as DNA repair and apoptosis, which is, however, inconsistent with previous reports supporting a higher repair activity and apoptosis for the epidermis or keratinocytes.[34–37] This discrepancy might be solved by supposing an efflux of UVR-damaged fibroblasts from the UVR-exposed skin area along with an influx of undamaged ones from the surrounding intact dermis by post-irradiation cell migration, or by postulating an induction of premature senescence of damaged fibroblasts,[38] which should stop DNA replication and prevent mutation induction in those cells because the fixation of mutation in DNA requires a UV photolesion bypass by DNA synthesis.[39–41]

We also estimated the amounts of UV photolesions produced in the epidermis after exposure to 25 and 500 Jm^{-2} of UVC and UVB, the minimum doses to induce the MIS response in mouse epidermis, namely MMISD, as shown in Table 3 and Fig. 4D. Since MIS is an immediate response in the epidermis after UVR exposure, the amounts of DNA lesions instantly produced by UVR could be one of the determinants in the induction of the MIS response. The estimates in Table 3 suggest that about 100 CPDs or

15 64PPs per million bases in DNA could induce the MIS response, although the lesion amounts necessary for the induction by UVB were roughly twice as high as those by UVC (Fig. 4C and Table 3). It would be an important question which photolesion, CPD or 64PP, triggers the MIS response, or to what extent each photolesion contributes to the induction of the MIS response, which is, however, difficult to solve decisively with the data provided in the present study.

The origin of the different mutagenicities of CPDs produced by UVC and UVB

We found that UVB-induced CPDs were more mutagenic than UVC-induced ones in both the epidermis and dermis. This observation might suggest that CPDs were eliminated more efficiently by some biological processes such as DNA repair and apoptosis in the skin after exposure to UVC than to UVB. However, such differential responses to CPDs have not been demonstrated yet between UVC- and UVB-exposed skin or cells.

Although CPDs can be formed at four different dipyrimidine sequences, TT, TC, CT and CC, only the latter three, cytosine-containing dipyrimidines can induce UVR-specific C $\rightarrow$ T mutations. The importance of cytosine-containing CPDs for the UVR mutagenesis has also been suggested previously.[12,42] Cytosines in CPDs are unstable and easily deaminate to produce uracils (or thymines if their 5-position is methylated),[4,43,44] which are known to produce C $\rightarrow$ T mutations if translesion DNA synthesis (TLS) polymerase η bypasses these deaminated CPDs.[4,45,46] The propensity of cytosine to deaminate is known to vary by the sequence context in which CPDs are placed. Cytosines in TC-CPDs formed in the 5'-TCG-3' context have been shown to deaminate more easily than other TC- or CC-CPDs in other contexts as far as examined

in naked and chromatin DNA,[47–49] thus making CPDs in the TCG context highly mutagenic compared to CPDs in the other sequence contexts. The TCG context includes a CpG motif, the target sequence of mammalian DNA methylation, with which the cytosine in the motif is methylated to produce 5-methylcytosine,[50] and CpG methylation at dipyrimidine sites is known to enhance CPD formation by longer wavelength UVRs such as UVB and solar UVR,[51–53] but not by UVC.[53–55] This methyl CpG preference of the CPD formation by longer wavelength UVRs should cause more CPDs at the TCG context, resulting in the production of more mutagenic CPDs after skin exposure to UVB than to UVC, as observed in the present study (Fig. 4C). Accordingly, we reported previously that UVB induced in mouse epidermis the UVR-specific C → T mutations in the TCG context at a higher ratio than did UVC.[18] Thus, UVB could produce more mutagenic CPDs than UVC in the mammalian skin genome.

Conclusion

The introduction of calibration DNA to ELISA facilitated our estimation of the genotoxicity of UV photolesions on a molecular basis, revealing a remarkable variation of CPD mutagenicity depending on the UVR sources and the type of skin tissues. The UVR source-dependent variation would reflect the wavelength-dependent methyl CpG preference of CPD formation and the sequence context-dependent cytosine-deamination tendency of CPD. The tissue-dependent variation might result from a difference in the response to the genotoxicity of UVR photolesions between the epidermis and dermis: the former is known to respond with the MIS response, whereas the latter might respond with a premature senescence of damaged cells or a post-irradiation cell exchange between damaged and undamaged dermal areas. The ELISA analysis with calibration

DNA could help provide not relative but absolute quantitative estimations of UVR genotoxicity at a reasonable cost.

Conflicts of interest

There are no conflicts of interest to declare.

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Table 1 Quantification of UV photolesions in UVC/UVB-exposed mouse skin

UVR source	Tissue/sample	Unit-dose CPD formation [a] (CPDs/Mb/Jm^{-2})	Unit-dose 64PP formation [a] (64PPs/Mb/Jm^{-2})	64PP/CPD ratio (64PPs/CPD)
UVC	Epidermis	2.59×10^{0}	4.59×10^{-1}	0.177
	Dermis	3.70×10^{-1}	3.64×10^{-2}	0.098
UVB	Epidermis	2.99×10^{-1}	4.14×10^{-2}	0.138
	Dermis	1.97×10^{-1}	1.56×10^{-2}	0.080
	DNA [b]	1.33×10^{0}	8.91×10^{-2}	0.067

[a] Slopes of regression lines estimated in Fig. 2A and 3B. CPD, cyclobutane pyrimidine dimer; 64PP, pyrimidine(6-4)pyrimidone photoproduct; Mb, 10^6 bases. [b] Calf thymus DNA solution directly exposed to UVB and used as a standard in the present study.

Table 2 Evaluation of the mutagenicity of CPD induced in UVC/UVB-exposed mouse skin

Tissue	UVR source	Unit-dose CPD formation per *lacZ* transgene [a] (CPDs/lacZ/Jm^{-2})	UVR mutagenicity [b] (MF/Jm^{-2})	CPD mutagenicity (MF/CPD)
Epidermis	UVC	1.56×10^{-2}	1.20×10^{-5}	7.71×10^{-4}
	UVB	1.79×10^{-3}	3.27×10^{-6}	1.82×10^{-3}
Dermis	UVC	2.22×10^{-3}	1.85×10^{-7}	8.32×10^{-5}
	UVB	1.18×10^{-3}	8.55×10^{-7}	7.24×10^{-4}

[a] Estimates of the number of CPDs in a *lacZ* transgene formed by a unit dose of UVB or UVC, which were calculated from the values in Table 1, estimating the size of a *lacZ* transgene as 6000 bases. [b] Initial slopes of mutation induction estimated in Fig. 4A. MF, mutant frequency.

Table 3 Evaluation of UV photolesion amounts produced by minimum MIS-inducing dose of UVC/UVB in mouse epidermis

Photolesion	UVR source	Unit-dose photolesion formation [a] (Lesions/Mb/Jm^{-2})	MMISD [b] (Jm^{-2})	MIS-inducing photolesion amount [c] (Lesions/Mb/MMISD)
CPD	UVC	2.59×10^{0}	25	64.8
	UVB	2.99×10^{-1}	500	149
64PP	UVC	4.59×10^{-1}	25	11.5
	UVB	4.14×10^{-2}	500	20.7

[a] Slopes of regression lines estimated in Fig. 3B. [b] Minimum MIS dose: minimum doses to induce the response of mutation induction suppression (MIS). [c] Calculated by multiplying the unit-dose photolesion formation by MMISD.

Figure legends

Fig. 1 Profiles of percent spectral outputs of UVC and UVB lamps used in the present study. Spectral energy outputs of each lamp were measured with a spectroradiometer (USR-45DA, Ushio Inc., Japan) at 1-nm intervals.

Fig. 2 Quantification of UV photolesions in standard UVB-exposed DNA. (A) The amounts of CPDs and 64PPs at each dipyrimidine of TT, TC, CT and CC in DNA samples exposed to a series of UVB doses were determined with HPLC-MS/MS, summed up by each photolesion, and plotted. Error bars show standard deviations. Lines with an equation show linear regression curves (slopes: $P < 10^{-10}$ for CPDs, $P < 10^{-8}$ for 64PPs). (B) ELISA reactions with the standard DNA samples were monitored by the absorbance at 450 nm at every 2 min up to 30 min for CPDs (left) and 64PPs (right). (C) Regression analysis was performed for the values obtained at 30 min in Fig. 2B excluding the value at the highest dose for 64PPs. Error bars indicate standard deviations. Lines with an equation show regression curves ($R^2 > 0.99$ for CPDs; $P < 10^{-7}$ for the slope of 64PPs).

Fig. 3 Quantification of UV photolesions in mouse skin exposed to UVR from UVC or UVB lamps. (A) Dose response of UV photolesion formation estimated by ELISA. The amounts of CPDs and 64PPs in genomic DNA from mouse skin epidermis and dermis exposed to UVR from UVC (left) and UVB (right) lamps were estimated with ELISA and shown by the values of absorbance at 450 nm (A_{450}). Each point is derived from a single mouse. (B) Dose-dependent UV photolesion formations shown by absolute

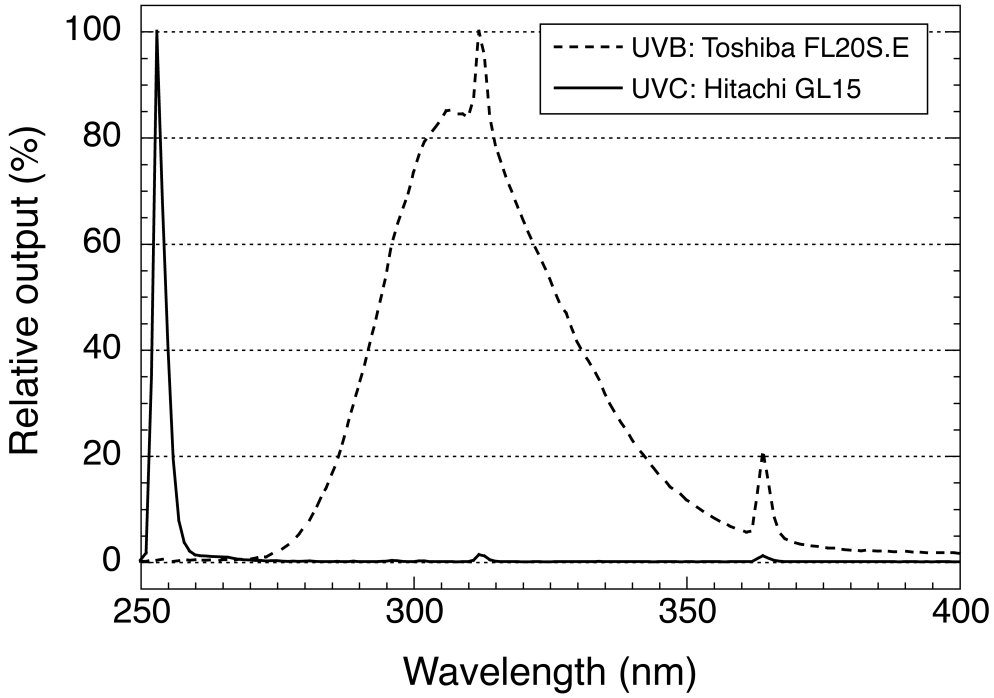
molecular amounts of CPDs and 64PPs. The A_{450} value for each data point given in A was converted to a molecular amount per million bases using the standard UVB-exposed DNA, and given separately by CPDs (upper panels) and 64PPs (lower panels). The efficiencies of photolesion formation were estimated from the slopes of the regression lines for the data points labeled by colors (red and blue for epidermis and dermis, respectively). The equation for each regression line is given in the same color in each panel. (C) Unit-dose formation of UV photolesions in mouse skin exposed to UVR from UVC and UVB lamps. Molecular amounts of CPDs (left) and 64PPs (right) formed in the epidermis (dark) and dermis (light) by a unit-dose of UVC and UVB are given along with those in naked DNA (the standard DNA used for calibration) by UVB (oblique striped). Error bars show standard errors. (D) Ratios of 64PPs to CPDs formed in mouse skin and naked DNA exposed to UVR from UVC or UVB lamps. Symbols are the same as those in C.

Fig. 4 Quantitative evaluation of the mutation induction kinetics in UVR-exposed mouse skin. (A) Quantification of mutation induction in UVC/UVB-exposed mouse skin. Mutation induction kinetics for UVC-exposed (upper) and UVB-exposed (lower) mouse skin were evaluated for the efficiencies of mutation induction by regression analysis. Data points used for the analysis are color-labeled (red for epidermis and blue for dermis), and equations for the regression lines are provided in the same colors in each panel. Error bars show standard deviations. (B) Mutagenicity of CPDs produced by UVC (dark) and UVB (light) in skin tissues. (C) Amounts of photolesions produced by the minimum dose of UVC (dark) and UVB (light) to induce the MIS response, which were estimated as 25 and 500 Jm^{-2}, respectively, from the MIS induction kinetics

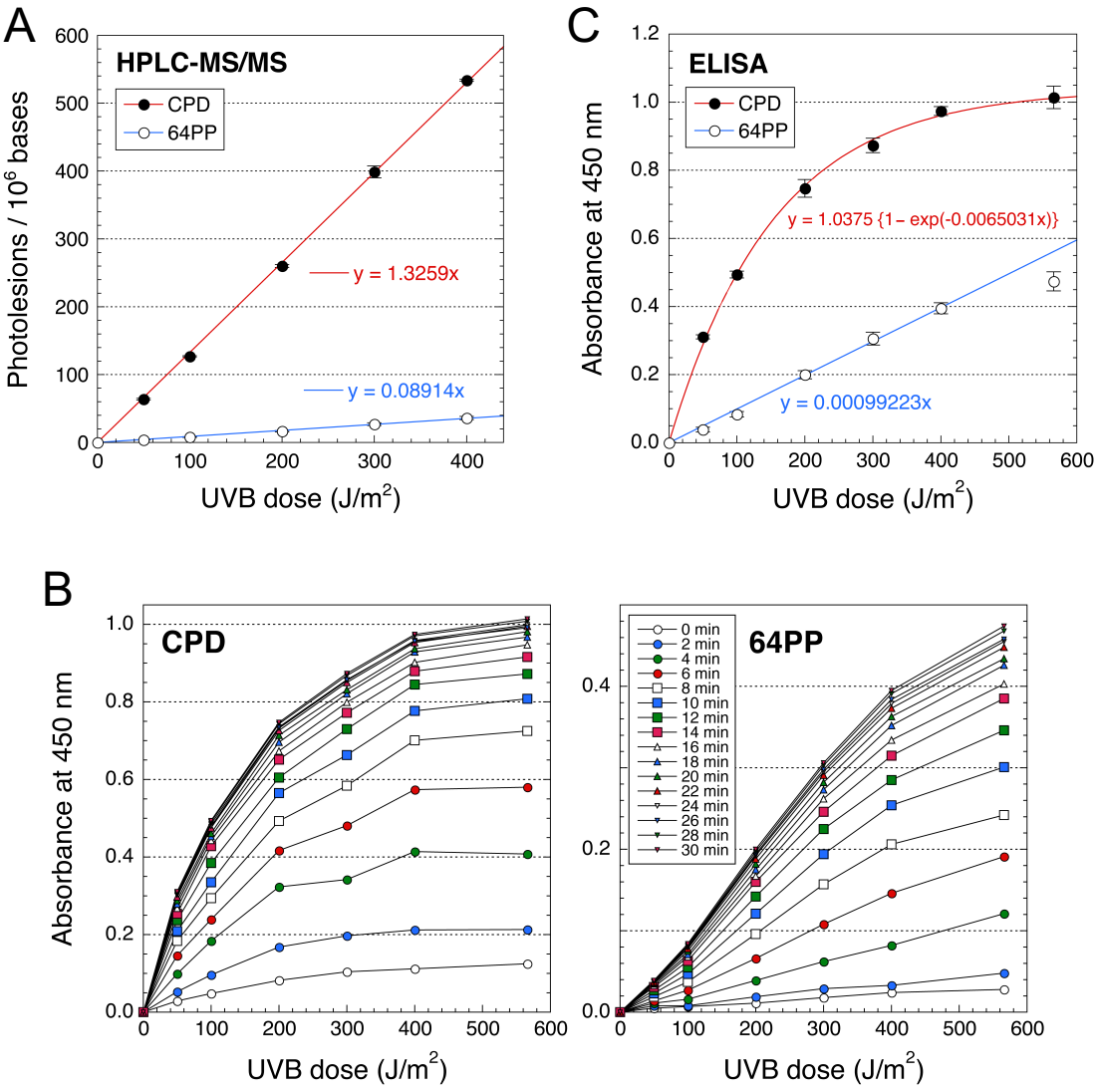
in the epidermis given in A. Error bars in B and C show standard errors. MF: mutant frequency, MMISD: minimum MIS dose.

Graphical abstract

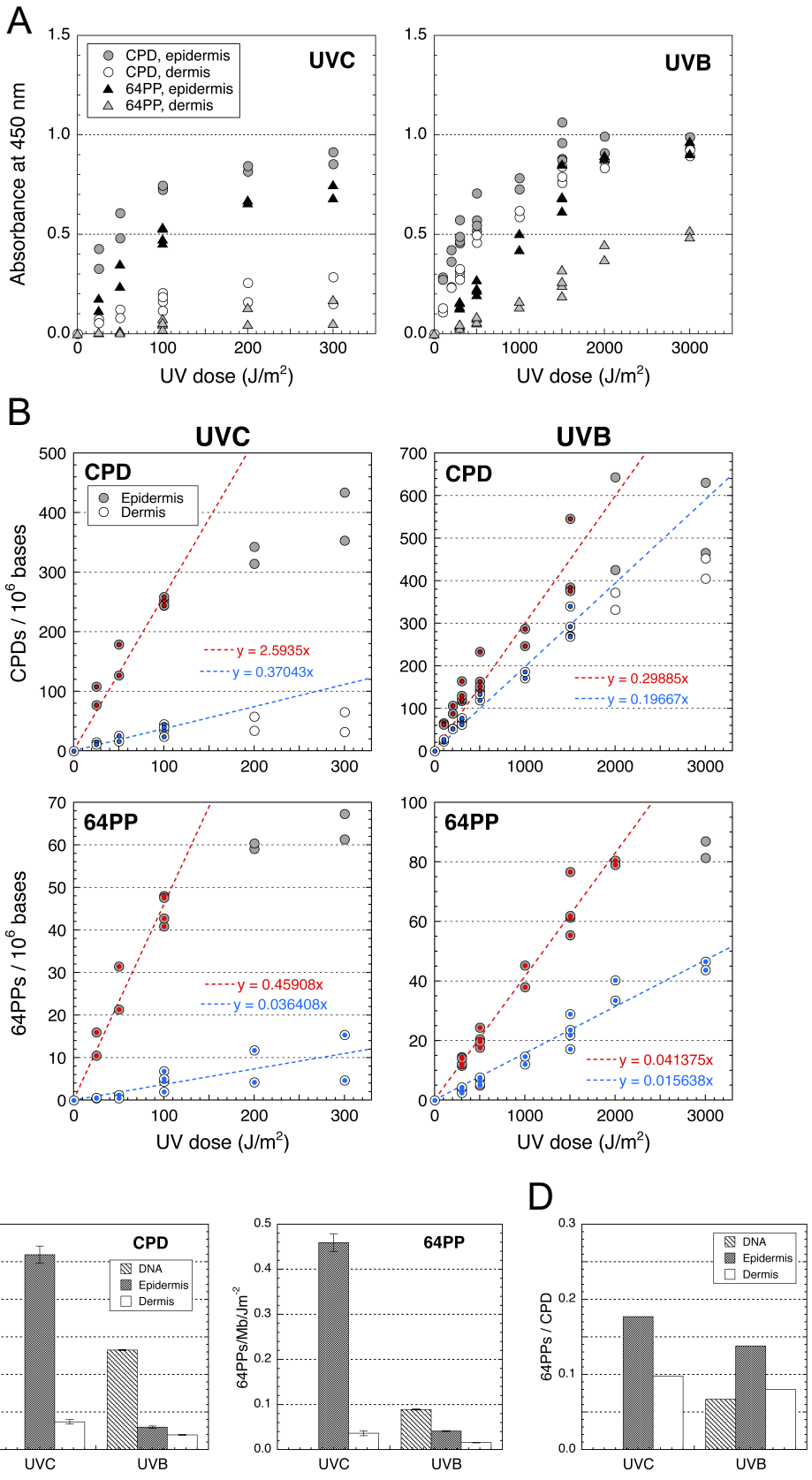
The absolute quantification of UV photolesions with a calibrated ELISA and the mutagenicity assay of UVR-exposed transgenic mice revealed that the molecular genotoxicity of CPDs depends on the UVR components and skin tissues.



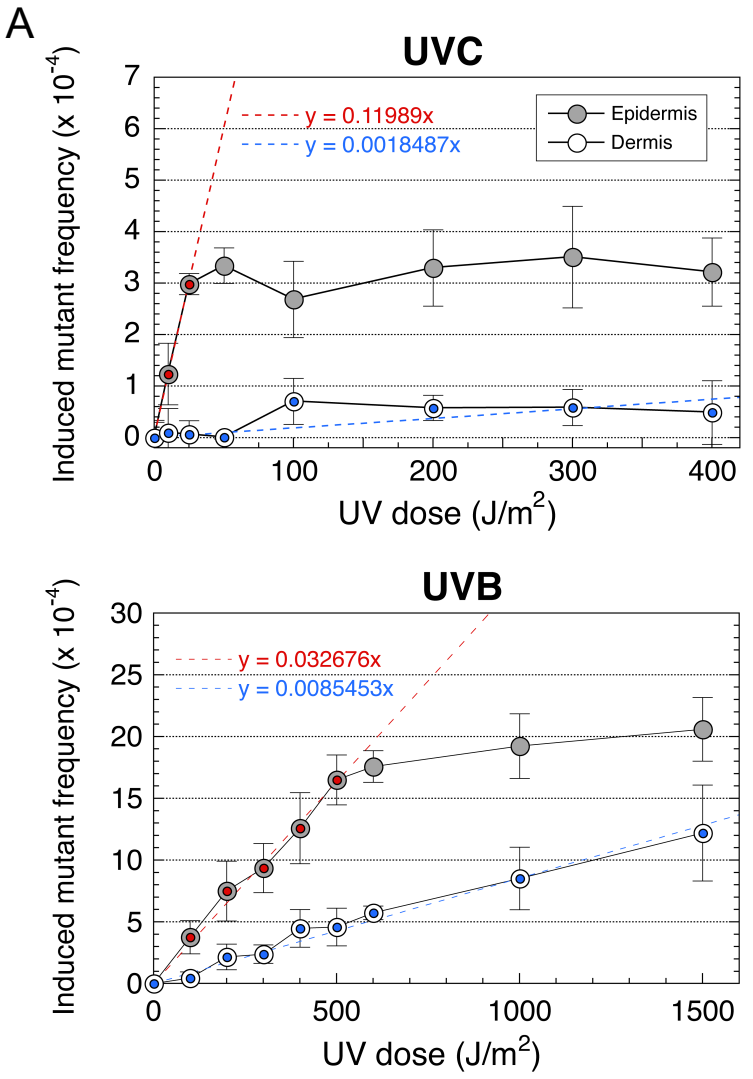
H. Ikehata *et al.* Fig. 1

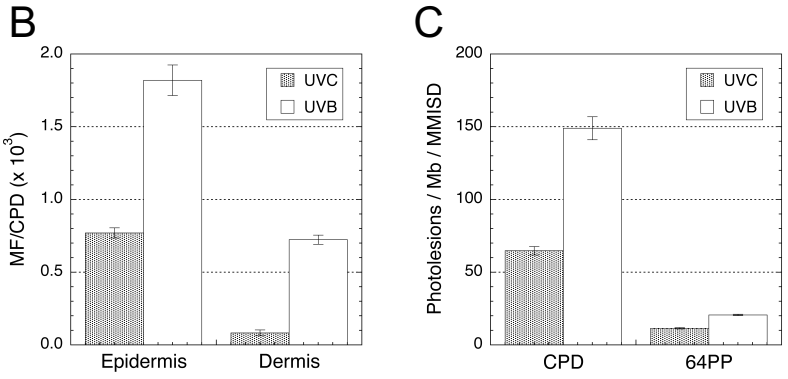


H. Ikehata *et al.* Fig. 2

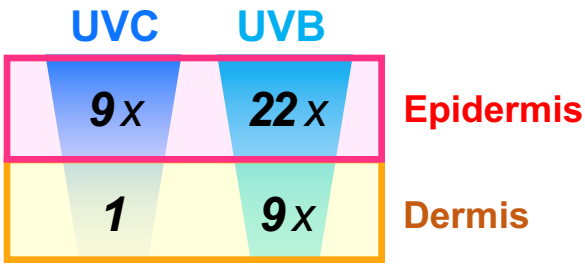


H. Ikehata *et al.* Fig. 3





H. Ikehata *et al.* Fig. 4



Molecular mutagenicity of CPDs in skin **(relative)**

The absolute quantification of UV photolesions with a calibrated ELISA and the mutagenicity assay of UVR-exposed transgenic mice revealed that the molecular genotoxicity of CPDs depends on the UVR components and skin tissues.

H. Ikehata *et al.* Graphical abstract