

Narrow-Band UVB Induces More Carcinogenic Skin Tumors than Broad-Band UVB through the Formation of Cyclobutane Pyrimidine Dimer

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Phototherapy with narrow-band UVB (NB-UVB), with a peak exclusively at 311 nm wavelength, has been found to be more effective in treating a variety of skin diseases than conventional broad-band UVB (BB-UVB). To assess the difference in carcinogenic activity between NB-UVB and BB-UVB, we investigated skin tumor formation by irradiating albino hairless, *Ogg1* knockout mice and C57BL/6J wild counterparts with these two UV sources. We found that the ratio of malignant skin tumors induced by NB-UVB was significantly higher than that induced by BB-UVB. There was no significant difference in carcinogenicity of skin tumor induced by NB-UVB between *Ogg1* knockout and wild-type mice. To investigate the possible cause of different carcinogenic activity by the different UV sources, we examined three types of DNA damage: cyclobutane pyrimidine dimer (CPD), (6-4) photoproduct, and 8-oxoguanine (8-oxoG) induced by each UV source. We found that CPD formation following a minimum erythema dose (MED) by NB-UVB was significantly higher than that following 1 MED by BB-UVB, whereas the formation of (6-4) photoproducts and 8-oxoG following BB-UVB was significantly higher than those following NB-UVB exposure. These results suggest that CPD formation is closely related to the higher carcinogenic characteristics of NB-UVB.

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INTRODUCTION

Phototherapy, particularly UVB treatment, is widely used to effectively treat various skin diseases. Narrow-band UVB (NB-UVB) treatment, with a peak wavelength of 311 nm, has been shown to be effective for a variety of skin diseases such as psoriasis vulgaris, vitiligo vulgaris, and atopic dermatitis. Some reports indicate that NB-UVB is more therapeutic than broad-band UVB (BB-UVB), which has a wavelength range between 280 and 320 nm (van Weelden *et al.*, 1988; Coven *et al.*, 1997; Walters *et al.*, 1999; Gathers *et al.*, 2002; Samson Yashar *et al.*, 2003) (Figure 1a). However, the effectiveness of NB-UVB needs to be considered along with its adverse effects, mainly skin carcinogenesis, since UVB causes the development of skin cancers in humans and in other animals (Kraemer, 1997). There are several reports

about skin carcinogenesis following NB-UVB therapy compared with BB-UVB, showing that NB-UVB is more carcinogenic than BB-UVB (van Weelden *et al.*, 1988; Flindt-Hansen *et al.*, 1991; Wulf *et al.*, 1994; Gibbs *et al.*, 1995), which is now considered to be tentative consensus (Young, 1995; el-Ghorr and Norval, 1997). In this study, we performed a comparative study of skin tumor formation following exposure to NB-UVB or BB-UVB, using different strains of mice. We also investigated the specific mechanism involved in skin carcinogenesis caused by NB-UVB or BB-UVB, particularly focusing on the types of UV-induced DNA damage such as cyclobutane pyrimidine dimer (CPD), (6-4) photoproduct, and oxidative DNA damage.

Reactive oxygen species, which are generated endogenously by cellular oxygen metabolism or exogenously by UV, environmental mutagens, produce various types of DNA damage (Hattori-Nakakuki *et al.*, 1994; Aburatani *et al.*, 1997). Among many oxidative DNA base modifications, 8-oxoguanine (8-oxoG) can pair with adenine as well as cytosine during DNA replication, which results in GC→TA transversion mutations (Kasai *et al.*, 1991; Maki and Sekiguchi, 1992). 8-OxoG is one type of oxidative DNA damage that can result in stable mutations. In mammalian cells, the *Ogg1* gene encodes 8-oxoG-DNA glycosylase, a repair enzyme, which removes the oxidized base from DNA. As we have reported, 8-oxoG formation plays an important role in the development of skin cancers following BB-UVB exposure (Kunisada *et al.*, 2005). In this study, we also

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Abbreviations: 8-oxoG, 8-oxoguanine; BB-UVB, broad-band UVB; CPD, cyclobutane pyrimidine dimer; NB-UVB, narrow-band UVB; MED, minimal erythema dose

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studied whether oxidative DNA damage involves in the carcinogenesis by NB-UVB using *Ogg1* knockout mice. Overall, we found that NB-UVB induces more malignant skin tumors through the formation of CPD rather than (6-4) photoproduct or 8-oxoG. This finding provides early evi-

dence of the types of DNA damage closely related to UV carcinogenesis caused by NB-UVB or BB-UVB, which have different photocarcinogenic features.

RESULTS

Comparative study: difference of skin carcinogenicity between NB-UVB and BB-UVB

We compared the formation of skin tumors following chronic exposure to NB-UVB or BB-UVB (Figure 1a) using albino hairless, hairy C57BL/6J, and *Ogg1* knockout mice (C57BL background), with minimum erythema dose (MED) doses of each type of UVB. MED in albino mice induced by BB-UVB and NB-UVB was 170 and 370 mJ/cm², respectively (Figure 1b). MED in C57BL/6J induced by BB-UVB and NB-UVB was 250 and 850 mJ/cm², respectively. We performed histological analyses of all skin tumors that developed. The total numbers of skin tumors induced by NB-UVB are not significantly different from that by BB-UVB (albino hairless mice: *P*=0.20; C57BL/6J mice: *P*=0.43). However, the ratios of malignant skin tumors produced by NB-UVB were 80.0 and 91.6% in hairless mice and in C57BL/6J mice, respectively. That is a significantly higher rate than that produced by BB-UVB, in which 61.2 and 50.0% were malignant tumors in hairless mice and in C57BL/6J mice, respectively (albino hairless mice: *P*=0.026; C57BL/6J mice: *P*=0.022) (Tables 1 and 2 and Figure 2a and b). Since we previously reported the involvement of oxidative stress in photocarcinogenesis by comparing the BB-UVB induced formation of skin tumors between wild-type and *Ogg1* knockout mice (Kunisada *et al.*, 2005), we then also evaluated the involvement of oxidative DNA damage in the development of skin tumors following chronic NB-UVB exposure. The number of skin tumors and rates of malignant tumors induced by NB-UVB was not significantly different irrespective of *Ogg1* genotypes (Table 2 and Figure 2b).

Immunohistochemistry: accumulation of DNA damages in the epidermis following NB-UVB or BB-UVB

We then investigated the relevance of UV-induced DNA damage, such as CPD, (6-4) photoproduct, and 8-oxoG, for

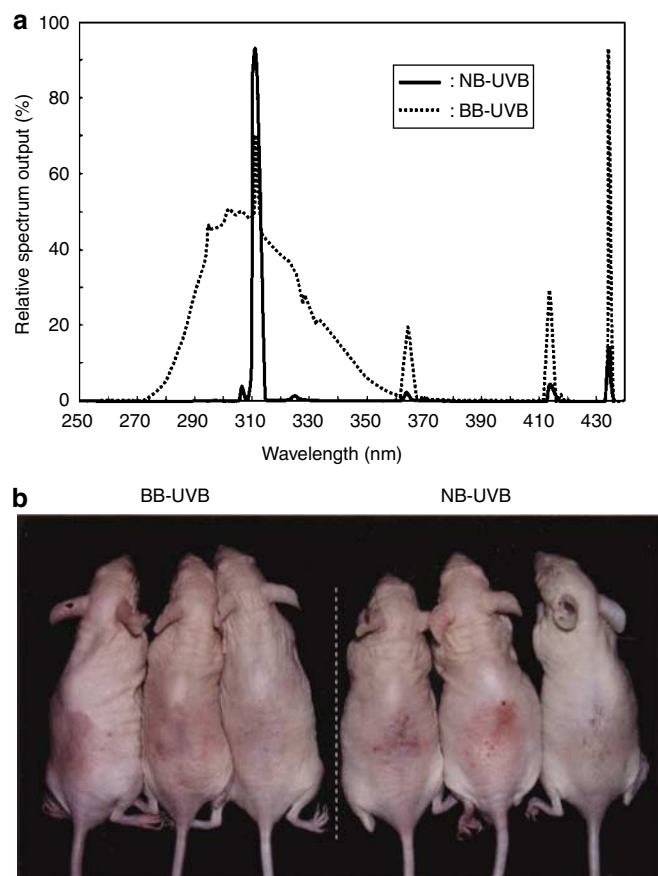


Figure 1. Comparison of two UVB sources. (a) Spectra of NB-UVB and BB-UVB. (b) MED of mice induced by NB-UVB or BB-UVB. The redness of back skins for albino hairless mice at minimal erythema dose of BB-UVB and NB-UVB; left three mice: BB-UVB with 170 mJ/cm²; right three mice: NB-UVB with 370 mJ/cm².

Table 1. Tumor formation by chronic NB-UVB or BB-UVB irradiation of albino hairless mice

UVB source	Total number of tumors			Mean number of tumors/mouse	Histological analysis %						
	Male	Female	Total		Malignant tumors %			Benign tumors %			
					Squamous cell carcinoma	Sarcoma	Total	Papilloma	Cyst	Total	Unidentified ¹
Narrow Band	13 (2) ²	37 (7)	40 (9)	4.44±2.79 [†]	80.0 (32/40) ³	0 (0/40)	80.0 (32/40) [*]	10.0 (4/40)	5.0 (2/40)	15.0 (6/40)	5.0 (2/40)
Broad Band	6 (2)	43 (6)	49 (8)	6.13±5.00 [†]	57.1 (28/49)	4.1 (2/49)	61.2 (30/49) [*]	36.7 (18/49)	0 (0/49)	36.7 (18/49)	2.0 (1/49)

BB-UVB, broad-band UVB; NB-UVB, narrow-band UVB.

¹Unidentified tumors were those which we were not able to make the diagnosis due to the failure of the embedding the specimens.

²The number of skin tumor-bearing mice at the end of the experiment.

³The number of skin tumors/total skin tumors histologically.

[†]*P*, not significant.

^{*}*P*<0.05.

Table 2. Tumor formation by chronic NB-UVB irradiation for *Ogg1* knockout mice and its wild counterpart in comparison with that by BB-UVB

UVB source	<i>Ogg1</i> genotype	Total number of tumors			Mean number of tumors/mouse	Histological analysis %					
		Male	Female	Total		Malignant tumors %			Benign tumors %		
						Squamous cell carcinoma	Sarcoma	Total	Papilloma	Total	Unidentified ¹
Narrow band	Wild	3 (1) ²	9 (6)	12 (7)	1.71 ± 0.76 [†]	67.7 (8/12) ³	25.0 (3/12)	91.6 ^{†,*} (11/12)	8.3 (1/12)	8.3 (1/12)	0 (0/12)
	Knockout	4 (2)	5 (3)	9 (5)	1.80 ± 0.84 [†]	66.7 (6/9)	22.2(2/9)	88.9* (8/9)	11.1 (1/9)	11.1 (1/9)	0 (0/9)
Broad band ⁴	Wild	2 (1)	10 (6)	12 (7)	1.71 ± 0.76 ^{†,***}	41.7 (5/12)	8.3 (1/12)	50.0 ^{†,***} (6/12)	41.7 (5/12)	41.7 (5/12)	8.3 (1/12)
	Knockout	5 (1)	21 (6)	26 (7)	3.71 ± 1.38 ^{**}	73.1 (19/26)	15.4 (4/26)	88.5 ^{***} (23/26)	11.5 (3/26)	11.5 (3/26)	0 (0/16)

BB-UVB, broad-band UVB; NB-UVB, narrow-band UVB.

¹Unidentified tumors were those which we were not able to make the diagnosis due to the failure of the embedding the specimens.

²The number of skin tumor-bearing mice at the end of the experiment.

³The number of skin tumors/total skin tumors histologically.

⁴Broad band UVB data is on previous proof for comparison.

[†]*P*, **P*, not significant.

[†]*P*, ****P* < 0.05.

***P* < 0.01.

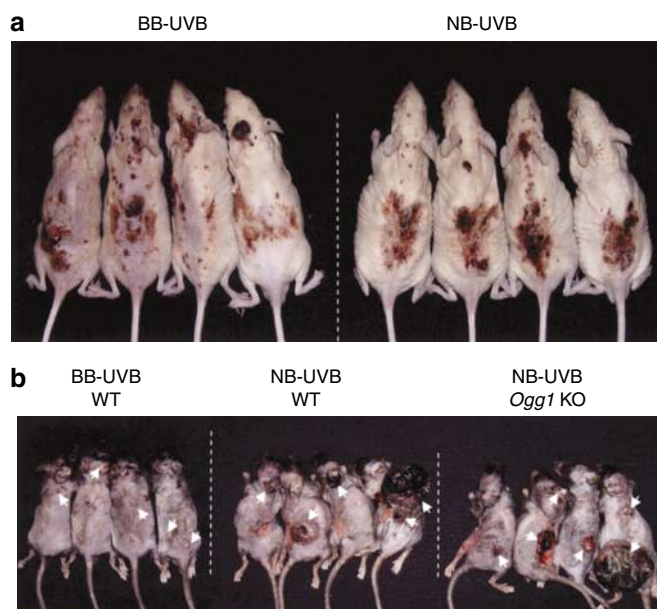


Figure 2. Developing skin tumors induced by NB-UVB or BB-UVB.

Representative features of skin tumor formation in (a) hairless mice and in (b) C57BL/6J mice irradiated with BB-UVB (left four mice), irradiated with NB-UVB (middle four mice), or *Ogg1* knockout mice (right four mice) at the end of the observation period. Arrows indicate the developing tumors. (b) C57BL/6J mice irradiated with BB-UVB (left four mice) are on previous proof for comparison.

the different ratio of malignant skin tumors following NB-UVB or BB-UVB irradiation. We immunohistochemically quantified the accumulation of those DNA damage in epidermis and dermis following NB-UVB or BB-UVB exposure using Image J analysis software. The strong

accumulation of CPD was observed in epidermal cells at both 3 minutes and 3 hours after NB-UVB or BB-UVB exposure at similar levels. The lesser amount of immunofluorescent staining was observed in dermis at 3 minutes and 3 hours after NB-UVB or BB-UVB exposure at similar level. However, a greater amount of immunofluorescent staining was observed in epidermis at 72 hours after NB-UVB exposure than after BB-UVB exposure (*P* < 0.0001) (Figure 3a). There was no significant difference in intensity of CPD irrespective of *Ogg1* genotype at any time after NB-UVB or BB-UVB exposure (data not shown). Conversely, the staining intensity of (6-4) photoproduct was more significant in both epidermal and dermal cells at 3 minutes (epidermis: *P* < 0.0001; dermis: *P* < 0.001, respectively) and 3 hours (epidermis: *P* < 0.0001; dermis: *P* < 0.05, respectively) after BB-UVB exposure than after NB-UVB exposure. Staining signal was low in both epidermal and dermal cells 72 hours after exposure to either NB-UVB or BB-UVB (Figure 3b). There was no significant difference in the intensity of (6-4) photoproduct in epidermal cells irrespective of *Ogg1* genotype at any time after either NB-UVB or BB-UVB exposure (data not shown). The accumulation of 8-oxoG was significantly strong for epidermal cells 3 hours after BB-UVB exposure than after NB-UVB exposure in both *Ogg1* knockout mice and wild-type mice (*P* < 0.0001) (Figure 3c). The lesser amount of immunofluorescent staining was observed in dermis after 3 and 72 hours after NB-UVB or BB-UVB exposure with no significance. There was no significant difference in intensity of 8-oxoG for epidermal cells irrespective of *Ogg1* genotype at any time after NB-UVB exposure. However, the intensity of 8-oxoG in the epidermis was much higher in *Ogg1* knockout mice than in wild-type mice 72 hours after BB-UVB exposure (*P* < 0.0001) (Figure 3c), which confirms our previous studies (Kunisada et al., 2005).

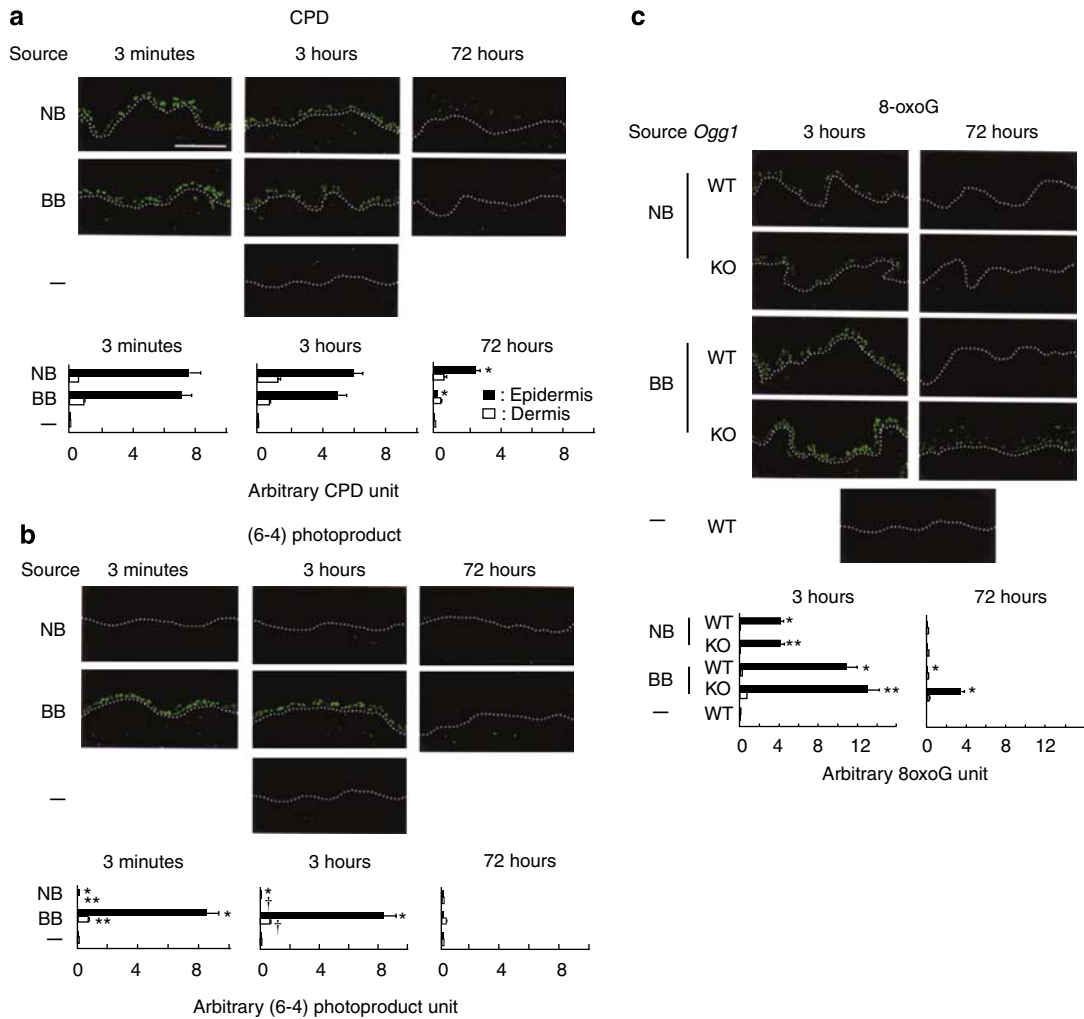


Figure 3. Kinetic study of epidermal DNA damages after NB-UVB or BB-UVB. Immunostaining of CPD, (6-4) photoproduct, and 8-oxoG in skin specimens using TDM-2, 64M-1, N45.1, at upper panel of (a-c), respectively, were measured 3 minutes, 3, and 72 hours after NB-UVB or BB-UVB exposure at the dose of 1 MED along with *Ogg1* knockout (KO) mice. The boundary between epidermis and dermis is marked with a broken line. Representative photomicrographs of at least three independent experiments are shown. Bar = 100 μ m. Relative levels of CPD, (6-4) photoproduct, and 8-oxoG in epidermis and dermis were determined by densitometry in lower panel of (a-c), respectively. **P*, ***P*<0.0001, †*P*<0.05.

Induction of skin tumors: comparison between NB-UVB and BB-UVB in *Ogg1* wild and knockout mice

Furthermore, we evaluated the skin tumor induction by chronic NB-UVB or BB-UVB exposure in relevance of oxidative DNA damage caused by *Ogg1* gene disruption. As shown in Figure 4, in wild-type mice, the first appearance of tumor was 3 weeks earlier and the time of 100% incidence was 3 weeks earlier in NB-UVB-irradiated mice than in BB-UVB-irradiated mice. Tumor induction by NB-UVB was not significantly different irrespective of *Ogg1* genotype.

DISCUSSION

In this study, we showed that chronic exposure of 1 MED of NB-UVB induces malignant skin tumor in mice at significantly higher frequency than that of BB-UVB (albino hairless mice: *P*=0.026; C57BL/6J mice: *P*=0.022) (Tables 1 and 2 and Figure 2a and b). However, there was no significant

difference in the number of tumors induced by these two UV sources. To begin this comparative photocarcinogenesis study between NB-UVB and BB-UVB irradiation on mice, we had to determine a MED for each strain of mouse. Setting a MED for NB-UVB or BB-UVB for mice or humans is the most critical parameter, which might lead to different outcomes. There are, however, several reports which have determined the optimal ratios of doses for NB-UVB or BB-UVB to induce erythema (Wulf *et al.*, 1994; Gibbs *et al.*, 1995). Originally, the ratio of BB/NB-UVB was calculated by weighting the spectral irradiance according to the Commission Internationale de l'Eclairage (CIE) erythema action spectrum for human skin and was 1:4.2 (Diffey *et al.*, 1984). After that report, Flindt-Hansen *et al.* reported that the ratio of BB/NB-UVB was 1:10.8 and that NB-UVB was more carcinogenic. The ratio of 1:7.4 and 1:6.3 was also reported for pigmented hairless and albino hairless mice, respectively

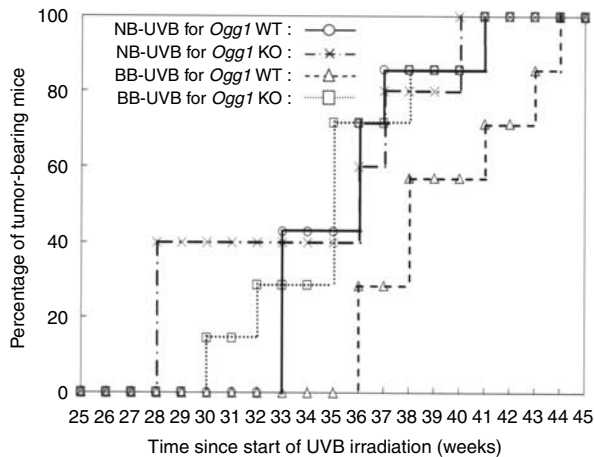


Figure 4. Difference of tumor induction following NB-UVB or BB-UVB with *Ogg1* genotype. Effects of chronic NB-UVB and BB-UVB exposure on the onset and incidence of tumors along with *Ogg1* genotype. Mice that died during the experiment were not included. The graphs of BB-UVB were overlaid from the data in our previous work.

(Wulf *et al.*, 1994; Gibbs *et al.*, 1995). In contrast, there was another report that the ratio was 1:2.8 for humans (Hansen *et al.*, 1994). Our data on the ratio of BB/NB-UVB that induced a MED was determined in our laboratory, which simulated the clinical situation for humans (Figure 1b). We obtained a ratio 1:2.2 for albino hairless mice and 1:3.4 for C57BL/6J mice. Our measurement of the BB/NB-UVB-induced MED is similar to a report by Hansen *et al.*, who speculated that wavelengths below 298 nm or more than 328 nm would be overestimated from the CIE erythema action. The ratio of malignant tumor induced by NB-UVB versus that by BB-UVB is higher in C57BL strain (91.6 vs 50.0%) in comparison with that of albino (80.0 vs 61.2%). The reason for this is possibly because the ratio of NB/BB-UVB dose irradiated to C57BL is higher (3.4 times) than that irradiated to albino hairless (2.2 times) if the dose is determined by MED.

To elucidate the specific mechanism of the higher carcinogenicity of NB-UVB, we focused on types of DNA damage induced by UVB, such as CPD, (6-4) photoproduct, and 8-oxoG. One reason why we focused on the DNA damage is that there have been reports indicating scale differences in the action spectrum between the induction of CPD and (6-4) photoproduct observed in the UVB spectrum (Mitchell, 1988; Matsunaga *et al.*, 1991; Mizuno *et al.*, 1991). Another reason is that recently the involvement of reactive oxygen species in carcinogenesis has been suggested (Nishigori *et al.*, 2004; Kunisada *et al.*, 2005). Although UVB has been shown to induce 8-oxoG, we do not know the action spectrum for 8-oxoG formation. Thus, we consider it is of importance to see whether NB-UVB is highly productive of 8-oxoG or not. We characterized the accumulation of such DNA damage caused by NB-UVB or BB-UVB irradiation using monoclonal antibodies and found that significantly fewer (6-4) photoproduct were produced by NB-UVB,

whereas BB-UVB produced both CPD and (6-4) photoproduct (Figure 3a and b). Moreover, CPD produced by NB-UVB remained even 72 hours after exposure, which implies that CPD induced by NB-UVB are more abundant in epidermal cells, since the repair ability should be the same among the inbred strain. The mice were irradiated with the UVB dose inducing 1 MED both for tumor induction and DNA damage detection, and this dose seems to be too high to accurate quantification of CPD fluorescence intensity at post-24 hours UVB exposure. Thus, time course studies were performed to assess the accumulation of CPD at 3 minutes, 3, and 72 hours after exposure with precision. These suggest that the more efficient induction of malignant skin tumors by NB-UVB can be attributed to CPD formations in epidermal cells rather than to (6-4) photoproduct formation. There are many reports indicating the link between CPD induced by UV exposure and the UV-induced mutations in tumor-related genes such as *p53* (Giglia-Mari and Sarasin, 2003), which explains our results. Our results showed that the ratio of malignant tumor formation is higher in NB-UVB-exposed group although the number of tumors are not different, which might imply the larger amount of CPD is involved not only in the initiation phase (the formation of mutation) but also modulate the immunological response in a way that favors tumor development, since there are several pieces of evidence that CPD play a very important role in UV-induced immune suppression (Nishigori *et al.*, 1996). This is early evidence that shows the higher amount of CPD is formed by NB-UVB, which partly explains the higher incidence of malignant tumors induced by NB-UVB. Our immunofluorescence time course studies also revealed that much higher accumulation of (6-4) photoproduct 3 hours after BB-UVB than that after NB-UVB exposure result from production of larger amounts of (6-4) photoproduct by BB-UVB than NB-UVB, since there are significant differences in the accumulation of (6-4) photoproduct as early as 3 minutes after irradiation.

Recently, the involvement of 8-oxoG has been suggested in photocarcinogenesis (Basset-Seguín *et al.*, 1994; Agar *et al.*, 2004; Nishigori *et al.*, 1994, 2004). Indeed, we previously found that BB-UVB exposure generates 8-oxoG in epidermal cells and that *Ogg1* knockout mice, which are not able to remove the oxidized base from DNA, are more prone to having skin cancers following chronic BB-UVB irradiation, which indicates that oxidative DNA damage caused by BB-UVB exposure, as well as pyrimidine photoproducts, plays an important role in the development of skin cancers (Kunisada *et al.*, 2005). There was no significant difference in either mean number of tumor nor in ratio of malignant tumor formation induced by continuous NB-UVB exposure between *Ogg1* knockout mice and wild-type counterpart (Table 2 and Figure 2b). Although the onset of developing skin tumor with chronic NB-UVB irradiation in *Ogg1* knockout mice appears earlier than that in wild-type mice, the tumor induction lines afterwards are similar irrespective of *Ogg1* genotype, which indicates that oxidative DNA damage is rarely responsible for NB-UVB-induced carcinogenesis. As for the amount of 8-oxoG produced by NB-UVB and BB-UVB, there are several controversial reports (Budiyanto *et al.*, 2002; Orimo *et al.*,

2006). Therefore, we assessed accumulation of 8-oxoG by single NB-UVB exposure using each *Ogg1* genotype mice as well. Immunohistochemically no significant difference in intensity of 8-oxoG staining was observed after NB-UVB exposure irrespective of *Ogg1* genotype (Figure 3c), indicating that very little amount of 8-oxoG is produced by NB-UVB. Figure 3c also showed that much less amount of 8-oxoG was formed after 1 MED by NB-UVB in comparison to that by BB-UVB, which is consistent with those reported by Orimo *et al.* (2006).

As for tumor induction, wild-type mice irradiated with NB-UVB began to develop tumors 3 weeks earlier than with BB-UVB, and 100% incidence was at 41 weeks, 3 weeks earlier than with BB-UVB (Figure 4). This shows that NB-UVB also promotes the onset of tumor development earlier than BB-UVB, which is compatible with the report by Flindt-Hansen *et al.*

In conclusion, we demonstrate that CPD formation in epidermal cells, rather than formation of (6-4) photoproduct or 8-oxoG, is closely related to the carcinogenic activity of NB-UVB. The most important factor to be considered is the balance between the efficiency and carcinogenicity of NB-UVB treatment required for treating several skin diseases (Gibbs *et al.*, 1995; Weischer *et al.*, 2004). If the tumor incidence is plotted to the total accumulative dose, BB-UVB induces skin tumors by smaller dose (Supplementary data). However, we usually use higher dose in treating with NB-UVB when we decide the initial therapeutic dose being standardized by MED. There are reports that the effectiveness of NB-UVB treatment has an advantage over BB-UVB treatment for psoriasis (Coven *et al.*, 1997, Walters *et al.*, 1999). NB-UVB phototherapy has to be considered in the balance between the higher carcinogenic characteristics and the higher therapeutic benefits. Thus, it will be ideal if we will be able to determine the optimum dose and wavelength to obtain the effective immunomodulation without carcinogenicity.

MATERIALS AND METHODS

Mice

Albino hairless albino mice (Balb/cA Kud-hr) were used for the comparative study of NB-UVB and BB-UVB continuous irradiation. C57BL/6J *Ogg1* knockout mice (Sakumi *et al.*, 2003), together with wild-type mice, were used to study skin tumor production following NB-UVB exposure. We inbred *Ogg1* heterozygous mice (C57BL/6J, $N=12$) and genotype was determined as described previously (Sakumi *et al.*, 2003). These mice were also used for DNA damage detection by UVB exposure using immunohistochemical study. Mice aged 12–15 weeks were selected and divided into groups of 10 mice each corresponding to each type of UVB source and *Ogg1* genotype, in wild-type and *Ogg1* knockout mice. The mice were housed under special pathogen-free conditions, and all animal experiments were conducted according to the Guideline for Animal Experimentation at Kobe University School of Medicine.

NB-UVB and BB-UVB irradiation

Banks of six TL 20W/01RS and TL 20W/12RS fluorescent lamps (Philips, Eindhoven, Holland) were used to irradiate the mice with

NB-UVB or BB-UVB, respectively. TL 20W/01RS lamps emit in a narrow peak around 311 nm exclusively. TL 20W/12RS lamps emit a continuous spectrum from 275 to 390 nm, with a peak emission at 313 nm; approximately 65% of that radiation is within the UVB wavelength range (Figure 1a). The irradiance was 7.6 J/m²/second for TL 20W/01RS lamps and 3.8 J/m²/second for TL 20W/12RS lamps at a distance of 40 cm, as measured by an IL1400A radiometer/photometer (International Light Inc., Peabody, MA), or an UVR-305/365D digital radiometer (Tokyo Kogaku Kikai KK, Tokyo, Japan), respectively. For skin tumor production, the mice were placed 40 cm below the bank of lamps and were irradiated three times per week over 30 weeks (for albino hairless mice) and over 40 weeks (for C57BL/6J mice, after shaving their backs). For the TL 20W/01RS lamps, exposures of 370 or 850 mJ/cm² are the approximate sub-MEDs for albino hairless mice and for C57BL/6J mice, respectively. Exposures with 170 or 250 mJ/cm² are the approximate MEDs for albino hairless mice and for C57BL/6J mice, respectively, for the TL 20W/12RS lamp (Figure 1b). For immunohistochemical detection of CPD, (6-4) photoproduct and 8-oxoG after UVB irradiation, mice aged 12 weeks were irradiated with the same doses of MED irradiance corresponding to each type of UVB source.

Immunohistochemistry

For detection of CPD or (6-4) photoproduct, skin specimens were obtained at 3 minutes, 3 and 72 hours after NB-UVB or BB-UVB irradiation. For detection of 8-oxoG, skin specimens were taken at 3 and 72 hours after NB-UVB or BB-UVB irradiation. Skin specimens were subjected to immunohistochemical staining as described previously (Kunisada *et al.*, 2005) using primary mouse monoclonal antibodies against CPD (TDM-2), (6-4) photoproduct (64M-1) (Mizuno *et al.* 1991), or 8-oxoG (N45.1) (Hattori *et al.*, 1996). Specimens were observed using an Olympus FluoView confocal laser scanning microscope (Olympus Co. Ltd., Nagano, Japan).

Observation and measurement of cumulative tumor incidence

After the chronic UVB exposure, we observed tumor formation until all mice developed skin tumors. The numbers of tumors with diameters larger than 2 mm in diameter were counted. Tumors less than 2 mm in diameter, or those that regressed, were not counted. The numbers of tumors on mice that died during the experiment were not included. All mice were killed at the final observation and all skin tumors were excised and examined histologically with hematoxylin and eosin staining. In addition, all mice including mice that died during the experiment were subjected to autopsy to confirm whether they had internal spontaneous tumors macroscopically.

Histological analysis

Tumors examined histologically were classified as malignant or benign. Malignant tumors were classified as squamous cell carcinomas (tumors with atypical epithelial differentiation) or sarcomas (tumors with atypical mesenchymal differentiation), whereas benign tumors were classified as papillomas (tumors with papillomatous growth of epidermal cells without atypicality) or cysts.

Statistical analysis

Results are expressed as the mean \pm SD. Statistical differences were determined using an un-paired *t*-test for mean number of tumors/

mouse and the χ^2 test for malignant tumor rate; $P < 0.05$ is considered to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Figure S1. Difference of tumor induction following NB-UVB or BB-UVB with *Ogg1* genotype according to total dose of irradiation.

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