

**Fig. 6** RT-PCR analysis of mRNA expression of cytokines and chemokine receptors in immune LNC. LNC were taken from mice 1 h (day 1), 48 h (day 3) and 96 h (day 5) after sensitization with KP plus UVA. Each assay was performed four times using two independent samples. Representative data were shown. Veh: vehicle alone.

responder haplotype [4]. Thus, the susceptibility to photocontact dermatitis in individuals appears to be different depending on each photocontactant. The majority of exogenous photoallergic substances have a photohaptenic property [2,17]. Photohaptens are capable of binding to MHC class II molecules/self peptides on LC upon exposure to UVA [18]. In this context, the T cell response is likely controlled by MHC molecules.

In the adoptive transfer study, injection of CD4<sup>+</sup> T cells was crucial to evoke the sensitivity, but transfer of both CD4<sup>+</sup> and CD8<sup>+</sup> cells resulted in a higher response. In accordance with the present study, cutaneous photoallergy to exogenous agents is mediated by CD4<sup>+</sup> T cells [4,17,18]. The roles of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in ordinary contact hypersensitivity remains disputed. Several independent studies have shown mediation of the sensitivity by CD8<sup>+</sup> T cells [19–21]. On the other hand, the contribution

of CD4<sup>+</sup> cells has been variously reported, as CD4<sup>+</sup> cells are unnecessary [21], helpful [22,23], or suppressive [24,25]. Circumstantial evidence may indicate that CD4<sup>+</sup> cells participate more profoundly in photocontact hypersensitivity than ordinary contact hypersensitivity. For example, in vitro stimulation of immune LNC with photohaptens results in the preferential propagation of CD4<sup>+</sup> cells, and the sensitivity can be transferred to naïve mice with CD4<sup>+</sup> T cell line [17]. In such a case, CD8<sup>+</sup> T cells may be required for the full development of the sensitivity.

In the draining LNC, mRNAs for not only IFN-γ and CXCR3 but also IL-4 and CCR4 were increasingly expressed. Rather, the expression levels of these Th2-relevant molecules were higher than those of Th1. Such a Th2 dominant state was also found in photosensitivity to TCSA [26]. In the skin, keratinocyte-derived chemokines initiate migration of T cells. mRNAs for TARC (a ligand for CCR4) and Mig

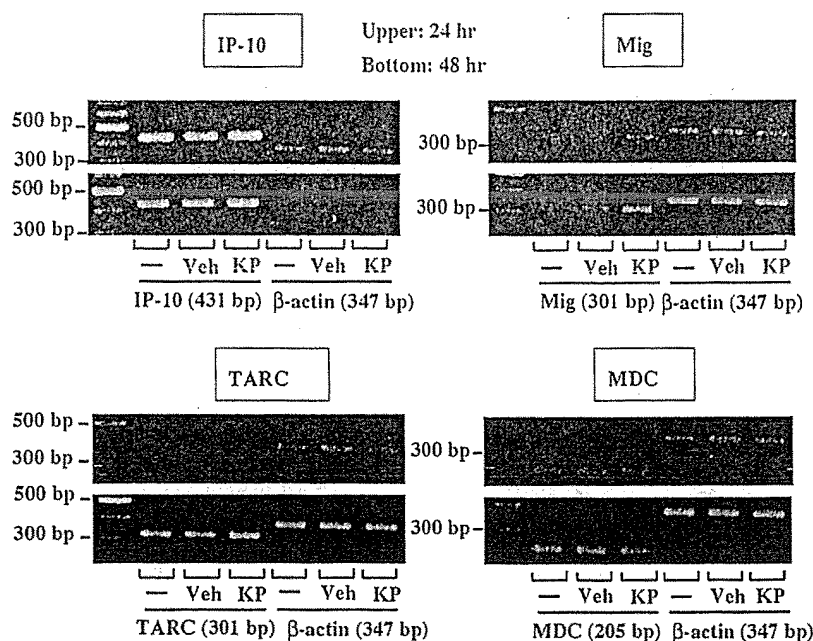


Fig. 7 RT-PCR analysis of chemokine mRNA expression in epidermal cells. Epidermal cell suspensions were prepared from earlobes of KP/UVA-sensitized mice 24 or 48 h after challenge with KP plus UVA. Each assay was performed four times using two independent samples. Veh: vehicle alone.

(a ligand for CXCR3) were also enhanced in the epidermal cells from earlobes of mice sensitized and challenged with KP plus UVA. This chemokine expression is different from that of ordinary contact sensitivity to picryl chloride, which shows apparent Th1 chemokine mRNA expression but no TARC expression in the challenged ears [27]. Of particular importance is whether these Th2 cells serve as effectors or suppressors in the sensitivity. We measured the percentage of CD4<sup>+</sup>CD25<sup>+</sup> cells, indicative of regulatory T cells [24,25], in immune LNC from KP- or TCSA-photosensitized AKR/N or BALB/c mice, and found no increment of cells bearing this phenotype (data not shown). Together with the ability of CD4<sup>+</sup> T cells to transfer the sensitivity, these findings implies that CD4<sup>+</sup> cells play a helper or effector role in photocontact dermatitis to KP.

Photocontact dermatitis to KP is known to prolong at the applied skin site even several months after cessation of application [9–11]. This enigmatic phenomenon cannot be clarified from the present study. In addition to its photohaptenic moiety, KP might exert its pharmacological effect on LC [28], possibly leading to the prolongation. Elucidation of this phenomenon may characterize this photosensitivity more specifically.

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# 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

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Psoralen plus ultraviolet A (PUVA) and narrowband ultraviolet B (UVB) are widely used in skin disease phototherapy. Recently, the efficacy of UVB therapy has been greatly improved by narrowband UVB, compared to conventional broadband UVB. The objectives of the current study were to evaluate the influence of UVB-induced and PUVA-induced oxidative stress on cultured keratinocytes. We analyzed 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in human keratinocytes (HaCaT cell line) using a high-performance liquid chromatography system equipped with an electrochemical detector. Non-irradiated human keratinocytes contained a baseline of  $1.48 \pm 0.22$  (mean  $\pm$  SD) 8-OH-dG per  $10^6$  deoxyguanosine (dG) residues in cellular DNA, which increased linearly with higher doses of UVB. When their abilities to induce 8-OH-dG were compared to each other, based on the minimal erythema and therapeutically used doses, by irradiating them with broadband UVB at  $100 \text{ mJ/cm}^2$ , the amount of 8-OH-dG increased to  $3.42 \pm 0.46$  residues per  $10^6$  dG, while a narrowband UVB treatment at  $1000 \text{ mJ/cm}^2$ , with biological effects comparable to those elicited by  $100 \text{ mJ/cm}^2$  broadband UVB, increased it to  $2.06 \pm 0.31$  residues per  $10^6$  dG. PUVA treatment, with  $100 \text{ ng/mL}$  8-methoxypsoralen and  $5000 \text{ mJ/cm}^2$  UVA, increased the 8-OH-dG level to  $4.52 \pm 0.42$  residues per  $10^6$  dG. When HaCaT cells treated with  $2000 \text{ mJ/cm}^2$  narrowband UVB were cultured and the amount of 8-OH-dG was monitored in the living cells, 65.6% of the residues were repaired 24 h after treatment. Our study provides a warning that widely used narrowband UVB and PUVA induce cellular oxidative DNA damage at the therapeutically used doses, although to a lesser degree than broadband UVB with the same clinically effective dose. (*Cancer Sci* 2006; 97: 99–105)

**E**ight-hydroxy-2'-deoxyguanosine (8-OH-dG), also known as 7,8-dihydro-8-oxo-deoxyguanosine (8-oxo-dG),<sup>(1)</sup> has been proposed as a key biomarker of oxidative DNA damage relevant to carcinogenesis<sup>(1,2)</sup> and pathogenesis of autoimmune disorders.<sup>(3,4)</sup> This DNA damage is induced by the reactions of reactive oxygen species (ROS), such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), superoxide anions ( $\text{O}_2^-$ ), singlet oxygen and hydroxyl radicals ( $\cdot\text{OH}$ ).

Human skin is constantly exposed to environmental stresses, and is vulnerable to the effects of ROS generated by exposure to ultraviolet (UV) radiation.<sup>(5)</sup> Yamamoto *et al.*<sup>(6)</sup> reported that

the formation of 8-OH-dG in DNA might be one of the mechanisms of daylight-induced mutagenesis. In fact, irradiation with a fluorescent sun lamp or with UVB does induce 8-OH-dG in the epidermis of hairless mice.<sup>(7,8)</sup>

Parrish and Jaenicke<sup>(9)</sup> found that 313 nm UVB radiation is the most effective wavelength for the treatment of psoriasis. This finding provided the impetus for developing the Philips TL-01 fluorescent bulb, a narrowband UVB light source that produces a spectral emission between 310 and 315 nm. Narrowband UVB phototherapy has thus significantly improved the therapeutic efficacy of conventional broadband UVB (290–320 nm) phototherapy for skin diseases such as psoriasis, atopic dermatitis, vitiligo and others.<sup>(10–13)</sup>

Narrowband UVB is widely used in the treatment of skin disease, and the current trend toward the increased use of narrowband UVB phototherapy is justified.<sup>(14)</sup> Its carcinogenic potential is judged to be substantially less than that of psoralen plus UVA (PUVA) photochemotherapy.<sup>(15)</sup> Although the results of studies in mice indicate that narrowband UVB could induce more skin cancers than broadband UVB therapy,<sup>(16)</sup> the participants in a workshop on the use of narrowband UVB in phototherapy concluded that the long-term human cancer risk should be no greater than that with broadband phototherapy.<sup>(17)</sup>

When the DNA damage in keratinocytes induced by narrowband or broadband UVB was measured by single cell gel electrophoresis (comet assay), narrowband UVB produced less DNA damage than broadband UVB at equal doses.<sup>(18)</sup> The formation of 8-OH-dG has also been reported in fibroblasts after UVA irradiation<sup>(19–21)</sup> and in normal human epidermal keratinocytes after broadband UVB exposure.<sup>(22)</sup> Using immunofluorescence staining methods, Budiyo *et al.*<sup>(23)</sup> observed that in both mouse skin and organ cultured human skin cells, 250 and 500  $\text{mJ/cm}^2$  narrowband UVB yielded levels of cyclobutane pyrimidine (CPD/Py-Py) dimers similar to those induced by 25 and 50  $\text{mJ/cm}^2$  broadband UVB, respectively, which have biological effects comparable to 250 and 500  $\text{mJ/cm}^2$  narrowband UVB, respectively. However, the yields of 8-OH-dG after irradiation with 1000 and 3000  $\text{mJ/cm}^2$

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narrowband UVB were 1.5–3 times higher than those obtained using 100 and 300 mJ/cm<sup>2</sup> broadband UVB, respectively.

The ratio of Py–Py dimers to 8-OH-dG formation in cellular DNA after UVB irradiation is 80–100:1,<sup>(19,24)</sup> while the ratio of psoralen-adducts to 8-OH-dG formation by PUVA treatment is 25:1.<sup>(25)</sup> Although UVB and PUVA treatments induce Py–Py dimers and psoralen-DNA adducts, respectively, as major cellular DNA modifications, our study focused on the analysis of 8-OH-dG as a marker of cellular oxidative stress for the following reasons: (1) not only initiation but also chronic inflammation-induced promotion and progression may be involved in UVB-induced skin carcinogenesis;<sup>(26,27)</sup> (2) antioxidants inhibit both UVB-induced 8-OH-dG formation and carcinogenesis in mouse skin;<sup>(28,29)</sup> and (3) in *Ogg1* (8-OH-Gua glycosylase) knockout mice, UVB irradiation induced both 8-OH-dG formation and an increase in skin tumors, suggesting that 8-OH-dG is involved in UVB-induced skin carcinogenesis.<sup>(30)</sup>

The purpose of the present study was to assess the oxidative stress induced by clinically used UV wavelengths, doses and apparatus. There has been no accurate analysis reported using a high-performance liquid chromatography (HPLC) system equipped with an electrochemical detector (ECD) of 8-OH-dG in human keratinocytes irradiated with narrowband UVB. PUVA is another modality whose potential to form 8-OH-dG should be investigated, because PUVA is the most widely used phototherapy for skin diseases. However, the PUVA-induced formation of 8-OH-dG has been reported only for human epidermoid carcinoma cells.<sup>(31)</sup>

In the present study, we quantified the 8-OH-dG formed in keratinocytes (HaCaT) after irradiation with clinically used doses of broadband and narrowband UVB, and PUVA. Our results provide information about the oxidative DNA damage-inducing potencies of these three phototherapies and the repair of 8-OH-dG.

## Materials and Methods

### Cells and culture conditions

The HaCaT cell line<sup>(32)</sup> was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan), supplemented with 10% fetal bovine serum, L-glutamine (2 mM), 100 units/mL penicillin, 100 µg/mL streptomycin sulfate and sodium pyruvate (1 mM), and was maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. Unless otherwise mentioned, all culture supplies were purchased from Gibco-Invitrogen (Carlsbad, CA, USA).

### Ultraviolet irradiation of cells

The cells were seeded into 100-mm tissue culture dishes and allowed to attach for a period of 16–24 h at 37°C. Before UV irradiation, the culture medium was removed and 5 mL of phosphate-buffered saline (PBS, pH 7.4) were placed over the monolayer, so that the depth of the solution was always 0.1 cm, to prevent cell drying and reflection of UV. A total of approximately 5 × 10<sup>6</sup> keratinocytes in a 100-mm dish were exposed to UV irradiation at room temperature. Broadband UVB irradiation was applied at a wavelength range of 280–370 nm, peaking at 305 nm, using a bank of five FL20SE.30 medical sun lamps (Toshiba, Tokyo, Japan) emitting mainly

UVB, but also small amounts of UVA and UVC. The irradiation was 1.0 mW/cm<sup>2</sup> at a distance of 33 cm, as measured with a radiometer (UVR-3036/S; Toshiba). Narrowband UVB irradiation was carried out with a bank of four TL-20 W/01 lamps (Philips, Eindhoven, Holland) at a wavelength range of 310–315 nm (emission maximum at 313 nm, almost monochromatic) housed in a luminaire (type UV801 KL-1; Waldmann, Villingen-Schwenningen, Germany). For PUVA treatment, keratinocytes were exposed to UVA produced by six 40-watt CLEO lamps (Philips) at a wavelength range between 315 and 400 nm with a peak emission at 355–365 nm, housed in a Waldmann luminaire. The distance from the light source was maintained at 25 cm. The dosimetry was monitored with a UV meter (type 585200000; Waldmann) equilibrated for the UV sources according to the manufacturer's instructions. Control cells were incubated in PBS without irradiation. At several time points after irradiation, the adherent cells were harvested, washed with ice-cold PBS and processed immediately for DNA isolation.

### Cell viability

A portion of each cell suspension obtained from the control and irradiation experiments was used to determine cell viability. Cell viability was determined using the trypan blue dye exclusion test (0.4%) (Gibco-BRL, Grand Island, NY, USA). Due to the toxicity of UV light, we collected the adherent cells immediately after irradiation unless otherwise mentioned. As damaged cells gradually became detached during the culture period, depending on the UV irradiance, only adherent cells with viability above 90% were subjected to the analysis in the time course experiments.

### PUVA treatment

Stock solutions were prepared by dissolving crystalline 8-methoxypsoralen (8-MOP) (Sigma, St Louis, MO, USA) in absolute ethanol (100 µg/mL). Before UVA irradiation, 10 µL of the 8-MOP stock solution were added to 10 mL of PBS for the keratinocyte culture. A final 8-MOP concentration of 100 ng/mL was chosen, as the mean plasma concentration in humans receiving PUVA therapy is approximately 100 ng/mL.<sup>(33)</sup> After an incubation at 37°C for 30 min in the dark, the cells were irradiated with UVA.

### Determination of 8-OH-dG in cellular DNA

Cellular DNA was isolated using a DNA extractor WB kit containing NaI (Wako, Osaka, Japan).<sup>(34,35)</sup> Desferal (deferrioxamine mesylate; Sigma) was added to the lysis solution (1 mM) to prevent DNA oxidation.<sup>(36)</sup> The isolated DNA was digested with 8 units of nuclease P1 (Yamasa, Choshi, Japan) in a 100 µL solution containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM sodium acetate (pH 4.5), and was then treated with alkaline phosphatase (2 units) in a 250 mM Tris-HCl (pH 8.0) buffer. This solution was filtered with an Ultrafree-Probind filter (Millipore, Bedford, MA, USA) and a 70 µL aliquot of the sample was injected onto an HPLC column (Shiseido Fine Chemicals, Tokyo, Japan 5 µM, 4.6 × 250 mm, 27°C, flow rate 1.0 mL/min) equipped with an ECD (Coulchem II, ESA, Chelmsford, MA, USA; electrode 1, 150 mV; electrode 2, 300 mV; guard cell, 350 mV). The mobile phase consisted of 10 mM phosphate buffer (pH 6.7)

containing 8% methanol. As the standard samples, 20- $\mu$ L aliquots of the deoxyguanosine (dG) (0.5 mg/mL) and 8-OH-dG (5 ng/mL) solutions were injected. The concentration of test samples was determined by comparison to the standards. The 8-OH-dG level in the DNA was expressed as the number of 8-OH-dG per  $10^6$  dG.

#### Efficiency of DNA synthesis in HaCaT cells after UVB irradiation

HaCaT cells were cultured in 96-well plates (Corning Glass Works, Corning, NY, USA) until semiconfluent. After the culture medium was replaced by PBS, the cells were irradiated with UVB. The irradiated cells were further cultured in medium for 24 h, and  $^3$ H-thymidine (1  $\mu$ Ci/well; Amersham International, Amersham, UK) was added for the last 12 h. Adherent cells were detached with EDTA/trypsin and collected on glass fibers using a cell harvester, and radio-uptake was measured in a scintillation counter.

#### Statistical analysis

All analyses were carried out using the StatView-J® 5.0 program (SAS Institute, Cary, NC, USA). All of the data are expressed as the mean  $\pm$  SD from four to five independent measurements. Statistical significance was determined by the Student's *t*-test, using  $P < 0.05$  as the level of significance.

## Results

#### Quantification of 8-OH-dG in HaCaT cells irradiated with broadband or narrowband UVB

HaCaT cells were exposed to broadband or narrowband UVB at various doses, and the 8-OH-dG formed in the cells was measured. Figure 1 shows a representative 8-OH-dG analysis. The hatched peak in Fig. 1a is derived from authentic 8-OH-dG. Untreated cells had a small but discernible amount of 8-OH-dG (Fig. 1b). Irradiation of cells with 1000 mJ/cm<sup>2</sup> narrowband UVB increased the amount (Fig. 1c).

As shown in Fig. 2, the level of 8-OH-dG in untreated cells was  $1.48 \pm 0.22$  per  $10^6$  dG. Irradiation of cells with broadband UVB (50–500 mJ/cm<sup>2</sup>) induced 8-OH-dG formation in a dose-dependent manner (Fig. 2a), and 0.0113 residues per mJ/cm<sup>2</sup> were estimated to be increased by broadband UVB on the per-dose basis.

At the low doses of 250 and 500 mJ/cm<sup>2</sup> of narrowband UVB, the amount of 8-OH-dG was not increased compared to that of the non-irradiated control (Fig. 2b). A significant augmentation of 8-OH-dG was found at 1000 mJ/cm<sup>2</sup> of narrowband UVB. The amount of 8-OH-dG produced by narrowband UVB at 2000 mJ/cm<sup>2</sup> ( $3.51 \pm 0.83$ ) was comparable to that generated by  $3.42 \pm 0.46$  of broadband UVB at 100 mJ/cm<sup>2</sup>. Therefore, broadband UVB seemed to induce approximately 20-fold higher oxidative DNA stress than narrowband UVB when compared at the same exposure dose.

The minimal erythema doses (MED) of broadband and narrowband UVB were 70–150 and 500–1200 mJ/cm<sup>2</sup> in Japanese normal subjects and patients with psoriasis or cutaneous T-cell lymphoma. Thus, approximately 10-fold higher doses of narrowband UVB than broadband UVB are used clinically. Given this 10-fold difference in the biological activities of the two UVB sources, the level of 8-OH-dG in

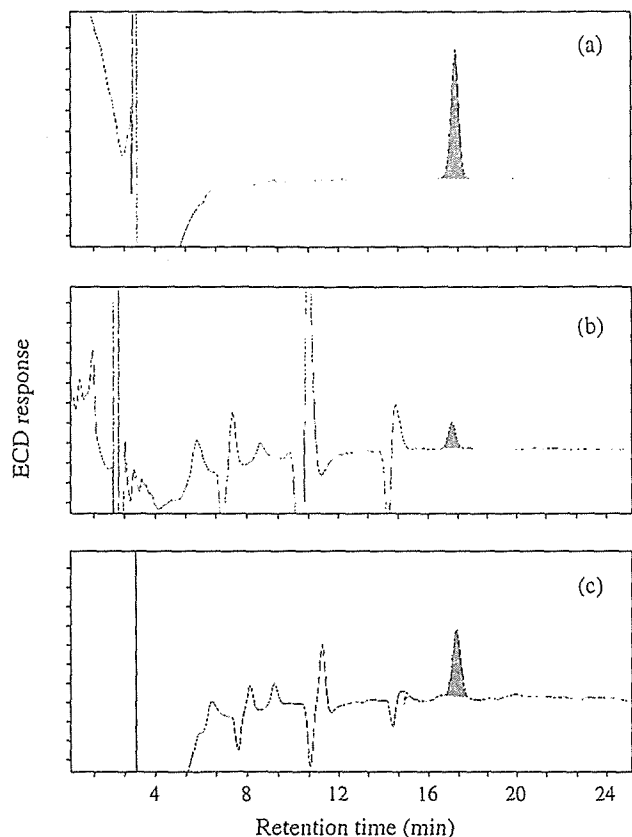


Fig. 1. Representative high-performance liquid chromatography (HPLC)-electrochemical detector (ECD) analyses of 8-OH-dG. DNA isolated from HaCaT cells was treated with the nuclease P1 protein, and a 70- $\mu$ L aliquot of each sample was subjected to HPLC-ECD analysis. (a) Authentic 8-OH-dG (100 pg), (b) DNA from unirradiated cells and (c) DNA from 1000 mJ/cm<sup>2</sup> narrowband ultraviolet B-irradiated cells. The amount of DNA in the injected samples (b,c) was adjusted.

1000 mJ/cm<sup>2</sup> narrowband UVB-treated cells ( $2.97 \pm 0.44$ ), for example, was less than that in 100 mJ/cm<sup>2</sup> broadband UVB-treated cells ( $3.42 \pm 0.46$ ). However, it should be considered that narrowband UVB yields considerable amounts of 8-OH-dG in clinical settings.

#### Quantification of 8-OH-dG in HaCaT cells treated with 8-MOP plus UVA

HaCaT cells were treated with 100 ng/mL of 8-MOP and various doses of UVA, or UVA alone. As shown in Fig. 3, UVA alone produced low levels of 8-OH-dG in a dose-dependent manner. Endogenous photosensitizers, such as porphyrins and flavins, which have UV absorption in the UVA range (320–400 nm), may be involved in this process. In contrast, the incubation of cells with 8-MOP before UVA irradiation (2000–10000 mJ/cm<sup>2</sup>) significantly enhanced 8-OH-dG formation. Because PUVA therapy usually starts with 100% of the minimal phototoxic dose (ranging from 500–5000 mJ/cm<sup>2</sup> UVA),<sup>(37)</sup> the amount of 8-OH-dG produced by narrowband UVB exposure is considered to be lower than that generated by PUVA therapy.

In the control experiments without UVA and with 8-MOP, the 8-OH-dG levels were higher than in those without UVA

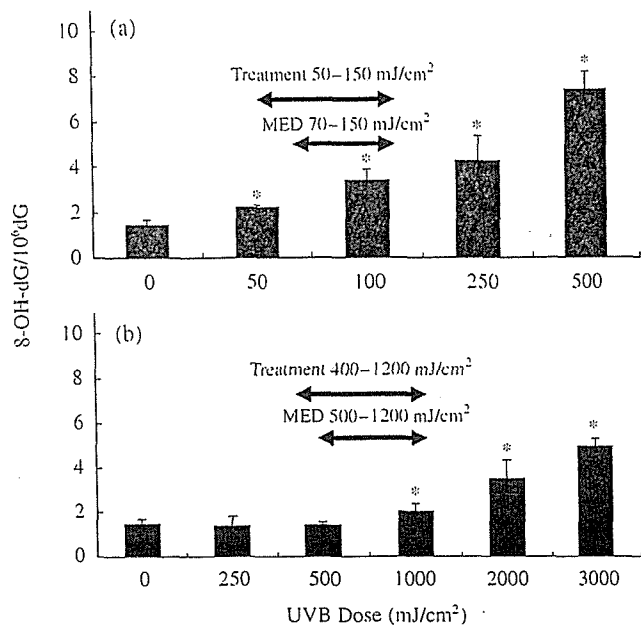


Fig. 2. Formation of 8-OH-dG in the cellular DNA of HaCaT cells irradiated with (a) broadband and (b) narrowband ultraviolet (UV) B. Data are expressed as the mean  $\pm$  SD of determinations on four to five independently irradiated dishes of keratinocytes. \* $P < 0.05$ , compared with the unirradiated samples (0 mJ/cm<sup>2</sup>, the background level). MED, minimal erythema doses of Japanese individuals whose skin types were III to IV, defined according to the Fitzpatrick classification.<sup>(54)</sup> Treatment: broadband UVB and narrowband UVB doses for psoriasis vulgaris in our institution.

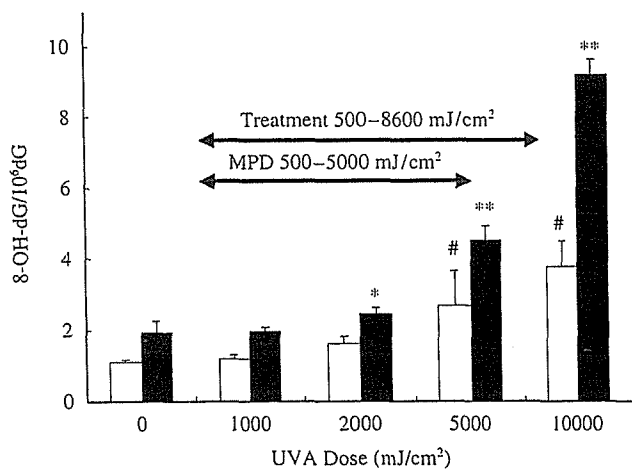


Fig. 3. Formation of 8-OH-dG in HaCaT cells treated with ultraviolet (UV) A or psoralen plus UVA (PUVA). HaCaT cells were incubated with 100 ng/mL 8-MOP and irradiated with various doses of UVA. The data represent the mean  $\pm$  SD of four to five experiments. \* $P < 0.05$ , \*\* $P < 0.001$ , compared without UVA and with 8-MOP. # $P < 0.01$ , compared without UVA and 8-MOP; MPD, minimal phototoxic doses. Skin was exposed to UVA 2 h after the ingestion of 8-MOP tablets. The MPD is defined as the dose that induced minimally perceptible erythema 72 h after irradiation.<sup>(55)</sup> Treatment: oral PUVA therapy doses for psoriasis vulgaris.<sup>(55)</sup>

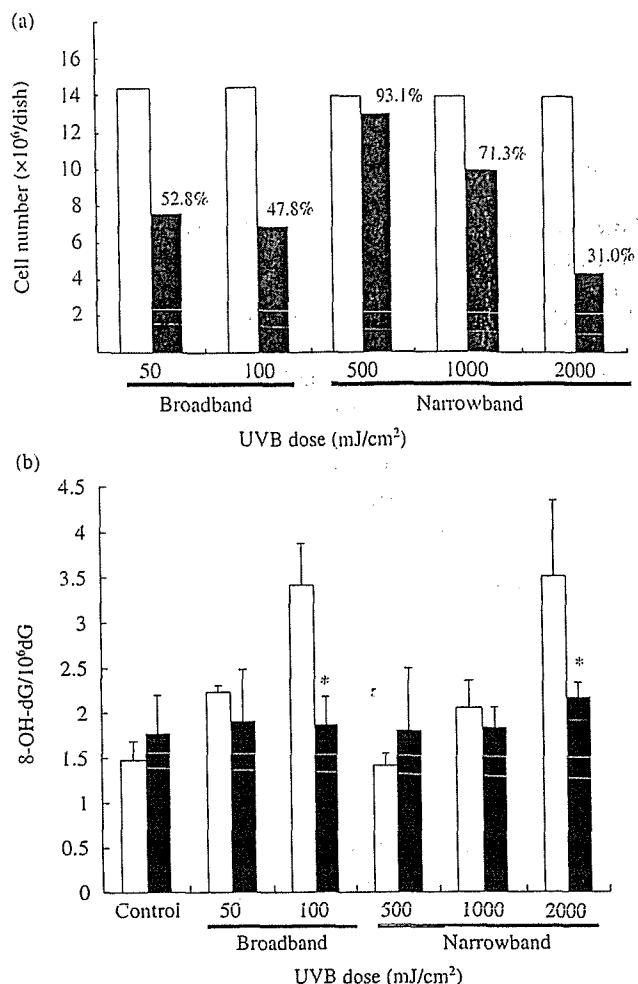


Fig. 4. (a) Viability levels of cells 24 h after ultraviolet (UV) B irradiation. □, Non-irradiated cells; ■, irradiated cells. (b) 8-OH-dG levels immediately (□) and 24 h after (■) UVB irradiation. HaCaT cells were irradiated with the indicated doses of broadband or narrowband UVB. \* $P < 0.05$ , compared with the value immediately after UVB irradiation. The results represent the mean  $\pm$  SD of four to five experiments.

and 8-MOP (Fig. 3). This may be due to artifactual formation of 8-OH-dG during DNA isolation under light.

#### Removal of 8-OH-dG in UVB-irradiated HaCaT cells

We compared the 8-OH-dG levels in HaCaT cells immediately after and 24 h after UVB irradiation. Viability levels of HaCaT cells 24 h after UVB irradiation are shown in Fig. 4a. As UVB exposure induced the detachment of HaCaT cells from the dish, depending on the UVB dose, we quantified the 8-OH-dG in the attached cells, so that only living cells were analyzed. As shown in Fig. 4b, the discernibly elevated 8-OH-dG amounts in the 100 mJ/cm<sup>2</sup> broadband and 2000 mJ/cm<sup>2</sup> narrowband UVB-irradiated HaCaT cells were significantly decreased after 24 h of culture. Therefore, the oxidative DNA damage in living keratinocytes seemed to be repaired within 24 h of UVB irradiation.

To further confirm the lack of influence of cell proliferation on the 8-OH-dG reduction, the <sup>3</sup>H-thymidine incorporation

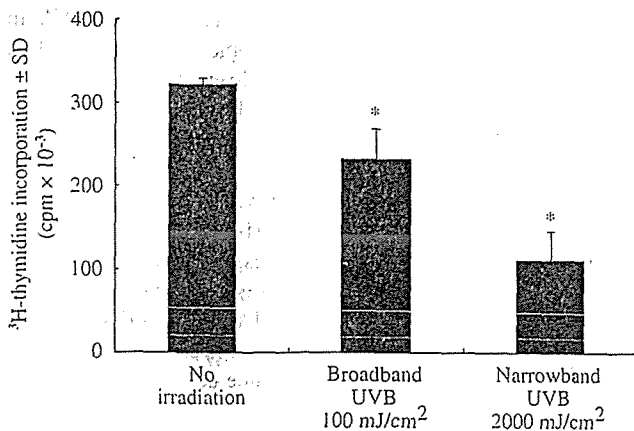


Fig. 5. Reduction of <sup>3</sup>H-thymidine incorporation in ultraviolet (UV) B-irradiated HaCaT cells. After irradiation with broadband UVB at 100 mJ/cm<sup>2</sup> and narrowband UVB at 2000 mJ/cm<sup>2</sup>, HaCaT cells were cultured for 24 h, and were pulsed with <sup>3</sup>H-thymidine for the last 12 h. \**P* < 0.05, compared with no irradiation.

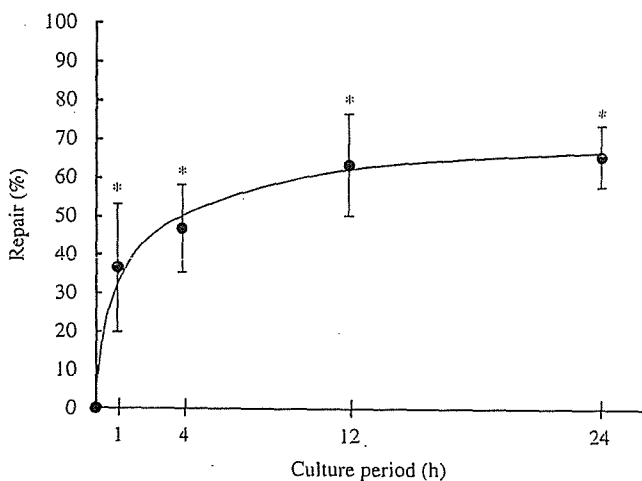


Fig. 6. 8-OH-dG repair rate after irradiation with narrowband ultraviolet (UV) B. HaCaT cells were irradiated with narrowband UVB at 2000 mJ/cm<sup>2</sup>, cultured for the indicated period, and subjected to the analysis. Repair (%) =  $(3.51 [\text{8-OH-dG level immediately after irradiation}] - X [\text{8-OH-dG level 1-48 h after irradiation}]) / (3.51 - 1.48 [\text{8-OH-dG level without irradiation}]) \times 100 = ((3.51 - X) / 2.03) \times 100$ . \**P* < 0.01, compared with the value immediately after irradiation.

by UVB-irradiated HaCaT cells was measured. The DNA synthesis levels in the cells treated with broadband UVB at 100 mJ/cm<sup>2</sup> and narrowband UVB at 2000 mJ/cm<sup>2</sup> were decreased after 24 h of culture (Fig. 5). Therefore, the 8-OH-dG formed in HaCaT cells was probably repaired during the cultivation.

The amount of 8-OH-dG was monitored in HaCaT cells at 1–24 h after 2000 mJ/cm<sup>2</sup> narrowband UVB irradiation and the repair rate was calculated. The viability of the cells attached to the dish was similar to that of the control. As shown in Fig. 6, the level of 8-OH-dG was reduced with time, and 65.6% of the 8-OH-dG was repaired at 24 h after UVB exposure.

## Discussion

Ultraviolet radiation produces ROS by photodynamic action,<sup>(38,39)</sup> which causes several kinds of DNA damage, such as 8-OH-dG, and eventually leads to mutations and abnormal cell proliferation.<sup>(8,40)</sup> Several techniques have been developed to detect 8-OH-dG. The measured background levels of 8-OH-dG differ, depending on both the DNA isolation technique and the 8-OH-dG analysis method.<sup>(41)</sup> To measure the steady-state level of DNA oxidation, HPLC-ECD is particularly useful because of its selectivity, sensitivity and ease of quantification. During the past two decades, improved DNA isolation techniques and enhanced HPLC-ECD sensitivity have considerably lowered the assayed background levels of 8-OH-dG.<sup>(34)</sup> Reliable data have been obtained mainly by an improved method that uses an iron chelator, desferal, in the lysis step.<sup>(36)</sup> In the present study, we also analyzed 8-OH-dG by HPLC-ECD, after DNA was isolated by the improved method.

Previous studies revealed that the number of 8-OH-dG residues in murine keratinocytes treated with UVB increases in an irradiance-dependent manner.<sup>(42–44)</sup> However, those techniques had a limitation derived from the artifactual oxidation of DNA during its extraction. It was recently reported that relatively low doses of UVB (62.5–500 mJ/cm<sup>2</sup>) cause dose-dependent increases in 8-OH-dG, and DNA from unirradiated normal human epidermal keratinocytes contains  $1.49 \pm 0.11$  8-OH-dG residues per  $10^6$  dG.<sup>(22)</sup> This is similar to the background level of 8-OH-dG observed in our study using HaCaT cells ( $1.48 \pm 0.22$ ). Furthermore, we report that narrowband UVB at a dose of more than 1000 mJ/cm<sup>2</sup> increases the amount of 8-OH-dG, but to a lesser degree than broadband UVB with the same clinically effective dose. The maximum recommended dose of narrowband UVB for atopic dermatitis and psoriasis is 1500 mJ/cm<sup>2</sup>.<sup>(45–47)</sup> We found that at the highest narrowband UVB dose, such as 1500 mJ/cm<sup>2</sup> used in clinical treatment, 8-OH-dG increased to 2.82 per  $10^6$  dG.

When the biological effects of broadband and narrowband UVB were assessed by the inhibition of macrophage-derived chemokine production, 10-fold higher doses of narrowband UVB than broadband UVB exerted a comparable inhibitory effect.<sup>(48)</sup> This is consistent with the observation that the MED and the therapeutic dose of narrowband UVB are approximately 10-fold higher than those of broadband UVB. Even when narrowband UVB at 1000 mJ/cm<sup>2</sup> was compared with broadband UVB at 100 mJ/cm<sup>2</sup>, the former induced fewer 8-OH-dG residues than broadband UVB.

Ultraviolet A-induced formation of 8-OH-dG has been observed in human skin fibroblasts,<sup>(19–21)</sup> and has been detected immunohistochemically in human keratinocytes.<sup>(49)</sup> Our study demonstrated that UVA induced a dose-dependent increase in 8-OH-dG with a fixed concentration of 8-MOP in keratinocytes. PUVA produced both singlet oxygen and superoxide anions in an *in vitro* system.<sup>(50)</sup> PUVA has already been reported to induce 8-OH-dG in the human epidermoid carcinoma cell line A431.<sup>(31)</sup> Upon irradiation of A431 cells with a fixed dose (2500 mJ/cm<sup>2</sup>) of UVA, the level of 8-OH-dG increased, depending on the concentration of 8-MOP. However, the background 8-OH-dG level was as high as 27 per  $10^6$  dG,<sup>(31)</sup> compared with 1.48 per  $10^6$  dG in our study.



The amount of 8-OH-dG formed by UVB was reduced in living cells during cultivation. As the cell number and the rate of DNA synthesis were decreased after UVB irradiation, the reduction in 8-OH-dG does not seem to result from cell proliferation and division. Therefore, it is likely that 8-OH-dG is successfully repaired in keratinocytes. The repair rate of 65.6% in 24 h is slightly lower than that of Py-Py dimers<sup>(51)</sup> and higher than that of 8-MOP-DNA photoproducts.<sup>(25)</sup> The kinetics of 8-OH-dG repair in the present study seem to be slower than those determined in the previous study by Osterod *et al.*<sup>(52)</sup> This may be explained by the presence of an overwhelming amount of Py-Py dimers in the irradiated DNA.

Clinically, narrowband UVB is as effective as PUVA in patients with psoriasis<sup>(37,45)</sup> and atopic dermatitis when administered in equi-erythemogenic doses.<sup>(46,53)</sup> The highest final doses of narrowband UVB and PUVA for these treatments were 2450 and 8600 mJ/cm<sup>2</sup>, respectively.<sup>(37)</sup> In the present study, narrowband UVB at 2000 mJ/cm<sup>2</sup> induced 3.51 8-OH-dG per 10<sup>6</sup> dG, and PUVA at 5000 mJ/cm<sup>2</sup> (8-MOP, 100 ng/mL) induced 4.52 8-OH-dG per 10<sup>6</sup> dG. Thus, narrowband UVB seems to induce less oxidative stress than PUVA at the clinically effective doses. However, this study provides a warning that widely used narrowband UVB and PUVA at the therapeutically used doses induces cellular oxidative DNA damage, which may induce cancer.

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# Prostaglandin E<sub>2</sub> is required for ultraviolet B-induced skin inflammation via EP2 and EP4 receptors

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Keratinocytes are the major target of sunlight, and they produce prostaglandin (PG) E<sub>2</sub> upon ultraviolet (UV) exposure. Although indomethacin, one of cyclooxygenase inhibitors, is known to suppress UV-induced acute skin inflammation, it remains uncertain whether endogenous PGE<sub>2</sub> is responsible for UV-induced skin inflammation, and which subtype of PGE<sub>2</sub> receptors mediates this process. UV-induced skin inflammation was investigated by using genetically and pharmacologically PGE<sub>2</sub> receptor-deficient mice. We applied UV-induced skin inflammation model to genetical and pharmacological PGE<sub>2</sub> receptor-deficient mice. We exposed UVB on these mice at 5 kJ/m<sup>2</sup>, and examined the ear swelling and the histological findings. We also measured the blood flow using a laser doppler device to assess the intensity of UVB-induced inflammatory change. The UV-induced ear swelling at 48 h after exposure was significantly reduced in EP2<sup>-/-</sup>, EP4<sup>-/-</sup> or wild-type mice treated with the EP4 antagonist compared to control mice. Consistently, inflammatory cell infiltration into the local skin, and local blood flow after UV exposure were significantly reduced by EP2 or EP4 signaling blockade. These data suggest that PGE<sub>2</sub>-EP2/EP4 signaling is mandatory in UV-induced acute skin inflammation, presumably by enhancing blood flow in the microenvironment.

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**Keywords:** EP2; EP4; PGE<sub>2</sub>; skin inflammation; ultraviolet; vasodilatation

Ultraviolet light (UV) radiation has a wide variety of actions, such as sunburn formation, immuno-suppression, skin aging and cancer development. Acute skin inflammation is another effect of UV radiation and is characterized by erythema and edema. Vasodilatation at the early phase after UV exposure is a critical event, leading to skin accumulation of inflammatory cells, such as neutrophils and T cells.<sup>1</sup> In addition, UV exposure to the skin triggers the release of lipid mediators such as prostaglandins (PGs), which are produced via sequential pathways involving cyclooxygenase (COX) and each PG synthetase. Among PGs, PGE<sub>2</sub>

is known to be produced abundantly by keratinocytes in the skin on UV exposure.<sup>2,3</sup>

PGE<sub>2</sub> exerts its actions by binding to four different types of G-protein-coupled receptors, known as EP1, EP2, EP3 and EP4.<sup>4</sup> EP2 and EP4 receptors bind to G<sub>s</sub> and increase cAMP. EP1 receptors are coupled to G<sub>q</sub> and EP3 receptors mostly to G<sub>i</sub>.<sup>4</sup> We have previously generated mice individually deficient in each of the four subtypes of PGE<sub>2</sub> receptors and studied the *in vivo* significance of each PGE<sub>2</sub> receptor with novel findings in various aspects.<sup>5–11</sup>

It is well known in humans that indomethacin, one of COX inhibitors, inhibits UV-induced acute skin inflammation,<sup>12</sup> and an exogenous intradermal injection of PGE<sub>2</sub> increases skin microenvironmental blood flow.<sup>13</sup> Although these observations have suggested that PGE<sub>2</sub> serves as a mediator in the UV-induced skin alterations, it remains uncertain whether endogenous PGE<sub>2</sub> is responsible for UV-induced skin inflammation, and which subtype of PGE<sub>2</sub> receptors mediates this process. Using both

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genetical and pharmacological approaches in mice, we examined *in vivo* the potential role of PGE<sub>2</sub> in UV-induced skin inflammation.

## Materials and methods

### Animals

Mice lacking the EP1, EP2, EP3 and EP4 receptors individually (EP1<sup>-/-</sup>, EP2<sup>-/-</sup>, EP3<sup>-/-</sup> and EP4<sup>-/-</sup> mice, respectively) were generated as described.<sup>5-8</sup> With the exception of EP4<sup>-/-</sup> mice, each mutant was backcrossed 10 times to C57BL/6CrSlc (Japan SLC, Shizuoka, Japan), and females of the F<sub>2</sub> progenies of N10 mice were used with C57BL/6 female mice as their controls. Mice were maintained on a 12-h light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto University Faculty of Medicine.

### Reagents

EP4 antagonist, ONO-AE3-208, 4-(4-Cyano-2-(2-(4-fluoronaphthalen-1-yl) propionylamino) phenyl) butyric acid (AE3-208) was kindly provided by Ono Pharmaceutical Co., Osaka, Japan. AE3-208 was administered (10 mg/kg/day) orally in the drinking water 2 days before UV exposure and through the experiment. A volume of 20  $\mu$ l of 1% wt/vol indomethacin in acetone was topically applied on the ears of mice immediately after UV irradiation.

### UVB Irradiation

Sunlamps emitting 280–360 nm with a peak emission at 312.5 nm (Toshiba FL 20SE; Toshiba Electric Co.) were used as a source of UVB. The irradiance was 5.5 J/m<sup>2</sup>/s at a distance of 40 cm, as measured by an UVR-305/365D digital radiometer (Tokyo Kogaku Kikai KK, Tokyo, Japan). For UV-induced skin inflammation, mice were exposed to 5 kJ/m<sup>2</sup> of UVB. The ear swelling was measured at the indicated time points after irradiation using ear thickness gauge, and the ear thickness change was shown.<sup>14</sup>

### Determination of PGE<sub>2</sub> Content in the Mouse Skin

The amounts of PGE<sub>2</sub> in the mouse abdominal skin at 0 and 48 h after irradiation with 5 kJ/m<sup>2</sup> of UVB were determined by enzyme immunoassay basically as manufacturer's protocol (Cayman Chemical). In brief, abdominal skin was excised and immediately dropped into liquid nitrogen. Frozen skin were weighted and homogenized with a polytron homogenizer in 10 ml ethanol containing 0.1 ml of 5 N HCl, which was precooled at -20°C. After centri-

fugation, PGE<sub>2</sub> in the ethanol extract were applied on SEP-PAK C18 cartridges (Waters Associates, Milford, MA, USA). The amounts of PGE<sub>2</sub> were measured by enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI, USA).

### Histology

The skin of the ear was excised and fixed in 10% formaldehyde. Sections of 5  $\mu$ m thickness were prepared and subjected to staining with hematoxylin and eosin. The numbers of neutrophils and lymphocytes per field ( $\times$ 20) were counted at five randomized spots using microscopy. The diameter of randomly selected 50 blood vessels in the ear was measured.

### Measurement of Skin Blood Flow

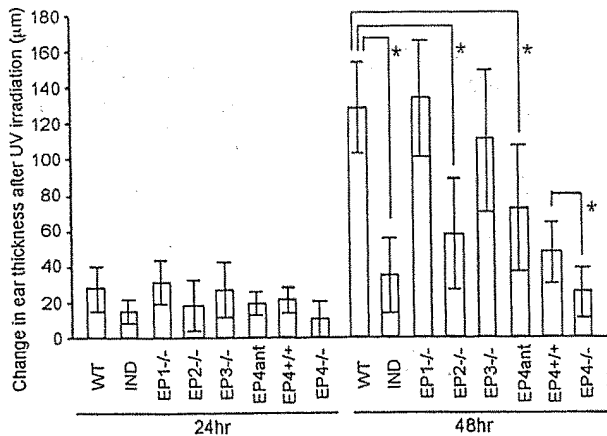
Abdominal area of mouse skin was shaved and irradiated with UVB. Blood flow was assessed using an Advance Laser (model ALF21R) Doppler flowmeter. The probe was held at six spots of abdominal area, and each reading was the mean of the six measurements.

### Statistical Analysis

Data were analyzed using an unpaired two-tailed *t*-test or one-way ANOVA followed by Dunnett multiple comparisons. *P*-value of <0.05 was considered to be significant.

## Results

It is known that a single exposure of UVB to mice causes marked skin inflammation, most remarkably on the ears. The extent of inflammation was evaluated by measuring the ear swelling responses. When control C57BL/6 mice were exposed to at 5 kJ/m<sup>2</sup> to UVB, the ear swelling became detectable within 24 h after irradiation, and the response progressed over the next 24 h as reported previously.<sup>15</sup> Mice receiving topical application of 1% indomethacin in acetone immediately after UV exposure showed significantly reduced ear swelling responses at each time point tested, as compared to the control mice that received acetone alone (Figure 1). As indomethacin inhibits COX, an enzyme to produce PGs, it is assumed that PGs play a pivotal role in the induction of UV-induced skin inflammation. As PGE<sub>2</sub> is the major PG produced by keratinocytes, we tested the magnitude of swelling responses in mice lacking each receptor subtype. The responses were significantly reduced in EP2<sup>-/-</sup> mice, EP4<sup>-/-</sup> mice and EP4 antagonist-treated C57BL/6 mice compared to the control C57BL/6 or EP4<sup>+/+</sup> mice at 48 h, although the response was similar in EP1 and EP3 mice (Figure 1). The baseline



**Figure 1** Impaired UV-induced ear skin swelling response by EP2 and EP4 receptor signaling blockade. C57BL/6 mice were treated without (WT,  $n = 15$ ) or with indomethacin (IND,  $n = 10$ ), or pretreated with EP4 antagonist (EP4 ant,  $n = 16$ ). Along with these mice, EP1<sup>-/-</sup> ( $n = 11$ ), EP2<sup>-/-</sup> ( $n = 8$ ), EP3<sup>-/-</sup> ( $n = 9$ ), EP4<sup>+/+</sup> ( $n = 6$ ) and EP4<sup>-/-</sup> ( $n = 6$ ) mice were exposed to 5 kJ/m<sup>2</sup> of UV radiation. They were examined in ear swelling responses 24 and 48 h later, and the increment in ear thickness was calculated (ear thickness after irradiation—ear thickness before irradiation). Data are a representative of three independent experiments and presented as the means  $\pm$  s.d. A Student's *t*-test was performed between the indicated groups and an asterisk indicates  $P < 0.05$ .

ear thickness of C57BL/6 with or without the EP4 antagonist, and EP-deficient mice, all without UV radiation, was comparable between these groups (data not shown). These results suggested that endogenous PGE<sub>2</sub> mediates UV-induced skin inflammation through EP2 and EP4 receptors. As EP4<sup>-/-</sup> mice were F<sub>2</sub> progeny and individual mice possessed mixed genetic backgrounds, we examined the response of mice administered with the selective EP4 antagonist for further evaluation of EP4 serving as critical receptors.

We then examined the histology, which showed that the ear of C57BL/6 mice treated with or without the EP4 antagonist, and EP2<sup>-/-</sup> mice, all without UV exposure, were similar (Figure 1). And the histology of the ears of wild-type mice 48 h after UVB irradiation showed significant dermal edema, inflammatory cells infiltration in the upper dermis, and the marginal thickening of the epidermis. This edematous change in the dermis was milder in EP2<sup>-/-</sup> mice and C57BL/6 mice treated with the EP4 antagonist (Figure 2). Moreover, the infiltration of inflammatory cells was less intensive in those mice (Figure 2). Whereas neutrophils and lymphocytes in the ears of wild-type mice were increased after UVB irradiation, those numbers in EP2<sup>-/-</sup> mice or C57BL/6 mice treated the EP4 antagonist were lower than those in C57BL/6 mice after irradiation (Figure 3).

The edema response following UV irradiation is dependent on vascular blood flow and vascular permeability. Especially, vascular blood flow can alter fluid movement between the vascular lumen and the interstitial space by increasing the luminal hydrostatic pressure. It is known that increasing

local-microenvironmental blood overflow at the early phase after UV exposure is essential to establish UV-induced skin inflammation.<sup>1,16</sup> We therefore measured the diameter of blood vessels in the skin 48 h after UVB irradiation in histological specimens. While blood vessels in wild-type mice were enlarged after UVB irradiation, those in EP2<sup>-/-</sup> mice or wild-type mice treated with the EP4 antagonist were significantly smaller (Figure 4). We then assessed the blood flow of the abdominal skin at 0, 12, 24, 36 and 48 h after UVB (5 kJ/m<sup>2</sup>) irradiation using a laser doppler device, and found that the blood flow peaked around 24 h after UV exposure, and was impaired by the EP2 or EP4 signaling blockade (Figure 5). These data suggested that PGE<sub>2</sub>-EP2/EP4 signaling is critical in acute skin inflammation by enhancing blood flow in the microenvironment.

The above results suggested that both EP2 and EP4 are essential for the development of acute skin inflammation. However, the suppressed level of inflammation by each signaling blockade tended to be lower than the indomethacin treatment. We therefore explored the effect of simultaneous blockade of EP2 and EP4 signaling by administering EP2<sup>-/-</sup> mice with the EP4 antagonist. The administration of the EP4 antagonist further decreased the reduced ear swelling level of EP2<sup>-/-</sup> mice (Figure 6). To rule out the possibility that UV-induced PGE<sub>2</sub> expression is affected by EP signaling blockade, which eventually affect the skin inflammation, we measured the PGE<sub>2</sub> expression in the skin. PGE<sub>2</sub> level in the abdominal skin 48 h after irradiation from wild-type and EP2<sup>-/-</sup> mice with or without the EP4 antagonist were as follows (wild-type C57BL/6 mice; 90.8  $\pm$  10.5, EP2<sup>-/-</sup> mice; 80.4  $\pm$  12.8, EP2<sup>-/-</sup> mice with the EP4 antagonist; 76.3  $\pm$  9.0 ng/g tissue: average  $\pm$  s.d.,  $n = 4$  each). As a comparison, the baseline PGE<sub>2</sub> expression in the skin without UVB irradiation was 0.9  $\pm$  0.3 ng/g tissue: average  $\pm$  s.d.,  $n = 4$  each. These results suggest that a significant amount of PGE<sub>2</sub> was induced by UV exposure, and the concentration of PGE<sub>2</sub> in the skin was not affected by the EP signaling blockade.

## Discussion

In this study, we demonstrated that UVB-induced ear swelling response, skin infiltration of neutrophils and lymphocytes, and local blood flow were reduced in EP2<sup>-/-</sup>, EP4<sup>-/-</sup> or wild-type mice treated with the EP4 antagonist. These results suggest that endogenous PGE<sub>2</sub> mediates UVB-induced acute skin inflammation through EP2 and EP4 receptors.

The UVB-evoked acute swelling response seems to be derived mainly from vasodilatation and resultant exudates. PGE<sub>2</sub> exerts its physiological functions by binding to its specific receptors, EP1-EP4, which are well known to be expressed on blood vessels. Vasodilatation is performed through EP2

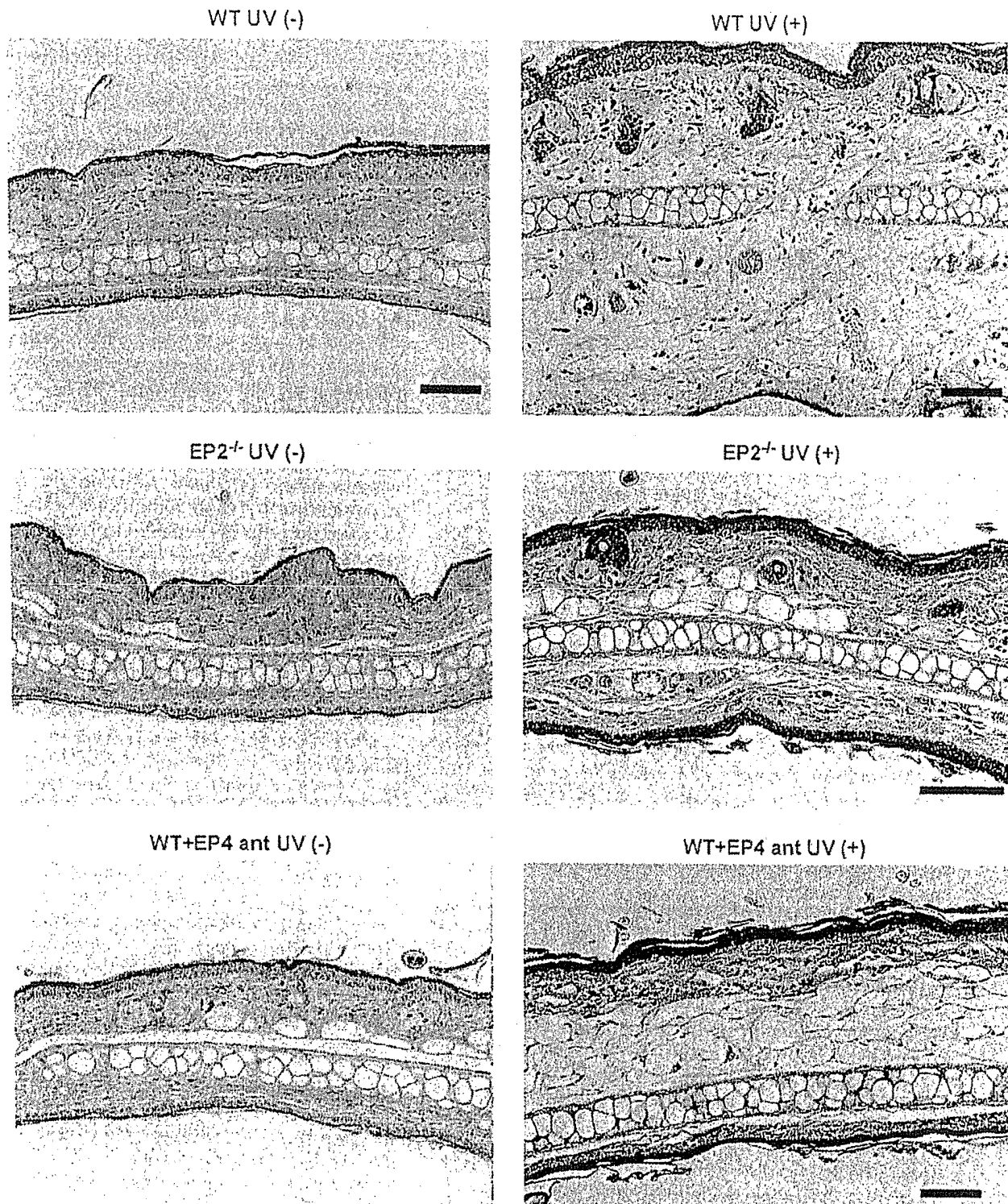
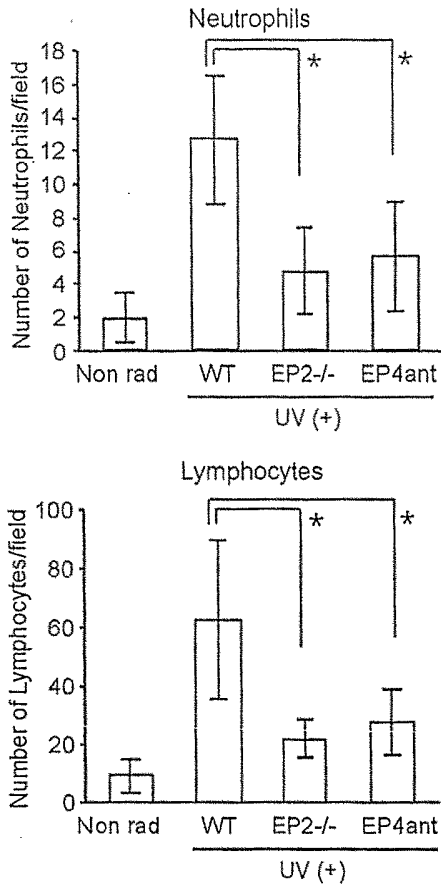


Figure 2 Histology of the skin after UV exposure. The ears of C57BL/6 mice treated with (EP4 ant) or without (WT) EP4 antagonist, and those of EP2<sup>-/-</sup> mice were excised before (UV (-)) and 48 h after UV irradiation (UV (+)). The edema and inflammatory cell infiltration was notified in WT mice after irradiation, which was less significant in WT mice treated with EP4 antagonist or EP2<sup>-/-</sup> mice. Sections of 5  $\mu$ m thickness were prepared and subjected to staining with hematoxylin and eosin. Scale bars, 50  $\mu$ m.

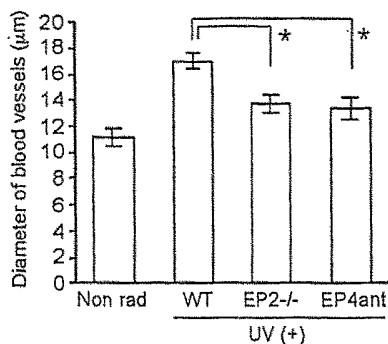
and EP4 receptors in association with increased cyclic AMP levels by coupling G<sub>s</sub>.<sup>17-19</sup> Moreover, PGI<sub>2</sub> receptor, IP and PGD<sub>2</sub> receptor, DP, are known to increase cyclic AMP levels via G<sub>s</sub>.<sup>4</sup> In mice,

however, PGD<sub>2</sub> was barely detected in the skin after UVB irradiation, and PGI<sub>2</sub> was not examined in that study.<sup>20</sup> We showed that the ear swelling response in EP2 mice (58 + 12.6  $\mu$ m) or C57BL/6 mice treated

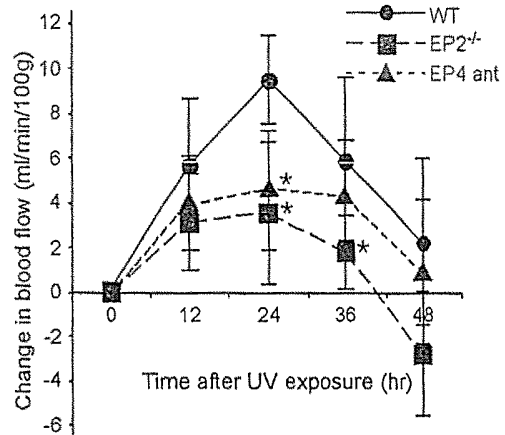




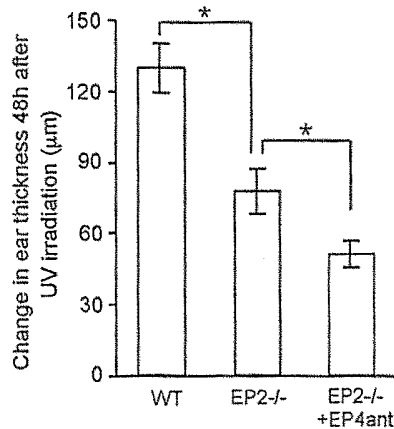
**Figure 3** Number of inflammatory cells in the skin. The ears of C57BL/6 mice treated with (EP4 ant) or without (WT) EP4 antagonist, and those of EP2<sup>-/-</sup> mice were excised 48 h after UV irradiation (UV (+)). As comparison, the ears of C57BL/6 mice without UV exposure were used (Non-rad). The numbers of neutrophils and lymphocytes infiltrating into the skin per field ( $\times 20$ ) were counted at five randomized areas, and are presented as the means  $\pm$  s.d. \*Statistically significant differences compared with the UV exposed WT group ( $P < 0.05$ , unpaired two-tailed *t*-test).



**Figure 4** Diameter of blood vessels in the skin. The ears of C57BL/6 mice treated with (EP4 ant) or without (WT) EP4 antagonist, and those of EP2<sup>-/-</sup> mice were excised 48 h after UV irradiation (UV (+)). As comparison, the ears of C57BL/6 mice without UV exposure were used (Non-rad). The diameter of 50 randomized blood vessels in the dermis were measured, and are presented as the means  $\pm$  s.e.m. \*Statistically significant differences compared with the UV exposed WT group ( $P < 0.05$ , unpaired two-tailed *t*-test).



**Figure 5** Blood flow of the skin after UV exposure. The blood flow of the abdomen of C57BL/6 mice treated with (EP4 ant,  $n = 5$ ) or without (WT,  $n = 5$ ) EP4 antagonist, and that of EP2<sup>-/-</sup> ( $n = 5$ ) mice were measured using laser Doppler flowcytometer at indicated time points. These data are a representative of three independent experiments and are presented as the means  $\pm$  s.d. \*Statistically significant differences compared with the UV exposed WT group ( $P < 0.05$ , Dunnett multiple comparisons).



**Figure 6** Enhanced impairment of UV-induced ear skin swelling response by combinational signaling blockade of EP2 and EP4 receptors. C57BL/6 (WT) mice and EP2<sup>-/-</sup> mice treated with or without EP4 antagonist were exposed to 5 kJ/m<sup>2</sup> of UV radiation ( $n = 5$ , each). They were examined in the ear swelling response 48 h later. These data are a representative of two independent experiments and are presented as the means  $\pm$  s.d. Student's *t*-test was performed between the indicated groups and an asterisk indicates  $P < 0.05$ .

with the EP4 antagonist ( $72 + 14.3 \mu\text{m}$ ) was not as low as mice treated with indomethacin ( $35 + 8.6 \mu\text{m}$ ). Therefore, we explored the effect of simultaneous blockade of EP2 and EP4 signaling by administering EP2<sup>-/-</sup> mice with the EP4 antagonist and found that the administration of the EP4 antagonist further decreased the ear swelling of EP2<sup>-/-</sup> mice ( $51 + 5.6 \mu\text{m}$ ). This extent of reduced ear swelling level was not as same as indomethacin-treated group, but quite similar. It might be interesting to investigate the role of other PGs, such as

PGI<sub>2</sub>, in the development of inflammation. In human, both PGD<sub>2</sub> and 6-keto PGF<sub>1α</sub>, stable metabolites of PGI<sub>2</sub>, were detected in the skin after UVB irradiation.<sup>21</sup> The differences may exist in the usage of PGs for the UV-induced skin response between mice and human. Nonetheless, the importance of PGE<sub>2</sub>, as documented in this study, is particularly notable. As cAMP is the downstream signaling in both EP2 and EP4, we expected that the differences in cAMP production are involved in the synergistic effect of EP2 deficiency and the EP4 antagonist treatment. However, the cAMP level in these mice were barely detected in the skin, and not elevated by UV exposure (data not shown). Moreover, there was no significant difference in cAMP production between these groups. We presume that cAMP is difficult to be detected in the skin *en bloc* even though it is induced in the blood vessels after UV exposure. One possibility is that PGE<sub>2</sub> is produced consistently after UV exposure and may not elevate cAMP levels robust enough to be detected *in vivo*. The local cAMP production around the vessels may be elevated, but it is difficult to detect this slight increase.

Keratinocytes are the major target of UV radiation and play a central role in the inflammatory and immune modulatory changes observed after UV exposure, at least partly via the UV-induced release of cytokines (IL-1, IL-6, IL-8, IL-10, GM-CSF, TNF-α)<sup>22</sup> and COX products.<sup>23</sup> IL-6 induces fever and the acute phase response and stimulates leukocytes infiltration in the skin.<sup>24</sup> PGE<sub>2</sub> plays a role in vasodilatation and in the erythral response of the skin after solar exposure.<sup>25</sup> It has been reported that nitric oxide is involved in the induction of vascular dilatation after UVB exposure.<sup>3,13,26</sup> As indomethacin treatment did not completely inhibit the UV-induced ear swelling response, there still remains the possibility that other factors are involved in this process. IL-6 and nitric oxide are the candidate in this process at present. So far, using normal human keratinocytes, Pupe *et al*<sup>27</sup> demonstrated that eicosapentaenoic acid decreased both PGE<sub>2</sub> and IL-6 secretion induced by UV-irradiation.<sup>27</sup> However, the relationship between PGE<sub>2</sub>, IL-6 and nitric oxide *in vivo* events remains unknown and is an interesting issue to be pursued in the future. In this study, we focused on the acute phase of UV-induced skin inflammation. During the acute process of fluid exudation, the role of histamine release from mast cell is important by increasing the vascular permeability.<sup>28</sup> Although PGE<sub>2</sub> reportedly suppresses mediator release by some mast cell subtypes *in vitro*,<sup>29</sup> it also enhances mediator release from mouse mast cells.<sup>30</sup> The mechanisms and EP receptor subtypes responsible for PGE<sub>2</sub>-mediated inhibition of mast cell activation in UV-induced skin inflammation are incompletely understood.

At cellular levels, UV radiation triggers cytokine production,<sup>22</sup> regulates surface expression of adhesion molecules,<sup>31</sup> affects cellular mitosis,<sup>32</sup> and

induces apoptotic cell death of skin components.<sup>33</sup> The UV-induced keratinocyte apoptosis is an important factor for preventing from skin cancer formation. EP2 and EP4 signaling blockade can be prophylactic for the acute sunburn reaction in which vasodilatation, exudation, and inflammation participate. On the other hand, it has been already reported that PGE<sub>2</sub>-induced skin cancer is mediated by EP1 or EP2 receptor signaling,<sup>34,35</sup> and there still remains the possibility that other PGE<sub>2</sub> receptor signaling may be involved in this process. The present study clearly demonstrated the deep relationship between PGE<sub>2</sub> and acute UV reactions. The involvement of PGE<sub>2</sub> and its receptors in more chronic UV-induced conditions, such as cancer development, is an issue to be elucidated.

## Acknowledgements

We appreciate Dr T Terui for special advice, K Deguchi and T Fujiwara for animal care, and ONO Pharmaceutical for the PGE receptor selective compounds, ONO-AE3-208. The authors have no conflicting financial interests. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, Shiseido Research Foundation, and Cosmos Technical Science Foundation.

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# Inhibition of T helper 2 chemokine production by narrowband ultraviolet B in cultured keratinocytes

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## Summary

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### Key words

chemokine, cytokine, keratinocyte, ultraviolet B

### Conflicts of interest

None declared.

**Background** Narrowband ultraviolet B (NB-UVB) has recently been used for the treatment of various skin disorders. Its effects on the production of cytokines and chemokines by keratinocytes are unknown.

**Objectives** To investigate the effect of NB-UVB on production of chemokines and proinflammatory cytokines by keratinocytes in comparison with broadband (BB)-UVB.

**Methods** Normal human epidermal keratinocytes (or the human keratinocyte cell line HaCaT in some experiments) at semiconfluency were irradiated with NB-UVB at 10, 100, 500 or 1000 mJ cm<sup>-2</sup> or BB-UVB at 10 or 100 mJ cm<sup>-2</sup>. The cultures were maintained in the presence or absence of interferon (IFN)- $\gamma$  at 200 U mL<sup>-1</sup>. The 72-h culture supernatants were analysed by enzyme-linked immunosorbent assay to quantify T helper (Th)1 chemokines (IFN-inducible protein 10 and monokine induced by IFN- $\gamma$ ), Th2 chemokines [macrophage-derived chemokine (MDC) and thymus and activation-regulated chemokine (TARC)] and proinflammatory cytokines [interleukin (IL)-1 $\alpha$  and tumour necrosis factor (TNF)- $\alpha$ ]. The expression of mRNA for these molecules was simultaneously assessed by reverse transcriptase-polymerase chain reaction. The culture supernatants were also tested for their chemotactic activity for Th1 and Th2 cells. The two UVB sources were compared on the basis of their minimal erythral doses and clinically used doses.

**Results** Although both NB-UVB and BB-UVB increased the production of IL-1 $\alpha$  and TNF- $\alpha$ , the augmentative effect of NB-UVB was less than that of BB-UVB. Both wavelength ranges of UVB enhanced or had no effect on Th1 chemokine production, but suppressed the production of Th2 chemokines MDC and TARC. This was confirmed by chemotactic assay, which showed decreased chemotactic activity for Th2 cells by the culture supernatants from NB-UVB-irradiated keratinocytes.

**Conclusions** NB-UVB reduces the production of Th2 chemokines without excess production of proinflammatory cytokines, suggesting its therapeutic effectiveness on Th2-mediated skin disorders as well as its relative safety in clinical usage.

Ultraviolet B (UVB) radiation has been used for the treatment of various skin diseases. As compared with psoralen UVA (PUVA) photochemotherapy, UVB phototherapy has an advantage in daily clinical use because it is not necessary to administer psoralen. Parrish and Jaenicke<sup>1</sup> reported that UVB of wavelength 313 nm is most effective for the treatment of psoriasis. This finding provided the impetus for developing the Philips TL-01 fluorescent bulb, the narrowband (NB)-UVB radiation source, which produces a spectral emission at 310–315 nm. NB-UVB phototherapy has thus significantly improved the therapeutic efficacy of conventional broadband

(BB)-UVB (290–320 nm) phototherapy for skin diseases such as psoriasis, atopic dermatitis, vitiligo and others.<sup>2–7</sup> Although a study in mice indicated that NB-UVB induced more skin cancers than BB-UVB therapy,<sup>8</sup> we have found that NB-UVB yields less oxidative DNA damage than BB-UVB when compared at clinically used doses.<sup>9</sup> In addition, the carcinogenic potential of NB-UVB is judged to be substantially lower than that of PUVA therapy.<sup>10,11</sup>

Keratinocytes are one of the major targets of UVB phototherapy. UVB irradiation induces not only the formation of DNA damage such as cyclobutane pyrimidine dimers in

keratinocytes but also the modulation of their immunological functions. The production of cytokines and the expression of adhesion molecules are profoundly changed by UVB in keratinocytes.<sup>12-14</sup> Although these alterations are concerned with the therapeutic effectiveness of UVB, the augmented production of proinflammatory cytokines, such as interleukin (IL)-1 $\alpha$  and tumour necrosis factor (TNF)- $\alpha$ , may induce skin inflammation as an adverse effect. Therefore, it is of importance to compare the effect of NB-UVB with that of BB-UVB on the production of proinflammatory cytokines. There have been two reports regarding the modulatory effects of NB-UVB on cytokine production. In one study, whole-body NB-UVB irradiation did not alter the levels of immunomodulatory cytokines in the serum of human volunteers.<sup>15</sup> However, because cytokine modulation was assessed by serum concentration in this study, detection of changes occurring in the skin appears to be difficult. The other study demonstrated that NB-UVB irradiation decreased the production of proinflammatory cytokines by stimulated T cells.<sup>16</sup> No published study to date has analysed the effect of NB-UVB on cytokine and chemokine production by keratinocytes.

Recent accumulated findings have revealed that cell migration driven by the interaction between chemokines and chemokine receptors is pivotal in the pathogenesis of various inflammatory disorders.<sup>17</sup> In the skin, external stimuli or cytokines such as interferon (IFN)- $\gamma$  and TNF- $\alpha$ <sup>18</sup> stimulate keratinocytes to produce various chemokines, which initiate migration of T cells and polymorphonuclear leucocytes. Among chemokines, macrophage-derived chemokine (MDC/CCL22) and thymus and activation-regulated chemokine (TARC/CCL17) are known as T helper (Th)2 chemokines that bind to CC chemokine receptor 4 (CCR4) on Th2 cells, whereas monokine induced by IFN- $\gamma$  (MIG/CXCL9) and IFN-inducible protein 10 (IP-10/CXCL10) are Th1 chemokines with affinity for CXC chemokine receptor 3 (CXCR3) on Th1 cells.<sup>19</sup>

In this study, we compared NB-UVB and BB-UVB in their modulatory effects on the production of proinflammatory cytokines and Th1 and Th2 chemokines. Results suggest that both wavelength ranges of UVB similarly alter the production of these cytokines and chemokines, but the levels of modulation are different between them. Preferential downmodulation of Th2 chemokines by NB-UVB is especially interesting.

## Materials and methods

### Cell culture

Normal human epidermal keratinocyte (NHEK) cells isolated from neonatal foreskin were grown in the serum-free keratinocyte growth medium KGM-2 (Clonetics, San Diego, CA, U.S.A.), and subcultured using trypsin-ethylenediamine tetraacetic acid (Clonetics). Hydrocortisone was omitted 48 h before experiments. Cells of the human keratinocyte cell line HaCaT<sup>20</sup> were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL Life Technologies Inc., Gaithersburg, MD,

U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U mL<sup>-1</sup> penicillin and 100  $\mu$ g mL<sup>-1</sup> streptomycin (all from Gibco-BRL Life Technologies Inc.).

### Ultraviolet B irradiation

Semiconfluent keratinocytes at third passage were obtained in six-well plates (Corning Glass Works, Corning, NY, U.S.A.) by plating the same number of keratinocytes. They were washed twice with 2 mL of phosphate-buffered saline (PBS; pH 7.4) and left in 300  $\mu$ L of PBS. Cells were immediately irradiated with BB-UVB (FL-20 bulbs, emission range 280-340 nm, 305 nm max.; Toshiba-Medical Systems Corp., Tokyo, Japan) or NB-UVB (UV801 KL; Waldmann Medical Division, Villingen-Schwenningen, Germany; equipped with Philips TL-20W/01RS NB-UVB tubes; Philips, Eindhoven, The Netherlands).

The minimal erythema dose (MED) of BB-UVB is 50-150 mJ cm<sup>-2</sup>, whereas that of NB-UVB is 500-1200 mJ cm<sup>-2</sup>. The clinically used doses for patients with psoriasis vulgaris or mycosis fungoides of BB-UVB and NB-UVB are 50-300 mJ cm<sup>-2</sup> and 300-1500 mJ cm<sup>-2</sup>, respectively.<sup>3,4,21</sup> Thus, 8- to 10-fold higher doses of NB-UVB are clinically equivalent to BB-UVB.<sup>9</sup> Therefore, to evaluate and compare the effects of NB-UVB and BB-UVB on keratinocytes, we chose the irradiation doses of 10 and 100 mJ cm<sup>-2</sup> for BB-UVB, and 100 and 1000 mJ cm<sup>-2</sup> for NB-UVB. In some experiments, cells were exposed to NB-UVB at varying doses of 10, 50, 100, 500 and 1000 mJ cm<sup>-2</sup> to see its maximal effect.

Immediately after UVB irradiation, PBS was replaced by DMEM in the presence or absence of 200 U mL<sup>-1</sup> of recombinant human IFN- $\gamma$  (Biogamma; Maruho Co., Osaka, Japan).

### Quantification of cytokines and chemokines in culture supernatants

Three-day culture supernatants from NHEK and HaCaT cells were collected, stored at -70 °C, and measured for IL-1 $\alpha$ , TNF- $\alpha$ , MDC, TARC, MIG and IP-10 by enzyme-linked immunosorbent assay (Genzyme-Techne, Minneapolis, MN, U.S.A.) according to the manufacturer's instructions. Optical density was measured with microplate reader Immuno-Mini NJ-2300 (Nihon InterMed, Tokyo, Japan).

### Reverse transcriptase-polymerase chain reaction of mRNA for cytokines and chemokines

NHEK and HaCaT cells treated or untreated with UVB were incubated for 2 h in the presence or absence of 200 U mL<sup>-1</sup> IFN- $\gamma$ . Total RNA was isolated from cells using the SV Total RNA Isolation System (Promega, Madison, WI, U.S.A.). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the SuperScript First-Strand Synthesis System (Invitrogen, San Diego, CA, U.S.A.) according to the manufacturer's instructions. IL-1 $\alpha$ , TNF- $\alpha$ , MDC, MIG and  $\beta$ -actin were amplified for 35 cycles, which achieved linear amplifica-

tion. The primers for human MDC have been described previously.<sup>18</sup> The other primers were as follows: IL-1 $\alpha$ , 5'-TTGAGTTTAAGCCAT-3' and 5'-GCATCATCTTTGATGACTT-3'; TNF- $\alpha$ , 5'-CCTTGGTCTGGTAGGAGACG-3' and 5'-CAGAGGGAAGAGTTCCCCAG-3'; MIG, 5'-TTAAACAATTTGCCCAAGC-3' and 5'-CTGTTGTGAGTGGGATGTGG-3';  $\beta$ -actin, 5'-GGCACCACACCTTCTACAATGAG-3' and 5'-CGTCATACTCC-TGCTTGCTGACT-3'. PCR products were visualized on 1.5% agarose gels containing ethidium bromide.

### Preparation of T helper (Th)1 and Th2 cells

Th1 and Th2 cells were established according to a previously reported method.<sup>22</sup> Briefly, for Th1-polarized cells, peripheral blood mononuclear cells (PBMC) from a normal subject were stimulated with anti-CD3 (Pharmingen, San Diego, CA, U.S.A.) and anti-CD28 (Immunotech SA, Marseille, France) monoclonal antibodies (mAbs) in 24-well plates (Corning Glass Works) for 3 days in the presence of recombinant IL-12, IL-2 (R&D Systems, Minneapolis, MN, U.S.A.) and anti-IL-4 mAb (Pharmingen) in RPMI-1640 (Gibco-BRL Life Technologies Inc., Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated FCS, 2 mmol L<sup>-1</sup> L-glutamine, 5  $\times$  10<sup>-5</sup> mol L<sup>-1</sup> 2-mercaptoethanol, 10<sup>-5</sup> mol L<sup>-1</sup> sodium pyruvate, 25 mmol L<sup>-1</sup> HEPES, 1% nonessential amino acids, 100 U mL<sup>-1</sup> penicillin and 100  $\mu$ g mL<sup>-1</sup> streptomycin (all from Gibco-BRL Life Technologies Inc.). The culture was maintained for 3 weeks with the same cytokines and mAbs. Half medium change was performed once a week. For Th2-polarized cells, PBMC were stimulated with anti-CD3 and anti-CD28 mAbs and maintained in the presence of IL-4 (R&D Systems), IL-2, anti-IFN- $\gamma$  mAb (BioSource International, Camarillo, CA, U.S.A.) and anti-IL-10 mAb (BioSource International).

### Chemotaxis assay

PBMC were plated in Transwell inserts with a pore size of 5  $\mu$ m and a diameter of 6.5 mm in 24-well plates (3421; Costar, Corning Life Sciences, Acton, MA, U.S.A.). PBMC (2  $\times$  10<sup>6</sup>) in 100  $\mu$ L were added to the upper wells and 590  $\mu$ L of culture supernatant was placed in the bottom wells, and plates were incubated for 3 h at 37  $^{\circ}$ C in 5% CO<sub>2</sub>. To determine Th1 and Th2 subsets of migrating cells, cells that moved to the lower chamber were stained with mAbs directed against chemokine receptors: CXCR3 expressed on Th1 cells and CCR4 on Th2 cells. The cells were double stained with fluorescein isothiocyanate (FITC)-labelled anti-CD4 mAb and phycoerythrin (PE)-labelled anti-CXCR3 or anti-CCR4 mAb (BD Biosciences Pharmingen, San Diego, CA, U.S.A.) and analysed on a FACSCalibur (BD Biosciences Pharmingen).<sup>18</sup> Either FITC-labelled mouse IgG1 or PE-labelled mouse IgG1 was used as an isotype-matched control. Duplicate wells were analysed for each condition. Data were expressed as % input, which indicates [(the number of migrating cells)/(the number of applied cells)]  $\times$  100. Th1 and Th2 cells were also used as migratory cells.

### Statistical analysis

Student's *t*-test was employed to determine the difference between means; *P* < 0.05 was considered to be significant.

## Results

### Background study

It is well known that production of proinflammatory cytokines IL-1 $\alpha$  and TNF- $\alpha$  by keratinocytes is enhanced by BB-UVB.<sup>12-14</sup> However, our preliminary study showed that the production of chemokines was differentially modulated by UVB, as MIG was enhanced, MDC and TARC were suppressed, and IP-10 was unchanged or slightly increased by BB-UVB. To evaluate the effect of UVB on each chemokine using the same experimental system, we therefore cultured UVB-irradiated NHEK cells in the presence (Figs 1-6) or absence (Table 1) of 200 U mL<sup>-1</sup> IFN- $\gamma$  in the experiments described below. When UVB suppressed the production of a given chemokine, this IFN- $\gamma$ -augmented system was helpful to assess its suppressive effect clearly. Inversely, even when UVB increased the production of a given chemokine, UVB enhancement was detected in the nonstimulated condition and could also still be observed in the IFN- $\gamma$ -stimulated condition.

Cell viability, as assessed by trypan blue dye exclusion test, was kept at 92-95% in NHEK cells treated with NB-UVB at 100 or 1000 mJ cm<sup>-2</sup>, or with BB-UVB at 10 or 100 mJ cm<sup>-2</sup>.

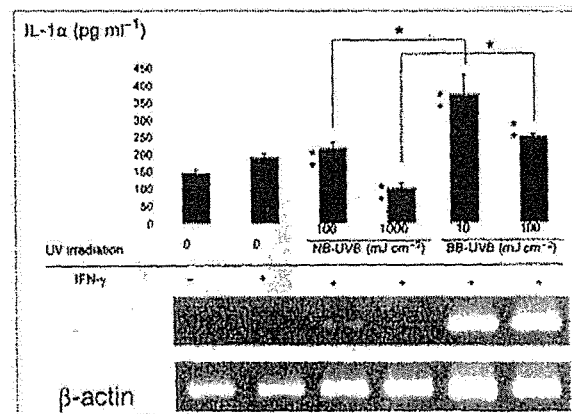


Fig 1. Interleukin (IL)-1 $\alpha$  production and expression by normal human epidermal keratinocyte (NHEK) cells irradiated with ultraviolet B (UVB). NHEK cells were irradiated with narrowband (NB)- or broadband (BB)-UVB at the indicated dose and cultured in the presence or absence of interferon (IFN)- $\gamma$  at 200 U mL<sup>-1</sup>. The concentration was measured by enzyme-linked immunosorbent assay in 3-day culture supernatants. The expression of mRNA was analysed by reverse transcriptase-polymerase chain reaction. Data are expressed as means  $\pm$  SD of triplicate cultures. \**P* < 0.05, between the means. \*\**P* < 0.05, compared with no UV with IFN- $\gamma$ .