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Quantitative Detection of Ultraviolet Light-induced Photoproducts in Mouse Skin by Immunohistochemistry

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UVB-induced cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4)photoproducts [(6-4)photoproducts] in mouse skin DNA were quantitatively measured using an immunohistochemical approach with a computer-aided color image analyzer. The skins of the C3H/HeN mice were irradiated with ultraviolet B (UV-B, 280–320 nm), and processed to give conventional formalin-fixed, paraffin-embedded histologic sections. Routine immunohistochemistry clearly demonstrated a dose-dependent induction of both photoproducts. CPDs were detectable at doses $\geq$ 125 J/m^2, while for (6-4)photoproducts, the minimal dose at which they were detectable was 250 J/m^2 in the present study. A time course study showed that the repair of (6-4)photoproducts was more rapid than that of CPDs, and that epidermal cells had a higher capacity for their removal than dermal cells. About half of the (6-4)photoproducts were excised within the first 24 h after the irradiation, and the process was essentially complete by 72 h. In contrast, there was no apparent removal (less than 10%) of CPDs in the first 24 h and they only completely disappeared from the epidermal cells at 120 h after irradiation. The effect of DNA dilution due to increased turnover of epidermal cells after UV-B irradiation was evaluated by quantitative immunohistochemical measurement of the time course of bromodeoxyuridine (BrdUrd) incorporated into nuclei at 2 days post irradiation when the proliferation reaches a peak. The removal of photoproducts was more marked than the decrease in BrdUrd staining. Our results suggest that mouse skin cells can repair both (6-4)photoproducts and CPDs, but with considerably lower efficiency, especially in the latter case, than human or monkey skin cells.

Key words: Immunohistochemistry — UV-photoproduct DNA repair — Mouse skin

Xeroderma pigmentosum (XP) patients have a high incidence of skin cancers on sun-exposed areas because their cells are deficient in DNA excision repair and, as a result, are highly sensitive to ultraviolet(UV) irradiation.[1)] UV light produces several classes of DNA photoproducts.[2)] It is a critical component of the solar spectrum for the induction of skin cancer,[3)] and levels of the more damaging shorter ultraviolet B (UV-B) wavelengths reaching the earth's surface are thought to be increasing due to the progressive depletion of stratospheric ozone by man-made chlorofluorocarbons.[4)] Among the photoproducts formed in cells, cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4)photoproducts [(6-4)photoproducts] are the most prominent, and appear to be involved in UV-induced mutagenesis and carcinogenesis.[2,5–7)]

The induction and removal of CPDs and (6-4)photoproducts in DNA have been investigated in rodents and man using *in vitro* and *in vivo* systems. The UV-induced photoproducts are deleted from nuclear DNA very efficiently by excision repair in normal human cells.[8–11)] In mouse cells it is generally accepted that only the repair of (6-4)photoproducts is active.[12)] There remains some controversy concerning the repair of CPDs, and generally mouse cells in culture have not been found to excise these photoproducts to any appreciable extent.[13,14)] Peleg *et al.*[15)] reported that cells of mouse embryos in earlier passage can excise CPDs. Concerning the repair of CPDs in mouse skin *in vivo*, there are substantial contradictions in the reported results,[16–21)] although this might be a reflection of the fact that the observations so far have mainly been limited to a short period after UV irradiation and based on the semiquantitative measurement of immunofluorescently stained frozen sections or biochemical assays that involved isolation of epidermal DNA.

In the present study we therefore evaluated the induction and repair time course of photoproducts in mouse skin *in vivo* using a highly sensitive immunohistochemical approach, developed in our laboratory,[22)] for the measurement of CPDs and (6-4)photoproducts. This method permits the quantitative analysis of UV-photoproducts with a computer-aided color image analyzer. We here document the dose-dependent induction and kinetics of repair of CPDs and (6-4)photoproducts in the mouse

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skin up to 120 h following UV-B irradiation, as well as differences in the repair capacity between epidermal cells and dermal fibroblasts.

MATERIALS AND METHODS

Chemicals Bromodeoxyuridine(BrdUrd), 3,3′-diaminobenzidine-4HCl(DAB), DNase I from bovine pancreas and phosphodiesterase I, type II, from *Crotalus adamanteus* venom were obtained from Sigma Chemical Co., St. Louis, MO. Hair remover cream (EBA cream) was purchased from Tanabe Pharmaceutical Co., Tokyo.

Antibodies Anti-BrdUrd antibody was purchased from Becton Dickinson, Mountain View, CA and a Histofine SAB-PO(M) kit was purchased from Nichirei Co., Tokyo. Monoclonal antibodies, TDM-2 and 64M-2, against CPDs and (6-4)photoproducts, respectively, were raised and characterized as previously described by Mori *et al.*[23] Specificity testing in the present study demonstrated that nuclear staining was negative with both the antibodies in sections from control mouse skin unexposed to UV-B or from mice locally exposed to a carcinogen, 4-hydroxyaminoquinoline 1-oxide (4HAQO) solution (75×10^{-6} *M*), and that nuclear staining was eliminated when sections were preincubated with phosphodiesterase I and DNase solutions before addition of the anti-photoproduct antibody (data not shown).

Ultraviolet source Three sunlamp fluorescent tubes (Toshiba FL 20 SE sunlamp, Toshiba Co., Tokyo), which deliver an average dose rate of 3.8 J/m^2/s at a distance of 30 cm over the wavelength range of 280 to 340 nm (this range includes approximately 90% of the total energy output of the lamp) with a main peak at 312 nm, were placed in parallel and used for animal exposure. The dose rate was measured with a Blak-ray Model J-221 ultraviolet intensity meter (Ultraviolet Products, Inc., San Gabriel, CA).

Animal treatment C3H/HeN female mice (8 week old) weighing 24 to 30 g were purchased from Japan SLC Inc. (Hamamatsu) and housed in plastic cages with wire tops and sawdust bedding in an air-conditioned room with a 12-h light/dark cycle. The animals were fed standard pelleted Laboratory Chow CE-2 (CLEA Japan, Tokyo) *ad libitum* and had free access to autoclaved tap water. The skins of the mice were prepared with an electric clipper and hair remover cream about one week before the experiments. The animals were anesthetized with sodium pentobarbital for exposure to UV-B at various doses. For the dose-response studies, the animals were killed immediately after irradiation, and skin samples were cut out and fixed in 10% neutral buffered formaldehyde solution at 4°C for 24 h. The fixed skin was cut into strips (5×20 mm), embedded in paraffin and sectioned at 3 μm. For the studies of photoproduct removal, mice were exposed to UV-B at a dose of 500 J/m^2. Skin samples were obtained at 2, 24, 48, 72, 96 and 120 h after the treatment and prepared as described above.

In order to study the dilution effect of epidermal cell turnover of mice on photoproducts after irradiation, we analyzed proliferation using the *in vivo* BrdUrd labeling approach. BrdUrd was injected i.p. at a dose of 50 mg/kg 2 h prior to killing of the animals. Immunohistochemical staining of BrdUrd was performed and 1000 basal cells were counted to generate labeling indices. We also investigated the time course of change in staining intensity of BrdUrd-positive cells. BrdUrd was injected 48 h post-irradiation, because at this time the increased proliferation in mouse skin induced by UV irradiation is known to reach its peak (Fig. 4). The labeling indices proved to be high and many basal cells could be labeled.

Immunohistochemical staining To obtain identical staining conditions, control and treated skins exposed to UV-B at various doses were embedded in the same paraffin blocks. Sections were mounted on glass slides coated with poly-L-lysine and after dehydration through a graded ethanol series, were incubated in 3% H_2O_2 for 10 min to inactivate endogenous peroxidase. They were then exposed to 0.1% $CaCl_2$-0.1*M* Tris-HCl buffer (pH 7.5) with 0.1% trypsin for 5 min at room temperature. Nuclear DNA was denatured using 70 m*M* NaOH in 70% ethanol for 2 min at room temperature, followed by washing twice for 30 s in 100 m*M* Tris-HCl (pH 7.5) in 70% ethanol, 2×10 min in 70% ethanol and 2×5 min in phosphate-buffered saline (PBS). After these pretreatments, the conventional avidin-biotin-peroxidase complex (ABC) method[24] was performed, using the monoclonal antibody TDM-2 (diluted 1:5000 in PBS) or 64M-2 (diluted 1:1000 in PBS) or the monoclonal anti-BrdUrd antibody (diluted 1:20 in PBS) at 4°C overnight. A 0.025% DAB solution in 0.05 *M* Tris-HCl buffer (pH 7.5) was applied as the chromogen. The group of slides constituting one sample were processed simultaneously.

Quantitative measurement of staining intensity Nuclear staining intensity was quantified using an IBAS color image analyzer (Carl Zeiss Co., Ltd, Germany). Microscopic images of the skin sections were selected in such a way that single cells could be analyzed. To record images, a 40× objective was used. For each tissue sample on the slides, the intensity of nuclear staining of consecutive cells was measured (in one direction), starting from a randomly selected point, for a total of 100 cells in the epidermis and 50 cells in the dermis. For dermal fibroblasts, cells located within about 250 μm underneath the epidermal basal layer were selected. Background counting, for subtraction from the intensity values of the UV-exposed nuclei, was performed for the control samples on each slide. The results of the image analysis were expressed as relative staining intensity using arbitrary

units (a.u.) and regression lines were calculated. The significance of differences was judged by use of the t test.

RESULTS

Dose-dependent induction of photoproducts in mouse epidermis Both photoproducts were clearly stained in the formalin-fixed and paraffin-embedded histologic sections (Fig. 1A and 1B). The relative staining intensities dose-dependently increased in the mouse epidermis, as shown in Fig. 1C. CPDs were detectable in skin sections obtained from the mice irradiated with the lowest dose (125 J/m^2) used in this study, while for (6-4)photoproducts, the minimal detectable dose was 250 J/m^2. The formation of both photoproducts increased at a relatively constant rate with irradiation doses up to 500 J/m^2. At doses above this value the increase rate of the immunohistochemical staining intensity slowed down considerably, although the levels continued to rise up to 2 kJ/m^2. This might be related to the fact that immunostaining intensity may not properly reflect the real photoproduct numbers in cellular nuclear DNA in the case of high-dose irradiation, because the density of antibody binding sites might be too high to be evaluated quantitatively by immunohistochemical staining. Regression analysis showed a highly significant correlation between the photoproduct formation and dose ($P<0.001$).

Time course of change in photoproduct levels in mouse skin The time course study of CPDs showed no apparent removal (less than 10%) from mouse skin in the first 24 h, but gradual repair was detectable afterwards with removal from the epidermal cells being more active than from the dermal fibroblasts (Fig. 2A). As shown in Fig. 3B, the staining was still apparent in dermal fibroblasts while it disappeared in epidermal cells. There was a significant difference at $P<0.01$.

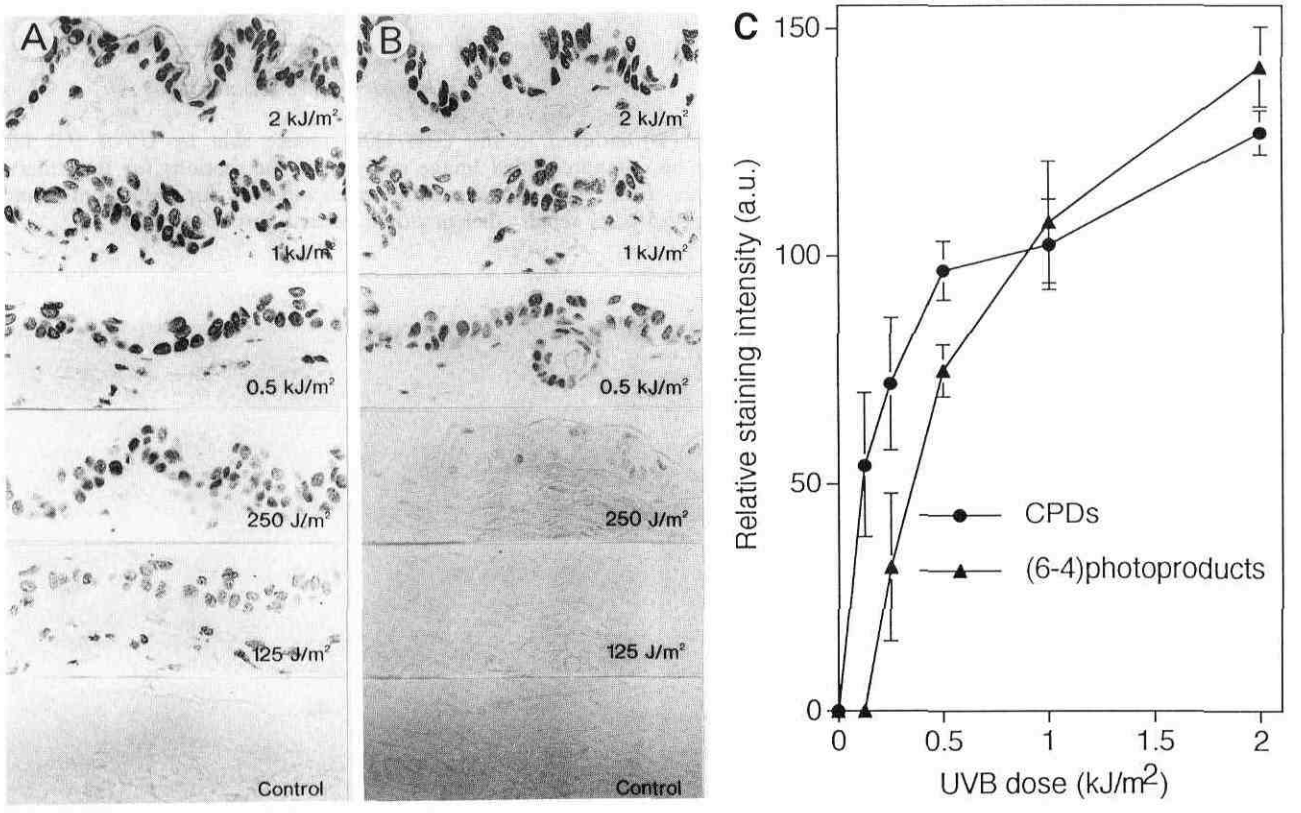

Fig. 1. Dose-dependent induction of UV-photoproducts in C3H/HeN mouse dorsal skin with UV-B irradiation. Nuclear immunohistochemical staining of CPDs (A) and (6-4)photoproducts (B) in skin sections from mice exposed to UV at various doses (from the top: 2 kJ/m^2, 1 kJ/m^2, 0.5 kJ/m^2, 250 J/m^2, 125 J/m^2 and control). A and B are serial sections from the same paraffin block. The original magnification of the microphotographs is ×200. The relative staining intensities (a.u.: arbitrary units), measured by computer-aided image analysis, were plotted for the different doses used (C). Each point represents the mean intensity for data from 5 mice. The bars indicate SD values. Regression analysis demonstrated very significant correlations between photoproduct formation and dose ($P<0.001$ for both photoproducts).

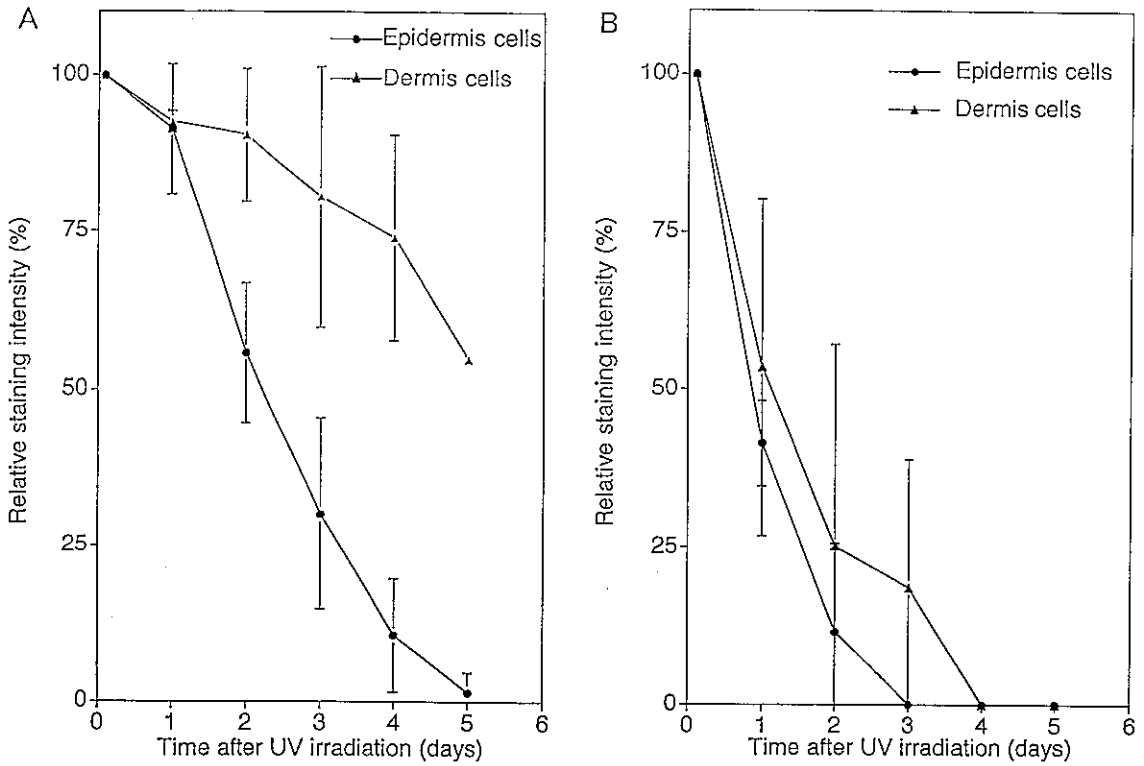

Fig. 2. Removal of CPDs (A) and (6-4)photoproducts (B) induced in the C3H/HeN mouse skin by UV-B 500 J/m^2 irradiation. The relative staining intensities were measured by computer-aided image analysis of the sections for the different time points which were processed together on the same slide. Each point represents the mean intensity for data from 5 mice. The bars indicate SD values. The epidermal cells demonstrated greater repair efficiency than dermal fibroblasts.

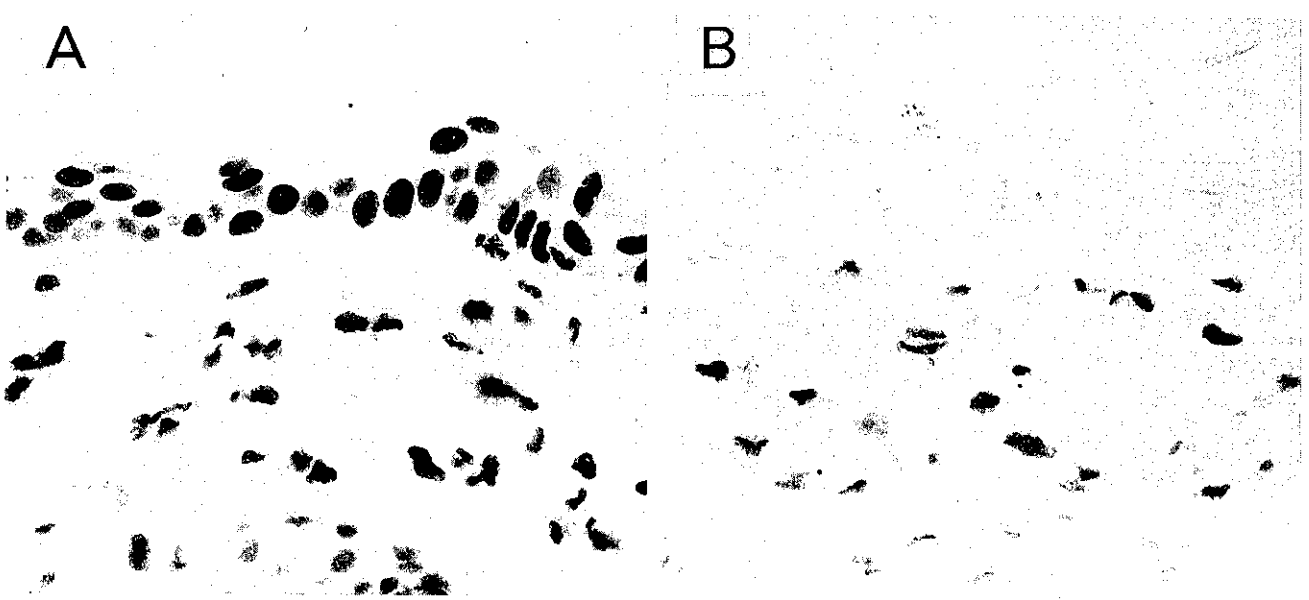

Fig. 3. Nuclear immunohistochemical staining of CPDs in skin sections from mice 2 h (A) and 5 days (B) after exposure to UV-B at 500 J/m^2. The original magnification of the microphotographs is ×200. The staining was still visualized in dermal fibroblasts, while it disappeared in epidermal cells (B).

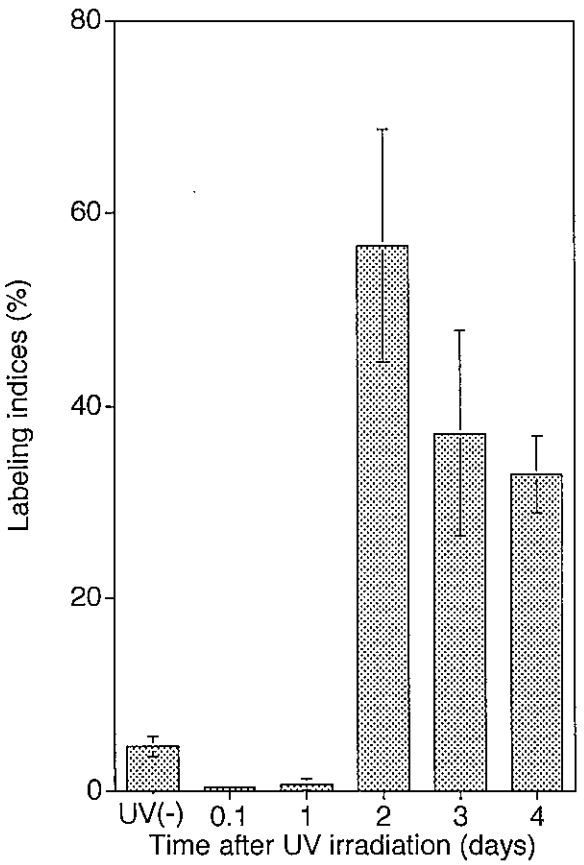

Fig. 4. BrdUrd labeling indices of mouse epidermal cells at different times after UV-B (500 J/m^2) irradiation. The bars indicate SD values.

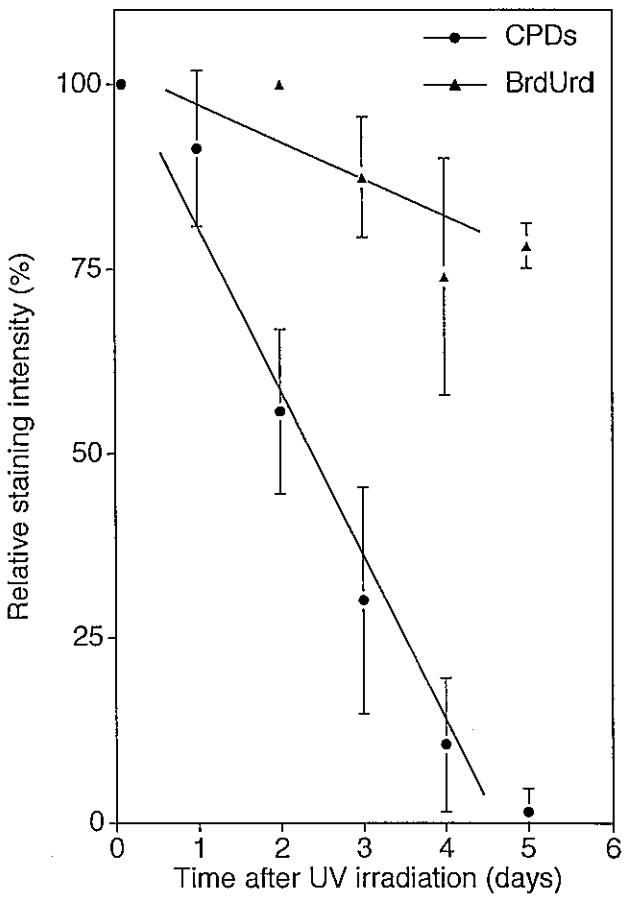

Fig. 5. Removal of BrdUrd incorporated at 48 h post UV-B (500 J/m^2) irradiation. The relative staining intensities were measured by computer-aided image analysis. Each point represents the mean intensity for data from 5 mice. The bars indicate SD values. The regression line of BrdUrd vs. time was $y=102-5x$ ($r=-0.511$, $P<0.01$). The slope was significantly different ($P<0.01$) from the slope of the regression line for CPD removal from the epidermal cells, the data for which are shown in Fig. 2(A) and reproduced here.

In the case of (6-4)photoproducts, there was more rapid repair compared with the removal of CPDs. About half of the (6-4)photoproducts were excised within the first 24 h after the irradiation and they were completely removed by 72 h (Fig. 2B). The difference between the removal of CPDs and (6-4)photoproducts in the epidermal cells was significant ($P<0.05$) with unilateral examination of probability, while the difference in the dermal fibroblasts was more significant ($P<0.01$). More active removal of photoproducts in epidermal cells than in dermal fibroblasts was also observed for (6-4)photoproducts.

Labeling indices and the time course of BrdUrd in mouse epidermis Values of the labeling indices of the basal cells of the epidermis of the mice irradiated with UV-B at a dose of 500 J/m^2 at various times after irradiation are summarized in Fig. 4. We evaluated the effects of DNA dilution due to increased cell turnover after UV-B irradiation by quantitative immunohistochemical measurement of BrdUrd which was incorporated into nuclei at 2 days post irradiation, when the proliferation reached its peak. The slope of the regression line of BrdUrd staining was significantly different at $P<0.01$ from that for CPDs in the epidermis (Fig. 5).

DISCUSSION

In the present study, we investigated the induction and repair of UV-photoproducts in mouse skin *in vivo*, using monoclonal antibodies, TDM-2 and 64M-2, specific for CPDs or (6-4)photoproducts. The specificity and the activity of the antibodies were described by Mori *et al.*[23] Briefly, TDM-2 can bind CPDs specially formed in TT and TC base sequences, and 64M-2 can detect (6-4)

photoproducts formed at least in TT and TC sequences. Linear induction curves of CPDs and (6-4)photoproducts in single-stranded DNA as a function of UV dose could be obtained using a direct enzyme-linked immunosorbent assay (ELISA) with these antibodies. The results demonstrated that 0.28 fmol of CPDs and 0.95 fmol of (6-4)photoproducts in single-stranded DNA could be detected. The sensitivity of detection of CPDs with TDM-2 antibody was almost the same as that of the immunoslot blot assay which was developed by Wani *et al.*[25] Furthermore, we found that the antibodies did not show any binding activity to control mouse skin unexposed to UV-B or treated locally with 4HAQO, and that when sections were preincubated with DNase before addition of anti-UV-photoproduct antibodies, nuclear staining was eliminated. These data indicate that recognition by the antibodies is highly specific for the type of DNA damage caused by UV irradiation *in vivo*.

We detected photoproducts in mouse skin at lower UV doses than those reported by other authors using immunofluorescent staining of frozen sections. This could be attributed to the use of paraffin sections for immunostaining. UV photoproducts (antigens) seemed to be preserved well during the procedures and reacted more efficiently with specific antibodies on paraffin sections. Nuclear staining of epidermal cells for CPDs was thus detectable at a UV-B dose of 125 J/m^2, and for (6-4)photoproducts at the dose of 250 J/m^2, due to the different binding capacities of the two monoclonal antibodies and the different yields. The yield of (6-4)photoproducts has been reported to be 10% to 50% of the yield of CPDs.[2]

With regard to the kinetics of repair of UV-photoproducts it has been reported that mouse cells *in vitro* and epidermal cells of mouse skin *in vivo* could repair (6-4)photoproducts.[12] But, there are substantial contradictions in the literature concerning CPDs. Bowden *et al.* found removal of 30% CPDs after 24 h.[16] Ley *et al.* reported that mouse epidermal cells *in vivo* had little or no capacity for the excision repair of CPDs after UV-B irradiation.[17] However, Yarosh and Yee reported the removal of CPDs from the epidermis of the hairless mouse with a half-life of 7.4 h at doses of 2.5–5 kJ/m^2 UV-B.[18] Ruven *et al.* demonstrated that, following the exposure of mice to a single dose of 2 kJ/m^2 UV-B, 60% of the CPDs was removed from active genes during the first 4 h, without further repair up to 24 h, and that in contrast, inactive genes did not show any removal of CPDs.[21] These repair studies mentioned above involved not only different detection methods, but also different doses of UV. Vink *et al.* suggested that a saturation of repair occurred at 2 kJ/m^2.[20] In the present study, since our immunohistochemical method was sensitive, we were able to choose a very low dose (0.5 kJ/m^2) of UV-B irradiation to study the repair of UV-photoproducts in mouse skin *in vivo*.

We can conclude that repair of (6-4)photoproducts starts soon after the irradiation and is almost completed by 72 h. Efficient removal of CPDs from the genome of mouse skin appears to take longer, with no appreciable removal of CPDs (less than 10%) within the first 24 h. Possible reasons are that (6-4)photoproducts are recognized more readily than CPDs, that (6-4)photoproducts are processed more efficiently, or that CPD excision repair is, for some reason, blocked. This latter could, for example, involve a photoreactivating enzyme binding to CPDs in the dark, as in yeast. However, the existence of a photoreactivating enzyme in mouse is still controversial.[26,27] Induction of the two classes of lesion is, however, not random, and (6-4)photoproducts are induced at a greater frequency in DNA that is more accessible to repair enzymes.[28–30] CPDs and (6-4)photoproducts could compete for rate-limiting enzymes involved in excision repair, as in ICR 2A frog cells.[2] Thus, repair of CPDs might have only become efficient following rapid repair of (6-4)photoproducts. Very recently, Galloway *et al.* reported that CPDs and (6-4)photoproducts are processed by distinct nucleotide excision repair pathways in human cells.[31] A similar situation could also pertain to the mouse skin case and explain the different efficiencies of repair of CPDs and (6-4)photoproducts.

In addition, as mentioned above, photoreactivation of CPDs might be involved, though no evidence is available as yet. Postreplication repair, on the other hand, is known to be an active process in mouse cells.[32] The disappearance of CPDs from the mouse skin demonstrated in the present study should be ascribed to the cooperative effects of excision repair and DNA dilution due to increased DNA turnover of the epidermis cells. It is well known that DNA damage resulting from a variety of chemical and physical agents, including UV irradiation, can block DNA replication and cause cell cycle arrest in the G1 phase of the cell cycle. This inhibition lasts at least 24 h and appears to be largely overcome by 48 h after exposure[33–35] (confirmed by the present data). Following recovery from the inhibition, rapid regenerative proliferation takes place, which results in DNA dilution and increases the excision repair capacity.[13] Comparison of relative rates of removal of BrdUrd and DNA photoproducts in the present study nevertheless showed that this dilution effect is not the main reason for the disappearance of photoproducts. Thus, it was found that the removal of CPDs was significantly more rapid than that of BrdUrd incorporated into the S phase cells at 48 h post irradiation, although BrdUrd can also be recognized as a steric encumbrance and so be repaired by nuclear DNA repair enzymes.[36] The results proved that at least the excision repair for CPDs was more efficient

than that for BrdUrd. An animal model of the disease XP was developed by using the gene targeting technique[37] and should be useful for clarification of these points in the future.

The present results also demonstrated that epidermal cells are more proficient than dermal fibroblasts at removal of photoproducts from DNA, in accordance with our former autoradiographic measurement of DNA repair at the single cell level in skin after *in vivo* treatment with UV[38] or a chemical carcinogen[39] and immunohistochemical detection of 4HAQO-DNA adducts in mouse skin.[40]

In conclusion, the present studies of formation and repair of UV photoproducts in mouse skin revealed that there is preferential repair of (6-4)photoproducts and that epidermal cells are more proficient than fibroblasts in DNA excision repair. Efficient removal of CPDs from the overall genome of mouse skin was observed, although the capacity of excision repair of the mouse skin appeared considerably less than that in man and the monkey.

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