Detection of Ultraviolet Photoproducts in Mouse Skin Exposed to Natural Sunlight

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In the present study, we for the first time investigated the formation of ultraviolet (UV) photoproducts, cyclobutane pyrimidine dimers (CPDs), pyrimidine-pyrimidone (6-4) photoproducts (64PPs) and Dewar isomers, in vivo in shaved and depilated C3H/HeN mouse skin exposed to natural sunlight (NSL) at noon for 5 min to 1 h in mid-summer, using a highly sensitive immunohistochemical method. This method permits the quantitative analysis of UV-photoproducts in formalin-fixed, paraffin-embedded sections with specific antibodies against CPDs, 64PPs and Dewar isomers. We demonstrated that the induction of CPDs in vivo in mouse skin by NSL was exposure time-dependent, but the accumulation of 64PPs or Dewar isomers was comparatively low in the skin sections from mice exposed to NSL in vivo. The results indicate that CPDs are the main photoproducts in vivo induced by sunlight and that their formation and repair may be important in connection with carcinogenesis in sun-exposed areas of human skin.

Key words: Immunohistochemistry — UV-photoproduct — Sunlight — Mouse skin

Ultraviolet (UV) radiation, which is present in the sunlight spectrum, produces several classes of photoproducts in DNA. Among these photoproducts, cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4)photoproducts (64PPs) are considered to be the major causes of skin cancer.[1] Xeroderma pigmentosum patients are deficient in repair of these photoproducts[2] and develop skin cancers at high incidence on sun-exposed areas.[3] UV-induced photoproducts are thus thought to play a crucial role in the pathogenesis of human skin cancers.

However, there has hitherto been no report on direct detection of photoproducts in vivo in skin exposed to natural sunlight (NSL). The solar spectrum impinging on the earth's surface consists of 0.3% UVB (280–320 nm), 5.1% UVA (280–400 nm), 62.7% in the visible region, and 31.9% in the infrared region[4] and only the shorter-wavelength UVB efficiently generates photoproducts in DNA.[1] Longer-wavelength UVA is reported to influence the tumorigenic efficacy of UVB,[5-8] besides exerting its own carcinogenic action at large doses,[9-12] with involvement in melanoma induction in fish.[13] This might be related to its effects on formation or repair of photoproducts induced by UVB. Photorepair of CPDs has been reported in mammals, although the data are to a certain extent contradictory.[14-17] In addition, 64PPs may be photoreversed to Dewar-type lesions by long-wavelength radiation[18] or to unmodified bases by the

64PP photolyase which was recently described.[19, 20] The DNA lesions induced by NSL might therefore be very different from those produced by artificial UV sources. In order to assess the biological significance of photoproducts in human skin carcinogenesis, it is important to measure the photoproduct formation and repair directly in skin exposed to sunlight under similar spectral conditions to those typically encountered by individuals in their natural surroundings.[21] Using specific antibodies against the major photoproducts (CPDs, 64PPs and Dewar isomers), we have developed a highly sensitive immunohistochemical method to detect photoproducts in formalin-fixed, paraffin-embedded sections.[22, 23] The present study was undertaken to determine the major photoproducts in mouse skin exposed to NSL at noon in mid-summer. Induction of UV-photoproducts could thus be demonstrated directly in vivo, using our immunohistochemical approach. The relative efficiency of formation of different photoproducts was compared.

MATERIALS AND METHODS

Antibodies A Histofine SAB-PO(M) kit was purchased from Nichirei Co., Tokyo. Monoclonal antibodies (mAbs), TDM-2, 64M-2 and DEM-1, against CPDs, 64PPs and Dewar-type photoproducts, respectively, were raised and characterized as previously described.[24, 25]

Solar source Noon sunlight in the campus of University of Tokyo on August 2nd and 3rd, 1994 was used for irradiation of the animals.

UVA A black light tube (National BL 15 lamp) was used

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as the source. The distance from the tube to the backs of the mice was 10 cm. The UVA radiation was filtered through a 5 mm thick plain glass plate selected to eliminate radiation below 320 nm. The dose rate, 8 J/m²/s, was measured with a Blak-ray Model J-221 ultraviolet intensity meter (Ultraviolet Products, Inc., San Gabriel, CA). **UVB** Three sunlamp fluorescent tubes (Toshiba FL 20 SE sunlamp, Toshiba Co., Tokyo), delivering an average dose rate of 3.8 J/m²/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 lamps) with a main peak at 312 nm, were placed in parallel and used for animal exposure. The dose rate was measured as for UVA.

Animal treatment C3H/HeN female mice (8 weeks 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 shaved with an electric clipper and treated with depilatory cream (EBA cream, Tanabe Pharmaceutical Co., Tokyo) about one week before the experiments. The mice were exposed to NSL on August 2 and 3, 1994 (cloudless fine weather with an air temperature of about 40 to 42°C in the sun). The mice were anesthetized with sodium pentobarbital and were kept in ice-cooled boxes with their heads covered by foil to avoid heating during exposure to sunlight at various doses. For each dose point, 5 mice were used. At one time, 10 to 15 mice were exposed to NSL. The mice were killed immediately after completion of the planned 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.

Immunohistochemical staining and quantitative measurement of staining intensity The methods applied were described in detail in previous papers.[22, 23] Briefly, to obtain identical staining conditions, control and treated skins exposed to sunlight at various doses were embedded in the same paraffin blocks. Sections were mounted on glass slides coated with poly-L-lysine and after deparaffinization and dehydration, then incubated in 3% H_2O_2 for 10 min, 0.1% trypsin solution for 5 min, and 70% ethanol with 70 mM NaOH for 2 min, and washed for 2 ×5 min in phosphate-buffered saline (PBS). After these pretreatments, conventional avidin-biotin-peroxidase complex immunohistochemistry was performed, with exposure to mAbs TDM-2 (diluted 1 : 5000 in PBS) or 64M-2 (diluted 1 : 1000 in PBS) or DEM-1 (diluted 1 : 200 in PBS) at 4°C overnight. A 0.025% solution of 3,3'-diaminobenzidine-4HCl (DAB, Sigma Chemical Co., St.

Louis, MO) in 0.05 M Tris-HCl buffer (pH 7.5) was applied as the chromogen. Nuclear staining intensity was quantitated using an IBAS color image analyzer (Carl Zeiss Co., Ltd., Germany). To record images, a 40× objective was used. For each tissue sample, 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. Background counting, for subtraction from the intensity values of the NSL-exposed nuclei, was performed with untreated control samples on the same slide. The results of the image analysis were expressed as relative staining intensity using arbitrary units (a.u.), which were arbitrarily decided to be equal to the absolute number of the differences between sample counting and background counting, and regression lines were calculated. Significance was judged by use of Student's t test.

RESULTS

CPDs could be clearly demonstrated in tissue sections from mouse skin exposed to sunlight for only 5 min (Fig. 1A). The increase in staining intensity of the nuclei was exposure time-dependent (Fig. 1A and B). Regression analysis revealed a highly significant correlation between photoproduct formation and exposure time (min) with a regression line of Y=24.7+1.85X (r=0.93, P<0.001). In terms of induction of CPDs, 1-h exposure to NSL was approximately equal to 2 kJ/m² artificial UVB irradiation according to the results of our previous experiment.[23] However, the production of 64PPs and Dewar isomers was very low, with no obvious exposure time-dependence, as shown in Fig. 1B. Examples of immunohistochemical staining for 64PPs and Dewar isomers on sections from mouse skin exposed to NSL for 1 h are shown in Figs. 2B and 3B, respectively. 64PPs were detected, but the staining was very weak, indicating a low production. As for Dewar isomers, little staining could be demonstrated on the sections. The staining intensity was similar to that of the control section (Fig. 3A).

For comparison, the stainings of 64PPs and Dewar isomers on skin sections from mice exposed to 2 kJ/m² UVB (Fig. 2C or 3C) or 2 kJ/m² UVB plus 34 kJ/m² UVA (Figs. 2D and 3D), both of which should be equivalent to the contents of 1-h NSL exposure, are illustrated in Figs. 2 and 3, respectively. As shown in Fig. 2, 2 kJ/m² UVB irradiation caused intense staining in nuclei of mouse skin (Fig. 2C), even with superimposition of 34 kJ/m² UVA radiation (Fig. 2D). Dewar isomers were clearly stained in the skin sections from mice exposed to 2 kJ/m² UVB alone (Fig. 3C) and were intensely stained in skin sections from mice exposed to 2 kJ/m² UVB with superimposition of 34 kJ/m² UVA radiation (Fig. 3D).
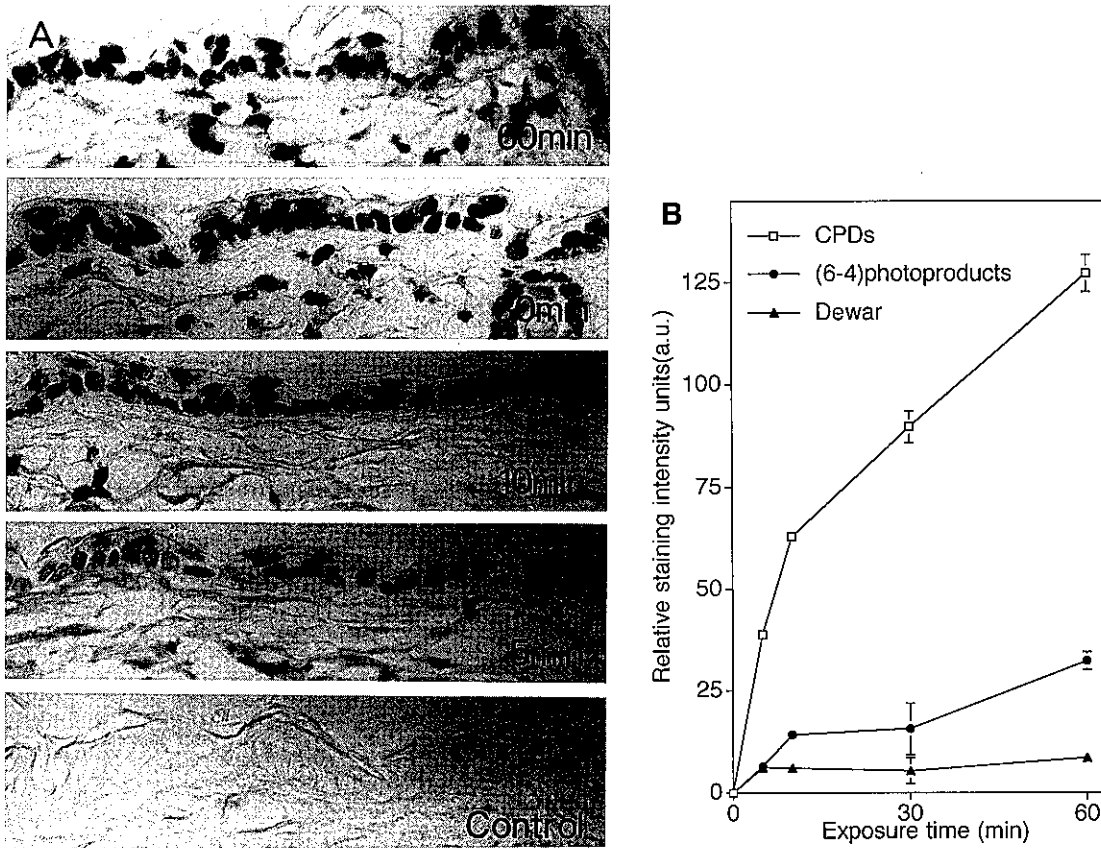
Fig. 1. Exposure time-dependent induction of CPDs in C3H/HeN mouse dorsal skin exposed to natural solar light. Note nuclear immunohistochemical staining of CPDs in skin sections from mice exposed to solar light for various time periods (from the top: 60, 30, 10, 5 min and control). The original magnification of the microphotographs is ×200 (A). Relative staining intensities, measured by computer-mediated image analysis of photoproducts and expressed in arbitrary units (a. u.), were plotted against the exposure period. Each point represents the mean of data from 5 mice. The bars indicate SD values. For CPDs, regression analysis demonstrated a highly significant correlation between photoproduct formation and exposure time, but this was not the case for either 64PPs or Dewar isomers (B).
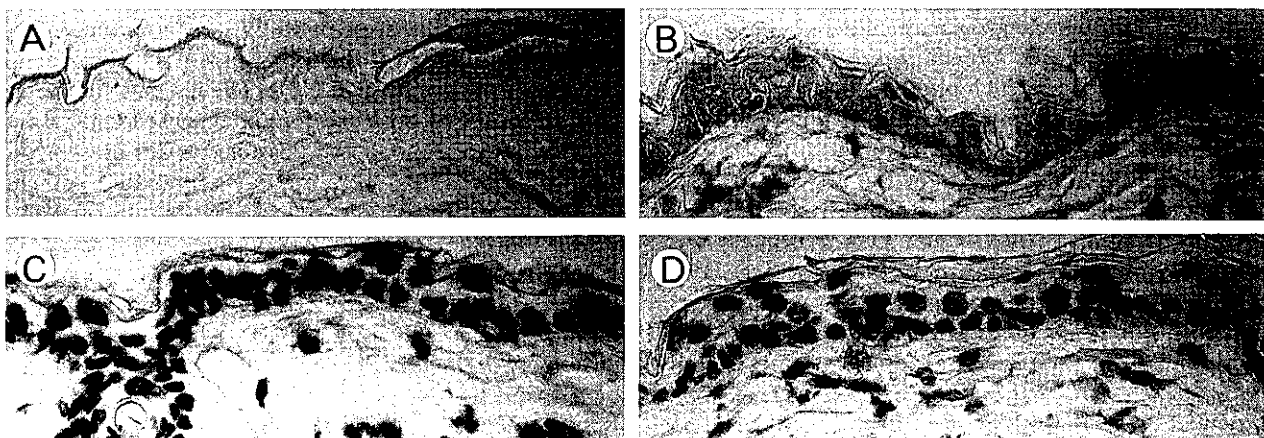

Fig. 2. Nuclear immunohistochemical staining of 64PPs in skin sections from mice exposed to solar light for 60 min (B) or 0 min (A). For comparison: C and D illustrate the staining of 64PPs in skin sections from mice exposed to 2 kJ/m^2 UVB(C) or 2 kJ/m^2 UVB plus 34 kJ/m^2 UVA (D), respectively. The original magnification of the microphotographs is ×200.
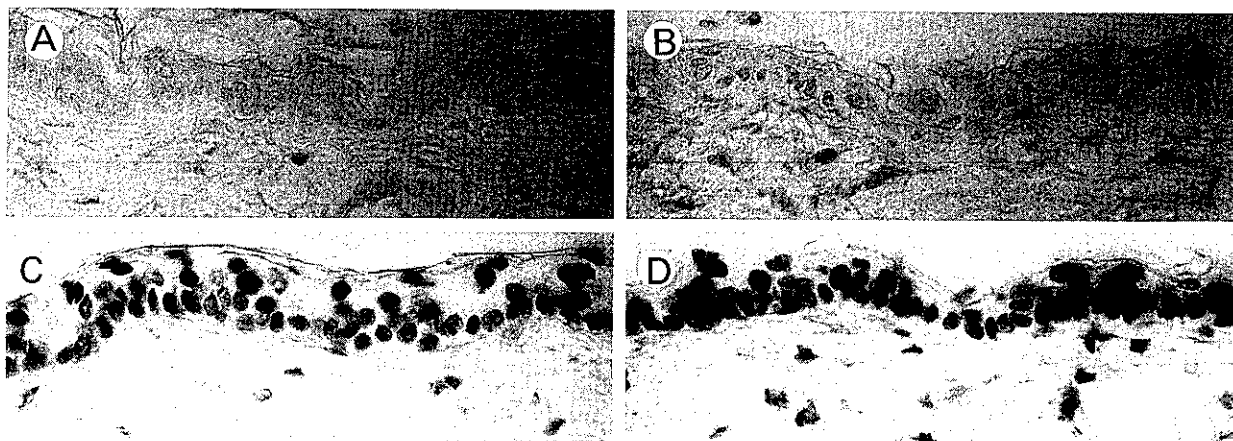
Fig. 3. Nuclear immunohistochemical staining of Dewar isomers of 64PPs in skin sections from mice exposed to solar light for 60 min (B) or 0 min (A). For comparison: C and D illustrate the staining of Dewar isomers in skin sections from mice exposed to 2 kJ/m² UVB (C) or 2 kJ/m² UVB plus 34 kJ/m² UVA (D), respectively. The original magnification of the microphotographs is ×200.

DISCUSSION

Epidemiologic evidence indicates that the increasingly popular habit of sunbathing is responsible for the documented increase in incidences of basal and squamous cell carcinomas and melanomas in man. Sunlight is thought to play a very important role in the pathogenesis of human skin malignancies, since the spectrun of sunlight includes UVB,[4] which has been unequivocally demonstrated to be carcinogenic in animal experiments. Two DNA photolesions, CPDs and 64PPs, have been proposed to cause mutagenesis and carcinogenesis.[1, 26–31]

However, the total spectrun of sunlight is made up of a large component of longer-wavelength light and only 0.3% UVB.[4] It should also be noted that interactions between the different wavelengths may influence the formation of UV-photoproducts.[1] Furthermore, there is a photoreversal phenomenon in which photoproducts induced by short-wavelength UV become converted to different structures following longer-wavelength irradiation.[14, 18–20]

In the present study, we have for the first time shown that CDPs are dose-dependently induced by NSL. However, the production of 64PPs and Dewar isomers was very low in mouse skin exposed to NSL for even 1 h, which corresponded to approximately 2 kJ/m² artificial UVB exposure in terms of CPD production. The fluence rate for sunlight in the present experiment appears consistent with the report that on the earth's surface at noon it is typically 0.2–0.5 kJ/m²/s.[32] The dose-dependent increase and consistency of the observed with the expected quantity of CPD production suggest that there is no photorepair of CPDs in mouse skin.

As regards 64PPs, as we have shown in our previous study, 250 J/m² UVB irradiation was sufficient to generate detectable levels, and 2 kJ/m² UVB resulted in intense staining in nuclei of mouse skin by our immunohistochemical method (Fig. 2C), even with superimposition of 34 kJ/m² UVA radiation (Fig. 2D), which should also be equal to the content of 1-h NSL exposure, according to our calculation. However, it is interesting to find that only a small amount of 64PPs could be demonstrated in skin sections from mice exposed to 1-h NSL in the present investigation. As for Dewar isomers, they could be clearly detected in skin sections from mice irradiated with 2 kJ/m² UVB (Fig. 3C) and were intensely stained in skin sections from mice irradiated with 2 kJ/m² UVB with superimposition of 34 kJ/m² UVA radiation (Fig. 2D). But, in the present study, we could not detect distinct formation of Dewar isomers in skin sections from mice exposed to 1-h NSL. The reasons for the very low detection of both 64PPs and Dewar isomers await further study. Among the possible causes are: 1) these two photoproducts were rapidly eliminated somehow during exposure; 2) the other components of NSL at longer wavelength than UVA may exert modifying effects on the formation and/or elimination of the photoproducts. As recently reported, 64PPs could converted to unmodified bases by a photolyase, which was first found in *Drosophila melanogaster*.[19, 20] The enzyme might exist in mouse skin tissue. A photorepair system for Dewar isomers may also exist, although no photoreactivating enzyme for them has yet been reported.

In conclusion, utilizing a sensitive immunohistochemical staining method, we have demonstrated that expo-

sure to NSL at noon in August induces substantial numbers of CPDs in the nuclei of mouse epidermal cells with an obvious dose-dependence. On the other hand, only comparatively low accumulation of 64PPs or Dewar isomers was found. The results indicate that CPDs are the main photoproducts generated in DNA due to sunlight. They might play an important role in the pathogenesis of human skin cancers.

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