Detection of Thymine Dimers in Suprabasal and Basal Cells of Chronically UV-B Exposed Hairless Mice

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An immunocytochemical method was developed to study induction and removal of DNA damage in specific cell populations in the epidermis of hairless mice during chronic ultraviolet (UV) exposure. Identification of mouse suprabasal cells was performed with an immunoperoxidase stain. This stain was shown not to affect the fluorescent nuclear stains, used to reveal DNA and DNA damage. In skin cells from hairless mice irradiated daily with 1500 J/m² UV-B for 11 consecutive days, cyclobutane thymine dimers accumulated in epidermal cells and reached a maximum level after 3 d. Thereafter dimer levels dropped to a lower, more constant level. So epidermal cells in vivo, both suprabasal and basal cells, remove dimers effectively, in contrast to cultured rodent cells, which display hardly any repair in genomic DNA. Dimer content in suprabasal cells was higher than that in basal cells, but initially the patterns of induction and removal of dimers in both cell types were rather similar. At days 4–11, however, after the drop in dimer content, the amount of dimers in basal cells prior to UV exposure was almost as low as that in non-exposed cells. The results presented here suggest important roles for both UV-induced DNA repair and cell proliferation in protecting epidermal cells against the mutagenic and carcinogenic effects of UV. J Invest Dermatol 100:795–799, 1993

UV radiation induces photoproducts in the DNA of cells, which are believed to play an important role in the process of photocarcinogenesis [1]. In studies on the effect of UV on skin, UV-B is often used because it is an effective inducer of skin carcinogenesis. As UV-B hardly penetrates into the dermis attention has been focused on epidermal cells. The epidermis consists of several layers containing various cell types: an outer layer of nucleated cells, the stratum corneum, the granular layer, a suprabasal cell layer with differentiated keratinocytes, and a basal cell layer with germinative, undifferentiated cells. In the process of carcinogenesis, DNA replication may play an important role in the fixation of mutations [2]. This may imply that DNA damage in the basal cells, the dividing cells in the epidermis, is more harmful than that in other epidermal cells, and that damaged basal cells play the major role in the induction of skin cancer. Therefore, the analysis of UV-induced DNA lesions in the different epidermal cell types—and in particular of their repair capacities—may give more relevant information than the study of overall DNA damage in the epidermis.

Induction and repair of DNA damage can be studied with biochemical and immunocytochemical methods (reviewed by Cadet and Vigny, 1990) [3]. The biochemical and certain immunocytochemical assays have the disadvantage that they require DNA isolation prior to the detection of lesions, which frustrates cell-type specific studies, whereas other immunoassays allow in situ detection. The latter assays are particularly suitable for the analysis mentioned above.

We previously studied DNA photoproducts, i.e., cyclobutane thymine dimers, in relation to photocarcinogenesis in the skin of hairless mice, by quantitative immunofluorescence microscopy with a dimer-specific monoclonal antibody [4]. We now present the results of a modified, extended assay that allows quantitative detection of cyclobutane thymine dimers in suprabasal and basal cells in hairless mouse skin.

In this study hairless mice were exposed to UV-B for 11 consecutive days. The dose regimen chosen is known to induce skin tumors in 50% of mice after daily irradiations during approximately 60 d [5]. Accumulation of thymine dimers was investigated both in suprabasal and in basal cells.

MATERIALS AND METHODS

Irradiation of Hairless Mice Female hairless mice (SKH-1 HR), 6–12 weeks old, were irradiated at a dose rate of 0.38 W/m² while allowed to move freely in a cage under a Westinghouse FS40 sunlamp (UV-B) [6]. The dose rate was checked with a Robertson-berger meter (Solar Light Company, Philadelphia, PA, USA) in combination with a micro-ammeter, calibrated to a Kipp E11 thermopile (Delft, The Netherlands). Mice were irradiated daily with 1500 J/m² UV-B (a dose resulting in a just perceptible erythema) for 11 consecutive days. On day 1 the irradiations were started with two mice, who received their first UV dose, and every next day two additional mice were entered into the procedure. So, on the eleventh day 22 mice were irradiated. On this day, before exposure, part of the dorsal skin of each mouse was covered with tape, to study the effect of the last UV dose. Immediately after the final irradiation all mice were sacrificed and two strips of skin (irradiated and non-irradiated) were excised from the mid-dorsal region. The epidermis was separated from the dermis by overnight trypsinization [7] (0.25% trypsin + 0.04% EDTA) at 4°C and cell suspensions were prepared. Epidermal cells were suspended in fetal calf serum (Gibco BRL, Life Technology, Grand Island, NY).

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Abbreviations: PBS, phosphate-buffered saline; UV, ultraviolet; AEC, 3-amino-9-ethylcarbazole; FCS, fetal calf serum.

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Technologies B.V., Breda, The Netherlands) and collected on slides by cytospin centrifugation. The cytospin preparations were stored dry at -20°C.

**Immunostaining Procedure** Epidermal cells were fixed for 10 min in acetone and air dried. Suprabasal cells were stained by overnight incubation at 4°C with an anti-cytokeratin 10 monoclonal antibody [RKSE60, Organon Teknika, The Netherlands, 1:125 diluted in phosphate-buffered saline (PBS)]. Slides were rinsed with PBS and incubated for 1 h at 37°C with peroxidase-conjugated rabbit-anti-mouse immunoglobulins (Dakopatts, Denmark, 1:50 diluted in PBS). After further washing with PBS, peroxidase activity was revealed as described by Graham et al [8], modified by Claassen and Adler [9]. In brief, 1.6 mg 3-amino-9-ethylcarbazole (AEC) was dissolved in 0.05 ml N,N-dimethylformamide and added to 4 ml sodium acetate buffer (0.05 M, pH 5.0). Just before use, 0.02 ml 3% H2O2 was added. Slides were stained at room temperature for 8 min, rinsed with PBS, and further immunostained for the detection of cyclobutane thymine dimers as described by Roza et al [10].

**Quantitative Immunofluorescence** The equipment and procedure used to measure the dimer-specific fluorescence have been described previously [11]. The software for the computer-assisted image-processing and analysis was adapted to separate the images of AEC-stained cells from non-stained cells.

**RESULTS**

**Triple Staining of Epidermal Cells** To distinguish between UV-B-induced DNA damage in suprabasal and that in basal cells of mouse skin, thymine dimer-specific immunofluorescence and DNA-specific fluorescence staining had to be combined with a cell-type specific immunohistochemical staining. For mouse epidermal cells, such discriminating staining procedures were not available. For the identification of specific human skin cells, on the other hand, numerous markers (monoclonal antibodies) were available. We therefore investigated the cross-reactivity of several human epidermal cell markers with regard to cells in mouse skin. Most of these markers turned out to be negative or only weakly positive. However, cross-reactivity of the monoclonal antibody RKSE60 with cytokeratin 10 in mouse skin resulted in satisfactory staining of suprabasal cells. The staining involved the use of a peroxidase-conjugated rabbit-anti-mouse immunoglobulin as second antibody, and the formation of a precipitate with AEC to reveal peroxidase activity (see Materials and Methods). This staining produced very good results with cytospin preparations. With cryostat skin sections it was less satisfactory due to somewhat high background staining. For this reason the former preparations were used in the further experiments.

First it was established that the AEC precipitate could withstand the subsequent treatments of the thymine dimer-immunostaining procedure. Next, to investigate a possibly negative effect of AEC on the dimer-specific fluorescence, AEC-stained and non-stained cytospin preparations of UV-exposed cells were immunostained via incubation with dimer-specific antibodies followed by fluorescently labeled anti-mouse IgG antibodies. No significant difference in dimer-specific fluorescence was measured in the presence or absence of the AEC stain (data not shown). Apparently, in the cytospin preparations studied, the cytoplasm that is present over the nuclei is so thin that nuclear fluorescence is not quenched by the AEC stain.

**Figure 1** presents the results of the combined staining procedures. Three images of the same epidermal cells are shown. It is clear that dimer-specific fluorescence (Fig 1A) and propidium iodide staining of DNA (Fig 1B) are located in the nucleus, whereas cytokeratin 10-specific AEC stain (Fig 1C) is located in the cytoplasm of suprabasal cells.

**Thymine Dimers in Suprabasal and Basal Cells After Repeated UV-B Irradiation** Cyclobutane thymine dimers were detected in suprabasal and basal cells of mouse skin after a single in vivo exposure to UV-B and after daily repeated in vivo exposures, up to 11 times. Twenty-two mice were involved in the experiment, two for each dose regimen. The final irradiations were given at day 11. Prior to the UV exposure on that day, part of the dorsal skin had been covered with tape. For the two mice that received their first UV dose on day 11, this irradiation was also the only one, so the skin sections of these mice that had been covered with tape represented non-exposed control skin. The results from one of these mice are shown in Fig 2. The suprabasal and basal cells from non-exposed skin (Fig 2, top) displayed the same low amount of background fluorescence. After the single exposure to UV-B, the thymine dimers induced in the epidermal cells were well detectable (Fig 2, bottom). Dimer-specific fluorescence was higher in suprabasal cells than in basal cells.

In the skin of the mice that had undergone 11 UV exposures, the suprabasal and —to a lesser extent—basal cells already contained cyclobutane dimers before the last irradiation (Fig 3, top), which indicates that the dimers induced by the 10 preceding irradiations had not been fully removed. The induction of dimers by the eleventh irradiation, on top of the unrepaired dimers still present, can be seen by comparing top and bottom panels in Fig 3.

The mean fluorescence of all epidermal cells resulting from the 1–11 consecutive irradiations is given in Fig 4. In the first days of irradiation the dimer-specific fluorescence increased. The mean fluorescence reached a maximum after 3 d of irradiation. Thereafter it dropped to a lower level, around which it fluctuated for the subsequent days. A similar phenomenon has been observed in preceding experiments as well [4]. Furthermore, it is noteworthy that the induction of dimers in the epidermal cells on days 2 and 3 was...
fluorescence on day 4 did not correspond to an increase in the fluorescence values ranging from Fe2Buore scence (per picture element, i.e., per area unit) is comparable to those substantially higher than that on the other days. The decrease of amount leveling off the UV -13 IIlc ntioned mention in. In. ntaneously 60 both exposures after 3 d appears fairly constant, whereas the amount of dimers i-r ra diation-i.e., days 1-3 and a drop in dimer content after day 3. The induction of dimers in suprabasal cells caused by each of the consecutive UV-B exposures after 3 d appears fairly constant, whereas the amount of dimers induced in basal cells seems to decrease after day 4. After 3 d the level of fluorescence in basal cells just prior to the next irradiation—i.e., 24 h after the previous UV-B exposure — was almost as low as the fluorescence level in non-exposed cells. Both cell-types show a strong induction of dimers on days 2 and 3.

**DISCUSSION**

In this study an immunochemical method is presented that allows cell-specific detection of UV-B-induced DNA damage in the skin of hairless mice. The quantitative immunofluorescence-microscopy method for the detection of thymine dimers described previously [4,11] was extended by the introduction of an extra cell-specific marker. The method presented here allows the quantitative detection of dimer-specific fluorescence in the nucleus to be applied separately on cytokeratin 10-positive and -negative cells.

As a keratinocyte leaves the basal layer, a number of changes in protein synthesis take place during terminal differentiation. The major change is the induction of large keratins [12]. Although basal cells (in human skin) express only two keratins, K5 and K14 [13], suprabasal cells express keratins K1, K2, K10, and K11 in addition to the basal cell keratins [12,14]. The RKSE60 antibody is directed against the 56.6-kD cytokeratin protein (cytokeratin 10) isolated from human skin, and it specifically recognizes keratinizing squamous epithelia.

It can be questioned whether RKSE60 recognizes all suprabasal cells. Basal cells, here defined as cells located on the basal membrane, may leave the basal layer to become suprabasal cells. Recognition of the differentiating cells by the RKSE60 antibody will depend on the amount of cytokeratin 10 synthesized. Consequently, some “early” suprabasal cells may not be scored. In this study we have regarded cytokeratin 10-positive cells as suprabasal and negative cells as basal cells.

In a previous study, induction and repair of cyclobutane thymine dimers in skin of UV-B-irradiated hairless mice were investigated by analysis of cryostat sections [4]. The results from the experiments with cytopsin preparations presented here are in line with the

![Figure 2](image2.png) Relative distribution of nuclear fluorescence over suprabasal cells (closed bars) and basal cells (open bars). Data are from one mouse exposed to a single UV-B dose (1500 J/m²). Dimer levels were determined in epidermal cells from a skin area that had been covered with tape during the irradiation (top) and in cells from uncovered skin (bottom). The classes 1-25 represent fluorescence values ranging from 0 to 255 arbitrary units. The nuclear fluorescence (per picture element, i.e., per area unit) is comparable to those substantially higher than that on the other days. The decrease of fluorescence on day 4 did not correspond to an increase in the amount of suprabasal cells. The amount of suprabasal cells increased from approximately 40% of all epidermal cells on day 1 to approximately 60-70% on day 11. Additionally, a gradual increase in the thickness of the epidermis could be observed over the whole experiment. In Fig 5 it is shown that the stratum corneum and the stratum granulosum had a considerably increased thickness on day 8 when compared to day 2.

![Figure 3](image3.png) Relative distribution of nuclear fluorescence over suprabasal cells (closed bars) and basal cells (open bars). Data are from one mouse irradiated daily on 11 consecutive days, with the same UV-B-dose as in Fig 2. Dimer levels were determined in epidermal cells from skin that had been covered with tape during the last irradiation (top) and in cells from uncovered skin (bottom).
previous results obtained with such skin sections. In the present chronic exposure experiment, dimer-specific fluorescence in epidermal cell nuclei increased on the first 3 d of irradiation. Thereafter, the DNA damage content per cell decreased to a lower, more constant level. Induction of dimers by the UV exposures was fairly constant throughout the experiment, except for days 2 and 3. From our experiments it can be concluded that thymine dimers are repaired to the same extent in suprabasal cells as in basal cells in the epidermis of mice.

The observed dimer removal in mouse skin epidermis confirms our previous experiments [4], in which ~60% of dimers induced were removed in 24 h. This is in contrast with the in vitro situation, in which there is hardly any removal of pyrimidine dimers [15–17]. A similar difference between in vivo and in vitro repair in rodent cells has been described by Mullhaart et al. [15], who concluded that the capacity of rat skin cells to remove pyrimidine dimers is almost completely lost upon transfer of these cells into culture. Our observations on substantial in vivo repair agree with those from other studies [15,18,19]; they disagree, however, with data from other investigators, who reported levels of dimer removal that were as low as those found for in vitro repair [16,17]. Studies are now ongoing to find out whether the use of different detection methods may be the cause of the discrepancy.

As expected, the dimer levels in basal cells were lower than those in suprabasal cells, owing to the fact that suprabasal cells are closer to the skin surface and therefore receive a higher dose of UV. As shown in Fig 6, the patterns of induction and subsequent removal of dimers were rather similar for both cell types. The slight decrease in induction observed in basal cells after day 4 may well be a consequence of increased shielding by the thickening of the epidermal layers.

Two other phenomena, the strong induction on days 2 and 3 and the sudden drop on day 4, are less readily understood. At the present time, we do not have a rational explanation for the increased induction. It can be speculated that after the first exposure the structure of the chromatin becomes more open and therefore the DNA more sensitive, due to the action of repair systems; it is unclear, however, why on day 4 the situation would change so drastically.

In our opinion, the variation in the dimer content of the cells over the subsequent days appears to be due to changes in the induction step rather than to alterations in the repair process. The return of the induction of dimers to the normal value from day 4 onward would already result in a somewhat lower dimer level than that seen on days 2 and 3. However, effects of UV irradiation on the exposed cells other than induction of dimers will enhance the lowering of this level. These effects are the consequence of cell proliferation evoked by the irradiation [20]. A clear result of the induced accelerated turnover of the epidermal cells is the gradual increase in the thickness of the stratum corneum observed over the 11 d irradiation period (Fig 5). The shielding effect [21] of this thickening on day 4 appears too small, however, to explain the drastic lowering of the dimer level on that day. But together with the other effects of increased cell proliferation a certain contribution might be expected.

The induction of cell replication in the basal cells will increase the number of epidermal cells, with the consequence that the proportion of “deeper” cells becomes larger. This will reduce the extent of dimer induction when averaged over all suprabasal cells, which effect precedes that of the thickening of the stratum corneum.

Furthermore, the cells that are terminally differentiated are the ones closest to the epidermal surface, which receive the highest UV dose. Accelerated turnover implies an increased rate of dying and disintegration of these cells. Consequently, the cells with the highest dimer content are preferentially removed from the population of suprabasal cells, which will be scored as disappearance (“repair”) of dimers.

It is not clear yet, however, at which stage of our experiment the induction of cell proliferation due to the irradiation really becomes effective. In our view, it appears likely that the induction of cell proliferation and its effects are UV dose dependent, i.e., depend on the cell’s DNA-damage content. It should be realized in this respect that in the present study only one dose regimen was applied. Other daily UV doses might well have resulted in a different pattern of variation of the dimer levels over this irradiation period. On the basis of the available data it appears plausible to assume that under the conditions used after the irradiation of day 3 a sufficient amount of dimers has accumulated, due to the only partial dimer removal in between the irradiations, to set off the proliferative process. A sec-

Figure 4. Accumulation of thymine dimers in mouse skin upon repeated irradiations with UV-B. Hairless mice were irradiated daily for 1 to 11 d. Two strips of skin were taken and cytospin-preparations were made for measurement of dimer-specific fluorescence: one from skin that had been covered with tape during the last irradiation (O,□), the other from uncovered skin (●,■). Symbols ○ and ■ represent samples taken from different mice. The mean nuclear fluorescence is given in arbitrary units (a.u.). Per datapoint about 100–200 nuclei were sampled.

Figure 5. Induction of hyperplasia. Haematoxylin-stained skin sections from a mouse after 2 d (A) and after 8 d (B) of irradiation. Bar, 12 μm.
UV-DNA DAMAGE IN SPECIFIC SKIN CELLS

Figure 6. Accumulation of thymine dimers in suprabasal (A) and basal cells (B) following repeated irradiations with UV-B. Experimental procedure as described in the legend to Fig. 4. Data ± SEM.

and consideration is based on the studies reported by Olsen [22], who found that a single dose of monochromatic UV-B has an immediate inhibitory effect on DNA synthesis followed by a regenerative response in which rapid cell proliferation occurs. This cell proliferation became evident 24 h after irradiation and was maximal another 12 h later. However, in our experimental set-up another UV exposure occurs after 24 h, which is expected to result in an additional inhibitory effect. It can be hypothesized that DNA synthesis is inhibited during the first 3 d of irradiation, whereas, on day 4, the inhibitory effect of UV on DNA synthesis is overcome by the stimulatory effect on cell proliferation.

Further studies are planned to investigate UV-induced cell proliferation in relation to the induction and removal of cyclobutane thymine dimers.

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