Effects of Chronic Low-Dose Ultraviolet B Radiation on DNA Damage and Repair in Mouse Skin

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ABSTRACT

Chronic exposure to sunlight causes skin cancer in humans, yet little is known about how habitual exposure to low doses of ultraviolet B radiation (UVB) affects DNA damage in the skin. We treated Skh-1 hairless mice with daily doses of suberythemal UVB for 40 days and analyzed the amount and distribution of DNA photodamage using RIAs and immunofluorescence microscopy. We found that DNA damage accumulated in mouse skin as a result of chronic irradiation and that this damage persisted in the dermis and epidermis for several weeks after the chronic treatment was terminated. Although the persistent damage was evenly distributed throughout the dermis, it remained in the epidermis as a small number of heavily damaged cells at the dermal-epidermal boundary. Rates of DNA damage induction and repair were determined at different times over the course of chronic treatment in response to a higher challenge dose of UVB light. The amount of damage induced by the challenge dose increased in response to chronic exposure, and excision repair of cyclobutane pyrimidine dimers and pyrimidine(6-4)pyrimidone dimers was significantly reduced. The sensitization of mouse epidermal DNA to photoproduct induction, the reduction in excision repair, and the accumulation of nonrepairable DNA damage in the dermis and epidermis suggest that chronic low-dose exposure to sunlight may significantly enhance the predisposition of mammalian skin to sunlight-induced carcinogenesis.

INTRODUCTION

Sunlight is a potent and ubiquitous carcinogen responsible for much of the skin cancer in the human population today with skin tumors in humans accounting for about 30% of all new cancers reported annually (1). Much of the human population is routinely exposed to low levels of solar UVB,3 the dose depending on geographical location, custom, occupation, and recreational behavior. Indeed, casual exposure to direct sunlight in the mid-United States latitudes is not trivial and may result in the accumulation of a mean lethal dose to unprotected human cells within approximately 30 min (2). Hence, chronic low-level UV exposure is an important consideration in studies directed toward determining the etiology and epidemiology of sunlight-induced skin cancer and in targeting high-risk populations.

An important but not well-attended facet of photocarcinogenesis is the effect that chronic sunlight exposure may have on the processes of DNA-damage induction and repair in the epidermis. Evidence strongly suggests that DNA photoproducts formed at sites of adjacent pyrimidine bases by the direct absorption of solar UVB (290–320 nm) are the predominant premutagenic events responsible for the initiation of human basal and squamous cell carcinomas (3). Transition mutations arising chiefly at the 3’ base of a thymine-cytosine dipyrimidine (C → T) have been found in the p53 tumor suppressor gene of ~50% of human basal cell carcinomas (4) and in the ras proto-oncogene at a much lower rate (5) and, along with the CC → TT tandem double mutation, are considered the “signature” mutations of UVB light. The predominant photoproducts occurring at these sites are the CPD, P(6-4)PD, and the Dewar photoproduc- somer of the P(6-4)PD (Fig. 1). The relative contribution of these lesions to mutation induction, tumor initiation, and tumor promotion is the subject of ongoing investigations.

As in humans, C → T transition mutations flourish in the p53 gene of murine skin tumors (5, 6), and it is probable that in mice, as in humans, the CPD and P(6-4)PD are important molecular determinants of skin cancer. The mouse-skin model has been used for many years to investigate the mechanisms of multistage carcinogenesis (7), and chronic exposure of the albino hairless (Skh-1) mouse to UVB has become the paradigm for laboratory studies on photocarcinogenesis (5) and photoaging (8). Although the effects of UVB light on the histopathology, growth kinetics, immune function, and other biochemical responses of the epidermis have been examined in some detail (8–12), little is known about how chronic UVB exposure affects DNA damage and repair. In the present study, we examine the effects of low-level chronic UVB exposure on DNA-damage induction and its removal by excision repair and photoreactivation in the hairless mouse-skin model. By establishing the frequencies and distributions of CPDs and P(6-4)PDs in epidermal cells at different times during and subsequent to exposure to low, chronic and high, acute UVB doses, we hope to better understand the etiology of photocarcinogenesis and to determine what role, if any, habitual exposure to sunlight has on DNA damage and damage-tolerance mechanisms in the skin.

MATERIALS AND METHODS

Animals and UVB Treatments. Female hairless mice (Skh:Hzr1, Charles River), 5–6 weeks old at the start of the experiment, were housed under yellow lights and periodically irradiated under a bank of eight 100-W TL01 fluorescent lights and periodically irradiated under a bank of eight 100-W TL01 fluorescent lamps (Philips) emitting predominantly UVB light [280–315 (88%), 315–340 (6%), and 340–400 nm (6%)] with a peak wavelength at ~313 nm. The light was filtered through UVT cast acrylic (Polycast Technology Corp., Stamford, CT), which excludes stray light below 280 nm. Transmittance spectra were identical for unirradiated UVT cast acrylic and that (irradiated) used during the chronic and acute exposure protocols (i.e., extended UV irradiation had no effect on the transmittance properties of the plastic). Under these conditions, the MED (i.e., the lowest dose that produces redness without edema or desquamation) was estimated to be between 3 and 4 kJ/m². The fluence rate was measured with an IL1400A Radiometer/Photometer coupled to a SEL240/UVB-1/TD detector (International Light, Inc., Newburyport, MA). Animals were exposed to UVB in an irradiation chamber of our design and manufacture to maximize incident fluence uniformity (StarchArt Corp, Smithville, TX). Individual mice were confined in a cassette constructed from cast acrylic (see above) containing 10 small ventilated chambers that allowed minimal movement. Six such cassettes were oriented along the circumference of a “carousel” that rotated the animals at 6.5 rpm through the outer portion of

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2 The abbreviations used are: UVB, UV-B radiation; CPD, cyclobutane pyrimidine dimer; IFM, immunofluorescence microscopy; kJ, kilojoule; mb, megabase; MED, minimal erythemal dose; NER, nucleotide excision repair; P(6-4)PD, pyrimidine-6-4pyrimidone dimer; TE buffer, 10 mM Tris (pH 8) and 1 mM EDTA; TPA, 12-O-tetradecanoylphorbol-13-acetate.

3 The abbreviation used is: solar UVB, UV-B radiation; CPD, cyclobutane pyrimidine dimer; IFM, immunofluorescence microscopy; kJ, kilojoule; mb, megabase; MED, minimal erythemal dose; NER, nucleotide excision repair; P(6-4)PD, pyrimidine-6-4pyrimidone dimer; TE buffer, 10 mM Tris (pH 8) and 1 mM EDTA; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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the circuclar transmittance field emitted by the lights. Two such carousels were used to irradiate 120 mice simultaneously. The average fluence rate at the level of the dorsa (−20 cm) was −5 J/m²/s, and the total incident dose for each treatment was determined by integrating the fluence over the time of exposure. Two fluences were used: (a) a daily low chronic dose of 0.5 kJ/m² (−2 min or 0.1 − 0.2 × MED); and (b) a challenge dose of 2 kJ/m² (−7.5 min or 0.5 − 0.75 × MED).

**NER and Photoreactivation.** For NER experiments, animals were irradiated with 2 kJ/m² UVB and either killed immediately or left for 6, 24, or 120 h free-running under a 12-h:12-h yellow-light:dark cycle before biopsy. For the short term, treated mice were kept under yellow lights and transported to the dissection area in light-tight containers. For photoreactivation experiments, mice were exposed free-running in cages for 3 h to four “Cool White” General Electric bulbs filtered through Mylar 500D (DuPont) to exclude light below 320 nm. The distance from the dorsa to the lamp surface was ∼20 cm. The temperature in the cage was periodically monitored and did not exceed 25°C. Comparable light conditions were shown to elicit a photoenzymatic response in whole organisms capable of such a response (13).

**Histology.** Tissue sections were prepared from all of the chronically irradiated mice and from the 0- and 5-day mice used in the repair experiments as described previously (14). Briefly, three to six 5-mm × 1.5-cm sections were excised from the dorsal surface and fixed in 10% neutral buffered formalin or 70% ethanol. Formalin-fixed samples were paraffin-embedded and 4-μm sections were cut and stained with H&E for the analysis of epidermal hyperplasia. For each section, the number of epidermal cells/mm was determined by counting cells at ×320 along a 2.5-mm length of skin. Five areas were counted per section, and the adjusted average and length of tissue (with high and low values omitted) were determined. Apoptotic epidermal cells were quantified as pyknotic nuclei in H&E-stained sections.

Ethanol-fixed sections were used for immunohistochemical detection of CPDs. Sections were extracted from paraffin blocks by incubation at 60°C for 1 h followed by four immersions in xyol for 5 min each. Sections were rehydrated by 2 min-incubations in 100, 96, and 70% EtOH, washed in PBS for 5 min, and denatured in 0.1 N NaOH/70% EtOH for 3 min. Sections were then dehydrated for 1 min each in 70, 90, and 100 EtOH, air-dried, and incubated with proteinase K (10 μg/ml) at 37°C for 10 min. After 5 washes for 5 min each in PBS, sections were incubated with 5% goat serum for 30 min at 23°C, rewashed five times for 5 min in PBS, and incubated overnight at 4°C with monoclonal antibody specific for incubated with 2 kJ/m² UVB and either killed immediately or left for 6, 24, or 120 h using an Ultra-Turrax T25 (IKA Laboratechnik). After homogenization, 40 μl of 20% SDS and 40 μl of RNase A (10 mg/ml; Sigma) were added. Samples were incubated for 1 h at 37°C after which, 200 μl of Proteinase K (10 mg/ml; Boehringer-Mannheim) were added, and incubation was continued for 3 h at 50°C with vigorous mixing. After deproteinization, samples were extracted sequentially with buffer-saturated phenol (Boehringer-Mannheim), phenol: Sevag (1:1), and Sevag (chloroform:isoamyl alcohol, 24:1). DNA was then precipitated with 90% volume (−100 μl) 3 M NH₄OAc (pH 5.2) and 1 volume (1 ml) ice-cold isopropanol and incubated overnight at −20°C. The DNA was collected by centrifugation at 10,000 rpm at 0°C for 20 min, washed twice with 70% ethanol, air-dried (30–60 min), and resuspended in 1 ml of 0.1× TE buffer. DNA concentration and purity were determined by reading the absorbance at 260, 280, and 320 nm.

**RIA of DNA Photoproducts.** Antisera were raised against DNA that was either irradiated with 100 kJ/M²-ultraviolet-C (254 nm) light for P(6-4)PDs or dissolved in 10% aceton and irradiated with UVB light under conditions that have been shown to produce CPDs exclusively. For the RIA, 2–5 μg of heat-denatured sample DNA was incubated with 5–10 pg of poly(2-deoxyxydylate-deoxyxythymidylic acid) (labeled to >5 × 10⁸ cpm/μg) for 20°C. The DNA was dissolved in tissue solubilizer (NCS, Amersham) and mixed with ScinitSafe (Fisher) containing 0.1% glacial acetic acid, and the 3²P was quantified by liquid scintillation spectrometry. Under these conditions, antibody binding to an unlabeled competitor inhibits antibody binding to the radiolabeled ligand. Sample inhibition is extrapolated through a standard (dose-response) curve to determine the number of photoproducts in 10⁵ bases [*i.e.,* CPDs or P(6-4)PDs/mb]. For the standard, we used double-stranded salmon tests DNA (Sigma) irradiated with increasing doses of ultraviolet-C light and heat-denatured, aliquoted, and kept frozen at −20°C. Rates of photoproduct induction were previously quantified using nonimmunological enzymatic and biochemical techniques and determined to be 8.1 CPDs and 1.56 P(6-4)PDs/mb/m², respectively. These details, as well as those concerning the specificities of the RIA, have been described previously (15).

**Data Analyses.** The experimental data were fitted to the model equation a(t) = a(0) exp(−t/r) + r using the Levenberg-Marquardt (least squares) algorithm where a is the amplitude, t is time, r is the exponential half-life (*i.e.,* 37% remaining), and r is the level of residual damage. The P(6-4)PD repair curves fitted the function well, and all of the data points were used. The kinetics for CPD excision at 20 days did not fit the least-squares algorithm and, hence, were not included in the results (Table 1).
RESULTS

DNA Damage Accumulation in Mouse Skin. Skh-1 hairless mice were exposed to 0.5 kJ/m² UVB light at 24-h intervals for 40 consecutive days (see flow diagram, Fig. 2). At 5-day intervals, animals were killed, and the epidermis was excised immediately after irradiation or at 5 and 10 days postirradiation for photoproduct analyses using RIA and immunohistochemistry. Unirradiated mice were used as controls and showed background levels of 10.4 (±2.6) CPDs and 0.8 (±0.2) P(6-4)PD/mb DNA (0 day in Figs. 3 and 4). Mice killed immediately after exposure showed a linear increase in CPDs over the 40-day treatment period (upper regression line in Fig. 3). When animals were allowed to recover from the chronic treatment for 5 and 10 days under yellow lights, fewer CPDs were evident, presumably

Table 1 Parameters for CPD and P(6-4)PD excision repair during chronic UVB treatment

<table>
<thead>
<tr>
<th>Chronic UVB (days)</th>
<th>Cyclobutane dimers</th>
<th>(6-4) photoproducts</th>
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<td>0</td>
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<td>10</td>
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<td>20</td>
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<td>4.5 (1.3)</td>
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<td>80.2 (7.7)</td>
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<td>90</td>
<td>33.0 (20.6)</td>
<td>7.4 (3.1)</td>
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a, Amplitude (total damage at t = 0).
b, t1/2, half-life for damage (t1/2 = t × 0.693).
c, r, residual damage (photoproducts/mb).
d, SD are shown in parentheses.
e, Degrees of freedom are in parentheses.
f, ND, not determined.

Fig. 2. Flow diagram of experimental protocols used for chronic and acute UVB experiments. Arrows above the time-line (in days), daily exposures to 0.5 kJ/m²; bold arrows below the time-line, acute exposures to 2.0 kJ/m². Closed and open symbols above the time-line refer to chronic data presented in Figs. 3 and 4; below the time-line, they refer to acute repair data presented in Figs. 8 and 9.

Fig. 3. Induction and accumulation of cyclobutane dimers during chronic UVB irradiation. CPD frequencies in epidermal DNA from duplicate mice killed immediately after, and at 5 and 120 days after, chronic irradiation are shown for 0 (○), 5 (□), 10 (●), 15 (○), 20 (□), 25 (○), 30 (●), 35 (○), 40 (□), and 90 (●) days. The upper regression line is for CPD data from mice killed immediately after the daily chronic treatment; the lower regression line is for data from mice killed 10 days later with no additional chronic exposures. The TRIMMEAN (Excel 97), excluding the upper and lower 25% of the data, and SE (S.E.M. = √(SD²/n)) were calculated from data from four RIAs (n ≥ 10).
because of DNA repair. As the chronic doses accumulated, however, the level of CPDs remaining after the 10-day recovery also accumulated, reaching ~20 CPDs/mb after 40 days of treatment or about 5-fold above the background (lower regression line in Fig. 3). The DNA damage remaining after 10 days seemed to be nonrepairable because 19.5 (±2.2) CPDs/mb were still evident 50 days after the final treatment. The pattern of P(6-4)PD accumulation in response to chronic UVB irradiation differed significantly from that of the CPDs, presumably because these lesions are repaired much more rapidly in mouse skin (see Table 1). When measured immediately or 10 days after chronic exposure, P(6-4)PDs did not differ significantly from background levels (Fig. 4).

IFM was used to quantify relative CPD frequencies in individual dermal and epidermal cell nuclei in chronically irradiated mouse skin. Consistent with the RIA data, little if any fluorescence was observed in skin cell nuclei from unirradiated mice (Fig. 5A). Daily irradiation with low UVB doses resulted in greater numbers of FITC-positive cells and a progressive increase in the average fluorescence/nucleus (Fig. 5, B–E). The increase in average fluorescence in epidermal nuclei was fit to an exponential growth curve and reached a maximum level at ~20 days (Fig. 6). CPD-associated fluorescence was detectable and uniformly distributed in the epidermis at 5 days but thereafter showed an increasingly nonrandom distribution. In samples biopsied at 15 days and later, the epidermis showed a somewhat uniform fluorescence field punctuated by very bright (heavily damaged) cells located primarily along the dermal-epidermal boundary. Surprisingly, 50 days after the last treatment, heavily damaged nuclei were still evident, even though the fluorescence in the remaining cells had returned to background levels (Fig. 5F). In contrast to the pattern displayed by fluorescence measurements, the number of apoptotic cells in the epidermis did not vary significantly in response to chronic irradiation (Fig. 7).

The spatial and temporal distribution of fluorescent nuclei in dermal cells was similar to that observed in the epidermis. After 5 days of chronic irradiation, CPDs were detected as positive fluorescence in a few dermal cells; by 15 days, the number of FITC-positive cells had increased measurably. In contrast to the epidermis, cells in the dermis seemed to accumulate damage in a large proportion of cells and several “very bright” cells were evident at days 15, 25, and 40 (Fig. 5, C–E). The damage in dermal fibroblasts seemed to be persistent (i.e., nonrepairable) because the fluorescence pattern at 50 days postchronic irradiation (Fig. 5F) was very similar to that seen during the later course of treatment. In addition, the average fluorescence determined for dermal cells was significantly greater than for epidermal cells at 90 days (Fig. 6).

Effects of Chronic Low-Dose UVB Exposure on Photoproduct Induction and Repair. At different times during the chronic treatment and 50 days after the last treatment, mice were challenged with an acute UVB dose approaching $1 \times \text{MED}$ (i.e., 2 kJ/m$^2$), and CPDs and P(6-4)PDs were quantified immediately and at 6, 24, and 120 h after irradiation using RIA (Figs. 2, 8, and 9). The acute dose was administered 24 h after the previous chronic treatment. First-order exponential decay functions [i.e., $a(t) = a(0)e^{-rt} + r$] were fitted to each repair curve using the Levenberg-Marquard (least-squares) algorithm and values for the amplitude (i.e., total damage frequency minus the calculated residual damage), residual damage (i.e., toward which the repair curves asymptotically approach), and half-life for damage were derived from the experimental data (Table 1). Because no CPD repair was observed within the first 6 h after irradiation, the exponential decay was fitted with a 6-h delay. In the absence of chronic irradiation (i.e., on day 0), the calculated amplitude was $32 (±18)$ CPDs/mb DNA; from the RIA, we measured $37 (±3)$ CPDs/mb after $2 \text{ kJ/m}^2$ UVB. Purified DNA, irradiated under the same conditions, yielded $78 (±9)$ CPDs/mb indicating that attenuation of UVB by shielding and absorption in the epidermis reduced the yield ~2-fold. The amplitude increased in response to the chronic treatment with $112 (±38)$ and $80 (±8)$ CPDs/mb DNA measured at 10 and 40 days, respectively. The
excision repair curve for 20 days showed negligible repair at 24 h postirradiation and did not fit an exponential decay function. Observed values (and SEs) for the number of CPDs/mb induced by 2 kJ/m² UVB after 10-, 20-, and 40-day chronic treatment were 60 (±11), 131 (±14), and 162 (±29), respectively (upper regression line in Fig. 8). At 50 days posttreatment 70 (±9) CPDs/mb were measured by RIA, and an amplitude of 33 (±21) was calculated from the repair curve. Differences between observed photoproduct frequencies and those derived from excision repair curves suggest that a significant component of induced damage is not repaired. In addition, these data indicate that 2- to 3-fold more CPDs were induced by an equivalent dose of UVB light while the animals were under chronic UV stress.

Although compared with the CPDs the P(6-4)PD levels were significantly lower, a similar pattern was evident in response to acute irradiation (Fig. 9). The number of P(6-4)PDs measured after acute exposure of control mice receiving no chronic treatment was 2.7 (±0.2) lesions/mb DNA or about 10% of that measured for CPDs. This value was comparable to the amplitude calculated from the exponential decay curve at 0 day [2.4 (±0.1) in Table 1]. Likewise, amplitudes determined from the exponential decay curves were very similar to the observed values measured over the course of chronic irradiation; that is, amplitudes of 2.0 (±0.9), 4.5 (±1.3), and 3.0 (±0.5) versus observed values of 3.1 (±0.3), 5.3 (±0.8), and 3.6 (±0.5) P(6-4)PDs/mb at 10, 20, and 40 days, respectively. At 50 days postchronic treatment, the observed frequency of 8.1 (±1.3) P(6-4)PDs/mb was very similar to the amplitude calculated from the exponential decay curve [7.4 (±3.1) in Table 1]. From the data in Figs. 8 and 9, the effects of chronic UVB irradiation on rates of excision repair were determined (t₁/₂ in Table 1). Because UV-induced hyperplasia would effectively dilute photoproduct concentrations (i.e., show a false positive), the number of cells per linear mm in the epidermis was estimated at 0 and 120 h postirradiation in H&E-stained skin sections (data not shown). No hyperplasia was observed; hence, the observed loss of damage was due to NER rather than cell division. Although rates of CPD excision showed considerable variation during the 40-day chronic treatment (note SDs in Table 1), the average half-life for CPD removal (i.e., 15–20 h) roughly correlated with the amplitude, suggesting that CPD excision repair is a first-order reaction dependent on substrate concentration. At 120 h after the acute dose, the amount of CPDs remaining in the epidermis was comparable to the amount of residual damage (r in Table 1) that accumulated as a consequence of chronic irradiation; the mean values for CPDs measured at 10 days postchronic treatment are shown (lower regression line in Fig. 8). Negligible excision repair was observed 24 h after acute irradiation at 20 days, but by 40 days, the repair rate resembled the control rate more. At both 20 and 40 days, a considerable level of

Fig. 5. Immunofluorescence micrographs of cyclobutane dimers in dermal and epidermal cell nuclei. A, unirradiated control and B, 5 d; C, 15 d; D, 25 d; E, 40 d; and F, 90 d after the initiation of the chronic treatment.
damage was unrepaired 120 h after acute exposure \[i.e., 55 (\pm 6)\) and 54 (\pm 5) CPDs/mb for 20 and 40 days, respectively]. From these data, it seems that chronic exposure to UVB adversely affected CPD NER.

Recent studies have described a photo-dependent repair mechanism in human epidermis under conditions in which a fractionated dose regimen was used, which suggests that this process is inducible (16). To test this response in chronically irradiated mouse skin, animals were exposed to 2 kJ/m² UVB and either killed immediately or exposed (or not) to photo-reactivating light for 3 h. Animals receiving visible-light treatments were killed at 6 and 24 h postirradiation (Table 2). Although a 24-h sample was not taken for the control mice, no effect of visible light on the level of photoproducts was observed by 6 h. Likewise, after 10, 20 and 40 days chronic treatment no light-dependent loss of photoproducts was observed.

Consistent with our previous studies (17), the rate of P(6-4)PD excision repair was much faster than for the CPD before, during, and after the chronic treatment (Fig. 9). Half-lives for P(6-4)PDs derived from exponential decay curves were 4.8 (\pm 0.6), 11.8 (\pm 11.8), 2.9 (\pm 2.2), and 10.1 (\pm 3.1) h for the 0-, 10-, 20-, and 40-day chronic

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**Fig. 6.** Cyclobutane dimer accumulation in dermis and epidermis. Closed symbols (■) and linear regression line (•—•) show CPD frequencies determined by RIA immediately after daily chronic exposure (same as upper regression line in Fig. 3). Open (○) and gray (■) symbols show average fluorescence/nucleus for epidermal and dermal cells, respectively. Curves were fitted to data to show exponential growth to a maximum level of fluorescence/nucleus (in arbitrary units) for epidermal (— —) and dermal (— —) cells.

**Fig. 7.** Relationship between apoptotic and heavily-damaged basal cells in the epidermis. Closed symbols (●) represent percentages of apoptotic cells at the dermal-epidermal boundary in tissue sections from duplicate mice at different times during the chronic exposure. Open symbols (○) show percentages of cyclobutane dimer-retaining cells determined from IFM of tissue sections (see Fig. 5).
treatments, respectively, and were, on average, 3-fold faster than for CPDs \((i.e., 16-17 \text{ h})\). The repair rate measured 50 days after chronic irradiation \((i.e., 2.7 (\pm 1.4))\) was comparable to the control. These rates were roughly proportional to the amplitudes at each time, indicating that P(6-4)PD repair, like CPD repair, follows first-order kinetics. As for the CPDs, there was considerable variation in repair rates during the chronic treatment (particularly at 10 days) but a significant reduction in rate was observed at 40 days. The amount of P(6-4)PDs remaining 120 h after the acute dose is comparable to background levels \((\text{lower regression line in Fig. 9})\).

From the exponential decay curves, a value, \(r\) (for residual damage), was calculated for each photoproduct that was an estimate of the amount of nonrepairable damage in epidermal DNA (Table 1). In the control mice that received no chronic treatments, approximately one-third of the...
damage was not repairable (i.e., remained at background levels). The \( r \) values for CPDs increased soon after the chronic treatments began (i.e., \( \sim 20 \) lesions/mb at 10 days) and continued to rise to 40 days (i.e., \( 50 \) lesions/mb). At 50 days after the treatment ended, there were \( \sim 20 \) residual lesions/mb. The number of residual CPDs calculated from the repair curves was approximately equivalent to the number of CPDs measured in chronically irradiated skin at each time point after a 10-day recovery. The fitted model also converged near the observed value at 120 h after the acute exposure. Hence, the amount of CPD damage measured either 120 h after an acute dose or 10 days after the last chronic dose is a good estimate of the residual level of damage produced in mouse skin by UVB. The increase in residual damage between 0 and 40 days suggests that CPD NER is reduced during this period of chronic exposure. In contrast to the CPDs, there was no significant accumulation of unrepaired P(6-4)PDs.

### DISCUSSION

Few studies have been published on DNA damage and repair in skin chronically exposed to low doses of solar UV light. Hattori et al. (18) described the increase of the photo-oxidative product 8-oxodeoxyguanosine in epidermal cells of hairless mice in response to chronic UVB exposure, and several other studies have shown induction of cellular processes involved in the quenching and scavenging of the reactive oxygen species responsible for this type of DNA damage (19–21). More pertinent to the work presented here is that of Vink et al. (22, 23) who analyzed the induction, repair, and accumulation of CPDs in the skin of UVB-irradiated hairless mice using immunohistochemical techniques. In the more recent study (23), mice were exposed daily for 11 days to 1.5 kJ/m² UVB light (i.e., \( 0.5 \times MED \)) from a broad spectrum source, and CPDs were quantified immediately and at 24 h after irradiation in suprabasal and basal cells in the epidermis. They found that CPDs accumulated in epidermal cells to a maximum level after three exposures (total dose of 4.5 kJ/m²) with the photoproduct content in suprabasal cells exceeding that in basal cells. After the third exposure, CPD levels decreased and the level of damage in basal cells before exposure (i.e., 24 h after the previous exposure) was at background levels.

Our studies complement earlier data and add a measure of complexity to the molecular and biological responses of mammalian skin to solar UVB. We analyzed DNA photodamage in Skh-1 hairless mice exposed to very low daily doses of UVB light (i.e., \( 0.2 \times MED \)) for several weeks and determined the fate of the lesions long after the treatment ended. We found that CPDs accumulate as a result of chronic irradiation and persist for a considerable time afterward, both in the dermis and epidermis. Several studies have shown that, in general, nondividing terminal differentiated cells display significantly less repair capacity than rapidly dividing proliferating cells (24). It is, therefore, not unexpected that dermal fibroblasts accumulate CPDs during the course of the chronic treatment and that this damage can persist for a considerable period thereafter, especially if the damage has reached a level that impairs normal cell function (e.g., repair).

The accumulation of nonrepairable CPDs in the epidermis is more problematic. Under conditions of constant low-level UVB exposure, we see the accumulation and persistence of DNA damage in \( \sim 5\% \) of the cells located along the dermal-epidermal boundary. Present models view the murine epidermis as a self-renewing tissue comprised of epidermal proliferative units consisting of slowly cycling stem cells, proliferative transit cells, and postmitotic keratinocytes (25, 26). The rate of renewal in the hairless mouse under normal conditions is approximately 1 week. Present dogma suggests that proliferating cells that have sustained DNA damage follow one of several paths: (a) they repair the damage and assume normal function; (b) they continue cell division using a damaged DNA template, in which case photoproducst are removed later or are diluted during cell division forming mutations in the process; or (c) they succumb to apoptosis or necrosis and are eliminated from the tissue.

Our present data point toward an alternate path, one in which some cells located at the dermal-epidermal boundary seem to excise-repair deficient and are nondoning (within the time frame examined). The behavior of these cells bears a striking resemblance to the “carcinogenic label-retaining cells” described by Morris and coworkers (27–29). Double-label experiments using \([\text{3H}]\text{thymidine and [14C]benz[al]pyrene}\) showed that 1–3% of the basal cells in the interfollicular epidermis did not divide and retained the carcinogen for at least 4 weeks after treatment. Their evidence suggests that this subpopulation of quiescent basal cells at the center of the epidermal proliferative unit is the target of tumor initiation. Should the CPD-retaining cells reported here correspond to those described by Morris, it is probable that these cells are the source of the signature mutations characteristic of sunlight-induced skin cancer as well as the mutant p53 clusters observed in murine epidermal whole mounts by de Grujil and coworkers (30). Our estimates of CPD-retaining cell and CPD frequencies in the epidermis (from Figs. 4 and 5) suggest that these cells may carry as many as 2000 CPDs/mb. An experiment was performed to test the ability of these cells to divide (data not shown). Mice were irradiated with 0.5 kJ/m² UVB for 40 days and, left untreated for an additional 33 days, and then topically treated with 200 \( \mul \) of a 100 \( \mug/ml \) solution of the tumor promoter TPA. IFM of skin sections taken at the time of TPA application showed the expected CPD-retaining cells at the basal layer. At 24 h, we observed significant hyperplasia. Although few bright fluorescent cells were evident at the dermal-epidermal boundary, several doublets and small clusters with reduced fluorescent signals were seen in transit. By 48 h, only background fluorescence was observed in the epidermis. Although preliminary, these data indicate that the CPD-retaining cells are capable of cell division.

RIA data from our chronic and acute irradiation protocols indicate that the accumulation of residual CPDs is dose-dependent. Regression analysis of the damage remaining after 10-days repair at different times during the chronic treatment (Fig. 3) shows a linear increase between 0 and 40 days. The frequency of CPDs present 10 days after the last chronic treatment at 40 days is comparable to the level measured at 90 days. Hence, the accumulated dose can be used to predict the amount of accumulated residual damage and, vice versa, the residual damage can be used to estimate exposure history (at least within the time constraints of this experiment). The rate of accumulation is approximately 1 CPD/mb/ kJ/m² UVB or about 6% of the number of lesions that would be induced by 20 kJ/m² (i.e., the accumulated dose). However, it is probable that the actual rate of accumulation is greater than that estimated due to the effects of chronic exposure on damage induction and repair. Residual damage estimates (\( r \) values) from the acute-dose repair curves suggest that the UVB fluence rate may influence the damage accumulation rate. The 40-day chronically exposed mice repair about two-thirds of the damage induced by the acute dose (i.e., amplitude), leaving one-third of the damage unrepaired. Because the irradiation protocol did not consider the long-term fate of the lesions induced by the acute treatments, future
Several factors may individually or in concert affect NER in chronically irradiated epidermal cells: 
(a) DNA damage may accumulate to such an extent that it affects the expression of genes important in excision repair. CPDs can both block the progression of RNA polymerase (31) and inhibit transcription factor binding (32); 
(b) chronic UVB may alter the spatial distribution of DNA photoproducts such that they occur preferentially in regions of the genome less accessible to repair enzymes. Because DNA-protein interactions can enhance photoproduct formation, it is conceivable that chromatin condensation in response to chronic stress (or apoptosis) may result in nonrandom induction of dimers in such regions; 
(c) daily exposure to low-level UVB may alter epidermal turnover kinetics such that the proportion of slowly repairing postmitotic keratinocytes increases relative to the proliferative transit cells. Although we observed no obvious effects of chronic irradiation on epidermal architecture (i.e., no hyperplasia), we did not attempt to describe proliferative profiles; 
(d) accumulation of free radicals generated by chronic UVB radiation may damage proteins involved in DNA repair. We recently showed (33) that topical treatment of UVB-irradiated Skh-1 mouse skin with \( \alpha \)-tocopherol, a hydroxyl radical scavenger, increased NER of CPDs. Because chronic UVB has been shown to produce reactive intermediates (19–21) that are capable of damaging proteins at very low concentrations, it is possible that the enhanced NER is due to the ability of \( \alpha \)-tocopherol to scavenge hydroxyl radicals that damage repair proteins.

Our data are consistent with previous reports (22, 23) showing that chronic UVB predisposes the DNA in epidermal cells to increased damage formation. Calculations of amplitude for both CPDs and P(6-4)PDs (\( a \) values in Table 1) as well as direct experimental data indicate that an acute dose of 2 kJ/m² UVB induces significantly greater levels of DNA damage in the epidermis of mice that have been repeatedly exposed to low levels of UVB light compared with those that have not. Surprisingly, several weeks after the chronic treatment (i.e., at 90 days), P(6-4)PDs but not CPDs are induced at frequencies significantly greater than in untreated controls. Vink et al. (23) speculated that “after the first exposure, the structure of the chromatin becomes more open, and, therefore, the DNA [becomes] more UV-sensitive.” Because of the magnitude of the effect, it is unlikely that changes in chromatin alone can account for the observed potentiation of photoproduct formation. Except for a 10.3 periodicity of CPD formation in nucleosome core DNA (34), there is no evidence that chromatin affects its formation. In contrast, the P(6-4)PD forms in linker DNA at a considerably greater frequency than in nucleosome core DNA (35, 36). Hence, localized dechromatization (in response to chronic irradiation) could contribute to increased induction of P(6-4)PDs, especially if formation of the damage potentiates the formation of additional damage nearby (i.e., clustering).

In addition to changes in chromatin structure, other mechanisms may contribute to the increase in photoproduct induction. First, signal transduction and gene activation in response to chronic UVB exposure may increase the probability of photoproduct formation by amplifying DNA-protein interactions. Pfeifer et al. (37), using ligation-mediated PCR to quantify DNA photoproduct formation in the phosphoglycerate kinase promoter at base resolution, found that photoproduct hotspots formed in the bound promoter region, presumably facilitated by the bending/unwinding of DNA associated with transcription factor binding. Although such hotspots could arise from the chromatin destabilization associated with transcription factor binding, it may also be that as yet uncharacterized photochemical interactions between DNA and bound protein are contributive.

Increased photoproduct formation may not represent a potentiation effect per se but rather reflect loss of a very early NER component undetected by our repair protocol. Present dogma suggests that little NER occurs in mouse skin during the 1st h postirradiation (14, 17, 22, 23). However, a recent report suggests that the initial rate of repair is dose-dependent, and that, after low UVB exposures (i.e., \(<1 \times MED\), \( \sim 40 \) and 65% of the CPDs and P(6-4)PDs, respectively, are excised from Skh-1 epidermis within 2 h (38). In the experiments reported here, the time between acute UVB exposure and fixation (by freezing) of dorsal biopsies was 30–40 min. To determine whether the increase in photoproducts measured at “zero hour” could be attributed to reduction in a veiled repair component occurring during the 1st h postirradiation, an additional experiment was performed in which dorsal tissue was frozen and 65% of the CPDs and P(6-4)PDs, respectively, are excised from Skh-1 epidermis within 2 h (38). In the experiments reported here, the time between acute UVB exposure and fixation (by freezing) of dorsal biopsies was 30–40 min. To determine whether the increase in photoproducts measured at “zero hour” could be attributed to reduction in a veiled repair component occurring during the 1st h postirradiation, an additional experiment was performed in which dorsal tissue was frozen (in liquid nitrogen) within 1 min and at 10 and 30 min after exposure. The data in Fig. 10 suggest that a significant portion of the CPDs and P(6-4)PDs may be excised within 30 min after formation, and that the loss of this repair capacity could partially contribute to the increased damage levels observed in our data (Figs. 8 and 9; Table 1). These results warrant further investigation.
Our results demonstrate that skin cells exposed to UV radiation slowly over a long period of time—conditions that mimic those associated with many occupational and recreational behaviors—respond in quite unexpected ways. The accumulation of DNA damage in cells at the dermal-epidermal boundary, reduction in excision-repair capacity, and potentialization of mouse epidermal DNA to photoproduct formation indicate that chronic low-dose exposure to solar UVB may result in a significant predisposition to sun-induced carcinogenesis. Because these experiments were performed in unpigmented hairless mice, it can be argued that the beneficial effects of casual exposure to sunlight (i.e., protection afforded by tanning) outweigh the harmful effects described here. However, considering the observation that melanin acts as a “double-edged sword,” not only absorbing UV radiation but also generating reactive oxygen species (9), such dividends may be refutable.

The participation of chronic low-dose and episodic high-dose exposures in photocarcinogenesis and particularly melanogenesis are a matter of some debate. Our data suggest that exposure to a high erythmal dose of UVB (i.e., sunburn) may be more injurious at the molecular level if preceded by consistent exposure to low levels of sunlight. The recent assertion that UV-A radiation may be primarily responsible for melanoma formation (40) and the ensuing controversy regarding the use of UVB-blocking sunscreens prompts us to investigate whether the molecular and biological responses to chronic UVB exposure described here are also elicited by UV-A. Of particular note is our observation of persistent heavily damaged cells at the dermal-epidermal boundary. These cells are stimulated to divide in the presence of a tumor promoter and may play a significant role in sunlight-induced carcinogenesis as well as providing a useful biomarker for estimating individual exposure history and skin-cancer risk. The dose-response relationship between UVB exposure and the frequencies of the persistent damaged cells as well as the cells’ identity (as stem cells or melanocytes?) is currently under investigation.

REFERENCES


Effects of Chronic Low-Dose Ultraviolet B Radiation on DNA Damage and Repair in Mouse Skin


Cancer Res 1999;59:2875-2884.

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