Solar-Simulated Skin Adaptation and its Effect on Subsequent UV-Induced Epidermal DNA Damage

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Repeated skin exposure to ultraviolet radiation leads to increased tolerance for erythema. Whether this tolerance is accompanied by a significant protection against epidermal DNA injury has never been thoroughly investigated. In a first set of experiments we irradiated 25 healthy volunteers three times a week for 3 wk using solar-simulating tanning lamps. In addition, all individuals were exposed to a (challenge) dose of three times the initial minimal erythema dose on a small area of skin before the first and after the final exposure. On both occasions, cyclobutane pyrimidine dimers were quantified in biopsies. As expected, repeated ultraviolet exposures resulted in increased epidermal pigmentation and thickness. The ultraviolet sensitivity for erythema decreased on average by 75%. The cyclobutane pyrimidine dimer formation was reduced on average by 60%. In a second set of experiments, with a group of 13 subjects, DNA repair kinetics were assessed. Within a period of 5 d after a single, slightly erythemal dose (1.2 minimal erythema dose), levels of cyclobutane pyrimidine dimer and p53-expressing cells were determined in skin biopsies. Both markers of DNA damage were elevated upon the single ultraviolet exposure and returned to background levels after 3–4 d. This information is important when trying to minimize the risk of DNA damage accumulation after repeated exposures during a tanning course. ***Key words: cyclobutane thymine dimers/erythema/p53/pigmentation/skin/skin cancer/UV radiation. J Invest Dermatol 117:678–682, 2001***

A variety of psychologic studies have shown that people are attracted to tanning because it helps them to relax and to look healthy, wealthy, and more attractive. In spite of increasing public awareness and knowledge of the unwanted effects of excessive sunlight exposure, many people still believe that the benefits of suntan outweigh the risk involved in getting the tan (Keesling and Friedman, 1987; Arthey and Clarke, 1995). This is also the reason why recreational and cosmetic use of artificial ultraviolet (UV) sources, tanning lamps, has become popular in recent decades, largely in countries where natural sunshine is relatively sparse. A survey among the adolescents in Sweden showed that more than 50% had used commercial sunbeds at least four times in 1 y (Boldeman *et al*, 1996). As a rule, young women are highly represented among the tanning lamp users (Lillquist *et al*, 1994).

A significant percentage of individuals using sunbeds believe that the use of tanning lamps is safer than sun exposure because the composition of UV radiation from the lamps is constant, and the dosage can be properly controlled. A large proportion of tanning bed users utilizes the sunbeds as a preparation for their holiday in sunny countries because they expect that the tan from UV lamps protects them from the harmful effect of outdoor sun exposure (Mawn and Fleischer, 1993; McGinley *et al*, 1998).

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Abbreviation: CPD, Cyclobutane pyrimidine dimer

That repeated solar irradiation of the skin not only results in increased pigmentation but also in skin thickening is generally recognized. The role of the thickened epidermis and stratum corneum is to increase the light path and consequently decrease the transmission of UV radiation to the vulnerable cells of the basal and suprabasal layers. In persons with ability to tan, photoprotection is achieved by the combination of tanning augmentation and increased thickening of epidermis. These two processes thus elevate the UV absorption abilities of the skin and, consequently, enhance sun tolerance. Based on the knowledge of absorption properties of the human skin and its components (Anderson and Jarrish, 1981) one may deduce that cutaneous thickening is more important for the UVB part of the solar spectrum whereas melanin pigmentation plays a role in the protection against both UVB and UVA photons. Recently, Sheehan *et al* (1998) demonstrated that tanning induced by solar-simulated radiation in skin type II or III individuals offered moderate protection against erythema. The authors, however, suggest that, in these two skin types, thickening of stratum corneum may afford even less photoprotection than tanning.

Scientific reports dealing with protection against epidermal DNA damage by photoadaptation are scarce. Gange *et al* (1985) reported that four UVB exposures administered over an 8 d period conferred significant protection against UVB-induced erythema and led to the reduction in yield of endonuclease-sensitive sites in epidermal DNA. UVA tan provided similar protection against DNA damage; however, it was not associated with protection against erythema. As many people worldwide use recreative tanning lamps in order to accommodate their skin to UV radiation, increased scientific knowledge on true effectiveness of such skin adaptation is warranted.

In this study we addressed two questions. First, what is the level of protection provided by repeated UV irradiation simulating a tanning course? Second, what are the kinetics of DNA repair after skin exposure to a single UV dose with intensity comparable to those used during tanning sessions. Answers to these questions will help to provide a sensible way of tanning. To address these, we have made use of novel UV lamps that emit radiation with a UV spectral distribution closely mimicking that of the solar UV spectrum. We show that the achieved skin adaptation provides measurable protection against UV-mediated DNA injury. In addition, we demonstrate that a single UV irradiation causes DNA damage in the epidermis that needs several days to be repaired. These results indicate that, for a safer way of tanning, special attention should be paid to the frequency of UV exposures.

MATERIALS AND METHODS

Subjects After obtaining permission from the Medical Ethics Committee 25 healthy volunteers were enrolled in the first part of the study. All persons gave their written informed consent for participation in the study. The group consisted of seven men and 18 women with age ranging from 18 to 34 y (mean 22 y). The volunteers were all Caucasians and they were divided into two groups according to their minimal erythema dose (MED) values. An MED value corresponding to a UVB dose of 250 J per m^2 was chosen as a margin between the two groups. An IL700 radiometer (International Light, Newburyport, MA) equipped with an SEE400 detector and a WBS-320 filter was used for the measurement of UV dosimetry. Nine participants were considered to have a lighter and 16 a darker skin phototype, as based on their personal MED value. The personal MED of the lighter skin type group was on average 185 ± 31 J per m^2 (range 105–200), whereas that of the darker skin type group was on average 339 ± 64 J per m^2 (range 275–450).

Another 13 healthy volunteers were enrolled in the second part of the investigation. All persons gave their written informed consent for participation in the study. The group consisted of two men and 11 women with an average age of 22 y, ranging from 18 to 27 y. According to the above-mentioned criterion, five participants were regarded as having a lighter and eight as having a darker skin phototype.

Radiation source Cleo Natural lamps, recently developed by Philips Lighting (Roosendaal, the Netherlands), emit the radiation that in the UV range simulates the spectrum of sunlight (**Fig 1**). These lamps were used throughout the study.

Procedure The volunteers filled in a questionnaire that allowed us to estimate their skin phototype. Subsequently, the MED values and the pigmentation score were assessed. For the first part of the study, the volunteers received a challenge dose of three times their individual MED on a small area (5 × 5 cm) of the skin on the buttocks or lower back. With regard to the induction of DNA damage this dose was on a linear part of a dose–response curve (Roza *et al*, 1988). Fifteen minutes later, 5 mm punch biopsies were taken from the irradiated and control sites under local anesthesia. Subsequently, the back skin of the volunteers was exposed to the tanning lamps three times a week (Monday, Wednesday, Friday) for 3 wk. The first dose was 0.5 MED, the second 1 MED, followed by doses with an increment of 20% MED. On the third day after the final exposure, the MED and pigmentation were determined again and a small area of skin (5 × 5 cm) was exposed to the same challenge dose of UV as at the beginning (i.e., three times the initial MED). Subsequently, 5 mm biopsies were taken from the irradiated and nonirradiated (control) parts of the skin.

In the second part, another group of volunteers received an individual dose of 1.2 MED on a 20 × 20 cm previously unexposed skin area of the buttocks or lower back, and 4 mm punch biopsies were taken from the irradiated and nonirradiated (control) sites under local anesthesia. Biopsies were taken from the irradiated site at 15 min, and 24, 48, 72, and 96 h after exposure.

Assessment of skin pigmentation A computer-controlled tristimulus color analyzer (Minolta Chromameter II Reflectance) was used to measure UV-induced pigmentation and erythema. This chromameter records color in a three-dimensional space designated $L^*a^*b^*$. First, the luminance (L^*) value expresses the relative brightness of the color ranging from total white (+) to total black (–). The second value, a^*, is the color hue ranging from red (+) to green (–), and the b^* value ranges from yellow (+) to blue (–) (Park *et al*, 1999). The color of the skin was measured before the irradiations and after the termination of the irradiation session. The same site was measured three times and care was taken to select the spots free of any pigmentation irregularities (e.g., nevi).

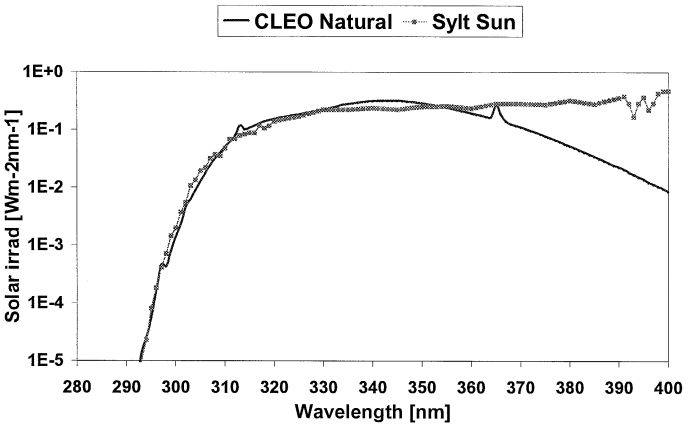

Figure 1. Comparison of the UV emission spectrum of Cleo Natural lamp with that of solar radiation demonstrates the similarity in spectral output of the two UV sources.

Determination of MED The MED values were determined with the use of the Cleo Natural lamps. The test sites (1.3 × 1.3 cm) were exposed to five 40% incrementing doses of UV. After 24 h the skin reaction was visually evaluated. The lowest dose of UV energy that caused a just perceptible, sharply demarcated erythema was considered as 1 MED.

Histology and immunohistochemical staining Each biopsy specimen was fixed in buffered 37% formaldehyde for 2 h and subsequently transferred to a 70% ethanol solution. Subsequently, they were embedded in paraffin and cut in consecutive sections with a thickness of 4 μm.

Quantification of viable epidermal thickness and stratum corneum thickness was accomplished with a Southern Microsystems computer-assisted image analysis system. Images were visualized with an Ikegami CCD color camera attached to an Olympus H40 light microscope. The thickness of stratum corneum and the whole epidermis was measured on three random chosen places in the histologic preparation and the mean value was calculated.

Monoclonal antibody H3 (IgG1-lambda subclass) developed against cyclobutane thymine dimers in single-stranded DNA (Roza *et al*, 1988) was used to detect these DNA photoproducts *in situ*. This antibody has high affinity for 5′T-containing dimers (Fekete *et al*, 1998). The bound H3 antibody was detected through binding of a second (anti-IgG1) antibody labeled with fluorescein isothiocyanate. Quantification of the fluorescent light emitted after the excitation of the fluorochrome was performed with a computer-assisted image-processing technique (Roza *et al*, 1991).

A slight adaptation of a method described by Krekels *et al* (1997) was used for the detection of p53 protein in the epidermal cells. The antibody staining was always done in one run within each part of the study.

Statistics A paired Wilcoxon–Kruskal–Wallis test was used to determine the difference before and after the repeated irradiations. To examine differences between darker and lighter skin types the unpaired Wilcoxon test was utilized.

RESULTS

Changes in skin pigmentation, thickness of epidermis and MED values Visual observations during the period of repetitive irradiations showed that the volunteers with lighter skin types frequently developed slight erythema and that they attained less pronounced pigmentation than those with darker skin types. This was also reflected by color changes as analyzed using the $L^*a^*b^*$ system before and after the UV irradiation session. At the end of the exposures, an 11% decrease of the L^* value (brightness) was recorded together with an increase of the a^* value (21%) and the b^*

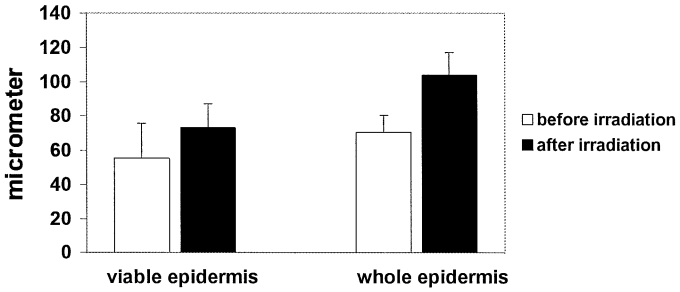

Figure 2. Increase in the epidermal thickness before and after nine UV exposures (n = 25). Bars represent means ± standard deviation.

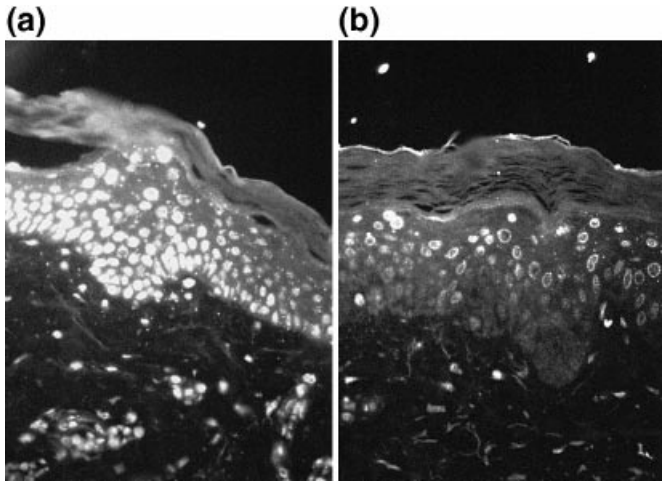

Figure 3. Solar-simulated skin adaptation leads to decreased CPD induction. (*a*) CPD immunoreactivity induced by three times the initial MED in unadjusted skin. The immunostaining is present throughout the epidermis. (*b*) CPD immunoreactivity induced by three times the initial MED in adjusted skin. The pattern of immunostaining is much less expressed. The basal and suprabasal cells stain negatively.

value (22%), which indicated that the skin was getting dark and that there was an intensification of the red and yellow color. The decrease of skin brightness was significantly more pronounced in individuals with darker skin (13%) compared to that in lighter-skinned (8%) individuals ($p = 0.03$).

As seen in **Fig 2**, the repeated irradiations caused a significant increase (42%, paired t test; $p < 0.0001$) in the thickness of the whole epidermis. When we evaluated both skin type groups, we did not find any statistically significant differences (36% ± 9% increase in the light skin type group *versus* 46% ± 6% increase in the darker skin type group; $p = 0.571$). The thickness of the viable epidermis was increased by 27% (paired t test; $p < 0.0001$). Also in this case we did not find differences between both skin type groups (26% ± 9% increase in the lighter skin type group *versus* 29% ± 6% increase in the darker skin type group; $p = 0.955$). The results of this investigation confirm the earlier findings that repeated UV exposures of the skin lead to epidermal hyperproliferation and that skin thickening can mainly be ascribed to the thickening of stratum corneum.

As a result of the adaptation processes, at the end of the UV exposure session, UV sensitivity with respect to the development of cutaneous erythema in 25 persons decreased on average 4-fold (4.04 ± 0.46). In the individuals with the lighter skin types MED values increased as follows: in two persons 3 MED, in seven 4 MED, and none 5 MED. In the group of volunteers with darker skin types 13 had an elevation of 4 MED and three individuals 5 MED. Overall changes in the skin UV sensitivity are statistically significant (paired Wilcoxon–Kruskal–Wallis test; $p < 0.001$). The

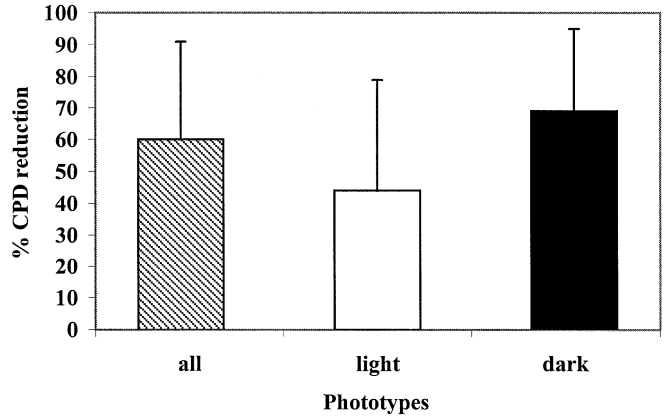

Figure 4. Decrease in UV-induced CPD formation after skin adaptation with repeated UV doses (n = 25). Bars represent means ± standard deviation.

difference between the increase of MED in lighter and darker skin types was also significant: 3.78 ± 0.44 and 4.19 ± 0.40 (mean ± SD), respectively ($p = 0.03$).

Occurrence of cyclobutane pyrimidine dimers (CPD) in the epidermis before and after solar-simulated skin adaptation **Figure 3**(***a***) shows the result of a typical CPD immunofluorescence staining in nonadapted skin irradiated with 3 MED. Visually, there seems to be an equal distribution of dimer induction throughout all epidermal layers including the basal layer. An example of CPD immunofluorescence after skin adaptation by tanning lamps is shown in **Fig 3**(***b***). There is considerably less CPD labeling and the fluorescence in the basal layer is absent. This may be of importance because the damage of basal cells with their proliferative potential is likely to have more consequences than the damage of the epidermal cells that are already committed to terminal differentiation.

The skin adaptation by tanning lamps resulted on average in 60% reduction of CPD formation after the 3 MED challenge (paired t test; $p < 0.0001$) (**Fig 4**). There was no significant difference between the basal (control) values before and after the UV adaptation (fluorescence before 5797 ± 967, after 6392 ± 1653; $p = 0.252$). Individuals with more pigmented skin tended to be better protected than the light-skinned people. This difference failed to reach statistical significance, however (unpaired t test; $p = 0.057$).

CPD removal and p53 expression after a single UV exposure In the second part of the study, the kinetics of CPD removal in the epidermis were addressed. As shown in **Fig 5**(***a***), the presence of CPD was clearly detectable after 15 min. After 72 h the presence of CPD became barely detectable. As shown in **Fig 5**(***b***), at 15 min after 1.2 MED, CPD levels in lighter and darker skin were similar. Also, at later time-points no (significant) differences were observed.

p53 expression was maximal at 24 h after exposure and declined to background levels in 3–4 d (**Fig 6***a*). In three out of the five light-skinned individuals, however, the highest number of p53 positive cells was found at 48 h after exposure (**Fig 6***b*).

DISCUSSION

CPD are well-recognized DNA photoproducts that have been shown to contribute substantially to the biologic consequences of UV exposure (Mitchell, 1988). We here demonstrate that our irradiation regimen provides protection against erythema and DNA damage formation: a 4-fold higher dose was required to cause erythema, and 60% less CPD formation was the result after a fixed dose of three times the initial MED. This protection, however, was achieved at the expense of 10 UV exposures that caused DNA damage as well. Due to individual variations there was no clear-cut

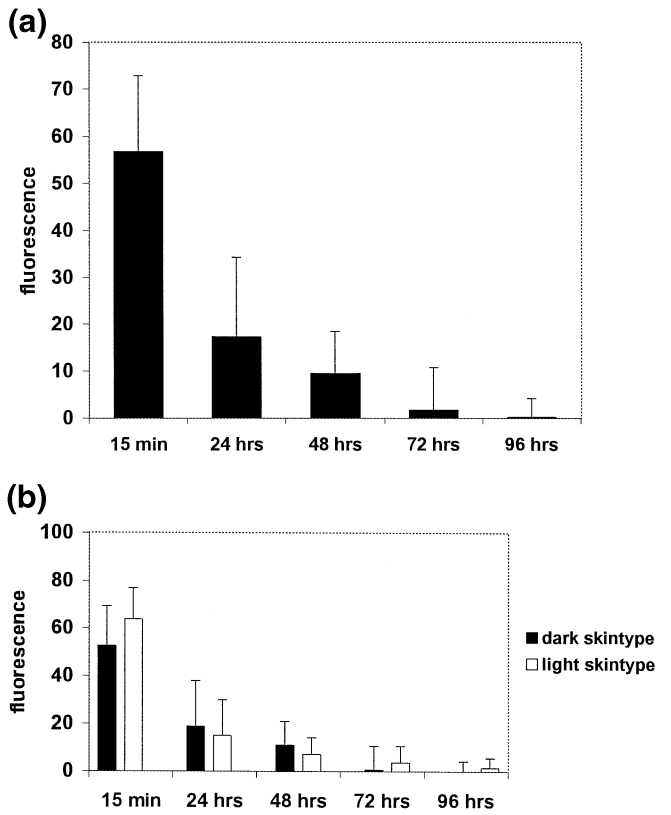

Figure 5. Decrease of CPD immunoreactivity in epidermis after a single UV exposure. (*a*) Time-course of the epidermal changes in CPD fluorescence after a single UV dose of 1.2 MED ($n = 13$). Bars represent means ± standard deviation. (*b*) Time-course of the epidermal changes in CPD fluorescence after a single UV dose of 1.2 MED in lighter ($n = 5$) and darker ($n = 8$) skin phototypes. Bars represent means ± standard deviation.

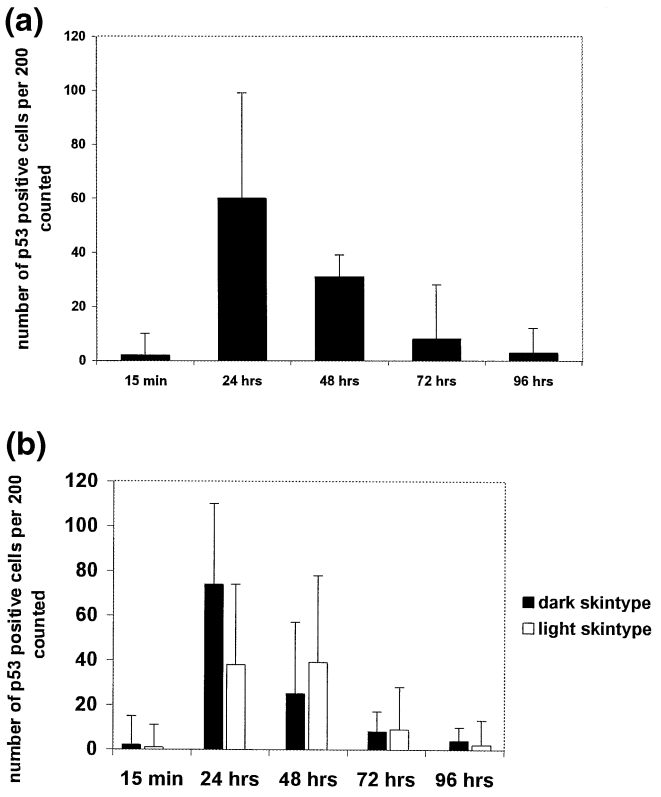

Figure 6. Decrease of p53 immunoreactivity in epidermis after a single UV exposure. (*a*) Time-course of the epidermal changes in p53 expression in epidermis after a single UV dose of 1.2 MED ($n = 13$). Bars represent means ± standard deviation. (*b*) Time-course of the epidermal changes in p53 expression in epidermis after a single UV dose of 1.2 MED in lighter ($n = 5$) and darker ($n = 8$) skin phototypes. Bars represent means ± standard deviation.

correlation between the increased UV tolerance with respect to erythema and protection against DNA damage (not shown).

There is no straightforward answer to the question whether people should be recommended a preholiday UV-induced skin adaptation. Individuals with very light, sun-sensitive skin, who always burn and never tan, should minimize any exposure to UV radiation because their skin is not capable of protecting them properly. The individuals who tan easily and who plan to visit warm sunny countries might consider increasing their UV tolerance by repetitive exposures to tanning lamps. We recommend that this UV adaptation should be done with only slightly erythematogenic UV doses that would be applied merely twice a week with an interval of at least 3 d in between. The expected, though not fully proven, advantage of this natural skin adjustment is to increase cutaneous UV absorption capacity, to facilitate repair of UV-induced DNA photoproducts, and to activate protective enzymes such as superoxide dismutase (Liu *et al*, 1999; Poswig *et al*, 1999). The induction of such photoadaptation has been reported by Bataille *et al* (2000) in 11 psoriasis patients (skin types II–IV) who received UVB therapy three times a week. Already at three exposures, levels of CPD in epidermal cells reached a plateau and were found to decrease for subsequent exposures despite increasing UVB doses. That chronic exposures to solar UVA and UVB radiation induces photoprotection even in the absence of melanin has recently been demonstrated by Mitchell *et al* (2001). The authors treated albino hairless mice with UVA and UVB radiation daily for 60 d and measured the frequency of CPD and 6–4 photoproducts induced by a single acute sunburn dose of UVB at different stages of the chronic treatment. They found that both UVA and UVB exposures produced a photoprotective response in the dermis and epidermis. The effects of chronic UVA irradiation on damage formation were much less pronounced than those of UVB irradiation. Moreover, the effect of chronic UVB irradiation appeared to be long lasting as significant attenuation of photodamage was observed after a recovery period of 60 d. The authors suggest that unknown substances that specifically block photoproduct formation may be induced during the adaptation processes.

All injured cells, including skin cells suffering from UV damage, must be given sufficient time to repair the damage before another attack may reach them. The timely repair of the injury can be of enormous importance with respect to the mutation susceptibility of cells. Young and coworkers reported that, in human skin, the removal of pyrimidine dimers was slow (half-life 33.3 h) whereas the elimination 6–4 photoproducts was completed within a few hours (Young *et al*, 1996). In our experiments the (almost) complete removal of CPD after a single irradiation with a slightly erythematogenic UV dose (1.2 MED) took approximately 72 h. By complete removal it is meant that CPD were no longer detectable above background, caused by the sum of the following activities: nucleotide excision repair, cell proliferation (dilution of DNA damage), and possible other activities that may hamper antibody recognition and/or binding. DNA repair rates are dependent on the applied UV dose: at high doses saturation of repair enzymes may occur (Vink *et al*, 1994). Moreover, Goukassian *et al* (2000) recently showed that there was a significant decrease with aging in the repair rates of both CPD and 6–4 photoproducts.

An important role in the repair of cellular damage has been ascribed to the tumor suppressor gene p53 (Oren, 1992). This gene is known to regulate cell cycle progression following the exposure to DNA-damaging events like UV irradiation (Yuan *et al*, 1995). In normal cells, the increase in p53 concentration is associated with G1 arrest, allowing time for the repair of DNA before DNA

synthesis and mitosis can progress (Lane, 1992). Another important role of the p53 protein is triggering apoptosis after UV irradiation. Hence, the p53 protein is involved in the decision process that determines the fate of the cells after UV-induced DNA injury (Brash *et al*, 1996). Recent reports also indicate that p53 might be involved in the nucleotide excision repair. Li and Ho (1998) were able to show that, in their model of human and murine fibroblasts, nucleotide excision repair was increased after low doses but not after high doses of UVB radiation. Conversely, apoptosis occurred only after the cells received high doses (over 200 J per m^2) of UVB. In control cells with a homozygote deletion of p53 gene no induction of repair and apoptosis was observed.

We applied doses that are frequently used in recreational tanning and found that the increased p53 levels returned to background within 96 h. Interestingly, there seemed to be some small (statistically nonsignificant) differences in the kinetics of p53 between darker and lighter skin phototypes. In the epidermis of light-skinned individuals fewer cells expressed p53 and the maximum level of expression was somewhat delayed; also, the removal of CPD was less efficient in the light-skinned epidermis. Our recent investigations of cultured melanocytes also suggested that lightly pigmented cells need more time to remove UVB-induced CPD (Smit *et al*, 2001). This was in line with report of Barker *et al* (1995) who found that exposure of melanocytes resulted in a block in G1, which was connected with the induction of p53 protein. In more pigmented melanocytes, p53 levels declined much faster than in the lightly pigmented cells. The authors speculated that the less pigmented melanocytes were arrested in G1 for a longer period by p53 in order to allow for more time to accomplish DNA repair. Taken all together, one can conclude that there is a theoretical possibility that lightly pigmented melanocytes need a somewhat longer time period for the removal of CPD. This important issue needs to be addressed in some future investigations.

High frequency UV exposures (e.g., daily exposures during sunny holidays) do not leave much time for repair of inflicted damage. This factor may logically play an important role in UV carcinogenesis. In almost all animal experiments documenting the carcinogenic properties of UV radiation, five to seven exposures a week have been applied (Strickland, 1986; Van Weelden *et al*, 1988; Kelfkens *et al*, 1991; De Gruijl *et al*, 1993; Wulf *et al*, 1994). There is no doubt that such frequent irradiations result in the accumulation of cellular injury (Vink *et al*, 1991) and, consequently, increase the risk of DNA mutations. The question remains whether UV radiation would be such a strong carcinogen if the irradiations were performed at reduced frequency.

An analogous situation applies when people daily expose their skin on sunny beaches and the skin does not get enough opportunity to rest. From the viewpoint of DNA repair kinetics this is a hazardous way of tanning. Indoor tanning is not safer than the sun but the use of timers and the possibility of easily regulating the exposure frequency could make it safer than the attitude of millions of people who want to get a tan during the first days of their sunny holidays.

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