

INVESTIGATIVE REPORT

Enhanced Epidermal Ultraviolet Responses in Chronically Sun-exposed Skin are Dependent on Previous Sun Exposure

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The p53 protein plays a key role in protecting cells from acquiring manifest mutations by inducing cell cycle arrest or apoptosis. The mechanisms for differences in epidermal responses to ultraviolet irradiation are unclear, although they have been shown to be related to both genetic events and environmental factors. In this study, we compared epidermal ultraviolet responses in chronically sun-exposed and non-sun-exposed skin using immunohistochemistry with antibodies recognizing thymine dimers and p53 protein. Six healthy volunteers were subjected to both artificial ultraviolet irradiation and natural sunlight, with and without photoprotection. A smaller number of thymine dimer-positive keratinocytes were detected 24 h after ultraviolet exposure in chronically sun-exposed skin compared to non-sun-exposed skin. Further, the p53 response was more variable in chronically sun-exposed skin. A significant correlation between total ultraviolet dose and number of p53-immunoreactive keratinocytes was found after natural sun exposure. Our findings suggest that repair of DNA damage is more efficient in chronically sun-exposed skin than in non-sun-exposed skin. *Key words: photoprotection; p53; thymine dimers; ultraviolet radiation.*

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Chronic exposure to ultraviolet (UV) radiation is known to be a major risk factor for development of non-melanoma skin cancer (NMSC). Crucial cellular events that precede formation of malignant tumours in skin include DNA damage, insufficient repair of DNA damage, establishment of UV-induced mutations in critical genes, and escape from apoptosis, which results in selective growth advantage of a precancerous clone. UV-induced DNA damage has been demonstrated in human epidermis both *in vitro* and *in vivo* (1, 2). Pre-mutagenic DNA damage includes the formation of cyclobutane pyrimidine dimers, most commonly thymine (TT) dimers and (6-4) photoproducts. To prevent

manifest mutations, the nucleotide excision repair (NER) system removes damaged DNA and restores the original base sequence. TT dimers are formed as a consequence of UV irradiation, and in studies using solar simulators approximately 30–50% of formed TT dimers were found to be repaired within 24 h (3, 4). The critical role of NER is well illustrated in patients with xeroderma pigmentosum (XP), in whom the risk of developing skin cancer is 1000 times greater than that in healthy individuals (5). The variability and finer dynamics of NER in non-XP patients is less well known and may be of importance for the risk of development of skin cancer. A decreased capacity for DNA repair of TT dimers in older populations (≥ 50 years of age) has been suggested (6), and in a recent study DNA damage in skin type IV was shown to have repaired more efficiently than that in skin type II (7).

The p53 gene plays a key role in cell cycle regulation, DNA repair and UV-induced apoptosis. It is well known that p53 protein accumulates in keratinocytes subsequent to UV radiation (8, 9). In human skin, a single erythemogenic dose of artificial UV irradiation induces a rapid accumulation of p53 protein, peaking at 8–24 h after exposure and subsiding within 5 to 15 days (9, 10). A physiological p53 response following genotoxic cell stress facilitates repair of pre-mutagenic DNA lesions, or induces apoptosis if the damage is severe. Mutations in the p53 gene resulting in a dysfunctional protein appear to be an early event in NMSC. Clones of morphologically normal keratinocytes that harbour a mutated p53 gene occur frequently in chronically sun-exposed skin and may represent a forerunner of NMSC (11, 12). Neoplastic skin lesions, e.g. actinic keratosis, invasive squamous cell cancer (SCC) and basal cell cancer (BCC), often contain p53 gene mutations carrying a typical UV signature (13).

Outdoor sun exposure can induce an accumulation of p53 protein, which can be significantly reduced by use of topical sunscreens and fabrics (14). Use of sunscreens has been shown to decrease the number of actinic keratosis lesions in patients (15, 16). A large 4.5 year randomized controlled study using a broad-spectrum sunscreen with a sun-protecting factor (SPF) of 16 showed a significant reduction in the total number of SCCs, but no reduction of BCCs (17). Such studies are difficult to perform for practical and ethical reasons,

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and there is therefore a need for other approaches with short-term surrogates for skin cancer, e.g. UV-induced DNA damage and subsequent mutations in cancer-related genes.

Previous studies have shown large interindividual variations in the epidermal p53 response (14, 18, 19), as well as in the kinetics of DNA repair (3, 18). The aim of the present investigation was to compare UV responses and effects of photoprotection between non-sun-exposed and chronically sun-exposed skin within and between individuals in order to determine intra- and interindividual differences. Following a single erythemogenic dose of artificial UV and exposure to natural sunlight for 6 weeks, skin biopsy specimens were immunohistochemically stained and the numbers of TT dimer- and p53-immunoreactive keratinocytes were calculated.

MATERIAL AND METHODS

Volunteers

Six healthy volunteers, V1-V6 (2 men, 4 women; age 47–75, mean 57 years), entered the study, which was approved by the local ethics committee. All were of Caucasian origin, two with skin type II and four with skin type III (20). The volunteers entering this study had not been bodily exposed to sun during the previous 8 months, except V4, in whom the upper part of the body had been exposed twice during outdoor activities 2 weeks prior to the start of the experiment.

Photoprotection

A topical sunscreen lotion with an SPF of 15 (Coppertone 15, Schering-Plough, Stockholm, Sweden), containing both UVB and UVA absorbers [benzophenone 3 (Eusolex 4360), butyl methoxydibenzoylmethane (Parsol 1789) and octyl methoxycinnamate (Parsol MCX)] (21), was applied 15 min before artificial UV irradiation on a 4 cm² area of a forearm and buttock, in a recommended dose of 2 mg/cm² (COLIPA) (22). Another circular area of 3 cm² was subjected to total photoprotection by coverage with blue denim fabric (SPF 1700), which was attached to the skin by a 20 cm² circular occlusive dressing (Actiderm, Convatec, Bristol-Meyers Squibb, Princeton, NJ, USA). During 6 weeks of natural sunlight (see below) the volunteers used photoprotection identical to that applied during artificial irradiation, i.e. topical sunscreen with SPF 15 and blue denim fabric. They were instructed to apply the sunscreen *ad libitum* every morning on a defined area of 4 cm². The denim fabric was changed two to four times a week.

Artificial UV irradiation with and without photoprotection

The UV source used was a SUPUVASUN 3000 device (Mutzhas, Germany) equipped with a sun filter emitting a broad band of UVB, UVA and near-infrared radiation. At an exposure distance of 50 cm the effect of UVB was 0.03 mW/cm² and of UVA 65 mW/cm². The artificial irradiation treatment was administered in May and the volunteers were irradiated with artificial UV on each skin area with and without photoprotection at a dose of 40 mJ/cm² of UVB and 85 J/cm² of UVA (Fig. 1). Twenty-four hours after the irradiation, 3 mm punch biopsy skin specimens were obtained under local anaesthesia from the dorsal aspect of a forearm and from a buttock, with and without photoprotection,

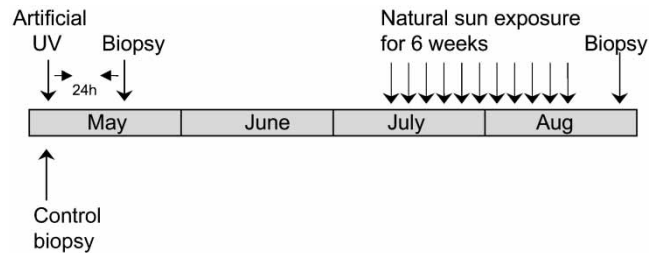


Fig. 1. Experimental protocol illustrating time-points of UV exposure and sampling of biopsy specimens.

representing chronically sun-exposed and non-sun-exposed skin, respectively. Biopsy specimens from non-irradiated skin of both sites were used as controls.

Natural sun exposure

Volunteers were exposed to natural sunlight during 6 summer weeks in Sweden (July–August) (Fig. 1). Twenty-four hours after the final day of sun exposure, 3 mm punch biopsy specimens were obtained from sun-exposed forearm skin with and without photoprotection.

Quantification of UV dose

The total UV dose received from natural sun exposure was measured with polysulphone badges, which the volunteers wore on sun-exposed forearms. The badges were changed every one to three days, depending on the weather. The total UV dose in each individual was measured and calculated at the Regional Medical Physics Department, Newcastle, UK using a conventional spectrophotometer at 330 nm. The results are given in Standard Erythema Doses (SED), one dose being equivalent to an erythemal effective radiant exposure of 100 J/m² (23). Exposure amounting to 1.5 SED is required to produce just perceptible erythema in the unacclimatized skin of sun-sensitive individuals who burn easily and never tan (skin type I), about 2 SED in subjects who burn easily but tan minimally (skin type II) and 3 SED in subjects who will burn but tan readily (skin type III).

Antibodies

A mouse monoclonal anti-p53 antibody of DO-7 (Dako; code M7001) was used as a primary antibody to detect p53 protein. The DO-7 antibody recognizes an epitope in the N-terminus of the human p53 protein and reacts with both wild type and mutant forms of the protein. To detect TT dimers, a mouse monoclonal anti-TT dimer antibody (Kamiya Biomedical Company; code MC-062) was used as primary antibody. A murine monoclonal anti-Ki-67 antibody of MIB-1 (Dako; code M7240) was used to detect Ki-67 antigen, which is expressed in all cells that are not in the G0 phase of the cell cycle. Biotinylated rabbit anti-mouse IgG (Dako; code E354) was used as a secondary antibody.

Immunohistochemical staining procedure

Biopsy specimens were fixed in 4% buffered formalin for one to three days, paraffin-embedded and cut into 4 µm thick sections. Sections were deparaffinized in xylene and rehydrated in a series of graded alcohol. The sections were permeabilized by microwaving at 750 W for 2 × 5 min in 0.01 M citrate buffer (pH 6). After 30 min treatment with 0.3% hydrogen peroxide solution to exhaust endogenous

peroxidase activity, slides were incubated with 1% bovine serum albumin in phosphate-buffered saline to block non-specific binding. After pre-incubation, sections were incubated with primary antibody, DO-7, to detect human p53 protein (dilution 1:200), TT dimer to detect thymine dimers (dilution 1:5000) or MIB-1 (dilution 1:50) to detect proliferating cells. After incubation of either of the three above-mentioned primary antibodies, a biotinylated rabbit anti-mouse IgG (dilution 1:200) was used as secondary antibody. The immune reaction was visualized by avidin/biotin complex (Dako, code K0355, dilution 1:200) with 0.004% hydrogen peroxide as a substrate and diaminobenzidine (Sigma-Aldrich; code D9542) as a chromogen. Negative controls were performed by omitting the primary antibody. Mayer's haematoxylin was used for counter-staining.

Scoring of p53, thymine dimers and Ki67

In each tissue section, p53-, TT dimer- and Ki-67-immunoreactive keratinocytes in the epidermis were recorded under the microscope at 400× magnification. Three random microscopic fields corresponding to 500–700 keratinocytes were counted per specimen. Counting was performed blindly by one person (CW). The results are presented as percentages of counted immunoreactive keratinocytes out of the total number of keratinocytes counted.

Statistical analysis

The Wilcoxon signed-rank sum test was used for pairwise comparisons between types of photoprotection, between unprotected and photoprotected skin and between UV responses in dorsal forearm and buttock skin. Spearman's correlation coefficients were calculated for TT dimer- and p53-positive cells in both forearm and buttocks skin after artificial UV irradiation and for SED and p53-positive cells after exposure to natural sunlight.

RESULTS

Twenty-four hours after artificial UV irradiation, strong erythema was observed in unprotected skin (forearm and buttock). Less pronounced erythema was seen in skin pretreated with sunscreen (SPF 15), and no erythema in skin covered with blue denim fabric. Artificial UV irradiation generated no visible tanning. However, 6 weeks of natural sunlight induced tanning in all volunteers both in skin with and without sunscreen, whereas skin covered with blue denim fabric showed a clear reduction in pigmentation compared to surrounding skin.

p53

All subjects showed increased proportions of p53-positive keratinocytes in unprotected skin of both the buttock (mean=30%) and the forearm (mean=33%) after artificial UV irradiation compared to control skin (mean=3%) (Fig. 2). The interindividual differences in p53 levels were more pronounced in the forearm (Fig. 2B) than in the buttock (Fig. 2A). The variability of the p53 response is well illustrated by the standard deviation (SD) values for the forearm (SD=14.1) and buttock skin (SD=7.6). The induced p53 positivity

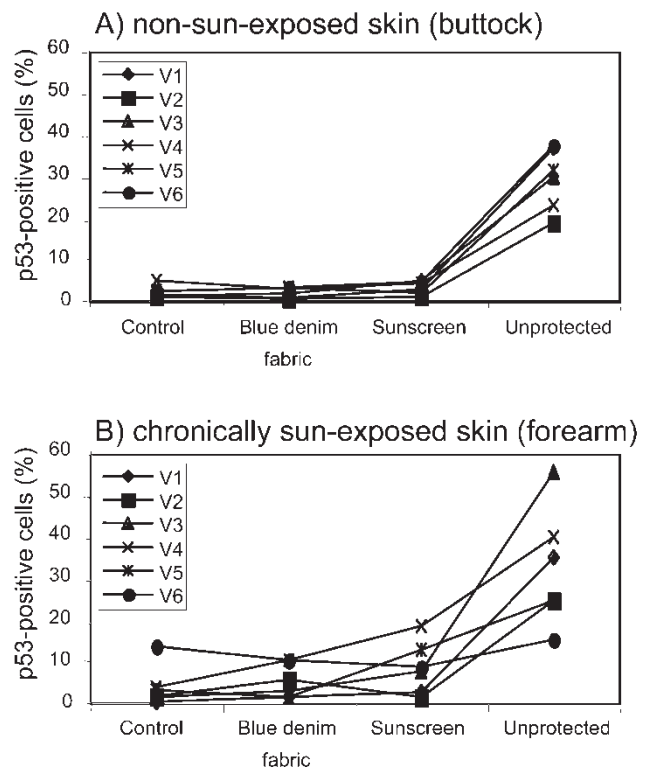


Fig. 2. p53-immunoreactive keratinocytes in non-sun-exposed (buttock; A) and chronically sun-exposed (forearm; B) skin with different degrees of photoprotection 24 h after artificial UV irradiation. Note the larger interindividual differences in chronically sun-exposed skin (B) compared to non-sun-exposed skin (A).

showed a typical reactive immunohistochemical staining pattern, with weak to moderate p53 immunoreactivity evenly distributed in all epidermal cell layers, except in the most superficial cell layers. Use of photoprotection (both sunscreen and denim fabric) reduced the number of p53 immunoreactive cells significantly ($p < 0.003$) (Fig. 3). The mean reduction in p53 protein level in forearms pretreated with sunscreen was 83% and in those protected with blue denim fabric 93%, while in buttock skin the corresponding reductions were 96% and 100%, respectively. A difference in p53 reduction resulting from photoprotection between the forearm and buttock was observed, but was not statistically significant ($p = 0.09$).

After 6 weeks of natural sunlight, increased p53 levels were seen in unprotected forearm skin compared to control skin in all volunteers except V4, who was working indoors during most of the study period (Fig. 4). The mean reduction in p53 levels was 70% for sunscreen and 74% for blue denim fabric compared to unprotected skin. Two p53 clones were found in both photoprotected and unprotected dorsal forearm skin in the two oldest subjects (V1 and V2).

The UV dose received per volunteer during the outdoor session expressed in SED varied from 24 to 98 (mean=48) SED. A positive association between p53

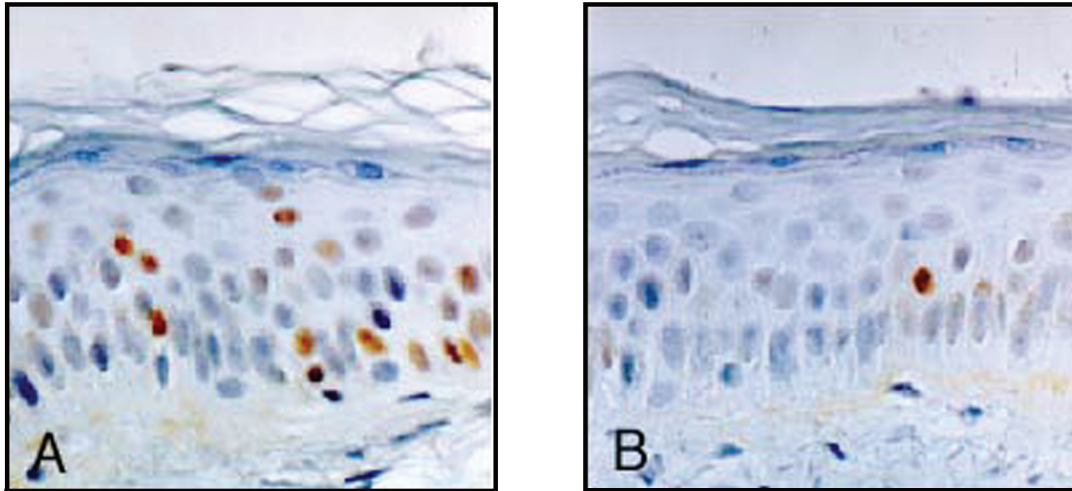


Fig. 3. High power view of skin samples from the same individual (V5) 24 h after artificial UV irradiation. Sections are immunohistochemically stained with antibodies for p53 protein. Immunoreactive nuclei are stained brown. Several p53-immunoreactive keratinocytes are present in unprotected skin (A) but only rare, single p53-immunoreactive keratinocytes are present in photoprotected skin (B).

level and UV exposure was found in unprotected skin after 6 summer weeks, with a Spearman correlation coefficient of 0.81 (Fig. 5)

Thymine dimers

High proportions of TT dimer-positive cells were detected in unprotected skin 24 h after artificial UV irradiation (Fig. 6). There were significantly more TT dimer-immunoreactive keratinocytes in buttock (mean = 32%) than in forearm skin (mean 17%) ($p < 0.03$). The difference in TT dimer immunoreactivity between forearm (Fig. 7A) and buttock skin (Fig. 7B) after artificial UV irradiation is shown for one subject (V1). Immunoreactivity for TT dimers displayed an epidermal gradient, with stronger staining intensity in the superficial cell layers of the epidermis compared to the suprabasal and basal cell layers (Fig. 8A). Both sunscreen and denim fabric gave almost total photoprotection (Fig. 8B). Very few (< 1%) TT dimer-positive keratinocytes

were found in both unprotected and photoprotected skin after 6 summer weeks in Sweden.

Scatterplots demonstrate the correlation between TT

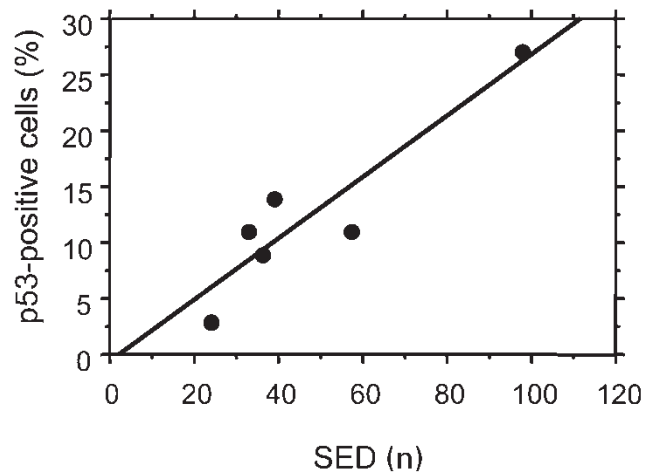


Fig. 5. Dose-response curve for p53 protein levels and UV dose (Standard Erythema Dose, SED) after 6 weeks of sun exposure in Sweden.

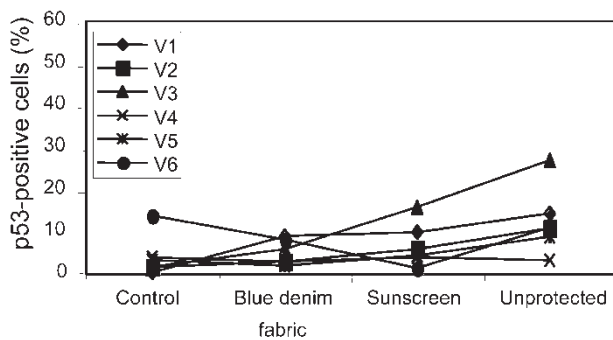


Fig. 4. p53-immunoreactive keratinocytes in chronically sun-exposed skin with different degrees of photoprotection after 6 summer weeks of sun exposure in Sweden.

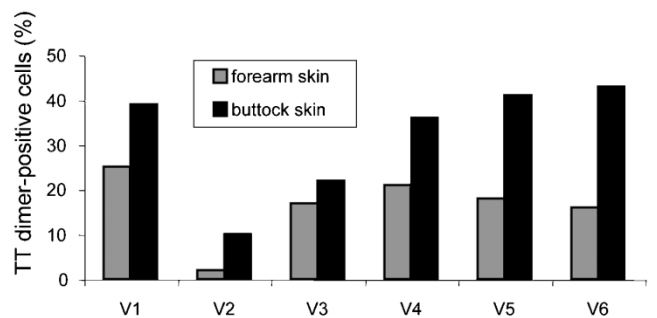


Fig. 6. Thymine (TT) dimer-immunoreactive keratinocytes in unprotected chronically sun-exposed and non-sun-exposed skin 24 h after artificial UV irradiation.

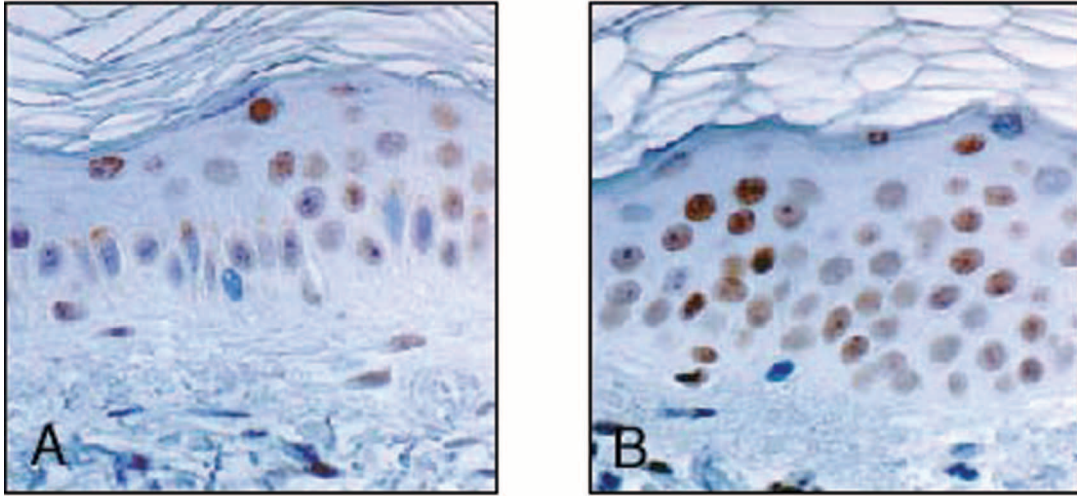


Fig. 7. High power view of skin samples from one individual (V1) 24 h after artificial UV irradiation. Sections are immunohistochemically stained with antibodies for thymine (TT) dimers. Immunoreactive nuclei are stained brown. Note the smaller number of TT dimer-immunoreactive cells in chronically sun-exposed skin (forearm; A) compared to non-sun-exposed skin (buttock; B).

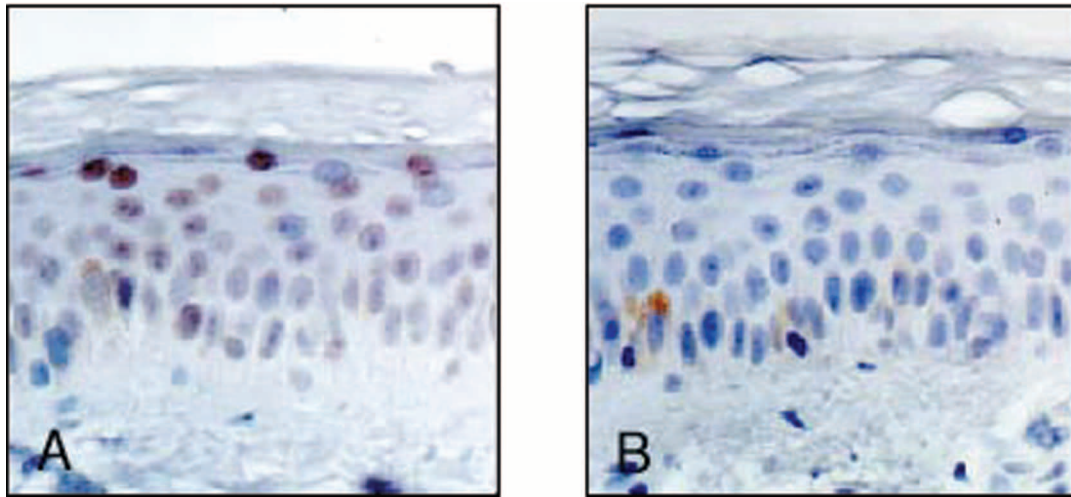


Fig. 8. High power view of skin samples from one individual (V1) 24 h after artificial UV irradiation. Sections are immunohistochemically stained with antibodies for thymine (TT) dimers. Immunoreactive nuclei are stained brown. TT dimer-immunoreactive keratinocytes are seen in upper layers of the epidermis in unprotected skin (A), whereas immunostaining is negative in photoprotected skin (B).

dimer-positive and p53-positive cells in buttock skin (Fig. 9A) and in forearm skin (Fig. 9B). A positive association between TT dimer and p53 was found in both types of skin. The Spearman correlation coefficient was higher in buttock skin (0.93) than in forearm skin (0.88).

Ki-67

No detectable difference in the proportion of Ki-67 immunoreactive keratinocytes between unprotected and photoprotected skin was observed either after artificial UV irradiation or 6 weeks of natural sun exposure. The mean percentages of positive cells varied between 7% and 12%. The immunoreactive cells were evenly distributed in the basal and suprabasal epidermal cell layers.

DISCUSSION

In this study we compared UV-induced responses in human non-sun-exposed and chronically sun-exposed skin in the same individuals. Epidermal responses, with and without photoprotection, were analysed 24 h after artificial UV irradiation and after natural sun exposure during 6 summer weeks. p53 and TT dimer immunoreactivity was used as the endpoint. The same dose of experimental UV irradiation was administered in each subject allowing analysis of each subject's individual UV response irrespective of skin type.

Novel findings in this study were: (i) that the p53 response after UV irradiation showed a more pronounced interindividual variation in chronically sun-exposed skin (forearm) than in non-sun-exposed skin (buttock) (Fig. 2); (ii) that TT dimer-positive keratinocytes

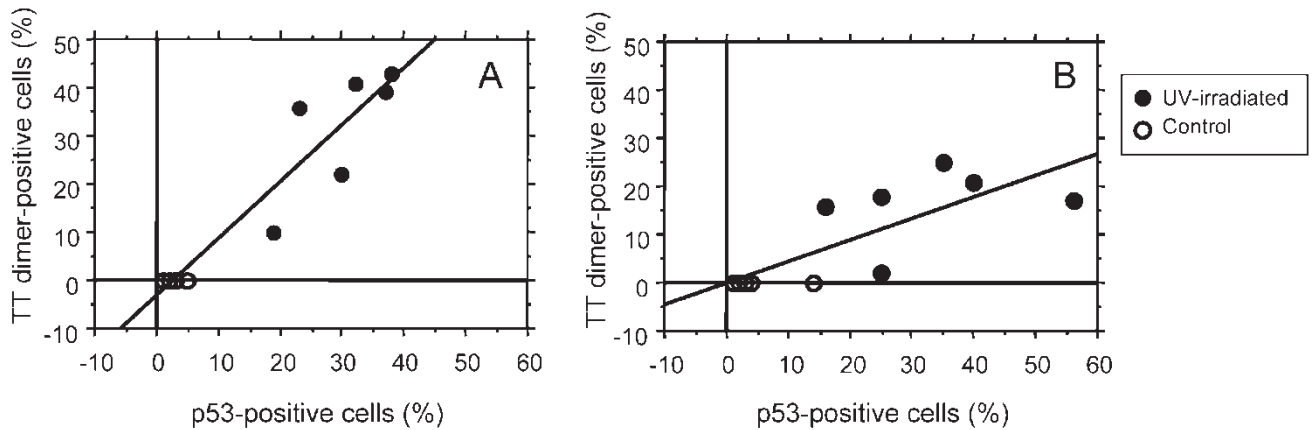


Fig. 9. Scatterplots of thymine (TT) dimer-positive cells versus p53-positive cells in buttock skin (A) and in forearm skin (B). Closed circles denote immunoreactivity 24 h after artificial UV irradiation and open circles denote control skin (non-irradiated).

were significantly fewer in chronically sun-exposed skin compared to non-sun-exposed skin 24 h after artificial UV irradiation (Fig. 6); (iii) that photoprotection in non-sun-exposed skin virtually abolished the epidermal p53 response, whereas a slight p53 response was evident in chronically sun-exposed skin (Fig. 2); and (iv) that a significant correlation between total dose of natural sunlight during 6 weeks and number of p53-immunoreactive keratinocytes could be established (Fig. 5).

It is well known that UV-induced DNA damage triggers an epidermal p53 response that subsequently can lead to cell cycle arrest or apoptosis. It has been reported that the p53 response and the DNA repair efficiency exhibit wide interindividual variations (3, 18). In the present study we show that in addition to these variations intraindividual differences also exist between chronically sun-exposed and non-sun-exposed skin. In a previous study, with analysis of p53 responses after UV irradiation using organ cultured skin, we found differences between chronically sun-exposed and non-sun-exposed skin (24). However, in that study the comparisons were not made in the same individuals. The underlying mechanisms for topographical differences in cellular responses to UV exposure of skin are unknown and might relate to the amount of previous accumulated sun exposure. It is possible that chronic sun exposure causes subtle differences in the way in which epidermal proliferative units are organized (25) or in other genetic/epigenetic events that render keratinocytes in chronically sun-exposed skin primed to react to cytotoxic cell stress, in a manner analogous to that in skin from individuals with darker skin types, i.e. skin types IV and V. Rare p53-immunoreactive keratinocytes observed in non-irradiated and photoprotected chronically sun-exposed skin could represent exceptional keratinocytes with a mutated p53 gene. Such p53-mutated single cells have been found in normal epidermis (26).

The smaller number of TT dimers found 24 h after

artificial UV irradiation in forearm skin could reflect enhanced repair of photoproducts. This could partly be due to differences in p53 response; i.e., skin areas subjected to chronic sun exposure could display an earlier or stronger p53 response resulting in a better DNA repair capacity. Results from an in vitro study in which skin explants were UV-irradiated and analysed at different time-points indicated that chronically sun-exposed skin repaired photoproducts more efficiently compared to non-sun-exposed skin (24). It was also shown in a recent study that UV-induced DNA damage is dose-dependent and is independent of skin type (7). Our finding with a smaller number of TT dimer-immunoreactive keratinocytes in forearm skin is in line with the conclusions by Sheenan et al. that TT dimer repair is more efficient in skin type IV than in skin type II (7). Gilchrest & Eller suggest that excised DNA fragments or DNA repair intermediates trigger melanogenesis (27). Is it possible that repair mechanisms are primed for faster repair after chronic exposure analogously to the faster induction of melanin synthesis in previously exposed skin? Chronically sun-exposed skin may thus share certain characteristics of darker skin types. As an alternative explanation, the differences could be due to physical factors, e.g. melanin pigmentation and/or skin thickness. However, the skin from the buttock was thicker and should in that case have shown fewer TT dimers. There is a difference in pigmentation between the buttock and forearm, but according to recently published data, formation of UV-induced DNA damage is independent of skin type (7).

Interindividual variations in p53 protein levels after exposure to natural sunlight using photoprotection could be explained by reasons discussed for artificial radiation or by the fact that the volunteers were instructed to apply sunscreen *ad libitum*, or factors such as compliance regarding attachment of fabric and different UV doses received. It has been shown that people in general apply only 20–50% of the recommended dose

of sunscreen (28, 29). The UV doses measured with polysulphone badges varied between 24 and 98 SED (Fig. 5). After artificial UV irradiation using photoprotection, both sunscreen and blue denim fabric almost totally abolished TT dimers, with <1% TT dimer-positive keratinocytes in the epidermis. Previous investigations of human skin have yielded similar results (30–33). After exposure to natural sunlight, no TT dimers were found even without the use of photoprotection, probably because the final days of the experimental period were cloudy, allowing time for TT dimer repair. A novel finding was that photoprotection virtually abolished the p53 response in non-sun-exposed skin compared to chronically sun-exposed skin, where more p53-immunoreactive keratinocytes were found (Fig. 2). One explanation for this could be that immunohistochemistry might not be sensitive enough for small numbers of TT dimers to be detected. Primed keratinocytes, such as in chronically sun-exposed skin, may react to small numbers of TT dimers and thus respond by inducing p53.

The variation in UV doses received was large (Fig. 5), and most likely due to differences in the amount of time spent in the sun. A correlation between UV dose and p53 response has not been reported previously for natural sun exposure. However, studies using artificial radiation have shown that the quantity and distribution of UV-induced p53 in human skin is highly dependent on the waveband and that the induction is dose-dependent (1, 8).

The present study, comprising only six individuals, has provided insight into differences between chronically sun-exposed and non-sun-exposed skin regarding UV responses. We suggest that the more pronounced and heterogeneous p53 response as well as the enhanced repair of UV-induced DNA damage in chronically sun-exposed skin is related to previous sun exposure. Such differences may have important clinical implications, such as in the choice of skin area for SPF testing. More research on the importance of previous sun exposure is required.

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