INVESTIGATIVE REPORT

Epidermal p53 Response and Repair of Thymine Dimers in Human Skin after a Single Dose of Ultraviolet Radiation: Effects of Photoprotection

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A cellular p53 response, DNA repair enzymes and melanin pigmentation are important strategies utilized by skin keratinocytes against impairment caused by ultraviolet radiation (UVR). In this study a double-immunofluorescence technique was used to investigate UVR-induced thymine dimers and p53 protein simultaneously. Four healthy volunteers were irradiated on both sides of their buttock skin with a single dose of solar-simulating UVR. One side was pretreated with a topical sunscreen. Biopsies from different time-points were immunostained for visualization of thymine dimers, p53 and proliferation. One single physiological dose of UVR generated widespread formation of thymine dimers throughout the epidermis 4h after irradiation. The level of thymine dimers decreased over time and was followed by a p53 response in the same cells. A late proliferative response was also found. The formation of thymine dimers, the p53 response and the late proliferative response were partially blocked by topical sunscreen. Large inter-individual differences in the kinetics of thymine dimer formation and repair as well as in the p53 response were evident in both sunscreen-protected and unprotected skin. Key words: DNA repair; sunscreens; keratinocytes.

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A robust and finely tuned defence system against ultraviolet radiation (UVR)-induced DNA damage is crucial to protect keratinocytes from malignant transformation and the development of skin cancer. It has been shown that UVR can induce specific DNA photo lesions such as cyclobutane dimers, e.g. thymine dimers. These photo lesions have been detected in human epidermis, both in vitro and in vivo (1). The cellular consequence of UVR-induced DNA damage includes repair subsequent to cell-cycle arrest, cell death due to apoptosis and acquired mutations, most typically with a UVR signature (2). UVR-induced DNA lesions are primarily removed by the process of nucleotide excision repair (3). The increased susceptibility to skin cancer in patients with xeroderma pigmentosum, who have impaired DNA repair, illustrates the strong association between DNA photodamage and human skin cancer (4). UVR also stimulates the proliferation of keratinocytes and melanocytes, and alters dermal collagen. Prolonged sun exposure leads to thickening of the epidermis as well as increased pigmentation.

Mutations in the p53 tumour suppressor gene are most frequently found in a wide range of human cancers including skin cancers, with over 50% in non-melanoma cancers (squamous cell carcinoma and basal cell carcinoma) (5–8). p53 protein plays a critical role in mediating cellular responses to DNA damage in mammalian cells (9). UVR induces accumulation of p53 protein in the human epidermis (10–13). The increased level of p53 protein transcriptionally activates the expression of an array of target genes facilitating DNA repair and enabling cell-cycle arrest or apoptosis.

Topical sunscreens have been widely used to protect against the harmful effects of UVR. Clinical studies have shown that sunscreens can prevent UVR-induced damage, including sunburn, photoageing (14), actinic keratosis (15) and squamous cell cancer, but not basal cell cancer (16). The protective effect on DNA damage has also been demonstrated by other groups (17, 18). The effectiveness of sunscreens, which is expressed by their sun protection factor (SPF), is usually determined by their ability to delay the development of erythema. A previous study showed that the amount of UVR-induced p53 during a summer of natural sun was reduced by 33% following treatment with topical sunscreen and 66% through protection with a blue denim fabric (19).

In this study, double-immunofluorescing labelling was used to visualize UVR-induced DNA damage, i.e. TT-dimers and p53 overexpression in the same histological tissue section. The aim was to investigate the induction of p53 expression and its relation to the formation and repair of cyclobutane dimers after a single dose of UVR, and to determine the photoprotective efficacy of sunscreens.

MATERIAL AND METHODS

Volunteers and ultraviolet irradiation

The study, which was approved by the local ethics committee, included 4 healthy volunteers, 2 men and 2 women, with age ranging from 28 to 68 years old. All subjects were of skin type II–III (20).

The minimal erythema dose (MED) on the untanned buttock skin was determined 24h after irradiation using a monochromator (Applied Photo Physics, UK) emitting a narrow band of UVB at 313 nm ( slit width 3 mm, corresponding to a band width of ± 4–6 nm) through a 1m liquid light guide with an aperture 9mm in diameter). The irradiance, measured with a thermopile before each session, was 15–17 mW/cm². Then, 2 MED of UVB was administered to the buttock skins as follows. In order to mimic daily sunlight, a SUPUVASUN 3000 (Mutschas, Germany) equipped with a SUN filter was used to generate a broad band of UVB (0.03 mW/cm²), UVA (65 mW/cm²)
and near-infrared radiation. In each volunteer, 1 area of 16 cm$^2$ was subject to topical sunscreen protection, while an equally sized area was left unirradiated. Fifteen minutes before irradiation, 2 mg/cm$^2$ of the sunscreen preparation (Coppertone 15%, Schering-Plough), containing both UVA and UVB absorbers [benzophenone 3 (Eusolex 4360), butyl methoxydibenzoylmethane ( Parsol 1789) and octyl methoxycinnamate Parsol MCX]), was applied to unexposed buttock skin. Punch biopsies (3 mm) were obtained from skin without sunscreen before irradiation as controls. Skin from both areas was biopsied at 4, 24, 48 and 120 h after irradiation. Biopsy at 48 h from 1 volunteer (B) was not performed because of technical difficulties.

Tissue samples were fixed in 4% buffered formalin for 1–3 days, embedded in paraffin and sectioned.

**Immunohistochemistry and immunofluorescent labelling**

Consecutive sections, 5 mm thick, were cut from each sample, dewaxed in xylene and rehydrated in graded alcohols (100%, 95% and 80%). Deparaffinized slides were permeabilized by microwaving in 0.01 M citrate buffer (pH 6.0) twice for 5 min. Slides were kept in phosphate-buffered saline for 10 min followed by immersion in 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Washed slides were incubated in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min.

P53 expression and DNA damage were visualized by double-immunofluorescent labelling using antibodies against p53 protein (DO-7, DAKO) and TT-dimers (KTM53, Kamiya Biomedical). Sections were first incubated with DO-7 (1:100) overnight at 4°C. Following incubation with biotinylated rabbit antimouse antibody (1:100, 30 min) and TRITC-conjugated streptavidin (1:100, 30 min), slides were incubated with the KTM53 monoclonal antibody (1:1000, 45 min). Thereafter, sections were again incubated with the biotinylated rabbit antimouse antibody (1:200, 30 min) followed by fluorescein isothiocyanate (FITC)–conjugated avidin (1:100, 45 min). Incubation with KTM53 and rabbit antimouse antibodies was performed at room temperature. Nuclei were stained by immersion in a solution of 4’6-diamidino-2-phenylindole (Sigma) at a concentration of 250 ng/ml in PBS for 10 min, and coverslips were mounted on to the tissue sections in an antifading medium (DAKO). Control slides were included in each analysis by performing the above procedures and substituting non-immune serum for primary and secondary antibodies individually.

Proliferation was visualized on parallel sections using a monoclonal antibody MIB-1 (Immunotech) recognizing the antigen Ki-67, which is expressed in most phases of the cell cycle (21). Sections were incubated at room temperature with MIB-1 (1:50, 30 min) followed by biotinylated rabbit antimouse antibody as secondary antibody (1:200, 30 min). The immunoreaction was visualized by avidin/biotin complex (DAKO), with 0.004% hydrogen peroxidase as a substrate and diaminobenzidine as a chromogen, which gave brown nuclear coloration. Slides were counterstained with hematoxylin.

**Assessment of staining results**

Quantification of fluorochromes was performed from 3 consecutive 400× magnification fields. All 3 fields were photographed for scoring of positive and negative cells. Each field was examined in succession for red (p53), green (TT-dimers) and blue (non-immunoreactive cell nuclei) fluorochromes using the appropriate filters, to assess the number of positive nuclei under respective filters. The percentage of keratinocytes showing immunoreactivity for p53 and TT-dimers was calculated. Sections stained with MIB-1 were visualized by light microscopy. Three consecutive 400× magnification fields were selected from each section and photographed. All cells, both negative and immunoreactive, were counted by one person (GL) from these photographs and the ratio of immunoreactive nuclei was calculated.

### Table 1. Percentage of immunoreactive keratinocytes using the 3 antibodies (TT-dimers, p53 protein and MIB-1)

| Subject | Time after irradiation (h) | TT-dimer-pos. cells (%) | p53-pos. cells (%) | MIB-1-pos. cells (%) |
|---------|---------------------------|-------------------------|--------------------|---------------------|
|         | No sunscreen | Sunscreen | No sunscreen | Sunscreen | No sunscreen | Sunscreen |
| A       | 0            | 1         | 3              | 8               |
|         | 24          | 35        | 25             | 7               |
|         | 48          | 28        | 13             | 9               |
|         | 120         | 10        | 9              | 7               |
| B       | 0            | 3         | 10             | 3               |
|         | 24          | 58        | 77             | 4               |
|         | 48          | nd        | nd             | nd              |
|         | 120         | 12        | 43             | 7               |
| C       | 0            | 29        | 17             | 10              |
|         | 4            | 80        | 42             | 3               |
|         | 24          | 74        | 48             | 8               |
|         | 48          | 67        | 25             | 10              |
|         | 120         | 55        | 16             | 18              |
| D       | 0            | 12        | 6              | 6               |
|         | 4            | 89        | 53             | 7               |
|         | 24          | 88        | 50             | 2               |
|         | 48          | 87        | 53             | 10              |
|         | 120         | 15        | 31             | 17              |
| Mean    | 0            | 11±13     | 9±6            | 7±3             |

The percentage of cells with and without pretreatment with topical sunscreen prior to ultraviolet irradiation of skin from the 4 analysed volunteers is displayed. The percentage of immunoreactive keratinocytes is given as a percentage of the total number of epidermal keratinocytes counted in 3 microscopic fields.

**TT-dimer:** thymine dimers; **MIB-1:** antibody recognizing proliferation associated Ki-67 protein; **nd:** not done.
RESULTS

The 4 volunteers in this study showed large inter-individual differences in TT-dimer formation, p53 induction and DNA repair kinetics after a single dose of UVR corresponding to 2 MED (Table 1). Figure 1A and B shows an example of double-immunofluorescence labelling in the epidermis at different time-points from one of the volunteers (D). Fig. 1A displays irradiated skin without sunscreen protection, whereas Fig. 1B shows skin treated with topical sunscreen. The codistribution of TT-dimers and p53 protein can be seen from overlaid fluorescence images.

Before irradiation, a mean of 11% (range 1–29%) of keratinocytes scored positive with the TT-dimer antibody. The peak of TT-dimer formation was found 4h after irradiation in all volunteers; however, the amount of positive cells differed (93–37%). The generated amount of TT-dimers at 4h was followed by high levels at 24 and 48h after irradiation, whereas levels resumed after 120h, yet still remained higher than before irradiation. Despite a general pattern, DNA repair kinetics varied between the volunteers, with different degrees of reduction at different time-points (Fig. 2).

Approximately 9% (range 3–17%) of epidermal keratinocytes showed a positive p53 staining in non-irradiated control skin. Four hours after UVR there was a 3–9-fold increase in p53-immunoreactive keratinocytes. The induced p53 response peak showed a mean of 47% immunoreactive keratinocytes. The time-point for reaching peak values varied, as did the degree of reduction at the following time-points. The number of p53-positive cells declined after 48h. In 3 cases a higher level of p53 remained 120h after irradiation compared with before irradiation (Fig. 3).

In general, sunscreen-treated skin showed a 2–3-fold decrease in the level of TT-dimers and there were reduced levels of p53 positivity at all time-points compared with untreated skin. The amount of reduced TT-dimers after sunscreen protection varied, with in one case (D) showing no significant reduction at 4h, and another (C) showing significant reduction at all time-points with approximately the same level (30%) of TT-dimers as in non-irradiated skin. The mean reduction in p53 was 4.4-fold (range 1.8–11.9), with individual variation in the kinetics of the p53 response after sunscreen (SPF 15) (see Figs. 2 and 3).

Proliferation as detected with MIB-1 was localized to basal and suprabasal cells. Individual variation was seen both before irradiation (3–10% positive keratinocytes) and at the different time-points after irradiation. A tendency towards a proliferative response with a higher number of positive cells was seen in 3 of the 4 subjects at 120h after irradiation. There was no obvious effect of sunscreen except at 120h, where a reduction in MIB-1-positive cells of between 35 and 96% was found (Table 1).

DISCUSSION

UVR-induced epidermal DNA photoproducts give rise to mutations, which become manifest if not repaired or if a cell which has acquired mutations fails to undergo apoptosis. Acquired mutations in genes controlling cell growth, apoptosis, senescence and genomic stability in epidermal stem cells are likely to be important and initial processes in UVR-induced skin cancer. The formation of cyclobutane pyrimidine dimers is an important step preceding UVR-induced mutations. The accumulation of TT-dimers and induction of p53 following different sources of radiation have been investigated, however the kinetics of p53 induction and TT-dimer repair simultaneously using a double-labelling technique has not been studied previously in human skin.

Despite large inter-individual differences, a general pattern could be seen, with a peak of TT-dimers 4h after irradiation which subsequently declined. This was mirrored by a delayed p53 response, consistent with the notion that an increase in p53 protein in epidermal keratinocytes following UVR is induced by DNA damage (Fig. 1A, B, overlay panels). DNA-damaged cells were distributed in all layers of epidermis 4h post-irradiation, whereas after 120h the remaining cells with TT-dimers were predominantly located in the superficial layers of the epidermis. These findings are in agreement with earlier work showing that TT-dimers persisted for more than 24h post-irradiation, with some keratinocytes in the upper epidermis still apparent at 7 days post-irradiation (12, 22).

Large inter-individual differences in the p53 response, independent of the type of radiation have previously been reported (23). The more variable and later p53 response is partly due to differences in the repair rates of TT-dimers. Up to 30-fold differences in the efficiency of repairing TT-dimers and 6-4 photoproducts in human skin have been shown (22, 24). The more persistent and high levels of TT-dimers seen in cases C and D were followed by a more delayed p53 response than in case B, where TT-dimers disappeared more rapidly with a peak of p53 at 4h post-irradiation. Owing to inconsistencies in the correlation between the p53 response and levels of TT-dimers, inter-individual differences in other factors regulating the intra-nuclear levels of p53 probably also play a role.

A previous study in mice showed that the application of sunscreen before UV irradiation resulted in a 67–91% reduction of cyclobutane dimers compared with unprotected skin (25). However, if other endpoints are examined the effects of sunscreens are not as clear. Recently, a large epidemiological study showed that long-term use of sunscreens protected subjects from developing squamous cell carcinoma but not basal cell carcinoma (16). In sunscreen-protected skin, similar inter-individual differences were seen in the levels of TT-dimers as well as p53-positive cells. The levels of positive cells followed the dynamic pattern found in corresponding unprotected skin. However, differences were found in the degree of protection. In general, topical sunscreen showed a stronger reduction in p53-positive cells compared with the reduction in TT-dimer-positive cells. The results suggest that DNA damage, in the form of cyclobutane dimers, is not the only factor triggering a p53 response. The large variability in protection by sunscreen found in these 4 volunteers is in agreement with other studies, where it was shown that protection by sunscreens was very individual and entirely independent of both DNA damage in unprotected skin and erythema response (24).

As shown in the overlay images in Fig. 1A, the same keratinocytes that overexpress p53 are also positive for TT-dimers. At all time-points, except for 120h after irradiation, there is an abundance of TT-dimer-positive cells compared with coexpressing cells, suggesting that not all cells with UV-induced DNA damage respond with p53 overexpression. Perhaps only cells in a certain phase of the cell cycle or differentiation state respond to cyclobutane dimers with stabilization of p53 protein. Another possibility could be that
Fig. 1. Visualization of TT-dimers (CPDs) (green) and p53 protein (red) in human epidermal keratinocytes. Sections were obtained from skin biopsies of buttocks before and 4, 24, 48 and 120h after a single dose of solar simulating UV radiation, corresponding to 2 MED. Keratinocyte nuclei show immunoreactivity for TT-dimers and p53 protein. Keratinocytes with TT-dimer and p53 coexpression (yellow colour of nuclei in overlay images) are seen in all layers of epidermis. Dotted lines outline the epidermal-dermal junction. (A) Skin without topical sunscreen from one volunteer (D); (B) corresponding skin pretreated with topical sunscreen (SPF 15) in the same volunteer.
Fig. 2. Percentage of keratinocytes showing TT-dimer immunoreactivity in skin biopsies from the 4 volunteers. Each grey bar represents the percentage of positive keratinocytes before irradiation and at 4, 24, 48 and 120 hours after a single dose of UVR in skin without sunscreen. The darker bars represent the percentage of positive keratinocytes found in skin pretreated with topical sunscreen (SPF 15).

Fig. 3. Percentage of keratinocytes overexpressing p53 protein in skin biopsies from the 4 volunteers. Each grey bar represents the percentage of positive keratinocytes before irradiation and at 4, 24, 48 and 120 hours after a single dose of UVR in skin without sunscreen. The darker bars represent the percentage of positive keratinocytes found in skin pretreated with topical sunscreen (SPF 15).

the coexpressing cells represent cells with a certain amount of DNA damage. The overlay also indicates the delay in the p53 response as seen at 120 hours post-irradiation, where there are more p53-positive cells than TT-dimer-positive cells, probably as a result of efficient repair of DNA-damaged cells. There was also a delayed (120 hours post-irradiation) proliferative response in 3 out of 4 cases. This proliferative response was blocked by sunscreen. The relatively wide range of cells expressing Ki-67 is in part due to normal clustering of cells in the cell cycle.

Inter-individual variability in response to 1 dose of UVR is reconfirmed in this study of only 4 volunteers and thus firm conclusions are difficult to draw. However, the technique of double staining provides a tool for studying the kinetics of both UVR-induced DNA damage and the response to that damage in the same cells. It is clear from the results that a topical sunscreen (SPF 15) has the ability, at least in part, to block DNA damage in keratinocytes as well as reduce p53 and proliferative responses. Further studies are needed to clarify the basis for the interindividual differences in responses to UVR and the effect of sunscreens.

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