DOI:10.1111/j.1600-0625.2008.00722.x
www.blackwellpublishing.com/EXD

Original Article

Cyclobutane pyrimidine dimer formation and p53 production in human skin after repeated UV irradiation

Yuji Yamaguchi[1]*, Sergio G. Coelho[1,2], Barbara Z. Zmudzka[2], Kaoruko Takahashi[1], Janusz Z. Beer[2], Vincent J. Hearing[1] and Sharon A. Miller[2]

[1]Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA;
[2]Center for Devices and Radiological Health, US Food and Drug Administration, Silver Spring, MD, USA
Correspondence: Dr Vincent J. Hearing, PhD, Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Building 37, Room 2132, MSC 4256, Bethesda, MD 20892, USA, Tel.: +1 301 496 1564, Fax: +1 301 402 8787, e-mail: hearingv@nih.gov
**Present address: Department of Geriatric and Environmental Dermatology, Nagoya City University Graduate School of Medical Sciences, Nagoya-shi, Aichi, Japan*

Accepted for publication 15 February 2008

Abstract: Substantial differences in DNA damage caused by a single UV irradiation were found in our previous study on skin with different levels of constitutive pigmentation. In this study, we assessed whether facultative pigmentation induced by repeated UV irradiation is photoprotective. Three sites on the backs of 21 healthy subjects with type II–III skin were irradiated at 100–600 J/m^2 every 2–7 days over a 4- to 5-week period. The three sites received different cumulative doses of UV (1900, 2900 or 4200 J/m^2) and were biopsied 1 day after the last irradiation. Biomarkers examined included pigment content assessed by Fontana–Masson staining, melanocyte function by expression of melanocyte-specific markers, DNA damage as cyclobutane pyrimidine dimers (CPD), nuclear accumulation of p53, apoptosis determined by TUNEL assay, and levels of p21 and Ser46-phosphorylated p53. Increases in melanocyte function and density, and in levels of apoptosis were similar among the 3 study sites irradiated with different cumulative UV doses. Levels of CPD decreased while the number of p53-positive cells increased as the cumulative dose of UV increased. These results suggest that pigmentation induced in skin by repeated UV irradiation protects against subsequent UV-induced DNA damage but not as effectively as constitutive pigmentation.

Key words: apoptosis – p53 – pigmentation – skin cancer – UV

Please cite this paper as: Cyclobutane pyrimidine dimer formation and p53 production in human skin after repeated UV irradiation. Experimental Dermatology 2008; 17: 916–924.

Introduction

The risk of skin cancer is inversely related to constitutive pigmentation of the skin due to its protective effects against ultraviolet (UV) damage (1–5). According to risk factors calculated for 2006 in the US, White/Caucasian (hereafter referred to as White) skin has a 70-fold higher risk for basal and squamous cell carcinomas compared to Black or African-American (hereafter referred to as Black) skin, and risks for melanoma are almost 20-fold higher in White subjects than in Black subjects (6–9). Irradiation by artificial sources of UV has similarly detrimental consequences in human skin, and the use of tanning devices has been associated with increased risk of skin cancers (6–8,10,11). Our group has published several articles on the importance of constitutive skin pigmentation for the reduction of UV damage resulting from a single irradiation to one minimal erythemal dose (MED) (12–14). It is sometimes argued that facultative tanning, i.e. skin pigmentation induced by UV irradiation, protects against damage caused by subsequent UV irradiation but no conclusive studies to measure that directly have been reported. We have begun to investigate the effects of repetitive UV irradiation, including effects on skin pigmentation (15,16), to assess the ability of facultative pigmentation to reduce DNA damage from subsequent UV irradiation.

Several studies have examined whether pigmentation induced by repeated low-dose UV irradiation is protective in human skin, but the findings have been inconclusive. One study reported that pigmentation induced by multiple UV doses protected against UV-induced DNA damage (17). That study showed increased epidermal pigmentation and thickness, and demonstrated that

Abbreviations: AU, arbitrary unit; CPD, cyclobutane pyrimidine dimer; MED, minimal erythema dose; SSR, solar simulated radiation; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-nick end labelling; UV, ultraviolet.

elevated post-irradiation levels of cyclobutane pyrimidine dimers (CPDs) and nuclear p53 return to background levels within a relatively short period of time (3–4 days). However, another study (18) reported that distinct DNA damage persisted in skin up to 3 weeks after a moderate dose of solar simulated radiation (SSR). Another study showed that DNA damage accumulates with repetitive UV irradiation and that comparable levels of damage (per physical dose) occurred in types II and IV skin (19); the authors proposed that the kinetics of DNA repair played an important role in controlling DNA damage and concluded 'that increased skin pigmentation may not be the major factor in natural photoprotection'. Thus, although it is well-accepted that excessive acute UV irradiation must be avoided (6,20,21) there is no consensus regarding whether and how photoprotection can be induced by moderate UV irradiation.

In this study, we examined how different doses and protocols of UV irradiation induce facultative pigmentation in White subjects (skin phototypes 2–3.5), what mechanisms are involved in melanocyte activation by UV and to what extent the induced pigmentation is protective against subsequent UV irradiation. DNA repair in epidermis repeatedly irradiated by UV is a very complex process (19,22,23). We compared various biomarkers in human skin irradiated with three different UV irradiation protocols, resulting in cumulative UV doses of 1900, 2900 and 4200 J/m^2 (Fig. 1) (16). The proteins examined included markers of melanocyte activation (e.g., tyrosinase, MITF and Pmel17) and also markers of cellular damage (e.g., CPDs, levels of p53, p21, apoptosis, etc). We previously reported that increases in CPD levels and the nuclear accumulation of p53 are significantly higher in White skin than in Black skin irradiated with a single 1 MED exposure (14). The results now reported indicate that UV-induced, facultative pigmentation in fair skin is less effective in protecting against subsequent UV damage than is constitutive pigmentation in the skin.

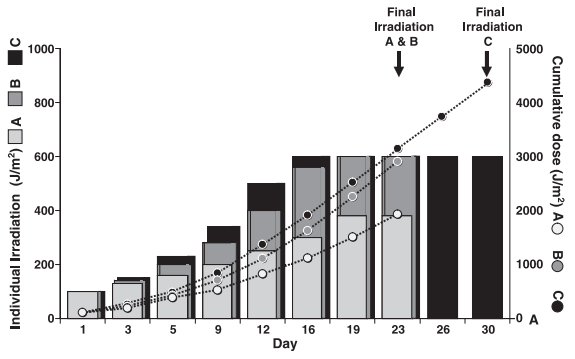

Figure 1. UV irradiation protocols used in this study. Histograms show individual UV irradiation doses on days indicated on the abscissa. Cumulative doses of protocols A, B and C are shown as circles connected by dashed lines using the axis to the right and were 1900, 2900 and 4200 J/m^2, respectively. Biopsies were taken 1 day after the final UV irradiation noted, i.e. on day 24 for protocols A and B, and on day 31 for protocol C and the unirradiated control.

Materials and methods

Study subjects

This study involved 21 volunteer subjects with skin phototypes 2–3.5 who were recruited from the local Washington, DC metropolitan area. Sex, age, phototype and MED of these subjects are given in Table 1. This study was approved by the FDA Research Involving Human Subjects Committee (#01-026R).

UV irradiation and dosimetry

Throughout this report, UV doses are expressed in erythema-effective J/m^2, i.e. J/m^2 weighted with the CIE reference action spectrum for erythema (24). The three UV irradiation protocols used in this study have been previously described and discussed (16). The MED for each subject was determined as previously described (16) using an array of 8 Kodacel- filtered (Eastman Chemical Products, Kingsport, TN, USA) FS lamps (FSX24T12/UVB/HO, National Biological Corp., Twinsburg, OH, USA). For repeated UV irradiation, we used a 12-lamp UV source canopy (SunQuest Model SQ 2000S; ETS, Indianapolis, IN, USA) equipped with 100 W lamps (Beach Sun; Light Sources, Orange, CT, USA) emitting 5% UVB, commonly

Table 1. Study subjects

Subject no.	Age	Sex	Phototype	MED (J/m^2)
T7	32	F	2	385
T8	40	F	2	205
T9	33	M	2.5	185
T10	28	F	2.5	330
T11	23	F	2.5	345
T12	24	M	2.5	285
T13	50	F	3.5	355
T14	40	M	3	170
T16	45	M	2.5	335
T17	38	F	2.5	330
T18	36	F	2.5	290
T19	34	M	2	195
T20	30	F	3	185
T21	38	F	2.5	320
T22	24	M	2.5	330
T24	22	F	3	615
T25	29	M	2.5	200
T26	26	F	2.5	295
T27	41	F	3.5	220
T28	31	M	2.5	210
T30	27	F	2	225

used in tanning salons. The spectrum of this source was previously shown in fig. 1 of Ref. (16).

We applied three different repeated UV irradiation regimens (Protocols A, B and C – Fig. 1) on three 3 × 3-cm areas on the back of each subject, as previously described (16). Cumulative doses were 1900 J/m^2, 2900 J/m^2 and 4200 J/m^2 for protocols A, B and C, respectively. The output of both UV sources used in this study was measured using a double-grating spectroradiometer (Model 754; Optronic Laboratories, Orlando, FL, USA), whose calibration is traceable to the National Institute of Standards and Technology. We used a low profile detector (SSD 001A; International Light, Newburyport, MA, USA) before each irradiation to determine the correct exposure time for each of the three areas of skin.

Skin biopsies

Shave biopsies (4 mm diameter) of skin were taken from the 3 UV-irradiated sites 1 day after the last UV irradiation, i.e., after eight irradiations (day 24) for protocols A and B, and after 10 irradiations (day 31) for protocol C, and from an adjacent unirradiated area as a control (on day 31). Each biopsy was placed dermis side down on a Millipore filter and was then fixed in 4% formaldehyde, embedded in paraffin, sectioned at 3 μm-thickness and mounted on silane-coated glass slides.

Melanin content

Melanin content was analyzed in sections fixed and sectioned as noted above. Specimens were stained for melanin using the Fontana–Masson method (25) and were quantitated as previously described (12,13). Melanin content was analyzed using a Leica DMRB/DMLD microscope (Leica Microsystems, Bannockburn, IL, USA), a Dage-MTI 3CCD 3-chip color video camera (Dage-MTI, Michigan City, IN, USA) and ScionImage software (Scion Corp, Frederick, MD, USA) from the integrated density in given areas of the epidermis in each section, as described previously (12–14). The Fontana–Masson stain correlates well with eumelanin content in the skin, but not with pheomelanin content (12). Using this approach, melanin contents were measured before and after repeated UV irradiation, and are reported in arbitrary units (AU).

Immunohistochemical analysis

Paraffin-embedded specimens were mounted on silane-coated glass slides and were processed as previously described (14,26,27). Briefly, specimens were deparaffinized twice with xylene for 5 min and were then dehydrated with a graduated series of ethanol, followed by antigen retrieval through boiling in antigen unmasking solution (Vector Laboratories, Inc., Burlingame, CA, USA) for 12 min. They were subsequently incubated with 10% goat serum (Vector Laboratories) for 30 min at 37°C, and then with primary antibodies in 5% goat serum at 4°C overnight. Secondary antibodies were appropriate for the primary antibody, Alexa Fluor 488/594 anti-mouse or anti-rabbit IgG (H+L) (at 1:500 dilution, Molecular Probes Inc., Eugene, OR, USA). Staining was analyzed and quantified using a Leica DMRB/DMLD fluorescence microscope and ScionImage imaging software. The fluorescence intensity was calculated for specific areas in the epidermis and 10 randomly selected areas were quantified for each data point. An internal control was used each time to control for reproducible antibody staining. Fluorescence intensities for antibodies detecting DNA damage and melanocyte-specific markers were normalized against DAPI staining. The following were measured:

Melanocyte density

Melanocytes were counted as cells positive for the expression of tyrosinase, MART-1, Pmel17 and MITF, and their density along the epidermal:dermal border is expressed as cells/mm. Primary antibodies used were αPEP7h (at 1:750 dilution) to detect tyrosinase (26), D5 (at 1:20 dilution, Abcam Inc, Cambridge, MA, USA) to detect MITF, HMB45 (at 1:100 dilution; Dako Inc., Carpinteria, CA, USA) to detect GP100/Pmel17, and Ab3 (at 1:100 dilution, NeoMarkers, Fremont, CA, USA) to detect MART-1. ScionImage software was used to semi-quantify the green and/or red fluorescence intensity of each primary antibody from 10 random images photographed in each section.

DNA damage

CPDs were detected by immunofluorescence using a thymine dimer mouse monoclonal-2 (TDM-2) antibody (at 1:40 000 dilution) (28). The TDM-2 antibody binds TT and CT cyclobutane dimers in a dose-dependent manner at UV doses as low as 0.5 J/m^2 (29).

p21 content

Primary antibodies used were anti-human p21$^{WAF1/Cip1}$ (#M7202, at 1:25 dilution, Dako, Glostrup, Denmark).

p53 content

Primary antibodies used recognized p53 (#9282, at 1:100 dilution, Cell Signaling Technology, Beverly, MA, USA), and phospho-p53-Ser46 (#2521, at 1:100 dilution, Cell Signaling).

Apoptosis

An ApopTag *in situ* apoptosis detection kit (Serologicals Corp., Norcross, GA, USA), based on the terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) assay, was used according to the manufacturer's protocol, as detailed in (14).

Statistical analyses

Values are reported as means ± SEM. All analyses, including Student's *t* test and the estimation of correlation coefficients and *P*-values, were conducted with SPSS 10.0 software (SPSS Inc., Chicago, IL, USA).

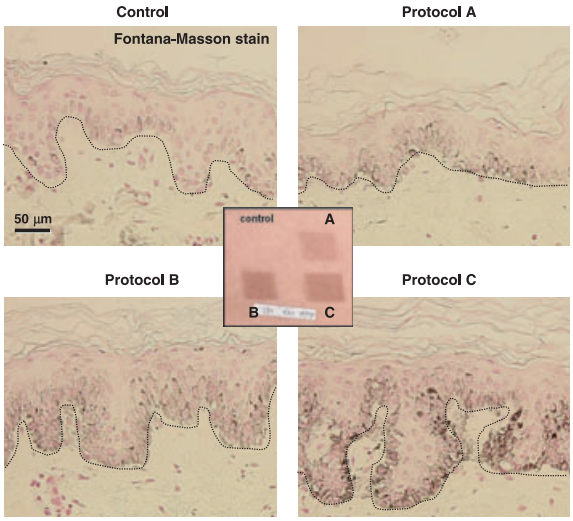

Figure 2. Melanin in the epidermis visualized by Fontana–Masson staining after repeated UV irradiations. Representative images of sections from subject T18. Dashed lines in all figures represent the epidermal:dermal border; scale bar = 50 μm. Inset: Example of facultative skin pigmentation elicited by the three UV irradiation protocols compared with the unirradiated control site (subject T20 at day 23).

Results

Melanin content

The skin phototype, gender, age and MED of the subjects in this study are reported in Table 1. The UV irradiation protocols are shown in Fig. 1, which gives individual doses and the cumulative doses over the course of the study. Note that the biopsies for protocols A and B were taken on day 24 while the biopsies for protocol C and the unirradiated control were taken on day 31 (1 day after the final irradiation). Representative micrographs of melanin stained in the epidermis and the pigmentation visible in the study areas are shown in Fig. 2. Increased melanin content was visible in all subjects after all three UV irradiation protocols, and correlated with increasing cumulative UV doses. Melanin content was quantified for 14 subjects and combined data show that after completion of protocols B or C, the epidermis contained ~2-fold more melanin than the control. Even the average 60% increase in melanin content after protocol A was statistically significant (Table 2). However, there was no significant difference in melanin content between protocols B and C suggesting that the pigmentary system was close to maximal stimulation at those doses.

Table 2. Quantitative measures of UV effects on human skin

		Repetitive UV Irradiation			Single UV Irradiation[1]	
	Unirradiated	A	B	C	Unirradiated	1 MED
Melanin content[2]	4.9 ± 0.4	7.9 ± 0.6***	10.6 ± 0.8***	11.5 ± 0.8***	4.7 ± 3.1	5.2 ± 3.7^{NS}
Melanocyte density[3]	12.6 ± 0.9	32.7 ± 3.2***	34.4 ± 3.5***	30.2 ± 1.9***	12.8 ± 1.2	14.3 ± 1.4^{NS}
Tyrosinase expression[4]	0.23 ± 0.05	0.71 ± 0.07***	0.72 ± 0.14**	0.52 ± 0.10*	9.89 ± 1.75[5]	18.90 ± 5.85**
DNA damage[6]	7.1 ± 4.3	308.3 ± 77.8**	243.9 ± 58.7**	196.2 ± 50.0**	Not done	Not done
DNA damage[7]	0.11 ± 0.01	0.86 ± 0.14***	0.70 ± 0.08***	0.42 ± 0.05***	0.05 ± 0.01	0.22 ± 0.02***
p53 expression[8]	0.0 ± 0.0	92.9 ± 7.0***	107.0 ± 7.7***	138.3 ± 6.8***	0.0 ± 0.0	59.2 ± 10.5**
p53 Ser46P[9]	0.0 ± 0.0	2.5 ± 0.7**	2.6 ± 0.7**	3.2 ± 0.5***	0.0 ± 0.0	0.0 ± 0.0
TUNEL stain[10]	0.3 ± 0.1	3.6 ± 0.7***	3.8 ± 0.5***	4.0 ± 05***	0.0 ± 0.0	3.3 ± 1.0**
p21 expression[11]	1.8 ± 1.4	8.0 ± 2.2*	10.0 ± 3.9^{NS}	16.2 ± 3.5**	Not done	Not done

Data are reported as means ± SEM; NS = not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.002$ compared to unirradiated controls.
[1]Data published for White subjects before and 1 week after a single 1 MED UV irradiation in Refs (12,13).
[2]Data for 14 subjects (T12–T14, T17–T22, T24–T28) reported in arbitrary units/field.
[3]Data for 15 subjects (T9, T12, T13, T17–T22, T24–T28, T30) reported as # melanocytes/mm skin.
[4]Data for 9 subjects (T9, T12, T13, T17–T22) reported in arbitrary units/field; similar changes in expression were found for MITF, Pmel17 and MART1.
[5]Units are reported as originally published in (13) and are not corrected for DAPI staining.
[6]Data for 10 subjects (T7, T9, T12, T13, T18–T22, T24) reported as CPD-positive cells/mm skin.
[7]Data for 18 subjects (T7–T14, T16–T22, T24, T27, T28) reported as arbitrary units/field.
[8]Data for 7 subjects (T12, T13, T24–T28) reported as # p53-positive cells/mm skin.
[9]Data for 7 subjects (T12, T13, T24–T28) reported as # cells positive for p53 phosphorylated at Ser46/mm skin.
[10]Data for 18 subjects (T7–T10, T12–T14, T17–T22, T24–T28) reported as # TUNEL-positive cells/mm skin.
[11]Data for 7 subjects (T12, T13, T24–T28) reported as # cells positive for p21/mm skin.

This contrasts with our observations on the effects of a single 1 MED UV irradiation [shown on the right side of Table 2, taken from (13)] which showed no significant increase in melanin content in White skin within 1 week.

Melanocyte density and function

Melanocyte density in the skin of the three UV-irradiated sites (assessed by counting cells in the basal layer of the epidermis that were positive for MITF, Tyr, Pmel17 and MART-1) also increased significantly (~3-fold) compared with the density of melanocytes in unirradiated skin (Table 2). There were no significant differences in melanocyte density among the three UV-irradiated sites in the 15 subjects analyzed. Again, this contrasts with our previous study which had shown that there was no increase in melanocyte density in White skin within 1 week following a single 1 MED UV irradiation [Table 2, taken from (13)].

Examples of expression of melanocyte-specific proteins in skin subjected to the three UV irradiation protocols are shown in Fig. 3. Increased expression of all four specific melanocyte markers examined (tyrosinase, MITF, Pmel17 and MART-1) following UV irradiation was dramatic (quantitative analysis of expression levels of tyrosinase is shown in Table 2). Tyrosinase expression in UV-irradiated sites A, B and C increased 2- to 3-fold compared with unirradiated skin but there were no significant differences in expression of tyrosinase among the three UV-irradiated sites. Similar increases were found for, Pmel17, MART-1 and MITF, although the latter protein is less abundant and as a transcription factor is primarily localized in the nuclei.

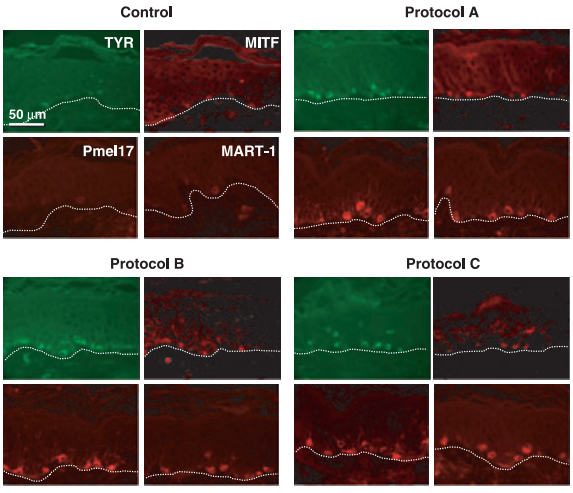

Figure 3. Expression of melanocyte-specific proteins after repeated UV irradiation. Representative sections stained for TYR (green, top left panel in each group) and for MITF (red, top right in each group) obtained from subject T27 at the three UV-irradiated sites and at the unirradiated control site. Also shown are representative sections stained for Pmel17 (red, bottom left in each group) and for MART-1 (red, bottom right in each group) from subject T28. Scale bar = 50 μm.

Our previous study had shown a slight, but not statistically significant, increase in expression of those four markers in White skin 1 week after a single 1 MED UV irradiation [Table 2, taken from (13)].

Markers of cellular function and damage

DNA lesions

To assess DNA damage in skin irradiated by the three UV irradiation protocols, we measured levels of CPDs in the epidermis. Examples of sections used for CPD analysis are shown in Fig. 4. Those same sections stained with DAPI show that the damage was localized in nuclei (as expected), and costaining for tyrosinase showed that melanocytes were among the cells that contained CPDs. Compared to unirradiated control skin, CPD levels of the 18 subjects measured were significantly higher in skin from all three UV-irradiation sites as were the number of CPD-positive cells (Table 2). There was no significant difference between CPD levels in UV-irradiation protocols A and B, but CPD levels in UV-irradiation protocols A and B were significantly higher than in protocol C ($P \leq 0.01$). We also analyzed the relationship between melanin content and CPD levels in the three UV-irradiation sites, and found that UV-induced CPD damage correlated inversely and significantly with the amount of melanin ($R^2 = 0.210$, $P < 0.02$, $n = 18$ subjects).

p53 expression

Representative images of p53 expression and its nuclear localization are shown for skin specimens taken 1 day after repeated UV irradiation (Fig. 5). Combined data for seven subjects (Table 2) shows that the number of p53-positive

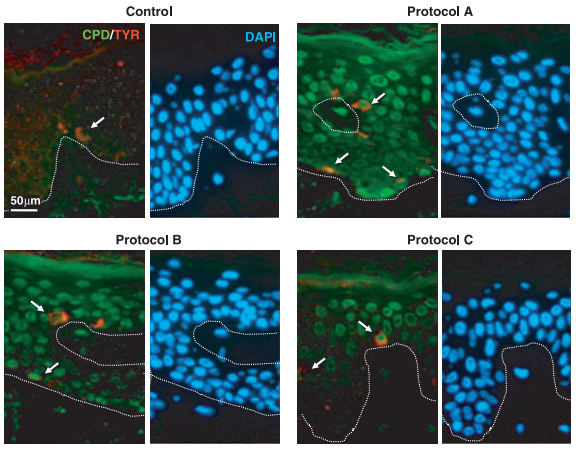

Figure 4. Levels of CPDs in the epidermis after repeated UV irradiation. Representative images of specimens stained for CPD (green) at the three UV-irradiated sites and at the control unirradiated site (taken from subject T12). Staining for TYR (red) is used to localize melanocytes; DAPI shows nuclei; scale bar = 50 μm.

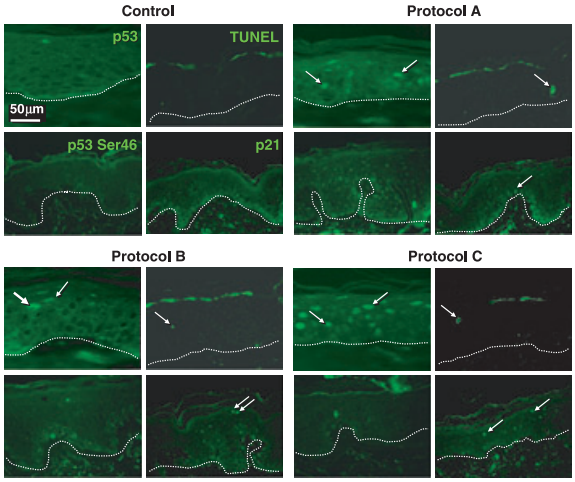

Figure 5. Expression of various markers of cellular damage and proliferation in the epidermis after repeated UV irradiation. Representative specimens stained for p53 (green, top left panel in each group) at the three UV-irradiation sites and the unirradiated control (taken from subject T28). Also shown are TUNEL (green, top right panel in each group), p53 Ser46 (green, bottom left panel in each group) and p21 (green, bottom right panel in each group); scale bar = 50 μm.

cells was significantly and dramatically higher in skin from all three UV irradiation protocols compared to unirradiated controls (which had almost undetectable numbers of p53-positive cells). There was no statistically significant difference between UV-irradiation sites A and B in the number of p53-positive cells/mm skin. However, the number of p53-positive cells in UV-irradiation site C was significantly and dramatically higher than in UV-irradiation sites A and B ($P < 0.01$). The number of p53-positive cells correlated positively with melanin content ($R^2 = 0.305$, $P < 0.05$, $n = 7$) but negatively with the number of CPD-positive cells ($R^2 = 0.912$, $P < 0.01$, $n = 10$).

Apoptosis

Results of TUNEL staining revealed that levels of apoptosis were significantly increased in response to repeated UV irradiation compared with unirradiated skin (Fig. 5, Table 2). However, the number of TUNEL-positive apoptotic cells was relatively low and did not differ significantly among the three UV-irradiation sites. In an earlier study, we observed similar TUNEL results for a single 1 MED UV irradiation (14).

Phosphorylation of p53 at Ser46 is an important post-translational modification that reflects the function of p53 to induce apoptosis in response to stress. While no p53-Ser46P- positive cells could be detected in unirradiated control skin, significantly increased numbers of those cells were found in skin irradiated by all three UV irradiation protocols (Fig. 5, Table 2) unlike in the samples from the previous single 1 MED UV irradiation (14).

p21 expression

Staining for the cell cycle marker p21 revealed that the number of p21-positive cells was extremely low in unirradiated control skin but increased significantly following all three protocols of repetitive UV irradiation (Fig. 5, Table 2).

Discussion

Previously, we reported the effects of a single UV irradiation at the level of 1 MED on human skin of different phototypes (5,12–14). However, in real life, human skin is repeatedly irradiated by UV emitted by the sun, by therapeutic devices and/or by indoor tanning equipment. Such exposures can be carcinogenic. The repetitive nature of the insults contributes to the complexity of the molecular and cellular phenomena elicited. This study explored the effects of three protocols of repetitive UV irradiations that result in increased skin pigmentation. We found that some cancer-related molecular and cellular phenomena correlate differently with pigmentation induced by repeated UV irradiations (i.e. facultative pigmentation) than with constitutive skin pigmentation.

We used three experimental UV irradiation protocols, A, B and C (Fig. 1), which resulted in cumulative doses of 1900, 2900 and 4200 J/m^2, respectively. UV irradiation according to each of those protocols stimulated skin pigmentation very effectively. Protocol B produced darker pigmentation of the skin than did protocol A, however the difference between pigmentation produced by protocols B and C at the end of the irradiations was small as assessed visually (inset of Fig. 2) and could only be detected instrumentally (30). The results of Fontana–Masson staining (Fig. 2) confirmed significant increases in pigment levels following each of the three UV irradiation protocols. Although histologically there seemed to be a difference in overall melanin content between protocols B and C, only small differences in melanin content were measured quantitatively (Table 1). This corroborated what was seen by visual evaluation following irradiation according to protocols B and C.

In our previous studies, a single dose of UVB-rich irradiation at the 1 MED level had negligible effect on melanocyte density in the skin within 1 week post-irradiation [data taken from (12,13) are included for comparison in Table 2]. In the present study, melanocyte density was significantly increased following repeated UV irradiation given over 3–4 weeks, and the three UV irradiation protocols tested were similarly effective (Table 2). It is possible that melanocytes in control unirradiated skin may be present but express their specific markers at levels too low to be detected. However, we do not believe this to be the case since we measured melanocyte density based on the

expression of a variety of melanocyte specific markers, all of which gave comparable numbers at the time points examined. Further, in our previous study (12), we used identical techniques and antibodies to show similar densities of melanocytes in unirradiated skin of the various racial and ethnic groups examined and those densities did not change within the first week after UV irradiation.

The amount of DNA damage measured as CPD in the epidermis was dramatically higher after repeated UV irradiation according to all three protocols compared to unirradiated skin (Fig. 4, Table 2). Interestingly, CPD levels were significantly lower after protocol C compared with protocols A and B, both when measured as overall intensity per area of skin or as the number of CPD-positive cells per mm of skin which suggests a photoprotective benefit from the facultative pigmentation induced by the repetitive UV irradiations. A similar phenomenon was reported by de Winter et al. (17). In their study, 9 irradiations given over 3 weeks resulted in lower CPD levels after a UV challenge than those observed in unirradiated controls. A recent study (31) reported that expression of DNA repair enzymes in the skin increases significantly following various types of stress, including UV irradiation. Hence, DNA repair kinetics may be significantly altered by different courses of UV irradiation. However, neither de Winter and co-workers nor our group assessed DNA damage during the course of repetitive UV irradiations to examine the kinetics of CPD generation and DNA repair. Such data are needed to fully assess differences in DNA damage caused by different UV irradiation regimens and for characterization of long-term effects of these UV doses. Additionally, many biomarkers other than CPD are required to elucidate the photoprotective effect of UV-induced pigmentation at occasionally sun-exposed sites.

An interesting recent study (32) showed that the type of UV used to irradiate human skin (types II and III) had important consequences on effects in the epidermis and dermis. That study used a repetitive UV irradiation protocol that irradiated skin to 0.5 MED UV (96% UVA/4% UVB) 19 times over 5 weeks. That is a similar UVA/UVB ratio and time course to our study but contrasts with respect to the increasing dose protocols in our study. That study also used buttock skin, normally considered a sun-protected site, compared to our study which used dorsal skin, considered an occasionally sun-exposed site. Nevertheless, that study reported significant increases in skin color, p53 nuclear accumulation, melanin staining (detected by Fontana–Masson), more dendritic DOPA-positive melanocytes and only a slight increase in apoptotic cells (sunburn cells). Those parameters are similar to what we observed in this study which suggests that UV responses are comparable in different anatomical locations regardless of UV exposure background. More recently, Young et al. (33) reported a study on human skin (types I and II) using repetitive UV irradiations (11 irradiations of 0.5–0.6 MED per day), again on sun-protected buttock skin. They reported the preventive effects of a sunscreen on DNA damage and p53 accumulation resulting from the repetitive UV irradiations and also noted that virtually no apoptotic cells (sunburn cells) were seen in the UV-irradiated sites. The low level of apoptosis in human skin following repetitive UV irradiation may result from decreased expression of the Fas ligand. In that regard, a recent study reported that while Fas ligand is relatively strongly expressed in the basal layer of the epidermis and weakly expressed in the upper layer, it became totally negative following chronic UV irradiation (34). The sum of those results is consistent with the observations and interpretations reported in our study.

The p53 tumor suppressor protein plays several important roles in the inhibition of photocarcinogenesis. It enhances the nuclear excision repair of UV-induced DNA damage, delays cell proliferation to provide time for DNA repair, and (following its phosphorylation at Ser46) stimulates apoptosis (20,35–37). p53 protein accumulates in the nuclei of skin cells after UV irradiation (38) and expression of p53 in the skin can serve as a risk marker for skin cancer in humans (39). We previously reported that following a single 1 MED UV irradiation: (1) increases in CPD levels and the nuclear accumulation of p53 are significantly higher in White skin than in Black skin despite the fact that the latter received up to 4-fold more UV in physical doses (13,14), but (2) the number of apoptotic cells is significantly lower in White skin than in Black skin (14). The results reported in this study (Fig. 5, Table 2) show that p53 expression increased significantly with increasing cumulative UV doses, i.e. it correlated well with increased skin pigmentation. Although this seems to contradict our previous report on p53 induction in skin, we think it simply highlights the fact that facultative skin pigmentation is distinct from constitutive skin pigmentation. The increase in skin pigmentation elicited by UV- irradiation protocol C showed significantly higher levels of p53 nuclear accumulation than seen after protocols A or B (Fig. 5) and p53 accumulation correlated with melanin content. However, neither the rate of apoptosis (measured as TUNEL-positive cells) or the level of p53 phosphorylation at Ser46 showed a dose-dependence, although levels of p53 and p21 were dose-dependent. p21 expression in the skin after UV irradiation can be regulated by p53-dependent and by p53-independent processes; a recent study showed the p53-dependent transcriptional activation of the p21 promoter which may be involved in this process (40). Again, lack of data regarding changes in these end points at earlier stages of the experiment should be noted. The extremely low level of TUNEL-positive cells is consistent with our

earlier single UV dose study (14). Very few TUNEL-positive cells are visible in White skin after repetitive UV irradiation, and this is consistent with other recent reports that found very low levels of apoptotic/sunburn cells in White skin after repetitive UV irradiation (32,33). Significantly increased numbers of TUNEL-positive cells were found in darker skin after a single 1 MED UV irradiation in our earlier study. The effects of repetitive UV irradiation on darker skin remain to be explored. These low levels of apoptosis, which potentially could reduce the risk of skin cancers from UV-mutated cells, would seem to be almost negligible in White skin due to the low numbers seen, and one could argue that they are too few to be physiologically relevant.

While analyzing these findings, it is particularly interesting to juxtapose the CPD and p53 dose-dependence data for repeated vs. acute UV irradiations. In both cases, increases in nuclear accumulation of p53 correlates with increases of UV dose. This is consistent with the findings of a study on subjects exposed to 6 weeks of natural sunlight (41). However, while higher acute UV doses lead to formation of more CPDs, the increases in cumulative repetitive UV doses result in lowering the CPD level (at least in the dose range explored in this study). It is tempting to speculate that the increases in p53 accumulation are mechanistically different in the two cases: within 1 day after a single UV irradiation it signifies an initial, perhaps relatively primitive response, of the cellular/nuclear defense system while after numerous repeated irradiations – and given more time – it may reflect development of a mature, efficient defense system. This is but one possible explanation for the decline of CPD levels at the higher cumulative repeated dose. The significance of such a putative mechanism for carcinogenesis would depend on the fidelity of the cellular defense system that evolved during repeated UV irradiations. Further investigations of DNA damage, changes in the p53 system (including p53 mutations) and apoptosis induced by repeated UV irradiation will be needed to fully assess the carcinogenic risk of such UV irradiations.

Acknowledgements and Disclaimer

This research was supported by the Office of Science and the Center for Devices and Radiological Health, Food and Drug Administration (FDA), by the Intramural Research Program of the National Cancer Institute at NIH, by a grant-in-aid from the Ministry of Education, Culture, Sports, and Technology of Japan, and by the FDA Office of Women's Health. The opinions and conclusions stated in this paper are those of the authors and do not represent the official position of the U.S. Department of Health and Human Services. The mention of commercial products, their sources, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products by the U.S. Department of Health and Human Services.

References

1 Kaidbey K H, Agin P P, Sayre R M, Kligman A M. Photoprotection by melanin – a comparison of black and Caucasian skin. J Am Acad Dermatol 1979: **1**: 249–260.
2 Kollias N, Sayre R M, Zeise L, Chedekel M R. Photoprotection by melanin. J Photochem Photobiol 1991: **9**: 135–160.
3 Slominski A, Tobin D J, Shibahara S, Wortsman J. Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev 2004: **84**: 1155–1228.
4 Kadekaro A L, Wakamatsu K, Ito S, Abdel-Malek Z A. Cutaneous photoprotection and melanoma susceptibility: reaching beyond melanin content to the frontiers of DNA repair. Front Biosci 2006: **11**: 2157–2173.
5 Miyamura Y, Coelho S G, Wolber R *et al.* Regulation of human skin pigmentation and responses to ultraviolet radiation. Pigment Cell Res 2007: **20**: 2–13.
6 Young A R. Tanning devices – fast track to skin cancer? Pigment Cell Res 2004: **17**: 2–9.
7 Levine J A, Sorace M, Spencer J, Siegel D M. The indoor UV tanning industry: a review of skin cancer risk, health benefit claims, and regulation. J Am Acad Dermatol 2005: **53**: 1038–1044.
8 Karagas M R, Stannard V A, Mott L A, Slattery M J, Spencer S K, Weinstock M A. Use of tanning devices and risk of basal cell and squamous cell skin cancers. J Natl Cancer Inst 2002: **94**: 224–226.
9 Lea C S, Scotto J A, Buffler P A, Fine J, Barnhill R L, Berwick M. Ambient UVB and melanoma risk in the United States: a case–control analysis. Ann Epidemiol 2007: **17**: 447–453.
10 Bataille V, Winnett A, Sasieni P, Newton Bishop J A, Cuzick J. Exposure to the sun and sunbeds and the risk of cutaneous melanoma in the UK: a case-control study. Eur J Cancer 2004: **40**: 429–435.
11 Gallagher R P, Spinelli J J, Lee T K. Tanning beds, sunlamps, and risk of cutaneous malignant melanoma. Cancer Epidemiol Biomark Prevent 2005: **14**: 562–566.
12 Tadokoro T, Kobayashi N, Zmudzka B Z *et al.* UV-induced DNA damage and melanin content in human skin differing in racial/ethnic origin and photosensitivity. FASEB J 2003: **17**: 1177–1179.
13 Tadokoro T, Yamaguchi Y, Batzer J *et al.* Mechanisms of skin tanning in different racial/ethnic groups in response to ultraviolet radiation. J Invest Dermatol 2005: **124**: 1326–1332.
14 Yamaguchi Y, Takahashi K, Zmudzka B Z *et al.* Human skin responses to UV radiation: Pigment in the upper epidermis protects against DNA damage in the lower epidermis and facilitates apoptosis. FASEB J 2006: **20**: 1486–1488.
15 Coelho S G, Miller S A, Zmudzka B Z, Beer J Z. Quantification of UV-induced erythema and pigmentation using computer assisted digital image evaluation. Photochem Photobiol 2006: **82**: 651–656.
16 Miller S A, Coelho S G, Zmudzka B Z, Beer J Z. Reduction of the UV burden to indoor tanners through new exposure schedules: a pilot study. Photodermatol Photoimmunol Photomed 2006: **22**: 59–66.
17 de Winter S, Vink A A, Roza L, Pavel S. Solar-simulated skin adaptation and its effect on subsequent UV-induced epidermal DNA damage. J Invest Dermatol 2001: **117**: 678–682.
18 Hemminki K, Xu G, Kause L, Koulu L M, Zhao C, Jansen T. Demonstration of UV-dimers in human skin DNA in situ 3 weeks after exposure. Carcinogenesis 2002: **23**: 605–609.

19 Sheehan J M, Cragg N, Chadwick C A, Potten C S, Young A R. Repeated ultraviolet exposure affords the same protection against DNA photodamage and erythema in human skin types II and IV but is associated with faster DNA repair in skin type IV. J Invest Dermatol 2002: **118**: 825–829.

20 Ziegler A, Jonason A S, Leffell D J *et al.* Sunburn and p53 in the onset of skin cancer. Nature 1994: **372**: 773–776.

21 Gilchrest B A. Sunscreens – a public health opportunity. New Eng J Med 1993: **329**: 1193–1194.

22 Hemminki K, Snellman E. How fast are UV-dimers repaired in human skin DNA in situ? J Invest Dermatol 2002: **118**: 699.

23 Young A R, Potten C S, Sheehan J M. Epidermal DNA repair under repeated exposure conditions is complex. J Invest Dermatol 2002: **119**: 700–702.

24 Erythema Reference Action Spectrum and Standard Erythema Dose. Vienna, Austria: CIE, 1999: 17166.

25 Bancroft J D, Stevens A. Theory and Practice of Histological Techniques. New York: Churchill Livingstone, 1982.

26 Yamaguchi Y, Itami S, Watabe H *et al.* Mesenchymal–epithelial interactions in the skin: Increased expression of dickkopf1 by palmoplantar fibroblasts inhibits melanocyte growth and differentiation. J Cell Biol 2004: **165**: 275–285.

27 Passeron T, Coelho S G, Miyamura Y, Takahashi K, Hearing V J. Immunohistochemistry and tissue in situ hybridization in the study of human skin melanocytes. Exp Dermatol 2007: **16**: 162–170.

28 Kobayashi N, Nakagawa A, Muramatsu T *et al.* Supranuclear melanin caps reduce ultraviolet induced DNA photoproducts in human epidermis. J Invest Dermatol 1998: **110**: 806–810.

29 Mori T, Nakane M, Hattori T, Matsunaga T, Ihara M, Nikaido O. Simultaneous establishment of monoclonal antibodies specific for either cyclobutane pyrimidine dimer or (6-4)photoproduct from the same mouse immunized with ultraviolet-irradiated DNA. Photochem Photobiol 1991: **54**: 225–232.

30 Miller S A, Coelho S G, Zmudzka B Z *et al.* Dynamics of pigmentation induction by repeated UV exposures: dose, dose interval and UV spectrum dependence. Br J Dermatol 2008: submitted.

31 Marionnet C, Bernerd F, Dumas A *et al.* Modulation of gene expression induced in human epidermis by environmental stress in vivo. J Invest Dermatol 2003: **121**: 1447–1458.

32 Seite S, Medaisko C, Christiaens F *et al.* Biological effects of simulated ultraviolet daylight: a new approach to investigate daily photoprotection. Photodermatol Photoimmunol Photomed 2006: **22**: 67–77.

33 Young A R, Orchard G E, Harrison G I, Klock J L. The detrimental effects of daily sub-erythemal exposure on human skin in vivo can be prevented by a daily-care broad-spectrum sunscreen. J Invest Dermatol 2007: **127**: 975–978.

34 Erb P, Ji J, Wernli M, Kump E, Glaser A, Buchner S A. Role of apoptosis in basal cell and squamous cell carcinoma formation. Immunol Lett 2005: **100**: 68–72.

35 Oren M. Decision making by p53: life, death and cancer. Cell Death Differ 2003: **10**: 431–442.

36 Oda K, Arakawa H, Tanaka T *et al.* p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 2000: **102**: 849–862.

37 Takeuchi S, Zhang W, Wakamatsu K *et al.* Melanin acts as a potent UVB photosensitizer to cause a novel mode of cell death in murine skin. Proc Natl Acad Sci U S A 2004: **101**: 15076–15081.

38 Hall P A, McKee P H, Menage H D, Dover R, Lane D P. High levels of p53 protein in UV-irradiated normal human skin. Oncogene 1993: **8**: 203–207.

39 de Graaf Y G, Rebel H, Elghalbzouri A *et al.* More epidermal p53 patches adjacent to skin carcinomas in renal transplant recipients than in immunocompetent patients: the role of azathioprine. Exp Dermatol 2007: **17**: 349–355.

40 Mouret S, Favier A, Beani J C, Leccia M T. Differential p53-mediated responses to solar-simulated radiation in human papillomavirus type 16-infected keratinocytes. Exp Dermatol 2007: **16**: 476–484.

41 Wassberg C, Backvall H, Diffey B, Ponten F, Berne B. Enhanced epidermal ultraviolet responses in chronically sun-exposed skin are dependent on previous sun exposure. Acta Derm Venereol 2003: **83**: 254–261.