Mechanisms of Skin Tanning in Different Racial/Ethnic Groups in Response to Ultraviolet Radiation

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Ultraviolet radiation stimulates pigmentation in human skin, but the mechanism(s) whereby this increase in melanin production (commonly known as tanning) occurs is not well understood. Few studies have examined the molecular consequences of UV on human skin of various racial backgrounds *in situ*. We investigated the effects of UV on human skin of various races before and at different times after a single 1 minimal erythemal dose UV exposure. We measured the distribution of DNA damage that results, as well as the melanin content/distribution and the expression of various melanocyte-specific genes. The density of melanocytes at the epidermal:dermal junction in different types of human skin are remarkably similar and do not change significantly within 1 wk after UV exposure. The expression of melanocyte-specific proteins (including TYR (tyrosinase), TYRP1 (tyrosinase-related protein 1), DCT (tyrosinase-related protein 2), MART1 (melanoma antigens recognized by T-cells) gp100 (Pmel17/silver), and MITF (microphthalmia transcription factor)) increased from 0 to 7 d after UV exposure, but the melanin content of the skin increased only slightly. The most significant change, however, was a change in the distribution of melanin from the lower layer upwards to the middle layer of the skin, which was more dramatic in the darker skin. These results provide a basis for understanding the origin of different skin colors and responses to UV within different races.

Key words: melanocyte/melanosome/photoprotection/pigmentation/ultraviolet
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Ultraviolet radiation (UV) stimulates pigmentation in human skin, but the mechanism(s) whereby this occurs is not well understood. The effect observed, commonly called the tanning reaction, is readily visible but few studies have examined differences in responses to UV of human skin in various racial groups and phototypes. To explore these differences, we used the Office of Management and Budget classification of races. This classification is subjective and based to some extent on geopolitical descriptions. Although it is not perfect, it is commonly accepted. There has been a recent call for more studies to address genetic and phenotypic differences among racial ethnic groups (Nature Genet Suppl. 11, 2004). Two types of tanning response are known, immediate pigment darkening which can occur within minutes after UV exposure and delayed tanning which takes several days or longer to become apparent. Human skin pigmentation is considered an important protective factor against skin cancer development following UV, and the constitutive color of the skin dramatically affects the incidence of various types of skin cancers (Preston and Stern, 1992; Kricker *et al*, 1994; Halder and Bridgeman-Shah,

1995). Previously, we reported that UV-induced DNA damage depends on racial/ethnic (R/E) origin (Tadokoro *et al*, 2003). In this report, we describe UV-induced DNA damage, melanin formation and redistribution, and the expression of various melanocyte-specific genes.

Skin pigmentation depends directly on the function of melanocytes, specialized dendritic cells localized in the skin at the epidermal:dermal junction (Levine, 1993; Nordlund *et al*, 1998). Melanocytes express specific proteins involved in the synthesis and deposition of the biopolymer melanin in melanosomes. Some of those proteins, e.g., tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1), and tyrosinase-related protein 2 (DCT), are enzymes whereas others, e.g., Pmel17/silver (gp100) are structural components of melanosomes (Kushimoto *et al*, 2001; Kushimoto *et al*, 2003). Specific antibodies to those melanocyte markers are now available as are chemical methods to analyze the type and amount of melanin in the skin. Microphthalmia transcription factor (MITF) is considered the master regulator of melanocyte function, and modulates the transcription of a number of melanocyte-specific genes, including those encoding many of the melanosomal proteins noted above (Shibahara *et al*, 2000; Tachibana, 2000).

Previously, we studied human subjects of various R/E origin exposed to a single 1 minimal erythema dose (MED) of UVA/UVB radiation, after which biopsies were taken at different times (Tadokoro *et al*, 2003). We used immunohistochemical approaches to analyze DNA damage and its

subsequent removal from the skin. In this study, we used the same skin specimens to examine the expression of melanocyte-specific proteins prior to UV exposure and at 7 min, 24 h, and 7 d after UV exposure. We have characterized the density of melanocytes at the epidermal:dermal junction in different types of human skin and analyzed the expression of melanocyte specific proteins as well as the distribution of melanin before and after UV exposure.

Results and Discussion

Melanocyte density in the skin and effects of UV Sections of skin were stained for melanocyte-specific markers (TYR, TYRP1, DCT, MART1, MITF, and gp100) and melanocytes were counted in those sections (Fig 1). Ten sections for each subject were analyzed for melanocyte density (stained by 6 different melanocyte specific markers) and the results are summarized in the top section of Table I. The densities of melanocytes in unirradiated skin of Asian, Black and White subjects were virtually identical ranging from 12.2–12.8 melanocytes per mm. This density agrees well with (Whiteman *et al*, 1999) who reported the density of melanocytes in White skin (as 17.1 ± 8.8 per mm). This number is also consistent with (Quevedo *et al*, 1987) who determined melanocyte density following L-3,4-dihydroxy-phenylalanine staining of en face sections of skin (890 ± 70 per mm^2, i.e., ~ 30 per mm). We should note that we counted melanocytes only when nuclei were visible in the cells, since smaller fragments could not be accurately distinguished from dendrites of melanocytes containing melanosomes. The similar densities of melanocytes in unirradiated skin of different races confirms earlier reports of the similar densities of melanocytes in various phototypes (Stierner *et al*, 1989). The latter study reported a doubling of melanocyte density after 17 d of chronic UV exposure, which

in the context of our results suggests that active proliferation of melanocytes in the skin is just beginning at 1 wk.

One day after UV exposure, the densities of melanocytes were actually slightly (but not significantly) reduced in all three R/E groups, perhaps because melanocyte functions had been impaired by the stress. At 7 d after irradiation, the densities of melanocytes in Asian and in White skin were slightly increased (Black skin had essentially returned to the constitutive level), but none of those changes were statistically significant.

Melanin content in human skin and effects of UV The melanin content prior to and following UV was determined by quantitative measurement of Fontana–Masson staining and is summarized in the middle section of Table I. The total amount of melanin in unirradiated skin from Asian and from White subjects was very similar, whereas in Black skin it was about 4-fold higher. These findings are consistent with our measurements of melanin contents by sensitive chemical methods (Tadokoro *et al*, 2003). The total amount of melanin was essentially unchanged in all three types of skin 1 d after UV exposure, and had a slight (6%–14%) but statistically insignificant increase 7 d later. As seen by eye (Fig 2) or by reflectance spectroscopy (bottom section of Table I), skin pigmentation, however, increased significantly by day 7 in Asians and was particularly noticeable in Black skin.

Melanin can be pseudocolored by computer software and quantitated in various layers of the skin. In the example shown (Fig 3), melanin in the lower (basal) layer has been pseudocolored blue, in the middle (spinous) layer red and in the upper (granulosum) layer green. Data obtained from such analyses are summarized in Table II. The total amount of melanin in all layers increased 5%–10% 1 wk after UV in all three races, but at the same time, melanin was redistributed among the three epidermal layers. The lower layer

Figure 1
Micrographs showing the identification of melanocytes at the epidermal:dermal junction (dotted white line) using immunohistochemistry for TYR (tyrosinase), TYRP1 (tyrosinase-related protein 1), and DCT (tyrosinase-related protein 2) (all stained green) and for gp100 (Pmel17/silver), MITF (microphthalmia transcription factor), and MART1 (all stained red), as detailed in the Materials and Methods. The right column of panels shows the colocalized images; note that MITF localization is nuclear whereas the others are cytoplasmic. Subject = S47.
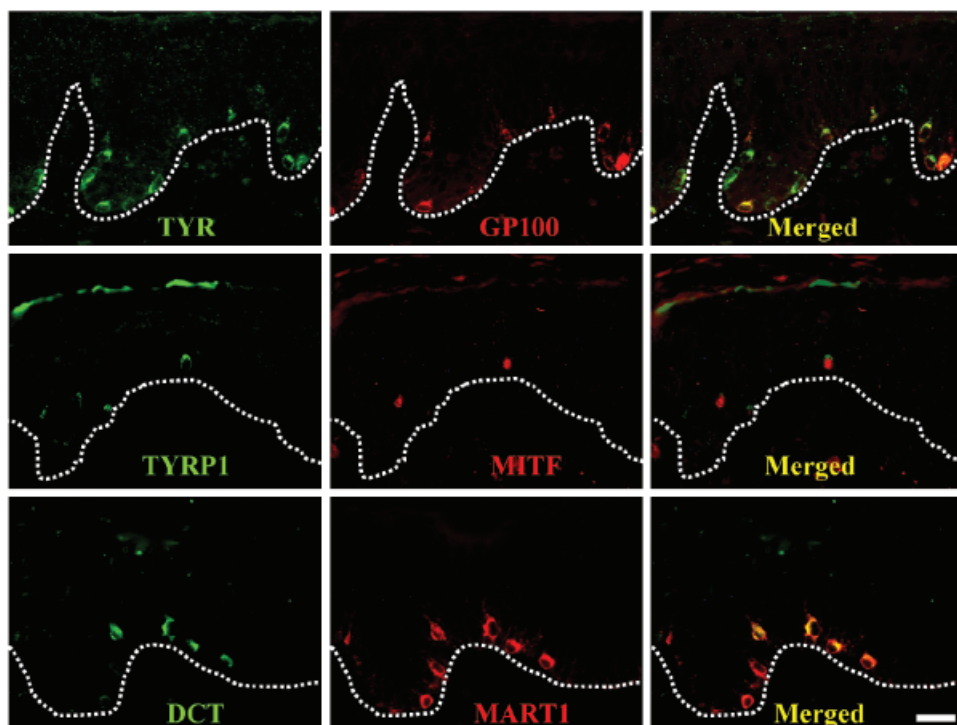
Table I. Melanocyte density and melanin content after UV exposure

Group	Unirradiated	24 h	7 d
Melanocyte density			
Asian	12.3 ± 0.7	11.3 ± 0.8[NS]	13.6 ± 0.7[NS]
Black	12.2 ± 0.7	11.1 ± 0.9[NS]	12.0 ± 0.7[NS]
White	12.8 ± 1.2	11.8 ± 1.1[NS]	14.3 ± 1.4[NS]
Melanin content (by Fontana–Masson stain)			
Asian	3.5 ± 0.9	3.5 ± 1.1[NS]	3.7 ± 1.0[NS]
Black	16.1 ± 3.9	17.3 ± 5.1[NS]	18.3 ± 6.9[NS]
White	4.7 ± 3.1	4.5 ± 2.9[NS]	5.2 ± 3.7[NS]
Change in melanin content (by reflectance spectrometry)			
Asian	0	0.09 ± 0.04[NS]	0.10 ± 0.04*
Black	0	0.09 ± 0.06[NS]	0.33 ± 0.06**
White	0	0.02 ± 0.02[NS]	0.04 ± 0.02[NS]

Melanocyte density is calculated based upon measurements of ten sections each from specimens stained at each time point for expression of MITF, TYR, TYRP1, DCT, MART1, and gp100; data are reported as means ± SEM (melanocytes per mm). Melanin content is based upon measurements of ten sections each from specimens stained with Fontana–Masson; data are reported as means ± SEM (in arbitrary units). Changes in melanin content were measured by diffuse reflectance directly on human skin; data are reported as means ± SEM relative to unirradiated skin. Subjects analyzed were: Asian = S5, S16, S21, and S47; Black = S27, S35, and S37; White = S19, S26, and S30.
*$p < 0.05$.
**$p < 0.001$.
UV, ultraviolet; NS, not significant; MITF, micropthalmia transcription factor; TYR, tyrosinase; TYRP1, tyrosinase-related protein 1; DCT, tyrosinase-related protein 2; gp100, Pmel17/silver.

of the epidermis contained 54%–68% of the melanin, the middle layer 25%–30%, whereas the upper layer contained only 7%–16% of the pigment. One week after the exposure to 1 MED UV, however, the percentage of melanin was decreased from 8%–13% in the basal layer in all three races, whereas it increased 4%–14% in the middle layer of the epidermis. Changes in the distribution of melanin in the upper layer was variable and ranged from a 3% decrease to a 14% increase. It is possible that more time is needed for preexisting melanin to reach the upper layers of the epidermis.

Expression of melanogenic proteins following UV MITF is considered the master regulator of melanocyte function since it regulates the expression, at least in part, of genes encoding the melanosome proteins TYR, TYRP1, DCT, gp100, and MART1 (Tachibana, 2000; Shibahara *et al*, 2001; Yasumoto *et al*, 2002; Du *et al*, 2003). Expression of those genes is regulated by melanocyte stimulating hormone (MSH), which functions through MC1R, and by ASP, an antagonist of that receptor (Sakai *et al*, 1997; Abdel-Malek *et al*, 2001), but their response to UV has not been previously reported. Immunohistochemical analysis showed that constitutive expression of MITF in unirradiated skin was highest in Black skin, but in Asian and in White skin was detectable in amounts >50% of that found in Black skin (Fig 4). All three types of skin responded to UV with similar increases in the expression of MITF within 1 d after UV. The increases in MITF were still present 7 d after this single moderate UV dose.

Constitutive levels of the melanosomal proteins (TYR, TYRP1, DCT, gp100, and MART1) were typically higher in Black skin, although not always dramatically so. Interestingly, expression of all five melanosomal proteins in skin from all three races was increased within 1 day after UV, and these responses were strongest in Black skin. TYR is the most critical enzyme for synthesis of pigment, and its levels showed a marked response to UV, even at day 1 after UVR, in all three races. DCT, an enzyme that modulates the type of pigment synthesized was the least responsive. Interestingly, gp100, the basic building block for melanosomes, was highly responsive to UV, even more so than TYR, in all 3 races. It is interesting to note that TYR levels are quite similar (within 2-fold) in all three races. Such observations support an earlier hypothesis that pigmentation of human skin reflects in large part the post-translational processing and activation of tyrosinase (Fuller *et al*, 2001). It has also been shown that many sequence variations/polymorphisms exist in the TYR gene (Oetting, 2000). Many of them do not affect transcription of the gene but do affect enzyme function (Shriver *et al*, 2003) and may thus also play important roles in regulating constitutive pigmentation.

Distribution of melanin following UV Several important parameters determine the appearance of skin color, and the amount and composition of chromophores are of primary importance. The two types of melanin, eumelanin and
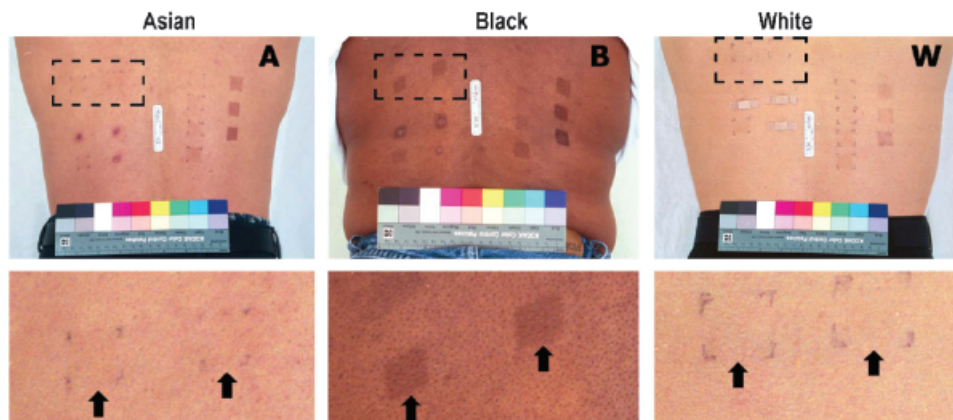
Figure 2
Photographs of a representative subject from each racial group; photos were taken 7 d after the single 1 MED (minimal erythema dose) ultraviolet (UV) exposure. Color scales are included in each photograph to ensure accurate color reproduction. (Top) The square areas on the right side of each back received varying doses of UV to establish the MED on day 1; the square areas on the left side of each back received 1 MED UV on day 0; marks from biopsies taken can be seen in some areas. (bottom). The areas outlined with the dashed boxes at the top show areas receiving 1 MED but without biopsy, and are magnified × 5 (arrows point to the UV exposed areas compared to surrounding skin). Asian = S21, Black = S35, White = S26. Gray ruler at waist is 8 inches wide.

Figure 3
Melanin distribution analysis in the epidermis before and 1 wk after a single 1 MED (minimal erythema dose) ultraviolet irradiation (subject S37 is shown). Melanin stained by Fontana–Masson is pseudocolored blue in the lower (basal) layer, red in the middle (spinous) layer and green in the upper (granulosum) layers of the skin. Data from such determinations are summarized in Table II.
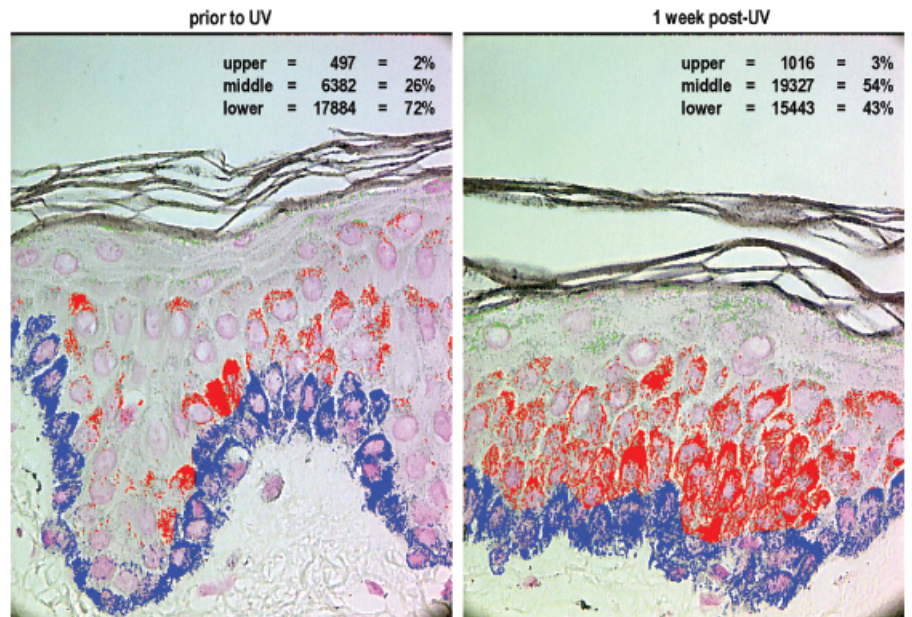
Table II. Analysis of melanin distribution in various layers of the skin

Subject	Group	Lower (basal) layer			Middle (spinous) layer			Upper (granulosum) layer		
		Day 0	Day 7		Day 0	Day 7		Day 0	Day 7	
S5	Asian	2025	5541		1830	6317		1385	597	
S21	Asian	11,628	7160		3765	7081		1017	3506	
S47	Asian	17,198	27,034		11,205	17,760		5931	6729	
Average melanin		10,283	13,245		5600	8491		2778	3611	
% total in skin		54	45	↓8	30	42	↑12	16	13	↓3
S27	Black	8673	9705		4019	4258		1513	982	
S35	Black	16,282	23,548		4940	14534		2034	4319	
S37	Black	17,884	15,443		6382	19,327		497	1016	
Average melanin		14,280	16,232		5114	12,706		1348	2106	
% total in skin		68	55	↓13	25	39	↑14	7	7	→0
S19	White	1975	878		1479	1805		467	1116	
S26	White	5408	5036		2247	5058		230	860	
S30	White	1959	2860		949	330		1200	1307	
Average melanin		3114	2925		1558	2398		632	1094	
% total in skin		56	44	↓12	30	34	↑4	14	22	↑14

pheomelanin as well as hemoglobin, are key factors in this regard (Quevedo and Holstein, 1998). Further, skin color is influenced by the distribution of melanin and melanosomes as well as the shape of melanosomes (Alaluf *et al*, 2002; Thong *et al*, 2003). The redistribution of melanin in the epidermis following UV exposure was noticed more than 80 y ago (Hausser and Vahle, 1922; Miescher, 1932; Hausser, 1938; Hamperl *et al*, 1939).

Three potential mechanisms could explain the phenomenon of tanning following UV exposure: (1) redistribution of existing melanin towards the surface of the skin, (2) changes in the shape and perhaps the intracellular localization of melanin (e.g., as seen in the supranuclear melanin caps), and/or (3) *de novo* melanin synthesis. The results of this study clearly support the first possibility as the major mechanism of tanning seen within 1 wk of UV exposure to Kodace filters FS lamps (Fluorescent FS Sun Lamp, National Biological, Twinsburg, Ohio), although mechanisms 2 and 3 are also involved. It should be noted that this redistribution occurs in all three races examined, and it is more dramatic in dark skin.

The data available suggest the time course of UV-induced changes in the skin as illustrated by Fig 5. Damage to
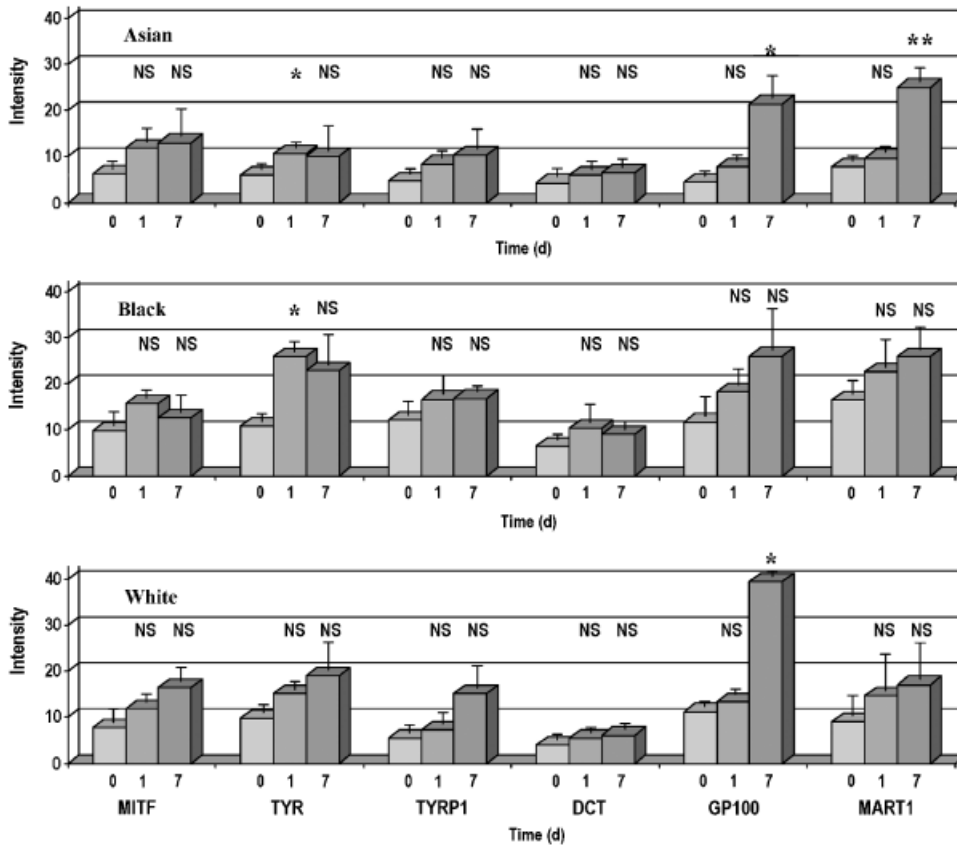
Figure 4

Quantitation of melanocyte-specific markers as identified on the abscissa, at 0, 1, and 7 d after the single 1 MED (minimal erythema dose) ultraviolet exposure. Data report staining intensity for each marker (MITF (micropthalmia transcription factor), TYR (tyrosinase), TYRP1 (tyrosinase-related protein 1), DCT (tyrosinase-related protein 2), gp100 (Pmel17/silver), and MART1) as averages of measurements of ten independent areas on three subjects each of Asian (S5, S16, and S21), Black (S27, S35, and S37) and White (S19, S26, and S30) subjects. Difference from unirradiated controls—NS, not significant; *p<0.05; **p<0.01.
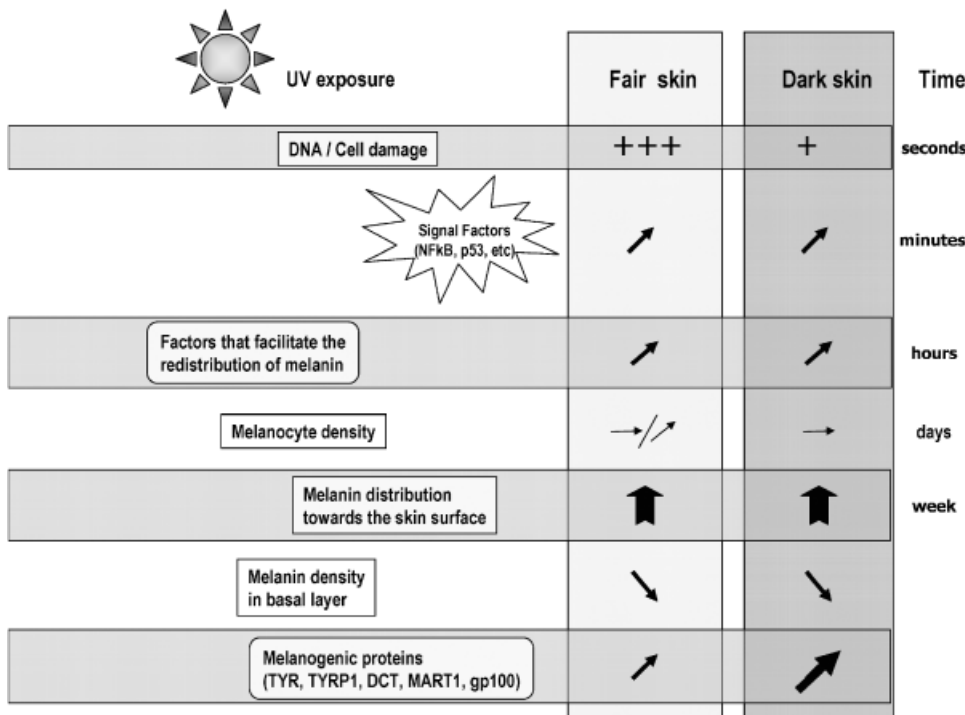

Figure 5

Proposed time course of ultraviolet-induced events related to tanning of human skin. The scheme shows the sequence of events following UV exposure of the skin. The time scale is shown to the far right, and the magnitude of responses in fair skin and in dark skin are indicated by the sizes and directions of the arrows.

DNA occurs immediately, and induction of intracellular signals (e.g., NFκB and p53) occurs within minutes thereafter. The induction of MITF and melanosomal proteins occurs within hours after UV exposure and significant increases in all of them occur within 24 h. Melanocyte proliferation begins to increase within 1 week and increases in melanocyte density would occur at later time points. The redistribution of melanin towards the surface of the skin occurs within the 1 wk time course after UV exposure. As the melanin content in basal layers of the skin declines, de novo melanin synthesis ensues. Normally, the time course for melanin preexisting in the basal layer to migrate to the surface of the skin and to be removed by desquamation is ~ 4 wk (Levine, 1993; Nordlund *et al*, 1998).

These responses are no doubt influenced by the UV spectrum and dose(s) used. Increases in pigmentation to a given dose of UV are quicker and more dramatic in dark skin than in light skin.

So what factors are critical for the increase in skin pigmentation that can be observed within several days after a single UV exposure? Obviously, the distribution of melanin in keratinocytes, particularly in the upper layers of the skin is quite important. We have previously reported that UV exposure stimulates the secretion of melanosomes by melanocytes, and concurrently increases the ingestion of melanosomes by keratinocytes (Virador et al, 2002). The mechanisms that regulate pigment transfer and the subcellular machinery involved in that process within melanocytes and keratinocytes are gradually being unraveled. Microarray or proteomic analysis of human skin after UV exposure might help further define genes and/or proteins that are critical to that process. The redistribution of melanin to upper layers of the skin following a single, relatively mild dose of UV is critical to the increased photoprotection of the skin, and is another example of the plasticity and adaptability of biological systems.

Materials and Methods

Study subjects, UV exposure, and biopsy methods This study involved human subjects representing six R/E groups (according to United States Office of Management and Budget Classification 0990-0208; American Indian or Alaska Native; Asian; Black or African American; Hispanic or Latino; Native Hawaiian or Other Pacific Islander; White). Details of the protocols, subjects of the study and UV doses have been published (Tadokoro et al, 2003). This study was approved by the FDA Research Involving Human Subjects Committee, and adhered to the Helsinki Guidelines; informed consent was obtained from each of the study subjects. In this study, we restricted our analysis to the three major R/E groups in the US—Asian (for this study we selected East Asians only), Black, or African American (hereafter referred to as Black) and White. There was a minimum of ten subjects in each R/E group and different numbers of specimens from each group were analyzed for different end points; the numbers of subjects examined by each procedure are noted in the table and figure legends.

An FS lamp (National Biological) was used as the source of UVA/UVB. The distance between the lamps and skin was 22–27 cm and was adjusted to deliver approximately 100 J per m^2-eff (CIE erythema effective, i.e., weighted with the CIE action spectrum for erythema (Diffey et al, 1997) per min (fluence rate of 1.67 W per m^2-eff, 250–400 nm). Kodacel filtration (Eastman Chemical Products, Kingsport, Tennessee) was used to remove the UVC component. The MED of each individual was determined to assess the sensitivity to UV by irradiating a series of 2 × 2 cm^2 on one side of the back of each subject. The following day, the MED dose was established and several 2 × 2 cm^2 on the other side of the back were irradiated with 1 MED ranging from 250 to 480 J per m^2 for Whites, 300–600 J per m^2 for Asians and 600–850 J per m^2 for Blacks. Shave biopsies, ∼ 4 mm in diameter, were taken prior to the UV exposure, immediately after (∼ 7 min), 1 d later and 7 d later. Each biopsy was placed dermis side down on a Millipore (Millipore, Bedford, Massachusetts) filter (pore size, 0.45 μm; diameter 13 mm), fixed in 4% formaldehyde, embedded in paraffin and sectioned using standard techniques.

Immunohistochemical staining Specimens fixed with formaldehyde and embedded in paraffin were sectioned at 3 μm thickness, then mounted on silane-coated glass slides. The expression of melanosomal proteins was measured in the sections by indirect immunofluorescence using polyclonal antibodies, αPEP7h specific for tyrosinase (Virador et al, 2001), αPEP1 specific for TRP-1 (Jiménez et al, 1989) and αPEP8h specific for TRP-2 (Virador et al, 2001), and monoclonal antibodies, HMB45 (DAKO, Carpinteria, California) specific for gp100, D5 (NeoMarkers, Fremont, California) specific for MITF, and MART-1 Ab-3 (NeoMarkers) specific for MART1. Samples were deparaffinized with xylene and dehydrated with ethanol, then treated with cold methanol and boiled in 10 mM citrate buffer (pH 6.0) for antigen retrieval. They were subsequently incubated with 10% goat serum (Vector Laboratories, Burlingame, California) containing 2% bovine serum albumin (Amersham Pharmacia Biotech, Piscataway, New Jersey) and 0.05% Tween20 for 30 min at 37°C, and then with αPEP7h at 1:4000, αPEP1 at 1:2000, αPEP8h at 1:15,000, HMB45 at 1:100, D5 at 1:100, or MART-1 Ab-3 at 1:400 dilution with 2% goat serum at 4°C overnight. The slides were incubated with Alexa Fluor(R)594 goat anti-rabbit IgG (H + L) and Alexa Fluor(R)488 goat anti-mouse IgG (H + L) (Molecular Probes, Eugene, Oregon) at 37°C for 30 min at 1:200 dilution with 2% goat serum, and covered by a drop of Prolong Antifade solution (Molecular Probes). The Alexa594 fluorescence (Eugene, Oregan) was superimposed over the Alexa 488 fluorescence to show co-localization. Alexa fluorescence was observed and analyzed using a Leica DMRB/DMLD laser microscope (Leica Microsystems, Bannockburn, Illinois) and ScionImage software (Scion, Frederick, Maryland). This system allows one to eliminate background level fluorescence and to quantitate fluorescence intensity from original images. Sections from subject S24 at 7 d after UV were stained each time as an internal control for antibody staining.

Melanocyte density, melanin content and distribution The numbers of melanocytes along the basement membrane were directly counted using the Leica DMRB/DMLD microscope after staining of melanosomal proteins. Melanocytes were only counted if the nucleus was visible.

Melanin content was analyzed following staining of sections by the Fontana–Masson method (Bancroft and Stevens, 1982) under the same controlled conditions. The Leica DMRB/DMLD laser microscope measures transmitted light intensity. Melanin quantity was analyzed from integrated density in the sections.

Melanin distribution was analyzed in digital images of Fontana–Masson stained sections using the image analysis software "KS 400" from Carl Zeiss (Thornwood, New York). First, the pictures were segmented according to the gray level, and dark pixels with a gray level <128 (threshold level for melanin-positive structures) were measured. Subsequently, the area of these detected spots was measured using the image analysis software for the different epidermal layers (basal, granulosum and spinous) in each image. The size of the detected area represents the melanin content. The threshold level (in this case 128, on a gray-value scale from 255 = white to 0 = black) was determined for each series of images; this was under exactly the same illumination conditions to allow comparisons between experiments.

Diffuse reflectance (DR) spectra in the 400–700 nm range were measured at 10 nm increments using a Minolta CM-2002 spectrophotometer (Minolta, Ramsey, New Jersey). Such spectra were obtained for two UV-exposed 2 × 2 cm skin areas and one adjacent unexposed control area. In each area, three measurements were performed in different locations and the averages of the three results were then used to generate reflectance spectra used in the analyses. These spectra were then transformed into apparent absorbance (AA) spectra, where AA represents the logarithm of the ratio of the DR of the control skin and the DR from UV-exposed skin, $AA = \log (DR_C/DR_{UV})$ (Kollias et al, 1994). The linear part of the 630-700 nm spectrum was used to calculate melanin content according to (Kollias et al, 1994) with modification of Jacques, 2001 (http://omlc.ogi.edu/spectra/melanin/opticaldepth.html).

Photography The study areas were photographed before exposure and at all post-exposure stages of the investigation. Photo-

graphs were taken under standardized conditions using a Canon Rebel 2000 35 mm camera (Canon, Lake success, New York) with a 28–80 mm lens set at 80 mm and f = 32. The shutter (1/90 s) was synchronized with a Speedotron Light (Chicago, Illinois) system consisting of two M11/CC light units, each with a 7 inch umbrella reflector and its own white umbrella diffuser. The lamps were 5500K color-corrected flash bulbs. Both lamps were directed at a 45° angle at the study area, i.e. the subject's back. A color scale (Kodak Color Separation Guide Q-13 Rochester, New York) was included in each image. The 35 mm film was processed by a commercial laboratory and was scanned at high resolution with no color or contrast correction. The digitized images were standardized to the Kodak color scale.

Statistical analyses The statistical software, JMP 5.1, was used to determine t values and correlation coefficients. A p value of < 0.05 is defined as significant using paired or unpaired *t* tests.

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