Melanin acts as a potent UVB photosensitizer to cause an atypical mode of cell death in murine skin

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Melanin protects the skin against DNA damage induced by direct absorption of sunlight’s UV radiation. Yet, irradiating melanin in vitro or in cultured cells also generates active oxygen species such as superoxide, which can indirectly induce oxidative base lesions and DNA strand breaks. This photosensitization is greater for pheomelanin (yellow and red melanin) than for eumelanin (brown and black). The in vivo photosensitizing ability of melanin is unknown. We used congenic mice of black, yellow, and albino coat colors to investigate the induction of DNA lesions and apoptosis after exposure to predominantly UVB (280–320 nm) or UVA (320–400 nm) radiation. Cyclobutane pyrimidine dimers induced by direct UVB absorption were equal in all three strains, as was apoptosis measured as sunburn cells or as keratinocytes containing active caspase-3. However, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL)-positive cells were 3- to 10-fold more frequent in black and yellow mice after UVB or UVA irradiation than in albino. In epidermal sheets, TUNEL-positive cells lined the upper portion of the hair follicle, consistent with UV-induced photosensitization by melanin in the hair shaft. Because the concentration of eumelanin in black mice was three times that of pheomelanin in yellow mice, pheomelanin had 3-fold greater specific activity. We conclude that UV-irradiated melanin, particularly pheomelanin, photosensitizes adjacent cells to caspase-3 independent apoptosis, and this occurs at a frequency greater than the apoptosis induced by direct DNA absorption of UV. Melanin-induced apoptosis may contribute to the increased sensitivity of individuals with blonde and red hair to sunburn and skin cancer.

Constitutive skin pigmentation dramatically affects the incidence of skin cancer. Fair-skinned individuals are more susceptible to UV-induced skin damage than individuals with darker skin, resulting in a 10- to 100-fold higher frequency of nonmelanoma and melanoma skin cancer (1, 2). UV-induced cutaneous cancers are frequent in patients with albinism subtypes caused by the absence of melanin (3), and albino mice appear to be susceptible to skin cancers (4). Melanin is thought to filter out UV radiation and scavenge active oxygen species, thereby reducing UV damage in the cutaneous cells. In addition, a supranuclear melanin cap structure minimizes photodamage to the nucleus (5, 6).

In contrast to these effects, melanin also is known to act as a photosensitizer that generates active oxygen species upon UV irradiation (1, 7). The tyrosine-derived aromatic rings of the melanin chromophore are excited to the singlet state, decay to the triplet, and transfer an electron to oxygen to yield superoxide (O2•−) (8). Some evidence also indicates transfer of excitation from the chromophore to oxygen, giving singlet oxygen (O2). Reaction of superoxide with iron (III) ions and hydrogen peroxide (created by dismutation of superoxide) can lead to the OH• radical, which is capable of causing DNA strand breaks. Melanins subsequently scavenge these active chemical species, but their scavenging capacity can be overwhelmed. In living skin exposed to UV, it is not known which of these opposing mechanisms predominates (7).

To determine the photosensitizing function of eumelanin and pheomelanin after UV irradiation in vivo, we examined cell death after UV exposure in skin having different melanin contents. In human skin, it would be difficult to determine the site of a photosensitization reaction leading to the death of nearby cells, because melanin is distributed throughout the interfollicular epidermis, hair follicle, and hair shaft. In the dorsal skin of mice, however, melanin is limited to the hair shaft and keratinocytes of the hair bulb and dermal papilla (9). This limited spatial distribution makes it possible to identify the source of melanin-based photoresponses. It also makes it possible to measure the damaging effects of melanin without complications from its shielding function. We therefore used three congenic strains having different coat colors: black (C57BL/6J), yellow (B6.Cg-Av; former name C57BL/6J-Av), and albino (C57BL/6J-Tyr−c2−/Tyr−c2−). The yellow strain carries the Av mutation in the A locus (agouti), resulting in production of pheomelanin instead of eumelanin in the murine hair (10). The albino strain carries a G-to-T base change at nucleotide 291 of the tyrosinase gene, changing the amino acid from arginine to leucine and destroying enzyme function. Three assays for apoptotic cells were used: sunburn cells (a morphological criterion), cells positive for active caspase-3 (an endstage effector of apoptosis), and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) (cells containing DNA double-strand breaks). We found that after UV exposure, the hair follicles of both strains of pigmented mice contain TUNEL-positive keratinocytes not seen in albino mice, and that the frequency of these cells exceeds that of the sunburn cells and active caspase-3-positive cells induced in all three genotypes by direct DNA absorption of UV.

Methods

Mice, UV Irradiation, and Tissue Sections. Female C57BL/6J, B6.Cg-Av, and C57BL/6J-Tyr−c2−/Tyr−c2− mice (The Jackson Laboratory) were used for experiments at 6–8 weeks of age. The coats of these congenic strains are black (C57BL/6J), yellow (B6.Cg-Av), and albino (C57BL/6J-Tyr−c2−/Tyr−c2−). No tyrosinase activity is found in hair bulb extracts from the albino mouse (11). Mice in the resting phase of the hair follicle growth-regression cycle were shaved on the back with clippers and a shaver; the next day, they were exposed to predominantly UVB (280–320 nm) from three FS20T12-UVB lamps (National Biological, Twinsburg, OH) or to UVA (320–400 nm) from eight F20T12BL lamps (Spectra Mini, Daavlin, Bryan, OH) passed through an 11-mm glass plate filter to remove UVB and UVC (100–280 nm).

Abbreviation: TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

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nm). The output of the UVB lamps was UVA, 27.1%; UVB, 72.8%; and UVC, 0.1%. The output of the filtered UVA lamps was UVA, 100%; UVB, 4 × 10^-4%; and UVC, 8 × 10^-4%. The UV output of the lamp was measured before each session by using a UVX meter (Ultraviolet Products). Mice were irradiated once with 1,250 J/m² UVB or 100 kJ/m² UVA. During irradiation, animals moved freely but were prevented from standing upright by a wire mesh with 1 × 1-cm openings. Animals were killed by isoflurane anesthesia and cervical dislocation immediately after irradiation for DNA lesion measurements, or 24 h later for examination of cell death in the epidermis. The animal protocol was reviewed and approved by the Yale Institutional Animal Care and Use Committee.

The central portion of the dorsal skin was removed and washed with PBS. A portion of the skin was embedded in OCT compound (Sakura Fine-Technical, Tokyo) on dry ice. Another portion was fresh-frozen, and sections were cut in a cryostat and fixed with 4% paraformaldehyde in PBS overnight at 4°C. Other portion was fresh-frozen, and sections were cut in a cryostat and fixed with 4% paraformaldehyde in PBS overnight at 4°C. Other portion was fresh-frozen, and sections were cut in a cryostat and fixed with 4% paraformaldehyde in PBS overnight at 4°C. Other portion was fresh-frozen, and sections were cut in a cryostat and fixed with 4% paraformaldehyde in PBS overnight at 4°C. Other portion was fresh-frozen, and sections were cut in a cryostat and fixed with 4% paraformaldehyde in PBS overnight at 4°C. Other portion was fresh-frozen, and sections were cut in a cryostat and fixed with 4% paraformaldehyde in PBS overnight at 4°C. 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fact that melanin in mouse dorsal skin is located solely in the hair follicles and the mice were shaved before the experiment. The number of dimer-positive cells also appeared similar in the three genotypes. We also examined paraffin or frozen sections for 8-oxo-deoxyguanosine and single-strand breaks in the three genotypes; although signal was detectable from H$_2$O$_2$-treated positive controls, no signal above background was observed immediately after UVB or UVA irradiation (data not shown). These lesions may be present but obscured by introduction of DNA lesions while the tissue and DNA are being processed. These events are difficult to suppress in fixed tissue.

**Apoptosis Induction: Sunburn Cells.** The sunburn cell is a morphological hallmark of skin overexposed to sunlight; it also appears in certain skin diseases (18). These cells have been shown to be late-stage apoptotic keratinocytes (21, 24). To determine whether melanin photosensitizes cell death, we counted sunburn cells in the dorsal epidermis of UVB-irradiated mice for each pigmentation genotype. Skin samples were taken 24 h after a single UVB irradiation (1,250 J/m$^2$). All three genotypes showed randomly distributed sunburn cells throughout both the interfollicular and perifollicular epidermis and the upper part of the hair follicle (Fig. 2 A, C, and E). The distribution of sunburn cells, penetrating almost to the middle level of the hair follicle, corresponded to the regions where cyclobutane dimers were present and is consistent with the involvement of UVB in sunburn cell induction. There was no apparent difference in location or distribution of sunburn cells among genotypes, nor were there differences after quantitation of sunburn cells located in the perifollicular plus interfollicular epidermis (Fig. 3E). After a single UVA exposure (100 kJ/m$^2$), sunburn cells were not seen (data not shown), consistent with reports that sunburn cells are absent or rare after UVA (25, 26), although UVA can induce apoptosis in cultured cells (27). We conclude that apoptotic keratinocytes in skin are induced by direct DNA absorption of UVB radiation, independent of melanin.

**Apoptosis Induction: Caspase-3.** As a molecular assay for early-stage apoptosis, we immunostained paraffin sections with an anticaspase-3 antibody that recognizes the active cleaved form. The distribution and frequency of the caspase-3-positive cells after UVB were similar to that of the sunburn cells (Fig. 2 B, D, and F). Typical caspase-3-positive cells showed the same con-
sunburn cell or as active caspase-3 assays. Pigmentation-dependent cell death revealed by TUNEL exceeds the pigmentation-independent apoptosis present in the albino strain or measured by the difference between pigmented mice and the albino (black vs. albino, yellow vs. albino, P < 0.01 by Student’s t test). Each value represents the mean of three mice, 1,250 J/m². Approximately 2 cm of epidermis was examined per mouse. (E) Quantitative comparison of the three apoptosis assays in interfollicular plus perifollicular regions of the three strains. TUNEL showed a 2.5-fold difference between pigmented mice and the albino (black vs. albino, P < 0.02; yellow vs. albino, P < 0.01 by Student’s t test). The contribution of pigmentation-dependent cell death revealed by TUNEL exceeds the pigmentation-independent apoptosis present in the albino strain or measured by the sunburn cell or active caspase-3 assays. (F) UVA-irradiated mouse skin (100 kJ/m²) also demonstrates a 3- to 4-fold increase in TUNEL-positive cells in hair follicles of black and yellow mice (black vs. albino, P < 0.01; yellow vs. albino, P < 0.02 by Student’s t test).

Induction of TUNEL in Follicles of Pigmented Mice. A molecular marker for late-stage apoptosis is the presence of DNA double-strand breaks (TUNEL) (24). The same UVB-irradiated samples studied above were therefore examined for epidermal cell death by using TUNEL. In all samples, most TUNEL-positive cells were seen in the interfollicular and perifollicular epidermis and in the upper end of the hair follicle (Fig. 3 A–C). Unexpectedly, TUNEL-positive cells were much more frequent in the black and yellow mice than in albino mice. Moreover, in the albino mice, TUNEL-positive cells were randomly distributed throughout the epidermis, whereas the positive cells in pigmented mice tended to concentrate around the hair follicle. Quantitation revealed that the frequency of TUNEL-positive cells in the follicle was 3-fold greater in black and yellow mice than in the albino (Fig. 3D); in perifollicular plus interfollicular epidermis, the difference was 2.5-fold (Fig. 3E). TUNEL-positive cells were almost exclusively keratinocytes, as indicated by keratin staining (data not shown).

It seemed possible that the excess TUNEL positivity in pigmented strains might represent DNA double-strand breaks induced nonenzymatically by melanin-sensitized photoreactions, rather than reflecting apoptosis. We therefore examined skin from black and yellow strains for TUNEL positivity at 0 h after UVB irradiation; no TUNEL-positive keratinocytes were seen (data not shown).

Numerical comparison of the three genotypes for each of the apoptosis assays is instructive (Fig. 3E). A UVB dose of 1,250 J/m² induced ~45 apoptotic cells per cm in albino mice and in pigmented mice assayed as sunburn cells or by active caspase-3. Superimposed on this basal level of “classic” apoptotic cells, pigmented strains sustained an additional ~75 cells per cm of TUNEL-positive cells that did not evince the other indications of apoptosis. These cells apparently represent apoptosis independent of active caspase-3 and not showing the classical sunburn cell morphology. Because the number of excess TUNEL-positive cells was approximately the same in black and yellow mice, whereas the concentration of eumelanin in black mice was 3-fold that of pheomelanin in yellow mice (Table 1), the specific activity of TUNEL induction per ng of melanin was 3-fold greater for pheomelanin.

We also performed TUNEL analysis after 100 kJ/m² UVA irradiation, less than a minimal erythema dose for UVA (23). Few TUNEL-positive cells (three or four per cm) were detected in the perifollicular plus interfollicular epidermis (data not shown). Within the hair follicle, more were seen and the number was greater in pigmented mice (Fig. 3F).

3D Distribution of TUNEL and Caspase-3-Positive Cells in Epidermal Sheets. In the paraffin cross sections, it appeared that the TUNEL-positive cells were more frequent along the hair shaft. Because the proximity to the hair shaft is critical to interpreting the role of melanin in generating the excess TUNEL-positive cells, we sought to confirm this result by performing the TUNEL assay on epidermal sheets. Strikingly, the TUNEL-positive cells in the pigmented mouse strains were indeed concentrated adjacent to the acellular hair shafts in the
hair follicles (Fig. 4 A and B). TUNEL-positive cells were not located along the entire length of the follicle, presumably because UVB penetrated only to the midpoint of the follicle (Fig. 1). In contrast, in the albino mouse, TUNEL-positive cells were less frequent and randomly distributed throughout the interfollicular epidermis and hair follicle (Fig. 4 C). These results indicate that keratinocytes located near hair shafts containing melanin are susceptible to cell death after UVB irradiation. We also examined caspase-3-positive cells in the epidermal sheets from UVB-irradiated mice. Consistent with the results in paraffin cross sections, caspase-3-positive cells were randomly distributed in the epidermal sheet of both pigmented and albino mice (Fig. 4 G–I).

Discussion
Melanin Photosensitizes Cell Death. The occurrence of UV-induced TUNEL-positive cells adjacent to melanin-containing hair follicles reveals that, in vivo, melanin is a photosensitizer as well as a photoprotector. This experiment was made possible by the absence of extrafollicular melanin in mouse skin. The frequency of perifollicular plus interfollicular TUNEL-positive cells in albino mice equaled the frequency of sunburn cells or caspase-3-positive cells in each of the three genotypes (Fig. 3 E). These results suggest that UVB (and, to a lesser extent, UVA) has two actions as diagrammed in Fig. 5: (i) Directly inducing classical apoptotic cells, which are identifiable by sunburn cell morphology, active caspase-3, or TUNEL. These are independent of melanin and evenly distributed throughout the region of epidermis penetrated by UVB. Sunburn cells are initiated by cyclobutane pyrimidine dimers (28) in actively transcribed genes (22) and require the p53 gene (24). Because typical caspase-3-positive cells resembled sunburn cells, they apparently also require a DNA damage signal. Extranuclear signals may be required in addition (18). (ii) Inducing a melanin-dependent mode of cell death detectable only by TUNEL. These sites are determined by UV penetration plus the distribution of melanin. In the mouse, this combination limits this mode of cell death to the upper half of the hair follicle. Melanin also is present in the hair bulb (data not shown), but UVB did not reach this site. Strikingly, the magnitude of the melanin-dependent cell death was approximately twice that of classic apoptosis.

Although black and yellow mice showed equal levels of TUNEL positivity, the level of eumelanin in black mice was 3-fold the level of pheomelanin in yellow mice, indicating a 3-fold greater specific activity of pheomelanin photosensitization. This activity is consistent with a report that pheomelanin produces almost five times as much superoxide as eumelanin after UV exposure (29). UV-irradiated melanin may thus also generate other consequences of superoxide, such as point mutations.

Caspase-Independent Cell Death from Melanin Photosensitization. Although apoptosis and necrosis are well known forms of cell death, several caspase-independent cell death modes have recently emerged. In these modes, cells undergo nuclear fragmentation without chromosome condensation or cell shrinkage. In one class of pathways, nuclear fragmentation results from nucleases which, unlike DFF45, do not require activation by caspases. These include AIF (apoptosis inducing factor) and endonuclease G (30, 31). Other modes of cell death achieve proteolysis without caspases, instead using lysosomal cathepsins [autophagy, resulting in double-membrane vesicles (32)], proteosomal degradation, or calpain proteases from the endoplasmic reticulum (30). The TUNEL-positive cells seen in the present experiments clearly exhibit nuclear fragmentation without chromosome condensation, otherwise they would have been scored as sunburn cells. We do not yet know whether...
the cells contain double-membrane vesicles or whether the process is inhibited by in vivo treatment with wide-spectrum caspase inhibitors or inhibitors of the autophagy pathway (32). TUNEL would also detect the DNA fragmentation in necrotic cells (33).

Potential Role of Melanin Photosensitization in Human Skin. These photosensitization processes may contribute to clinical manifestations in humans. Individuals with blonde or red hair are susceptible to sunburn and have a risk for skin cancer that is elevated 1.5-fold (blonde) to 3- to 6-fold (red), compared with Caucasian controls (34, 35). Skin cancer risk is correlated epidemiologically with the propensity to burn rather than tan. This “Celtic phenotype” is associated with red and blonde hair but also with light skin, so the greater transmission of UV through the skin has been thought responsible. The present studies indicate that melanin photosensitization may be an important contributor. If so, photosensitization would lead to a broader spatial distribution of TUNEL in human than mouse, because human epidermal melanocytes are distributed throughout the basal layer. The resulting sunlight sensitivity of individuals with any particular melanin type would reflect the balance between protection and damage, with pheomelanin contributing 3-fold greater damage than eumelanin and perhaps less efficient protection. In this scenario, when a pheomelanin-containing individual visits the beach, his melanosomes act as microscopic x-ray sources to generate superoxide and OH· radicals. The resulting TUNEL-positive response may be protective, with melanin acting as a screen at low UV doses and as a “poison pill” that removes damaged cells at higher doses (24, 36, 37). Yet when death-resistant mutant cells are already present, this cell death may instead be detrimental and favor clonal expansion of the mutants (12, 24).

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