Comparison of the Responses of Human Melanocytes with Different Melanin Contents to Ultraviolet B Irradiation

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ABSTRACT

Melanin is thought to serve in photoprotection. To investigate this, we have compared the responses of cultured human melanocytes derived from different pigmented phenotypes (skin types I-VI) to a single irradiation with different doses of UVB light, ranging between 11.7 and 70.1 mJ/cm². After UVB irradiation, heavily pigmented melanocytes had the same percent survival but a greater capacity to resume proliferation than their lightly pigmented counterparts. A significant increase in melanin content was observed in heavily pigmented but not in lightly pigmented melanocytes. Irradiation with UVB light blocked melanocytes, regardless of their melanin content, in G₁, and induced the expression of the tumor suppressor p53 protein within 4 h. This induction steadily increased up to 48 h in lightly pigmented melanocytes; however, in heavily pigmented melanocytes, p53 level peaked at 24 h after UVB treatment and declined thereafter. Additionally, DNA from lightly pigmented melanocytes contained significantly higher numbers of cyclobutane pyrimidine dimers than did DNA from heavily pigmented melanocytes after irradiation with increasing doses of UVB light. We speculate that the prolonged induction of p53 in lightly pigmented melanocytes arrests them in G₁ for a long time period in order to repair extensive DNA damage. The above described differences might partially explain the increased susceptibility of individuals with lightly pigmented skin compared to individuals with dark skin to the photodamaging and photocarcinogenic effects of sun exposure.

INTRODUCTION

Excessive exposure to the sun is implicated in the etiology of skin cancers (1–3). The UV light that reaches the surface of the earth is mostly in the form of UVA (320–400 nm). Some is in the form of UVB (290–320 nm). It is this latter spectrum of solar radiation that is most active in inducing genotoxic and photocarcinogenic effects (3, 4). The depletion of the ozone layer, which mainly filters out UVB and UVC rays, has become a major public health concern because it is projected to increase the penetration of UVB rays through the atmosphere of the earth, resulting in higher incidence of skin cancers, including malignant melanoma, the most lethal (5). Because of these concerns, we chose to investigate the effects of UVB light on, and to elucidate the cellular and molecular changes induced in, human epidermal melanocytes.

The mechanisms by which UV light induces DNA damage and mutations in humans is not well understood. Irradiation of mammalian cells in culture with UV light results in inhibition of DNA replication primarily due to the formation of cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts (6-8). Ultraviolet light and contain less cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts (6-8). Ultraviolet light and have a lower incidence of skin cancers than do individuals with fair skin (3, 21–24). It has been proposed by many that the main function of melanin is to protect against the damaging effects of UV radiation by quenching oxygen radicals and acting as a sunscreen (25–27). It has been shown that more UV rays are transmitted through the epidermis, and about five times more UV light reaches the upper dermis, of Caucasian than black skin (26, 28, 29). However, the question of whether melanin reduces the formation of photoproducts in epidermal cells still needed to be further addressed.

It is now established that normal human melanocytes in culture respond to UV light with increased melanin formation. Friedmann and Gilchrest (30) were the first to report on the inhibition of proliferation and increased melanogenesis of cultured human melanocytes after UV irradiation. We have recently reported that human melanocytes respond to multiple irradiations with UVB light with increased melanogenesis and growth inhibition (31). It was shown that melanotic melanocytes have a greater percent survival than do melanocytes with low melanin contents after irradiation with UVA light (32). Highly melanotic malignant melanoma cells were also found to be more resistant than were amelanotic human melanoma cells to killing by solar or UVB light (33). Moreover, the highly melanotic melanoma cells are more resistant to the killing effect of 254 nm wavelength UV light and contain less cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts (6-8). Ultraviolet light and have a lower incidence of skin cancers than do individuals with fair skin (3, 21–24). It has been proposed by many that the main function of melanin is to protect against the damaging effects of UV radiation by quenching oxygen radicals and acting as a sunscreen (25–27). It has been shown that more UV rays are transmitted through the epidermis, and about five times more UV light reaches the upper dermis, of Caucasian than black skin (26, 28, 29). However, the question of whether melanin reduces the formation of photoproducts in epidermal cells still needed to be further addressed.

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(6-4) pyrimidine photoproducts than do their less melanotic counterparts (34). Although these results clearly demonstrate the responsiveness of normal and malignant human melanocytes to UV light, comparison of the responses of normal melanocytes with different pigmentary phenotypes to treatment with UVB light has not been documented.

It is known that UV irradiation results in the induction of several genes that are involved in the regulation of cellular proliferation. One of these genes is the tumor suppressor p53, which acts as a cell cycle checkpoint, causing cells to be arrested in G1 (35–38). This arrest is thought to be essential to allow cells to repair the UV induced DNA damage before resuming proliferation.

To elucidate the role of melanin in dictating the responses of different pigmentary phenotypes to sun exposure, we have compared the responses of several lightly pigmented and heavily pigmented human melanocyte strains to irradiation with increasing doses of UVB light. Cell survival and resumption of proliferation, induction of p53 expression, melanin synthesis, and formation of cyclobutane pyrimidine dimers were compared among the above types of melanocytes after a single treatment with UVB light. This study represents an attempt to define some of the molecular mechanisms that account for the differences in the responses of individuals with different skin types to sun exposure, and ultimately in their susceptibility to develop skin cancers.

MATERIALS AND METHODS

Culturing of Human Melanocytes. Normal human melanocyte cell strains were established from individual neonatal foreskins (skin types I–VI) obtained from the nursery of University Hospital after routine circumcision, as described previously (19). One neonatal tyrosinase-negative albino cell strain was a generous gift from Dr. Raymond Boyssy (Department of Dermatology, University of Cincinnati). Melanocytes were maintained in a growth medium consisting of MCDB 153 supplemented with 5% FCS, 1% penicillin-streptomycin, 1 mg/ml human transferrin, 5 μg/ml insulin, 1 μg/ml α-tocopherol, and the melanocyte-specific mitogens, 0.6 ng/ml human recombinant basic fibroblast growth factor, 8 ng 12-o-tetradecanoylphorbol-13-acetate, and 13 ng/ml bovine pituitary extract (Clonetics) as described previously (39). All of the reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Irradiation of Melanocytes With Different Doses of UVB Light. The UV source that we have used consists of a bank of FS-20 fluorescent UV lamps manufactured by Westinghouse, and has a continuous emission spectrum with a peak at 313 nm. There is negligible emission in the UVC range, with 75% of the emission in the UVB and 25% of the emission in the UVA range of the spectrum. The source is equipped with a filter consisting of calcium, zinc, and thallium phosphate phosphor in a special glass envelope.

Tyrosinase Activity. Tyrosinase activity was measured in situ using a modification of the charcoal absorption method of Pomerantz (41). On day 6 after plating, melanocytes received [3H]tyrosine (specific activity, 52 μCi/ml; Amersham, Arlington Heights, IL) at a concentration of 0.5 μCi/ml of medium for 24 h. The medium was then collected, and the tyrosine hydratase activity of tyrosinase was measured by quantifying the amount of 3H2O released to the culture medium as tyrosine is converted to L-dihydroxyphenylalanine as described previously (42).

Cell Cycle Analysis. Melanocytes were plated in 100-mm dishes at a density of 5 × 10^5 cells. Three days later, cells were irradiated with 0, 23.4, 35.0, or 46.7 mJ/cm^2 of UVB light. Cell control untreated and UVB-irradiated monolayer cultures were incubated 14 h after UVB irradiation with 10^4 m BrdUrd for 60 min at 37°C. At the end of the incubation period, cells were harvested, fixed, and stained with FITC-conjugated mouse mAb against BrdUrd (20 μl/10^6 cells anti-BrdUrd–FITC, Becton Dickinson, San Jose, CA) for 1 h at room temperature, as described in detail previously (31). The cells were then washed twice, resuspended in PBS, and treated with 5 μg/ml propidium iodide (PI) and 1 μg/ml RNase A for 1 h at room temperature.

A negative control group consisting of cells that were not treated with BrdUrd was included for both the control and the UVB-irradiated groups and stained as above. Cells were analyzed by flow cytometry using an EPICS 753 flow cytometer (Coulter Cytometry, Coulter Corp., Hialeah, FL), as described previously (43). FITC and PI were excited with the 488-nm line of an argon ion laser. Fluorescence emission for FITC and PI was detected selectively by collecting 530 ± 15 and 575 ± 12.5 nm, respectively. Electronic gates were placed around the cells of interest using forward angle and 90° light scattering. When analyzing PI, gates were also set on the peak versus integral PI signals to eliminate doublets. The PI signals were analyzed using Multicycle (Phoenix Software, San Diego, CA), and the bivariate histograms were analyzed using Coulter XL software (Coulter Corp.).

Kinetics of Induction of p53 Protein. Melanocytes were seeded at a density of 5–7 × 10^5 cells/100-mm dish. Four days after plating, cells were irradiated with 0 or 35.0 mJ/cm^2, and 4, 8, 24, 32, or 48 h later were lysed in RIPA buffer containing the phosphatase and protease inhibitors: 10 mM Na_2VO_4, 200 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin (44).

Protein content per sample was determined using the Bio-Rad DC protein assay. Equal amounts of protein (80 μg/lane) were loaded on 7.5% SDS-PAGE gels. After electrophoresis, the gels were transblotted onto nitrocellulose membranes, which were blocked with 10% nonfat dry milk in 0.2% Tween 20 in PBS, then incubated with the p53 mouse mAb Ab-6 (Oncogene Science, Cambridge, MA), at a dilution of 1:100 overnight at 4°C. The membranes were blocked again as described above and incubated with horseradish peroxidase anti-mouse IgG (Amersham, Arlington Heights, IL) at a dilution of 1:15,000 for 1 h at room temperature. The immunoreactivity was detected using the enhanced chemiluminescence reaction (Amersham), followed by exposure of the membranes to Kodak AR 5 X-ray films.

Formation of Cyclobutane Pyrimidine Dimers in Genomic DNA. Melanocytes from a lightly or a heavily pigmented melanocyte strain were plated at a density of 7.5 × 10^5 cells/100-mm dish (triplicate dishes/group). Three days after plating, melanocytes were irradiated with 0, 23.4, 35.0, 46.7, or 70.1 mJ/cm^2 UVB light. The melanocytes were harvested immediately after irradiation, and the DNA was extracted as follows: cell pellets were lysed in 3–4 ml solution of 0.3 mg/ml proteinase K and 0.3% SDS overnight at 37°C. Genomic DNA was then extracted with an equal volume of chloroform-isomyl alcohol (24:1, v/v). The extract was treated with 0.5 mg ammonium acetate (2/5 of total volume), and the DNA was precipitated by adding 2.5 volumes of absolute ethanol at −20°C overnight. This was followed by centrifugation at 10,000 rpm for 4°C for 10 min. The DNA was finally resuspended in 1-ml 10× TE buffer.

To quantitate the number of cyclobutane pyrimidine dimers induced in lightly or heavily pigmented melanocytes, we used a RIA that has been described previously (7, 45). The antisera was raised in rabbits against

Lee et al. (40). Melanocytes were harvested, rinsed with PBS, and counted. Melanin was solubilized in 0.2 N NaOH (10^5 cells/ml) and measured spectrophotometrically at an absorbance of 475 nm against a standard curve of known concentrations of synthetic melanin (Sigma). Melanin content was expressed as μg/10^6 cells.
heat-denatured DNA, dissolved in a triplet sensitizer (acetophenone), and irradiated with high fluences of UVB light. These conditions produce a high yield of cyclobutane dimers and a low yield of (6-4) photoproducts. The RIA is a competitive inhibition assay in which approximately 2 μg of heat-denatured sample DNA are incubated with 10 pg of DNA (labeled to 2.5 × 10^8 cpm/μg by nick translation with [3^2P]dATTP) in a total volume of 1 ml 10 mM Tris (pH 7.8)-150 mM NaCl with 0.15% gelatin. Purified anti-cyclobutane cpm/pig by nick translation with ['"PjdTTP) in a total volume of 1 ml 10 mw carrier serum from nonimmunized rabbits (Calbiochem). After centrifugation, 30-60% binding to labeled ligand. After incubation overnight at 4°C, the immune complex was precipitated with goat anti-rabbit immunoglobulin and carrier serum from nonimmunized rabbits (Calbiochem). After centrifugation, the pellet was dissolved in tissue solubilizer, mixed with acid Scintiverse, and the ^3P was quantified by liquid scintillation spectrometry. Under these conditions, antibody binding to an unlabeled competitor resulted in reduced binding to radiolabeled ligand (i.e., inhibition). The percent inhibition of each sample was extrapolated through a standard (dose-response) curve to determine the cyclobutane dimer concentrations. Salmon sperm DNA irradiated at a dose rate that yielded 2.45 cyclobutane dimers/10^9 daltons/J/sec was used as a standard (46).

RESULTS

Constitutive Melanin Content of the Melanocyte Strains Used in the UVB Dose-Response Experiments. To compare the dose-dependent responses of melanocytes with different melanin contents to UVB irradiation, we tested the responses of 3 darkly pigmented (skin types IV-VI) and 4 lightly pigmented (skin types I-II) cell strains. To verify that these two types of melanocyte strains differ significantly in their constitutive pigmentation, we measured basal tyrosinase activity and melanin content in each. Our results clearly demonstrate that the heavily melanotic melanocytes from each of the 3 cell strains used expressed a higher tyrosinase activity and melanin content than did any of the lightly pigmented cell strains included in these experiments (Table 1).

Dose-dependent Effects of UVB Light on Cell Survival and Proliferation. Melanocyte cultures established from different skin types responded to a single irradiation with UVB light with a dose-dependent decrease in proliferation and cell survival. These findings are consistent with those of Friedman and Gilchrest (30) but differ from the results of Libow et al. (47), which showed stimulation of melanocyte proliferation by UV light. We observed that with increasing dose of UVB light there was an increase in the number of detached cells. By trypan blue staining we determined that the floating cells were dead, whereas those that remained attached to the plates were viable (data not shown).

After irradiation with the high dose of 35 mJ/cm^2 UVB light, we observed that the heavily pigmented melanocytes resumed proliferation, whereas the less melanotic melanocytes failed to proliferate even 8 days after irradiation (Fig. 1, a and b). This difference in the ability to resume proliferation after UVB irradiation was not due to differences in the proliferative rates of the heavily and lightly pigmented melanocytes because both cell strains tested had comparable replication rates (evident as similar control growth curves). The albino melanocytes had the lowest survival and recovery rates at the highest two doses of UVB light (Fig. 1c). The percent of survival, determined

Table 1 Constitutive tyrosinase activities and melanin contents of the cell strains used in the UVB dose response experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>Tyrosinase activity</th>
<th>Melanin content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPM/10^6 cells ± SEM</td>
<td>μg/10^6 cells</td>
</tr>
<tr>
<td>Group A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>96,540 ± 4757</td>
<td>23.5</td>
</tr>
<tr>
<td>2</td>
<td>157,600 ± 1426</td>
<td>19.6</td>
</tr>
<tr>
<td>3</td>
<td>308,341 ± 6480</td>
<td>37.5</td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5,023 ± 574</td>
<td>4.2</td>
</tr>
<tr>
<td>2</td>
<td>20,182 ± 665</td>
<td>5.3</td>
</tr>
<tr>
<td>3</td>
<td>19,547 ± 528</td>
<td>5.0</td>
</tr>
<tr>
<td>4</td>
<td>24,482 ± 189</td>
<td>7.9</td>
</tr>
</tbody>
</table>

Groups A and B refer to darkly pigmented and lightly pigmented cell strains, respectively. Melanocytes were plated at a density of 8 × 10^4 cells/60-mm dish and received medium containing 0.5 μCi/ml [3H]tyrosine on day 6 after plating. Twenty-four h later, the conditioned medium was collected and assayed for tyrosinase activity, and the cell number in each dish was determined using a Coulter counter. Melanocytes from each group (triplicate dishes/group) were collected and assayed for melanin content, as described in "Materials and Methods."
We have compared the induction of p53 in heavily pigmented versus lightly pigmented melanocytes after irradiation with 35.0 mJ/cm² UVB light. By Western blot analysis, we found that p53 expression was induced 4 h after UVB treatment in both types of melanocytes. In the more melanotic melanocytes, induction of p53 peaked at 24 h and gradually declined 32 and 48 h after UVB exposure, whereas in the lightly pigmented melanocytes, induction of p53 continued to increase steadily up to 48 h after UVB treatment (Fig. 2, a and b).

Formation of Cyclobutane Pyrimidine Dimers in Lightly and Heavily Pigmented Melanocytes. We have used a RIA to quantitate the number of cyclobutane pyrimidine dimers in genomic DNA purified from lightly or heavily pigmented melanocytes after irradiation with increasing doses of UVB light (23.4, 35.0, 46.7, or 70.1 mJ/cm²). We have found that at all the UVB doses used, DNA from lightly pigmented melanocytes contained more cyclobutane pyrimidine dimers/10⁷ daltons than their heavily pigmented counterparts (Fig. 3). The formation of cyclobutane pyrimidine dimers correlated directly with the doses of UVB light used to irradiate the melanocytes. These results are similar to those reported previously by Kobayashi et al. (34), who showed that more cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts are formed in UV-irradiated melanoma cell lines with a low melanin content than in cell lines that are very melanotic.

**DISCUSSION**

In humans, a primary target for solar radiation is the epidermis. The most visible effect of sun exposure is increased cutaneous pigmentation, or “tanning,” which results primarily from stimulation of melanogenesis in epidermal melanocytes. Other effects of solar radiation, particularly UVB light, include DNA damage and photocarcinogenesis. Skin cancers are more common in individuals with lightly pigmented skin than in those with dark skin. The molecular basis for this difference, based on the responses of human melanocytes with different melanin contents to irradiation with UVB light, is poorly understood. The studies hereby presented were intended to investigate the well accepted notion that melanin serves for photoprotection against the damaging effects of sun exposure.

To investigate the role of melanin, we have compared the responses of cultured human melanocytes derived from lightly pigmented (skin types I–II) to those of melanocytes derived from heavily pigmented...
was determined as described in “Materials and Methods.” Points, average of duplicate samples.

Fig. 3. Dose-dependent formation of cyclobutane pyrimidine dimers in heavily (•) and lightly (■) pigmented melanocytes after irradiation with increasing doses of UVB light. Melanocytes were plated at a density of 7.5 × 10^3/100-mm dish and irradiated on day 3 after plating with 0, 23.4, 35.0, 46.7, and 70.1 mJ/cm^2 UVB light. DNA was immediately extracted after irradiation, and the number of cyclobutane pyrimidine dimers/10^7 daltons was determined as described in “Materials and Methods.” Points, average of duplicate samples.

In our study, we found that lightly pigmented melanocytes do not respond to a single treatment with UVB light with a significant increase in melanin formation (Table 3). However, previously we showed that lightly as well as heavily pigmented melanocytes are stimulated to synthesize melanin after multiple irradiations with UVB light (31). This raises the question of whether the inability of the lightly pigmented melanocytes to mount a rapid increase in melanin formation further increases their susceptibility to DNA damage. A novel but nevertheless interesting notion is that melanogenesis induced by UVB light correlates directly with DNA repair. This was concluded from the observation that treatment of melanocytes with the repair enzyme, T4 endonuclease V, resulted in a dose-dependent increase in melanin content (49). This is consistent with the idea that the increase in melanin associated with postinflammatory hyperpigmentation is assumed to rid the cell of the damaging products of the inflammatory process (50). Whether melanogenesis correlates with repair of cyclobutane pyrimidine dimers will be investigated by comparing the rate of removal of these photoproducts in melanocytes with different constitutive melanin contents.

Because of the drastic environmental changes that allow for more penetration of UVB rays through the atmosphere of the earth and the prediction that this would lead to increased incidence of skin cancers, protection from the sun has become an important issue for skin cancer prevention (5). However, the effectiveness of melanin as a photoprotective sunscreen is still debatable. Whether it is melanin alone or additional cellular defense mechanisms, such as levels of glutathione, p53 induction, or the rate of DNA repair that are responsible for the differences in the susceptibility of individuals with different skin types to photaging and skin cancers needs to be further elucidated.

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