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Activation of the Cyclic AMP Pathway by α-Melanotropin Mediates the Response of Human Melanocytes to Ultraviolet B Radiation[1]

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ABSTRACT

A hallmark of sun exposure is increased melanin synthesis by cutaneous melanocytes which protects against photodamage and photocarcinogenesis. Irradiation of human keratinocytes or melanocytes with ultraviolet (UV) rays stimulates the synthesis and release of α-melanotropin (α-MSH) and adrenocorticotropic hormone (ACTH), which induce cyclic AMP (cAMP) formation and increase the proliferation and melanogenesis of human melanocytes. We report that stimulation of cAMP formation is obligatory for the melanogenic response of cultured normal human melanocytes to UVB radiation. In the absence of cAMP inducers, UVB radiation inhibited, rather than stimulated, melanogenesis. UVB radiation (28 mJ/cm^2) arrested melanocytes in the G_1 phase of the cell cycle, and concomitant treatment with 0.1 μM α-MSH enhanced their proliferation but did not increase the surviving fraction. Irradiation with UVB, with or without α-MSH, caused prolonged expression of p53 and p21(waf-1, cip-1), maintained pRB in a hypophosphorylated state, and reduced the expression of Bcl2. However, α-MSH allowed UVB-irradiated melanocytes to enter S phase, suggesting that α-MSH acts as a mitogen rather than a survival factor, and that overexpression of p53 is mainly a signal for cell death. Our results underscore the importance of the cAMP pathway and its physiological inducers in mediating the response of human melanocytes to UV radiation.

INTRODUCTION

The fact that sun exposure is the principle etiological factor for skin cancer and that cutaneous melanin is photoprotective has generated increasing interest in the response of epidermal melanocytes to UV light. Previous studies on the effects of UV radiation on human melanocytes *in vitro* showed that these cells respond with growth arrest, decreased survival, and increased melanogenesis (1–3). UV radiation affects human skin directly by producing DNA damage (4) and indirectly through stimulating the synthesis of biochemical mediators and/or influencing the expression of their receptors on target cells in the epidermis (5, 6). The classic physiological regulator of integumental pigmentation in many vertebrate species is α-MSH[4] (α-melanotropin; Ref. 7). Several reports have shown that α-MSH and ACTH are synthesized in the skin by epidermal melanocytes and keratinocytes (8–10). Synthesis of these and other keratinocyte-derived factors that affect melanocyte proliferation and/or melanogenesis, such as basic fibroblast growth factor and endothelin-1, is enhanced by UV radiation (11, 12). In addition, sun exposure was found to increase serum levels of melanotropins (13). Pawelek *et al.* (14) have proposed that the melanotropin receptor acts as a transducer for the pigmentary effects of UV radiation on the skin. These studies, which were carried out on the murine Cloudman melanoma cells, revealed that irradiation of these cells with UVB increases melanogenesis and up-regulates the expression of the MSH receptor, as well as the synthesis of proopiomelanocortin-derived peptides (15).

We and others have shown that human melanocytes respond to α-MSH and ACTH with increased proliferation and melanogenesis (16–19). α-Melanotropin was found to induce eumelanin formation in human melanocytes (20). Eumelanin is known to be more photoprotective and resistant to degradation by UV light than pheomelanin (21, 22). The mechanism of action of α-MSH and ACTH on human melanocytes involves binding to the melanocortin-1 receptor (MC1R), a G protein-coupled receptor, which activates the cAMP pathway (23, 24). Variants of the *MC1R* gene were identified in individuals with skin type I or II, a phenotype associated with poor tanning response and high risk for skin cancer (25). Recently, it was reported that one of these variants is associated with melanoma (26). Because the cAMP pathway is activated upon binding of α-MSH to MC1R and is pivotal for the stimulation of human melanocyte proliferation and melanogenesis, we compared the proliferative and melanogenic responses of melanocytes maintained in the presence or absence of α-MSH to UVB (290–320 nm) light. Additionally, we compared the effect of α-MSH on the UVB-induced expression of the tumor suppressor gene product p53 and its downstream target, the cyclin-dependent kinase inhibitor p21 (waf-1, cip-1), the hypophosphorylation of the retinoblastoma gene product pRb, as well as the Bcl_2 level. The above proteins are known to be involved in the response of cells to DNA damage and in determining whether cells undergo DNA repair or apoptosis (3, 27–33). Our results clearly demonstrate the importance of the cAMP pathway in mediating the response of cutaneous melanocytes to UVB radiation. The significance of this pathway in inducing melanogenesis implies that it might enhance photoprotection against further DNA damage caused by chronic UV exposure.

MATERIALS AND METHODS

Melanocyte Culture Conditions. Normal human melanocytes were derived from neonatal foreskins as described previously (34) and maintained in a growth medium consisting of: MCDB 153, 4% heat-inactivated FCS, 13 μg/ml BPE (Clonetics, San Diego, CA), 8 nM TPA, 5 μg/ml insulin, 1 μg transferrin, 1 μg/ml α-tocopherol, 0.6 ng/ml human recombinant basic fibroblast growth factor, and 1% penicillin-streptomycin (10,000 units/ml and 10,000 μg/ml, respectively), as described by Medrano and Nordlund (35). Unless stated otherwise, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The cultures were maintained in a humidified incubator with 5% CO_2 at 37°C.

For all of the experiments described here, melanocytes were maintained in a culture medium lacking BPE for 2–3 days prior to beginning and for the entire duration of the experiments. As we reported earlier, removal of BPE was essential for an optimal response to α-MSH or other cAMP inducers, because BPE contains high concentrations of α-MSH (16).

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[4] The abbreviations used are: α-MSH, α-melanocyte stimulating hormone; ACTH, adrenocorticotropic hormone; cAMP, cyclic AMP; BPE, bovine pituitary extract; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; db, dibutyryl; TRP-1, tyrosinase-related protein-1; PI, propidium iodide; PKC, protein kinase C; cdk, cyclin-dependent kinase.

Determination of Melanocyte Proliferation and Tyrosinase Activity. To investigate the effects of UVB and/or α-MSH on melanocyte proliferation and tyrosinase activity, cells were plated at a density of 1×10^5 cells/60-mm dish. On day 3 after plating, melanocytes were exposed once to 28 mJ/cm^2, every other day to 15 mJ/cm^2 for a total of 6 days, or daily for 6 days to 5 mJ/cm^2 UVB radiation. For this, the medium from each dish was removed, the cells were washed gently with sterile PBS, and in the presence of 2 ml PBS, each dish was irradiated with the lid removed. The UV source used consisted of a bank of FS-20 fluorescent UV lamps, with 75% emission in the UVB range, 25% emission in the UVA range, and peak emission at 313 nm. The growth medium was added to the culture dishes immediately after irradiation, and one set of UV-irradiated melanocytes was treated with 0.1 μM α-MSH (Fig. 1 and 4) or 10 μM db cAMP (Fig. 3). Fresh growth medium lacking BPE and fresh hormone were added every other day for a total of 6 days. In most experiments, cell number was determined every other day, and in experiments where melanocytes were irradiated every other day for a total of three treatments, cell number was determined at the end of the experiment.

In experiments where melanocytes were irradiated once or daily for 6 days with UVB, tyrosinase activity was determined on days 2, 4, and/or 6 after irradiation. In experiments where melanocytes were irradiated every other day for a total of 6 days, tyrosinase activity was determined at the end of the experiment. In all of these experiments, [L-,3,5-^{3}H]tyrosine (specific activity, 52 mCi/mmol; Dupont NEN, Boston, MA) was added at 0.7 μCi/ml media (2.8 μCi/dish) for 24 h prior to assaying for tyrosinase activity. The activity of tyrosinase was determined by measuring the tyrosine hydroxylase activity of the enzyme *in situ,* by using a modification of the charcoal absorption method developed by Pomerantz (36, 37), and expressed as dpm/10^6 cells.

Western Blot Analysis of Tyrosinase, Tyrosinase-related Protein-1, p53, p21, pRb, and Bcl2. For Western blot analysis of tyrosinase and TRP-1, cells were plated at a density of 7.5×10^5 cells/60-mm dish and irradiated once with 28 mJ/cm^2 UVB, as described above. One set of UVB-irradiated dishes was treated with 0.1 μM α-MSH. Fresh growth medium lacking BPE and fresh hormone were added every other day for a total of 6 days. Two, 4, or 6 days after irradiation, cell lysates were prepared as described previously (3), and equal amounts of protein (5–8 μg) were loaded on each lane of a minigel apparatus and separated on a 7.5% polyacrylamide gel. The proteins were transblotted onto nitrocellulose membranes, washed, blocked, with 10% nonfat dry milk in 0.2% Tween 20 in PBS, and then reacted overnight with αhPEP-7, a rabbit polyclonal antibody raised against the COOH terminus of the human tyrosinase (a gift from Richard King, University of Minnesota, Minneapolis, Minnesota) at a dilution of 1:1500, or TA99, a mouse monoclonal antibody raised against the human TRP-1 (kindly provided by Setaluri Vijayasaradhi, Wake Forest University, Winston-Salem, North Carolina), at a dilution of 1:1500, overnight at 4°C. The membranes were then reacted with horseradish peroxidase conjugated anti-rabbit IgG (Amersham Corp.; 1:3,000 dilution, following incubation with αhPEP-7) or anti-mouse IgG (Amersham; 1:15,000 dilution, following incubation with TA99) for 1.5 h at room temperature. The immunoreactive bands were detected by chemiluminescence, using the Renaissance kit (Dupont NEN).

For detection of p53, p21, pRb, and Bcl2 expression, melanocytes were plated at a density of 7.5×10^5 cells/dish (a single dish for control or α-MSH-treated groups and duplicate dishes for UV or UV + α-MSH treated groups) and were treated with UVB and/or α-MSH, as described above. Twenty-four h, 72 h, or 5 days after treatment, cell lysates were prepared, and 15 μg of protein were loaded per lane on a minigel apparatus; 7.5% gel was used for p53 and pRb, and 12% gel was used for p21 and Bcl2 detection. After electrophoresis and transblotting of the proteins onto nitrocellulose membranes, the membranes were washed, blocked as described above, and then incubated with p53 monoclonal antibody DO1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:150 overnight at 4°C, followed by incubation with anti-mouse horseradish peroxidase conjugated IgG at a dilution of 1:15,000 for 2 h at room temperature, and the immunoreactive bands were visualized by enhanced chemiluminescence. The same membrane was used for detection of pRb without stripping, using If8 monoclonal antibody (Santa Cruz Biotechnology), at a dilution of 1:150. The other membranes were reacted overnight with C-19 polyclonal antibody against p21 (Santa Cruz Biotechnology) at a dilution of 1:150, followed by incubation with anti-rabbit horseradish peroxidase conjugated IgG at room temperature and chemiluminescence reaction. The same membrane was used for detection of Bcl2, without stripping.

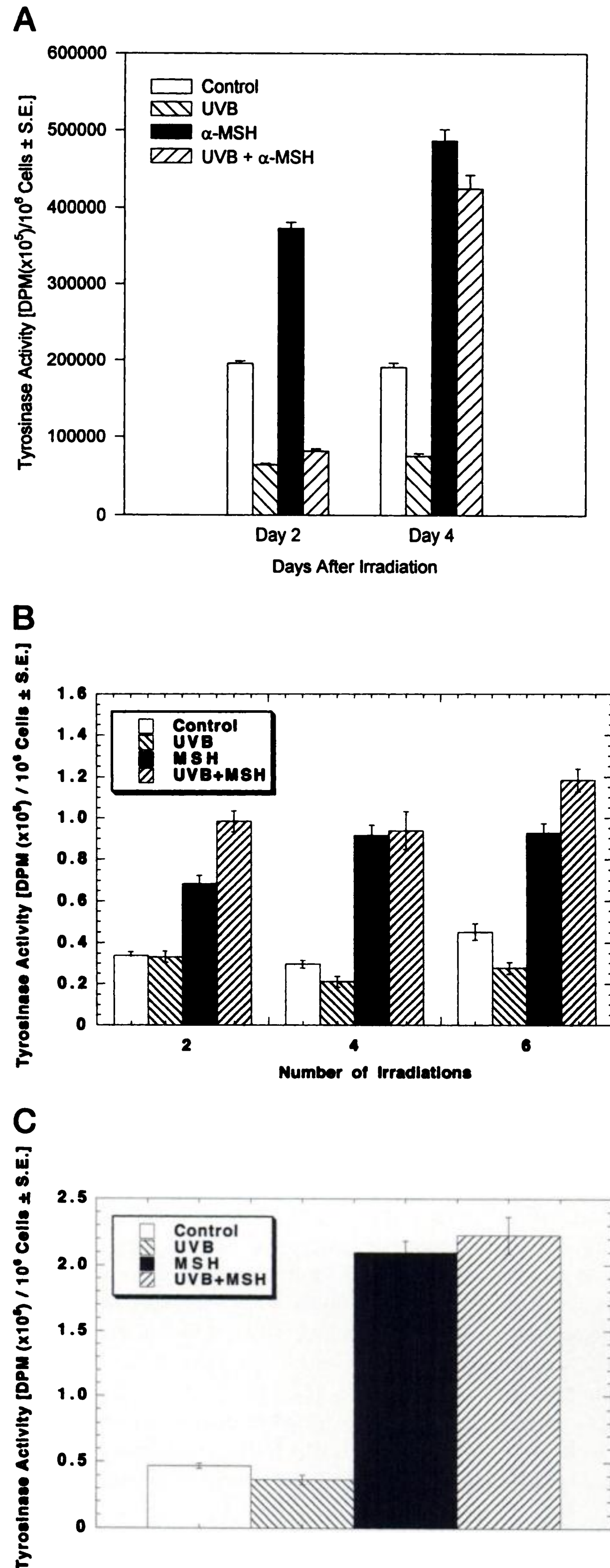

Fig. 1. Effect of different UVB irradiation protocols, with or without α-MSH treatment, on tyrosinase activity. In *A,* melanocytes were irradiated once with 28 mJ/cm^2 UVB and/or treated with 0.1 μM α-MSH. Tyrosinase activity was measured on days 2 and 4. In *B,* melanocytes were irradiated every day with 5 mJ/cm^2 UVB for a total of 6 days. One group was left unirradiated but treated with 0.1 μM α-MSH, and another was UVB irradiated and treated with α-MSH. Tyrosinase activity was determined after the second, fourth, and sixth irradiations. In *C,* melanocytes were irradiated once every other day with 15 mJ/cm^2 UVB for a total of 6 days, treated with 0.1 μM α-MSH, or irradiated and treated with α-MSH. Tyrosinase activity was determined 24 h after the last treatment with UVB and/or α-MSH. In *A–C,* each value for tyrosinase activity represents the mean of six determinations; *bars,* SE. Each experiment was repeated at least twice with similar findings.

For that procedure, the membrane was incubated overnight with mouse monoclonal antibody 100 (Santa Cruz Biotechnology) at a dilution of 1:150 at 4°C, followed by incubation with anti-mouse horseradish peroxidase conjugated IgG. The immunoreactive bands were visualized by enhanced chemiluminescence.

Determination of cAMP Levels following UVB Irradiation and/or α-MSH Treatment. Melanocytes were plated into six-well plates at a density of 5×10^5 cells/well. Seventy-two h later, cells were irradiated with 28 mJ/cm^2 UVB, and/or treated with 0.1 μM α-MSH. Cyclic AMP levels were determined by RIA at 0.75, 4, and 24 h after irradiation, as described in detail previously (23).

Cell Cycle Analysis of UVB and UVB plus α-MSH-treated Melanocytes. Melanocytes were plated into 100-mm dishes at a density of 7.5×10^5 cells/dish. Three days later, melanocytes were irradiated a single time with 28 mJ/cm^2 UVB light and/or treated with 0.1 μM α-MSH. Fresh growth medium lacking BPE and fresh hormone were added to the appropriate groups every other day. Twenty-four h, 72 h, or 5 days after irradiation, melanocytes were harvested, and their DNA was stained with PI, as follows. Bare nuclei were prepared by suspending melanocytes in 0.1% sodium citrate solution, containing 0.1% Triton X-100 and 1 mg/ml DNase-free RNase A, at a concentration of 2×10^6 cells/ml. Nuclei were incubated for 30 min at 37°C and then stained with 50 μg/ml PI. Flow cytometric analysis of the cell cycle was performed using a Coulter EPICS XL flow cytometer (Coulter Cytometry, Coulter Corp., Miami, FL). The PI signals were collected and analyzed using System II software (Coulter Corp.), and the DNA histograms were analyzed using Multicycle (Phoenix Software, San Diego, CA).

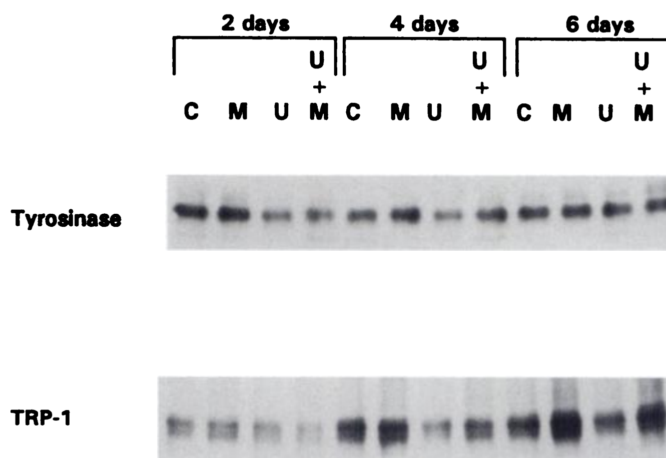

Fig. 2. Western blot analysis of tyrosinase and TRP-1 following a single UVB irradiation in the presence or absence of α-MSH. Melanocytes were irradiated once with 28 mJ/cm^2 UVB, treated with 0.1 μM α-MSH, or irradiated then treated with α-MSH every other day for a total of 6 days. The expression of tyrosinase and TRP-1 after 2, 4, or 6 days after irradiation and/or α-MSH treatment was determined by Western blot analysis, as described in "Materials and Methods." *C,* control; *M,* 0.1 μM α-MSH; *U,* UVB; *U + M,* UVB + α-MSH treatment. This experiment was repeated four times with similar results.

RESULTS

Effect of the cAMP Pathway on the Melanogenic Response of Human Melanocytes to UVB Radiation. We observed that in the absence of any cAMP inducer from the growth medium, a single irradiation of melanocytes with 28 mJ/cm^2 UVB resulted in a decrease in the activity of tyrosinase, the rate-limiting enzyme in the melanin synthetic pathway (Ref. 38; Fig. 1*A*). This was unexpected, based on data published previously showing that exposure of human melanocytes to UV radiation stimulated melanogenesis (1–3). However, we noted that melanocytes treated continuously with 0.1 μM α-MSH starting immediately after UVB treatment demonstrated a remarkable increase in tyrosinase activity 4 days after irradiation. Lack of stimulation of melanogenesis in the absence of any cAMP inducer was also observed in melanocytes exposed multiple times to different sublethal doses of UVB radiation. These included daily irradiations with 5 mJ/cm^2 UVB for a total of 6 days (Fig. 1*B*) or three irradiations, once every other day, at 15 mJ/cm^2 UVB (Fig. 1*C*). In these latter experiments, increased melanogenesis was detected only when α-MSH was added after irradiation.

To verify further the above results, Western blot analysis of the melanogenic enzymes tyrosinase and TRP-1, known to act as a dihydroxyindole carboxylic acid oxidase in the melanogenic pathway (39), was carried out (Fig. 2). The results obtained revealed that a single treatment with UVB radiation decreased the expression of both enzymes. As expected, melanocytes treated with α-MSH expressed slightly higher levels of tyrosinase, as well as increased TRP-1, compared with control or UVB-irradiated melanocytes (16). Treatment of UVB-irradiated melanocytes with α-MSH also increased tyrosinase and TRP-1 expression above the levels observed in melanocytes treated with UVB only.

To demonstrate further a role for the cAMP pathway in the melanogenic response of human melanocytes to UVB, melanocytes were irradiated once with 28 mJ/cm^2 UVB and treated with 1 μM db cAMP (Fig. 3*A*). We found that this agent mimicked the effects of α-MSH and stimulated tyrosinase activity following UVB treatment. To rule out the contribution of protein kinase C in mediating the UVB-induced melanogenic response, we compared the effect of α-MSH with that of the PKC activator TPA on the response of melanocytes to UVB radiation (40). The concentration of TPA typically used in our experiments (8 nM) has been shown to result in sustained activation of PKC (41). We found that although TPA contributed to melanocyte proliferation and basal tyrosinase activity, it did not induce melanogenesis following UVB treatment in the absence of α-MSH (Fig. 3*B*). However, the melanogenic response of UVB-irradiated melanocytes to α-MSH was maintained, even in the absence of TPA. These results underscore the importance of the cAMP pathway in mediating the pigmentary response to UVB and suggest that the PKC pathway activated by TPA is not pivotal for UVB-induced melanogenesis in human melanocytes.

Stimulation of cAMP Formation by UVB Radiation and/or α-MSH. Treatment with UVB radiation resulted in a delayed increase (observed after more than 4 h postirradiation) in cAMP level (Table 1). This increase, however, did not lead to stimulation of melanogenesis (Figs. 1–3). We found that the addition of α-MSH to nonirradiated or irradiated melanocyte cultures for 45 min had a very profound stimulatory effect on cAMP formation, indicating that adenylate cyclase activity was not perturbed by UVB treatment. However, 24 h after irradiation, the cAMP response to concomitant treatment with UVB and α-MSH was lower than the response to α-MSH alone. This decrease in cAMP level cannot be attributed to enhanced cAMP degradation, because these assays were performed in the presence of the phosphodiesterase inhibitor isobutyl methylxanthine.

Effect of α-MSH on the UVB-induced Growth Arrest. Irradiation with UVB radiation also resulted in a decrease in the surviving fraction and inhibition of proliferation of human melanocytes (Fig. 4). Continued treatment of UVB-irradiated melanocytes with 0.1 μM α-MSH (Fig. 4*A*) or 1 μM db cAMP (data not shown) resulted in increased cell number after 5–6 days of treatment. Similar stimulation of proliferation by α-MSH was observed when melanocytes were irradiated with sublethal fractionated doses of UVB and treated with α-MSH (Fig. 4, *B* and *C*). Comparison of the growth rates of UVB- and UVB plus α-MSH-treated melanocytes clearly showed that the latter had a higher proliferative rate. In these experiments, we found that stimulation of the cAMP pathway was mitogenic to human melanocytes but did not rescue these cells from UVB-induced lethality. In the absence, as well as in the presence, of α-MSH, there was no

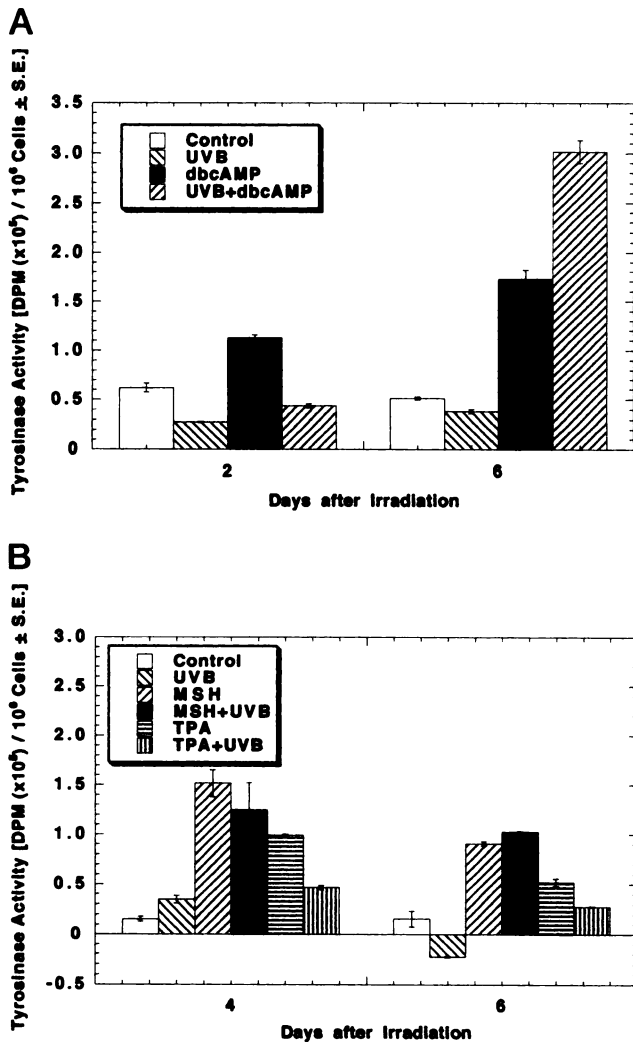

Fig. 3. Effect of db cAMP or TPA on tyrosinase activity of UVB-irradiated human melanocytes. In *A*, melanocytes were irradiated once with 28 mJ/cm^2 UVB and/or treated with 1.0 μM db cAMP for a total of 6 days, as described in "Materials and Methods." Tyrosinase activity ($n = 6$) was determined on days 2 and 6 after UVB treatment. *Bars*, SE. In *B*, the control, UVB (single irradiation with 28 mJ/cm^2 UVB), α-MSH (0.1 μM), and α-MSH + UVB groups were maintained in the absence of TPA, whereas the TPA (8 nM) and TPA + UVB (single irradiation with 28 mJ/cm^2 UVB) groups were maintained in the absence of α-MSH or any other cAMP inducer. Melanocytes were treated with α-MSH or TPA every other day for a total of 6 days. Tyrosinase activity ($n = 6$) was determined on days 4 and 6 after irradiation. *Bars*, SE.

difference in the number of detached cells, which were verified to be dead by trypan blue staining and their inability to attach and proliferate after replating.

Results of cell cycle analysis showed that irradiation of melanocytes with 28 mJ/cm^2 resulted in accumulation of cells in the G_1 phase of the cell cycle, an effect that was evident 24 h after irradiation (Table 2). Treatment of irradiated melanocytes with 0.1 μM α-MSH resulted in enhanced entry of melanocytes into S phase. In this group, comparison of day 3 to day 1 postirradiation revealed that there was a 10% decrease in the population of cells in G_1, concomitant with an 8% increase in the population of cells in S phase (Table 2). In the absence of α-MSH, only 4–5% of the cells progressed from G_1 to S phase. On day 5 after irradiation, the UVB-treated group continued to have a higher percentage of cells in G_1 and a lower percentage in G_2, compared with the UVB plus α-MSH-treated group.

Effects of α-MSH on the UVB-induced Expression of p53 and p21, Inhibition of Bcl2, and Lack of Phosphorylation of pRb. Previously, we reported that UVB-treated normal human melanocytes expressed high levels of p53 4 h after UVB treatment, an effect that persisted for at least 48 h after irradiation (3, 33). By Western blot analysis, we observed that high levels of p53 as well as the cyclin-cdk inhibitor p21 were overexpressed up to 5 days after irradiation with 28 mJ/cm^2 UVB in the presence or absence of α-MSH (Fig. 5). In melanocytes irradiated with 7, 14, or 28 mJ/cm^2 UVB, the extent and duration of p53 overexpression were dose dependent (data not shown). Melanocytes exposed every other day to 15 mJ/cm^2 UVB for a total of three irradiations expressed a high level of p53 in the absence of presence of α-MSH (data not shown). The level of Bcl2 was significantly reduced by treatment with 28 mJ/cm^2 UVB, an effect that was evident on day 3 and persisted for at least 5 days after irradiation (Fig. 5). Treatment of UVB-irradiated melanocytes with α-MSH had no effect on the UVB-induced inhibition of Bcl2 expression. Multiple exposures to 15 mJ/cm^2 UVB every other day for a total of three irradiations resulted in a minimal reduction of Bcl_2 level (data not shown).

It is known that phosphorylation of pRb is required for the progression of cells from G_1 into S phase of the cell cycle (30, 42, 43). Previously, we reported that pRb was expressed in the hypophosphorylated state in UVB-treated melanocytes that were blocked in G_1 (33). Here, we found that on days 3 and 5 after irradiation, the hypophosphorylated form of pRb continued to be expressed in UVB-irradiated melanocytes, regardless of whether they were treated or untreated with α-MSH (Fig. 6). Initially, control melanocytes expressed pRb in its hypophosphorylated state, consistent with the semiquiescent state of these cells due to their deprivation of cAMP inducers. pRb became hyperphosphorylated in control melanocytes on days 3 and 5 in response to the addition of fresh growth medium. Unirradiated melanocytes treated with α-MSH expressed the hyperphosphorylated form of pRb, an effect that is consistent with the ability of this hormone to induce the transit of melanocytes into S phase and stimulate proliferation.

The phosphorylation of pRb is brought about by cyclin-cdk complexes, which include cyclin D_1-cdk4 complex (30, 44). Twenty-four h after irradiation, there were no striking differences in the expression of cyclin D1 among the control, irradiated, and/or α-MSH-treated groups (Fig. 6). However, on days 3 and 5 after UVB exposure, both the UVB and UVB plus α-MSH-treated groups expressed an increase in the level of the slowest mobility form and a decrease in the intermediate mobility form of cyclin D1 (Fig. 6). In the control and α-MSH-treated groups, the most prominent form of cyclin D1 was the intermediate mobility form. As for cdk4, its expression was slightly reduced following 24 h of UVB treatment, in the absence or presence

Table 1 *Effect of UVB radiation on basal and α-MSH-induced cAMP levels*

Melanocytes were irradiated once with 28 mJ/cm^2 UVB and/or treated with 0.1 μM α-MSH for 0.75, 4, or 24 h. The increase in the control values over time reflects the accumulation and lack of degradation of cAMP due to the presence of 1-methyl-3-isobutylxanthine. This experiment was repeated three times using different melanocyte lines, with similar results.

	pmol cAMP/ml ± SE	% of control ± SE
45 min		
Control	0.84 ± 0.020	100 ± 2
UVB	0.85 ± 0.034	102 ± 4
α-MSH	15.65 ± 0.811	1874 ± 97
UVB ± α-MSH	14.28 ± 0.47	1710 ± 56
4 h		
Control	1.24 ± 0.075	100 ± 6
UVB	1.51 ± 0.039	122 ± 3
α-MSH	32.17 ± 1.94	2594 ± 157
UVB + α-MSH	31.41 ± 1.99	2533 ± 160
24 h		
Control	5.81 ± 0.16	100 ± 3
UVB	10.63 ± 0.56	183 ± 10
α-MSH	121.99 ± 10.72	2100 ± 85
UVB + α-MSH	74.21 ± 2.03	1277 ± 35

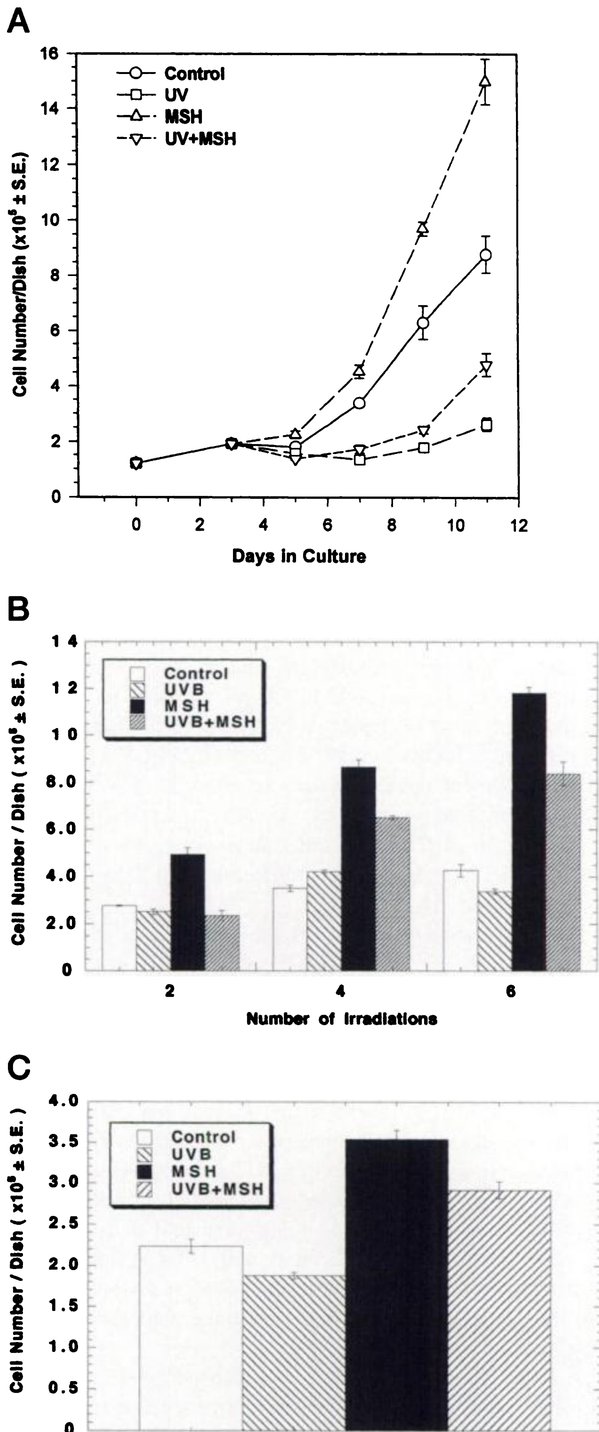

Fig. 4. Effects of α-MSH on melanocyte proliferation following a single irradiation with 28 mJ/cm^2 UVB (*A*) or multiple irradiations with 5 mJ/cm^2 (*B*) or 15 mJ/cm^2 UVB (*C*). The results in *A* were obtained from the experiment presented in Fig. 1*A*. Cells were irradiated on day 3 after plating, and cell numbers were determined on days 2, 4, 6, and 8 after irradiation. The cell numbers in *B* and *C* were obtained from the experiments presented in Fig. 1, *B* and *C*, respectively. Each value is the mean of triplicate determinations; *bars*, SE.

of α-MSH, but 24 h after treating unirradiated melanocytes with α-MSH, cdK4 expression increased (Fig. 6).

DISCUSSION

Sun exposure is the major etiological factor for nonmelanoma skin cancers (45–47). The frequency of skin cancer exceeds the sum of all other forms of cancer (48). The spectrum of UV radiation that has the most genotoxic and carcinogenic effects is in the UVB range (290–320-nm wavelength; Refs. 47 and 49). This spectrum is also the most effective in stimulating melanogenesis (50). The pigmentary response to UV radiation involves increased synthesis of melanin by epidermal melanocytes and transfer of melanin granules to surrounding keratinocytes. Melanin, particularly eumelanin, is thought to confer photoprotection to the skin by reducing the penetration of UVA and UVB rays through the epidermal layers and by quenching reactive oxygen radicals generated upon UV exposure (51, 52). Taking into account the importance of the melanocyte in the cutaneous responses to sun exposure, we have been investigating the signaling pathway(s) that mediate the effects of UVB radiation on normal human melanocytes.

It is known that UVB radiation induces the formation of DNA photoproducts, particularly in the form of cyclobutane pyrimidine dimers (4). Irradiation with UV results in cell death and in arrest of surviving cells in G_1. The latter effect is thought to be important for repair of DNA damage, and both effects are induced by increased levels of p53 (27, 32, 53). We found previously that in human

Table 2 *Cell cycle profiles of human melanocytes after a single irradiation with 28 mJ/cm^2 UVB and/or treatment with 0.1 μM α-MSH*

Melanocytes were irradiated once with 28 mJ/cm^2 UVB, left untreated, or immediately treated with 0.1 μM α-MSH, as described in "Materials and Methods." Flow cytometric analysis of the cell cycle was carried out 24 h, 72 h, or 5 days after irradiation.

	G_1	S	G_2–M
22 h			
Control	69.6	13.6	16.9
α-MSH	52.7	39.3	7.9
UV	83.3	3.9	12.8
UV + MSH	83.7	3.3	13.0
72 h			
Control	83.6	9.0	7.4
α-MSH	66.1	20.4	13.4
UV	78.0	8.2	13.9
UV + MSH	73.7	12.3	14.1
5 days			
Control	83.9	10.8	5.3
α-MSH	71.8	17.5	10.6
UV	64.8	14.5	20.7
UV + MSH	58.1	14.7	27.3

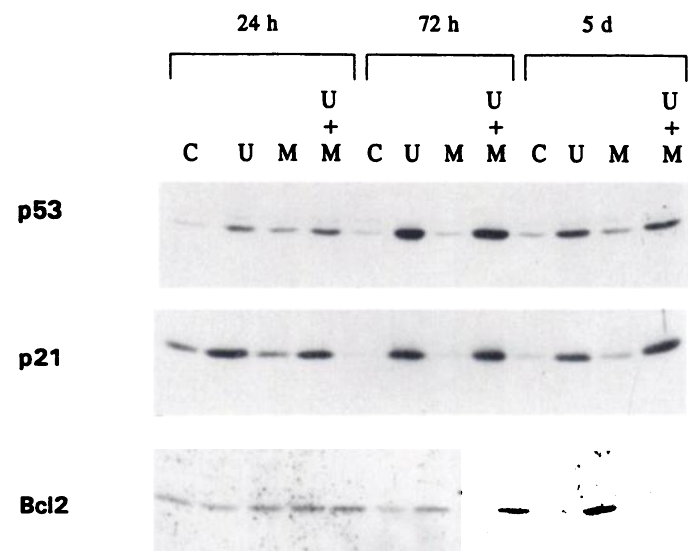

Fig. 5. Expression of p53, p21, and Bcl2 after UVB treatment in the presence or absence of α-MSH. Melanocytes were irradiated once with 28 mJ/cm^2 UVB and/or treated with 0.1 μM α-MSH. The expression of p53, p21, and Bcl2 was determined by Western blot analysis at 1, 3, or 5 days after irradiation and/or α-MSH treatment, as described in detail in "Materials and Methods." *C*, control; *U*, UVB; *M*, α-MSH; *U + M*, UVB + α-MSH treatment.

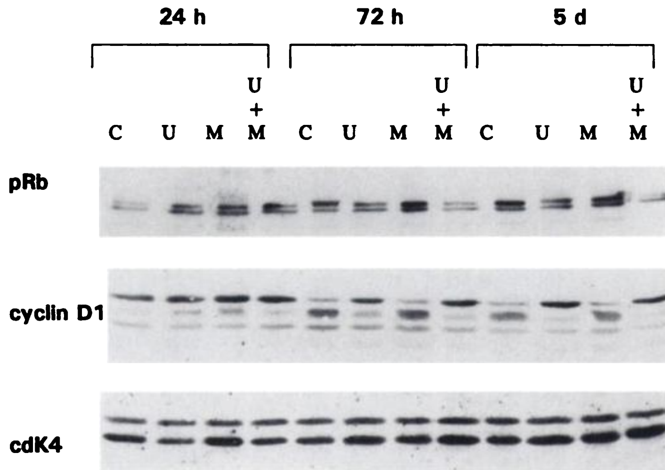

Fig. 6. Expression of pRb, cyclin D_1, and cdk4 after UVB treatment in the presence or absence of α-MSH. Melanocytes were irradiated once with 28 mJ/cm^2 UVB and/or treated with 0.1 μM α-MSH. Western blot analysis for pRb, cyclin D_1, and cdk4 was carried out as described in detail in "Materials and Methods." *C*, control; *U*, UVB; *M*, α-MSH; *U + M*, UVB + α-MSH treatment.

melanocytes, UVB radiation results in the formation of cyclobutane dimers and in G_1 arrest due to expression of high levels of p53 (3). It is known that some of the effects of UV radiation are mediated by stimulation of synthesis of biochemical mediators, such as basic fibroblast growth factor and endothelin-1, which act as paracrine regulators for melanocytes (5, 11, 34). UV radiation also increases the synthesis and release of α-MSH by human melanocytes and keratinocytes (9, 10). We have found that α-MSH, increased melanogenesis in melanocytes, and that in the absence of α-MSH or db cAMP, UVB-irradiated melanocytes failed to mount a melanogenic response after a single irradiation or multiple irradiations with a sublethal dose (5 or 15 mJ/cm^2) of UVB (Figs. 1 and 3). The activity, as well as the level, of tyrosinase were both decreased following UVB treatment in the absence of cAMP induction (Figs. 1–3). Activation of PKC following TPA treatment failed to stimulate melanogenesis in UVB-treated melanocytes (Fig. 3*B*), indicating that the melanogenic response of human melanocytes absolutely requires the activation of the cAMP pathway.

To determine whether or not UVB radiation perturbs adenylate cyclase and delays or inhibits the response of melanocytes to α-MSH, we compared cAMP levels in α-MSH-, UVB-, and UVB plus α-MSH-treated melanocytes (Table 1). We found that unirradiated as well as UVB-treated melanocytes responded to α-MSH with a similar initial increase in cAMP level. After 24 h of treatment, however, cAMP level in the UVB plus α-MSH-treated group was still high compared with control or UVB-treated groups but was significantly lower than the level detected in the unirradiated, α-MSH-treated group. Irradiation with UVB induced a delayed increase in cAMP, to about 80% above control level, at 24 h after irradiation. This increase, however, did not result in stimulation of melanogenesis. The delayed effect of UVB on cAMP level and the difference in cAMP levels between the α-MSH- and UVB plus α-MSH-treated groups might be due in part to the synthesis of autocrine factors, some of which, like the melanotropins, are stimulatory, whereas others are inhibitory to cAMP formation. The net effect of these putative factors is elevation of cAMP level and stimulation of melanin synthesis. *In vivo*, these factors might mediate the delayed tanning response to sun exposure. Previously, we showed that a sustained high level of cAMP induced by α-MSH treatment was important for induction of downstream events leading to increased replication and melanin formation (23). The difference observed between the levels of cAMP in the unirradiated α-MSH-treated and the UVB plus α-MSH-treated groups might partially explain why the former responded more vigorously to α-MSH with increased proliferation and melanin synthesis.

We showed previously that human melanocytes respond to UVB treatment with a dose-dependent inhibition of proliferation due to arrest in G_1 and an increase in cell death (3). Here, we found that treatment of UVB-irradiated melanocytes with α-MSH enabled these cells to recover partially from growth arrest and induced their transition into S phase (Table 2 and Fig. 4*A*). The proliferative effect of α-MSH could be mimicked by db cAMP or forskolin (data not shown), suggesting the involvement of the cAMP pathway in this mitogenic response.

The mitogenic effect of α-melanotropin is accompanied either by enhancement of DNA repair or fixation of DNA damage. These two possibilities have opposite implications on UV-induced carcinogenesis. It is known that DNA damage induces an increase in p53 level by increasing the stability of the protein (54). The accumulation of p53 seems to be a universal response to genotoxic stress (55). High levels of p53 up-regulate the transcription of the cyclin-cdk inhibitor p21 and inhibit *Bcl2* gene expression (29, 32, 56, 57). Increased expression of p21 results in G_1 arrest and inhibition of DNA synthesis by inhibiting the activity of cyclin D and cyclin E-dependent kinases and by associating with proliferating cell nuclear antigen (56, 58). By Western blot analysis, we found that UVB radiation induced a prolonged overexpression of the levels of p53 and p21 (Fig. 5), both of which were highly induced up to 72 h, then slightly decreased 5 days after UVB treatment in the presence or absence of α-MSH. These results were puzzling, particularly because α-MSH treatment enhanced the transit of UVB-irradiated melanocytes into S phase and resulted in an increase in cell number following 5 days of hormonal treatment (Table 2; Fig. 1*A*).

We observed that 28 mJ/cm^2 UVB, the dose we used in most of our experiments, resulted in cell death, beginning 48 h after irradiation. Treatment of irradiated melanocytes with α-MSH did not protect them from the lethal effect of UVB radiation. Western blot analysis of the viable UVB-treated cells revealed a decreased expression of Bcl2, an inhibitor of apoptosis, an effect that was not blocked by treatment with α-MSH (Fig. 5) (31, 59). These results suggest that UVB irradiation causes melanocytes to die by apoptosis, via a mechanism that involves p53 overexpression and reduction in Bcl2 level. Others have shown that treatment of rat ovarian follicles with vasoactive intestinal peptide or forskolin prevented apoptosis, suggesting that activation of the cAMP pathway suppresses cell death (60). Our results, however, imply that the cAMP pathway does not suppress apoptosis in melanocytes and that α-MSH acts as a mitogen rather than a survival factor for these cells.

It is known that pRb exists in a hyperphosphorylated state in late G_1, S, and G_2-M phases and becomes hypophosphorylated by a phosphatase in early G_1 (30, 42, 43). Treatment of unirradiated melanocytes with α-MSH induced hyperphosphorylation of pRb, an effect consistent with our findings that this hormone induces the progression of cells into the cell cycle (Fig. 6). Both UVB- and UVB plus α-MSH-treated groups expressed the hypophosphorylated form of pRb on days 3 and 5 after treatment. Lack of phosphorylation of pRb in UVB treated melanocytes might be due to inactivation of cyclin D_1-cdK4 complexes by p21. Upon comparing the expression of cyclin D_1 and CdK4 in control, α-MSH-, UVB-, and UVB plus α-MSH-treated groups, the most striking difference in cyclin D_1 expression was between UVB-irradiated melanocytes either treated or untreated with α-MSH on one hand and the unirradiated control or α-MSH-treated groups on the other. The implications of this difference on the activity of cyclin D_1-cdk4 complex remains to be determined.

In summary, the results of immunoblotting did not reveal any significant effects of α-MSH on the UVB-induced expression of p53, p21, Bcl2, pRb, or cyclin D_1. however, α-MSH enhanced the proliferation and induced the entry of UVB-irradiated melanocytes into S phase. This implies that α-MSH might drive only a minor subpopulation of melanocytes to undergo replication, and high levels of p53 might signal melanocytes with extensive DNA damage to die. Alternatively, α-MSH enhances the transition into S phase by a yet unknown mechanism that is p53 and pRb independent. We speculate that α-MSH provides photoprotection by stimulating the synthesis of eumelanin and enhances DNA repair of UVB-induced photoproducts, thus allowing UVB-irradiated melanocytes to resume proliferation. This possibility is being investigated because it has important implications on the role of α-MSH in UVB-induced carcinogenesis.

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