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α-Melanocyte Stimulating Hormone Potentiates p16/*CDKN2A* Expression in Human Skin after Ultraviolet Irradiation[1]

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ABSTRACT

The contribution of the UV component of sunlight to the development of skin cancer is widely acknowledged, although the molecular mechanisms that are disrupted by UV radiation (UVR) resulting in the loss of normal growth controls of the epidermal stem cell keratinocytes and melanocytes is still poorly understood. α-Melanocyte stimulating hormone (α-MSH), acting via its receptor MC1, has a key role in skin pigmentation and the melanizing response after exposure to UVR. The cell cycle inhibitor p16/*CDKN2A* also appears to have an important function in a cell cycle checkpoint response in skin after exposure to UVR. Both of these genes have been identified as risk factors in skin cancer, *MC1R* variants are associated with increased risk to both melanoma and nonmelanoma skin cancers, and p16/*CDKN2A* with increased risk of melanoma. Here we demonstrate that the increased expression of p16 after exposure to suberythemal doses of UVR is potentiated by α-MSH, a ligand for MC1R, and this effect is mimicked by cAMP, the intracellular mediator of α-MSH signaling via the MC1 receptor. This link between p16 and MC1R may provide a molecular basis for the increased skin cancer risk associated with *MC1R* polymorphisms.

INTRODUCTION

The role of UVR[4] as one etiological factor in the development of skin cancer, including melanoma, is well established (1), with UVR acting as both an initiator and promoter in the course of multistep carcinogenesis (2). UVR has a range of effects on epidermally derived cells *in vitro* and *in vivo*. It increases pigment production and the dendricity of melanocytes (3), and may also act as an independent mitogen for these cells (4). Studies have also demonstrated that UVR induces the production of keratinocyte-derived factors that regulate melanocyte growth and melanin synthesis, including α-MSH and related POMC-derived peptides and endothelin (5–7). These peptide factors synergize with UVR to increase the pigmentation response (8).

α-MSH increases melanin synthesis by up-regulating tyrosinase activity and the expression of TRP 1 to increase eumelanin synthesis (6). It also promotes an increase in the dendricity of melanocytes, which may facilitate their connection with surrounding basal layer keratinocytes and aid in the deposition of melanin (9). α-MSH also has a proliferative effect on melanocytes (10, 11). The biological action of α-MSH on melanocytes is mediated through its binding to the MC1R, the only melanocortin receptor expressed in melanocytes. MC1R is a G protein-coupled receptor that activates adenylate cyclase, and the increased intracellular concentration of cAMP mediates the melanogenic and morphological responses to α-MSH (reviewed in Refs. 8, 9). The *MC1R* gene has a number of naturally occurring polymorphisms that are related to skin color, although the functional significance of the these polymorphisms in terms of α-MSH binding and cAMP increase is still unclear. It is clear that some of the variants are associated with red hair and a poor tanning response, and an increased risk of melanoma (12); and this increased risk may not be solely a consequence of the red-hair phenotype (13). These variants also increase the risk of nonmelanoma skin cancers (14, 15).

p16/*CDKN2A* is a melanoma susceptibility gene, and up to 30% inherited melanoma families have mutant p16/*CDKN2A* alleles (16). The gene is also functionally inactivated in a high proportion of melanoma cell lines and in 10–30% of tumors (16). Mutations of p16/*CDKN2A* have also been identified in nonmelanoma skin cancers (17). We have previously demonstrated that the expression of the melanoma susceptibility gene product p16 is increased after UVR both in epidermally derived cell lines and in human skin (18, 19). The increased p16 expression in irradiated skin was restricted to basal and suprabasal layer melanocytes and keratinocytes, which suggested that it may be involved in a protective cell cycle response to UVR (19). In this study, we have examined the cooperativity of these two protective responses to UVR. We have used short-term whole-skin organ culture and demonstrated that α-MSH increases the proportion of basal layer keratinocytes and melanocytes that respond to UVR with increased p16 expression, and that this is likely to be via signaling through the MC1 receptor.

MATERIALS AND METHODS

Organ Cultures. Human neonatal foreskins were obtained immediately after circumcision. These were predominantly of Caucasian origin, with little melanin content or coloring. More heavily pigmented skin samples were not included in the analyses because of their low numbers. Organ cultures were established and maintained as described previously (19). Briefly, neonatal foreskins were cut into two; one piece was irradiated with 250 Jm^{-2} UVB and the other used as a control, maintained in specialized media supplemented with antibiotics, and incubated at 37°C in a humidified incubator with the epidermis exposed as described previously (19). For UVB irradiation, a single FS20T12 UVB lamp (Light Sources), with maximal output at 313 nm was used. Controls consisted of samples incubated in base medium alone, or supplemented with 150 nM α-MSH (Sigma-Aldrich) or 100 nM NDP-MSH (Sigma-Aldrich). Samples irradiated with 250 Jm^{-2} UVB were incubated in base medium alone, or supplemented with 150 nM α-MSH, 100 nM NDP-MSH, or 6 μM all-*trans*-retinoic acid (Sigma-Aldrich) immediately after irradiation, for periods of 24 h to 4 days as indicated.

BrdUrd Labeling. Tissue samples were incubated in base medium supplemented with 25 mM BrdUrd (ICN Biomedical Research Products) and 5% DMSO with the epidermis submerged, enabling maximal absorption and incorporation. BrdUrd labeling was undertaken between 0 and 24-h incubation and harvested at 24 h for controls and UVB-irradiated samples, with or without α-MSH, NDP-MSH, retinoic acid, or base medium alone.

Immunohistochemistry. Immunohistochemistry was performed as described previously (19), using a Zymed Histostain-Plus kit (Zymed) with the substrates AEC (3-amino-9-ethyl carbazole; Zymed) or Vector NovaRed (Vector Laboratories) to produce a red end product, or 3,3′-diaminobenzidine tetrahydrochloride (DAB) to produce a brown end product. Antibodies used were as follows: p16, 1:300 (20); PCNA, 1:200 (Novocastra); BrdUrd, 1:400 (Ab-2 NeoMarkers, LabVision), and TRP-1 (kindly provided by Dr. Peter Parsons). Antigen retrieval was performed for p16 and TRP-1 as described

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[4] The abbreviations used are: UVR, UV radiation; α-MSH, α-melanocyte stimulating hormone; POMC, proopiomelanocortin; MC1R, melanocortin-1 receptor; NDP-MSH, Nle^4DPhe^7 α-MSH; BrdUrd, bromodeoxyuridine; TRP-1, tyrosinase-related protein 1; PCNA, proliferating cell nuclear antigen.

previously (19). For BrdUrd staining, sections were incubated in 4 M HCl for 30 min at room temperature, followed by digestion with trypsin at 1 mg/ml in PBS for 10 min at 37°C. The sections were then blocked and stained as for p16.

Immunoblotting. Tissue samples were removed from the media 24 h after irradiation, snap-frozen and stored at −80°C until analysis. Tissue was cut into small pieces and placed in 1.2 ml of extraction buffer containing 300 mM NETN/NaCl [300 mM NaCl plus 1 mM EDTA, 0.5% NP40, and 20 mM Tris (pH 8)] supplemented with 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 10 mM NaF, 0.1 mM sodium orthovanadate, and 0.5 mM phenylmethylsulfonyl fluoride. Tissue was homogenized on ice using a Polytron homogenizer with a 7-mm aggregate (Kinematic AG, Switzerland), using 3–4 pulses at 5000 rpm for a duration of 5 s/pulse. Extracts were then ultracentrifuged in a TL-100 ultracentrifuge (Beckman), at 135,000 × *g* for 1 h at 4°C. Samples were boiled in SDS sample buffer and resolved on a 12% SDS-polyacrylamide gel, transferred to a polyvinylidene fluoride membrane (NEN Life Science Products), and immunoblotted for p16 at 4°C overnight. Protein-A-conjugated horseradish peroxidase (ICN Biomedical Research Products) secondary antibody was used for detection using chemiluminescence with a Western Blot Chemiluminescence Reagent Plus kit (NEN Life Science Products). Samples were equilibrated on the basis of the level of PCNA expression by SDS-PAGE and immunoblotting with a PCNA antibody (Novocastra).

Evaluation of Expression. The total number of foreskin samples tested consisted of 148 treated with UVB only, 17 with α-MSH only, 67 with UVB + α-MSH, 7 with NDP-MSH only, 28 with UVB + NDP-MSH, 17 with dibutyryl cAMP only, and 12 with UVB and dibutyryl cAMP. Sets of matched, untreated controls were also used. Five samples with each treatment were used in BrdUrd labeling experiments. A cell was considered to be immunolabeled and positive for the antibodies used if there was a visibly detectable signal within the nucleus for BrdUrd, or within both nucleus and cytoplasm of a cell for p16 and TRP-1. Five random fields (at ×40) of the epidermis on each section were selected and the number of cells staining positive were counted. An average field contained 170 cells within the epidermal layer. The statistical significance of changes in p16 expression was calculated using a Student's *t* test.

RESULTS

The biological efficacy of α-MSH, its potent long-acting analogue NDP-MSH (21), or dibutyryl cAMP, which can mimic the effects of α-MSH on the pigmentation pathway (6), were tested in the whole skin experimental system by examining their effects on the pigmentation pathway. Skin samples were either untreated or treated with α-MSH, NDP-MSH, or dibutyryl-cAMP for 4 days, then assessed for changes to one of the components of the pigmentation pathway, TRP-1, which has been shown to increase in expression in response to these stimuli (6). Immunohistochemical staining for TRP-1 showed a strong increase in both the TRP-1 levels and the dendricity of the melanocytes with each treatment (Fig. 1).

The effects of α-MSH treatment on p16 expression was examined after 24 h when the UVB induction of p16 was maximal (19). The level of p16 expressed in untreated skin is low to undetectable, and no increase in p16 expression was observed after treatment with α-MSH, NDP-MSH, or dibutyryl cAMP (Fig. 2), even after a 4-day treatment (data not shown). We have previously reported that suberythemal doses of UVB radiation induce a strong increase in p16 expression in discrete patches in the basal and suprabasal layer melanocytes and keratinocytes (19). When skin was treated with UVB and each of the agents, a strong increase in p16 expression was detected in the basal and suprabasal layer cells with both α-MSH and dibutyryl cAMP treatment, and this increase was seen more uniformly throughout the basal layer, in contrast to the discrete patches of staining seen in the absence of exogenous factors (Fig. 2). The increase was detected in both basal layer melanocytes, and the more abundant keratinocytes. Surprisingly, not only did the α-MSH agonist NDP-MSH not increase the p16 expression but it appeared to block the increase in p16 observed with UVB alone (Fig. 2), although this dose of NDP-MSH was equipotent to α-MSH at increasing TRP-1 expression and dendricity (Fig. 1).

Quantitation of the number of p16 staining cells in the epidermis of control, UVB-irradiated skin without or with the addition of α-MSH, dibutyryl cAMP, or NDP-MSH, revealed a 10-fold increase with UVB treatment alone compared with control-unirradiated skin, and this was increased an additional 3-fold with addition of α-MSH after UVB treatment (Fig. 3*A*). The effect with α-MSH was very robust, but the response with dibutyryl cAMP was more varied, with a relatively weak increase in the number and intensity of p16 staining cells in some skin samples, whereas others showed the same increase in staining as α-MSH; and this variability is reflected in the large SE. Addition of NDP-MSH after UVB treatment decreased p16 staining to unirradiated levels. The increase in p16 staining with either UV alone

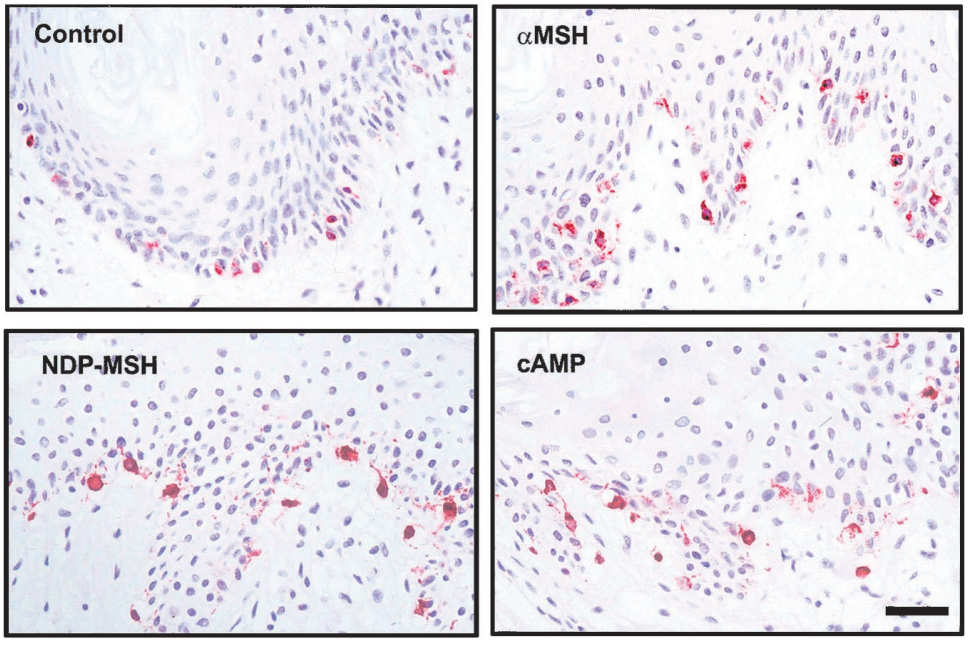

Fig. 1. α-MSH and analogues increase TRP-1 levels and dendricity in melanocytes. Skin was either untreated (*Control*) or treated with α-MSH, NDP-MSH, or dibutyryl cAMP (*cAMP*) for 4 days; then the samples were fixed and sections stained for TRP-1 (*red*) with a hematoxylin counterstain. Increases in the levels of TRP-1 staining in the basal layer melanocytes and an increase the dendritic projection from the melanocytes are clearly detected in the treated sections. The stains shown are typical of nine foreskin samples tested with each treatment. *Bar*, 50 μm.

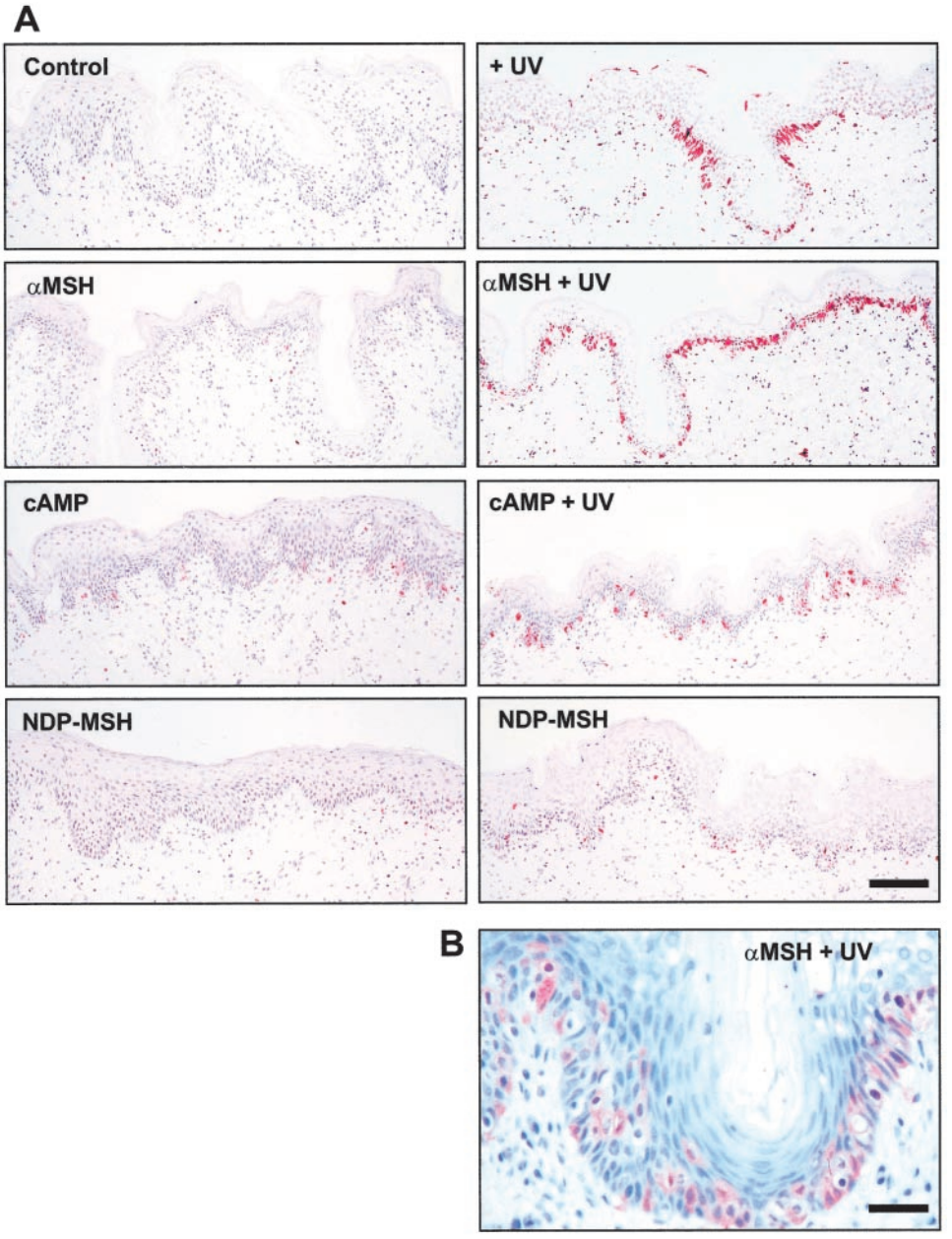

Fig. 2. αMSH potentiates UV-induced p16 expression. *A*, control skin or samples treated as in Fig. 1 were left unirradiated or irradiated with 250 Jm^{-2} UVB, then fixed after 24 h, and stained for p16 expression (*red*). *Bar*, 100 μm. *B*, higher magnification image of skin irradiated with UV and treated with α-MSH clearly shows the basal layer melanocytes and basal and suprabasal layer keratinocytes staining for p16 (*red*). *Bar*, 45 μm.

or in combination with α-MSH or dibutyryl cAMP was significant with a P of < 0.0001. There was no significant difference in p16 staining between control and UV + NDP-MSH treated samples ($P > 0.1$). The increase in p16 staining of combined UV and either α-MSH or dibutyryl cAMP treatment over UV alone was also significant ($P < 0.0001$ for UV + α-MSH and < 0.05 for UV + dibutyryl cAMP). The changes in p16 expression were verified by immunoblotting of tissue extracts from similarly treated skin samples. The increase in p16 expression with UVB combined with α-MSH or dibutyryl cAMP treatment is very clearly seen, whereas treatment with α-MSH or dibutyryl cAMP alone, or NDP-MSH with or without UVB did not produce this effect (Fig. 3*B*). The level of p16 in the α-MSH- and dibutyryl-cAMP-alone-treated samples showed a small increase over the untreated control, which suggested there was a small, global increased p16 expression that was below the detection sensitivity of the immunohistochemical staining. NDP-MSH alone also induced the low-level p16 expression seen with the two other agents, but no increase in p16 expression was observed in combination with UVB.

Both α-MSH and dibutyryl cAMP have been reported to be mitogens for melanocytes in culture (10), which suggests that the effect of these agents on UV-induced p16 expression was simply through increasing the number of proliferating melanocytes. No increase in p16 staining of melanocytes in either α-MSH- or dibutyryl-cAMP-only treated skin was observed (Fig. 2), but the low number of melanocytes in the basal layer (one melanocyte every 10–15 keratinocytes) may mask any effect. The strong increase in p16 staining of the majority basal and suprabasal layer keratinocytes demonstrated that these cells were also effected by UVR. To examine whether increasing the number of proliferating cells influenced p16 expression in response to UVR, skin samples were treated with all-*trans*-retinoic acid, which promotes proliferation in the basal layer keratinocytes (22), labeled with BrdUrd, and then stained for BrdUrd incorporation as a measure of proliferative activity. Retinoic acid produced a >3-

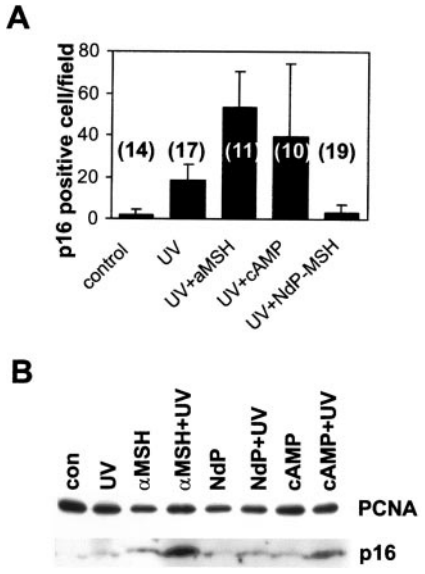

Fig. 3. α-MSH potentiates UV-induced p16 expression. *A*, the number of cells staining for p16 in the epidermis of either unirradiated controls, or irradiated with 250 Jm^{-2} UVB, without or with the addition of α-MSH, dibutyryl cAMP, or NDP-MSH. The mean and SE is shown. The number of sections counted for each treatment is shown in brackets. *B*, extracts from skin samples treated as those in Fig. 2 and harvested at 24 h after irradiation, were immunoblotted for the levels of p16 expression. PCNA was also immunoblotted as a loading control.

fold increase in the number of BrdUrd-positive nuclei (Fig. 4). Despite the increased proliferative capacity, no increase in p16 expression was observed (compared Fig. 4, *p16 + RA*, with Fig. 2, *Control).* When skin was treated with UVB and retinoic acid, not only was there was no increase in p16 expression as seen with α-MSH and dibutyryl cAMP, but retinoic acid treatment appeared to suppress the normal UVB-induced increase (Fig. 4).

The apparent suppressive effect of NDP-MSH on the UVB-induced p16 expression suggested that NDP-MSH was competing with endogenous α-MSH for MC1R binding. To test whether NDP-MSH could block α-MSH action, skin samples were irradiated with UVB and treated with α-MSH and increasing concentrations of NDP-MSH for 24 h, and the level of p16 expression was assessed by immunohistochemical staining. At high doses of NDP-MSH, the increased p16 expression caused by α-MSH was strongly suppressed, with decreases in both the number of p16-positive cells and the intensity of the staining detected (Fig. 5*A*), and lower doses of NDP-MSH also produced a quantitative reduction in the number of p16 expressing cells (Fig. 5*B*). The effect of NDP-MSH was reversed by the addition of dibutyryl cAMP, which increased the level of p16 expression to that observed with UVB and dibutyryl cAMP treatment only (Fig. 5*a*, NDP-MSH + cAMP + UVB).

DISCUSSION

The data presented in this report demonstrate that α-MSH potentiates UVB-induced p16 expression in basal and suprabasal layer cells in human skin. The mechanism appears to involve signaling via MC1R, mediated by elevated levels of intracellular cAMP, in combination with signaling directly responsive to UVR. The fact that neither α-MSH, nor NDP-MSH, nor dibutyryl cAMP alone increased p16 levels significantly demonstrates that these known mitogens were not acting by simply increasing the number of actively proliferating melanocytes, but that signaling in response to UVR was also necessary for the increased p16 expression. The potentiating effect of α-MSH appears to be specific because retinoic acid, a potent mitogen for keratinocytes, did not synergize with UVR to increase p16 levels. Indeed, retinoic acid reduced UV-induced p16 expression. This may be caused by the reported down-regulation of melanocyte MC1R expression by retinoic acid (23).

Does α-MSH have any role in the UV-induced p16 expression in the absence of exogenously added peptide? The observation that the α-MSH agonist NDP-MSH directly antagonized the effect of exogenously added α-MSH on UV-induced p16 expression suggests that NDP-MSH was competing with α-MSH for MC1R occupancy, because MC1R is the only melanocortin receptor expressed on melanocytes (24). NDP-MSH also inhibited UV-induced p16 expression in the absence of added α-MSH, and this is likely to be caused by NDP-MSH blocking endogenously expressed α-MSH binding to the MC1 receptors. Expression of POMC-derived peptides, including α-MSH, is increased by UVR (5, 8, 9), and the increased levels of these POMC-derived peptides, which can bind and activate MC1R, may stimulate the observed p16 expression.

The molecular basis for the unexpected inhibitory effect of NDP-MSH binding to the MC1R on p16 expression, but not TRP-1 expression and increased dendricity, is unclear but may have to do with the duration of the signal from the MC1R with NDP-MSH bound. The α-MSH signal has a duration of only a matter of hours after removal, whereas the NDP-MSH signal persists for up to 6 days (21), which suggests that, although both peptides bind the same receptor, they may elicit somewhat different intracellular signals.

The effects of combined UVR and α-MSH, NDP-MSH, dibutyryl cAMP, or retinoic acid treatment on p16 expression were observed not only on the melanocytes in the basal layer but also on the keratinocytes, which represent >90% of the basal layer cell population. These effects may still be through the MC1R because keratinocytes are also reported to express functional MC1R after exposure to moderate doses of UVR in culture (25), although this finding is yet to be independently confirmed. An alternative explanation is that the effects on the keratinocytes are mediated by melanocytes. The basal layer melanocytes are connected with up to 36 keratinocytes by dendritic projections to form the epidermal unit (26). After UVR exposure, both melanocytes and keratinocytes produce a range of cytokines, growth factors, and POMC-derived peptides that influence the proliferative and melanizing responses of the basal layer melanocytes (reviewed in Ref. 9). It may be possible that specific paracrine factors produced by melanocytes influence p16 expression in their associated keratinocytes.

The POMC-derived peptides mediate the increased melanin synthesis after UVR exposure in skin, providing a protective barrier against further cellular damage by subsequent irradiation. The ability of α-MSH, and its intracellular mediator cAMP, to potentiate the increased p16 expression after UVR may be an example of these peptides activating a more immediate protective mechanism involving a cell cycle arrest. Cell cycle checkpoint arrests are a common response to cellular damage that allow the cells to repair damage before proceeding through mitotic division (27). p16 acts as a cell cycle inhibitor, directly inhibiting the activity of the cdk4/cyclin D (28), and it has been implicated in checkpoint arrest after exposure to DNA-damaging agents, including UVR (18, 29–31). The α-MSH-mediated p16 expression may be involved in a checkpoint response to UVR in the basal layer cells. The p16 response appears to be specific for UVR (32) and is restricted to the basal layer cells (19), which respond to α-MSH. Increased melanin synthesis is also a response to DNA damage in melanocytes (33), but the existence of a connection between p16 expression and increased melanin synthesis is as yet unclear. Certainly it is possible to differentiate between the effects, because addition of α-MSH or its analogue alone was sufficient to increase TRP1 expression without effecting p16 expression. The role

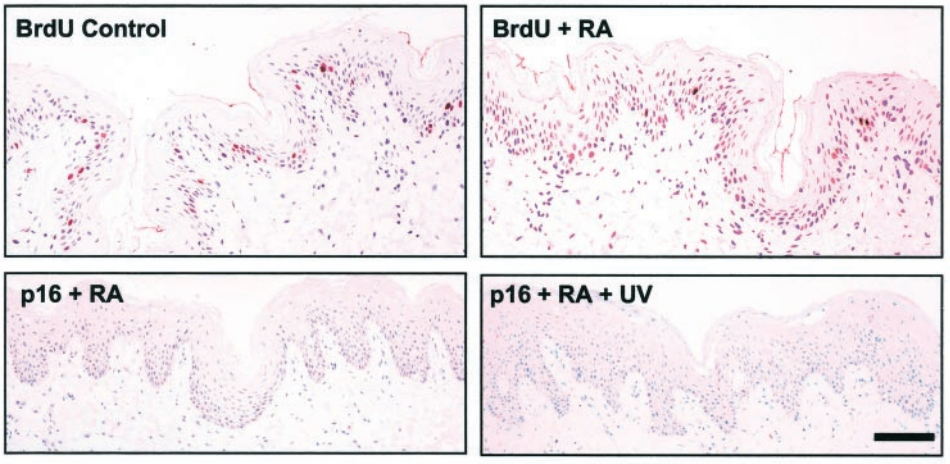

Fig. 4. Retinoic acid reduces UV-induced p16 expression. Skin samples were either untreated or treated with all-*trans*-retinoic acid (*RA*) and labeled with BrdUrd for 24 h, then fixed and stained for BrdUrd incorporation as a marker of proliferation (*red*). The level of p16 expression was assessed in retinoic acid treated skin after 24 h, either with or without exposure to UVR. The section shown was typical of triplicate samples. *Bar*, 100 μm.

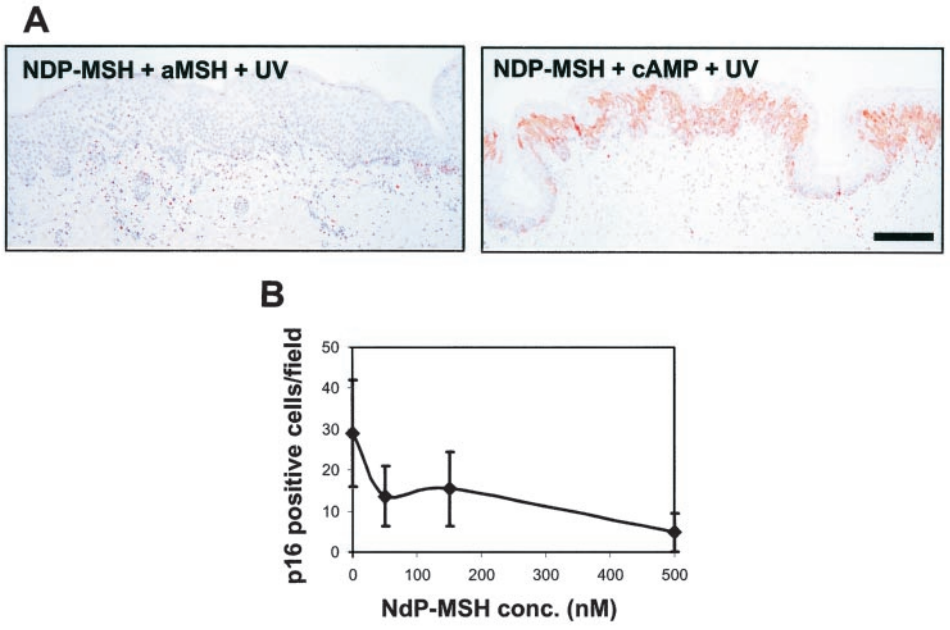

Fig. 5. NDP-MSH blocks α-MSH-induced p16 expression. Skin samples were irradiated with UVR and treated with 150 nM α-MSH and increasing concentration of NDP-MSH, then fixed after 24 h and immunostained for p16. *A*, at the highest concentration of NDP-MSH (500 nM), the expression of p16 was also completely blocked (NDP-MSH + α-MSH + UV). The effects of NDP-MSH on UV-induced p16 expression were overcome by addition of dibutyryl cAMP (NDP-MSH + cAMP + UV). Bar, 100 μm. *B*, quantitation of the inhibition of α-MSH potentiated UV-induced p16 expression with increasing concentrations of NDP-MSH. The data were from counting five random epidermal fields from triplicate samples, and expressed as mean and SE.

of p16 may be in the cell cycle arrest after UVR (31), which may allow increased melanin synthesis and transfer to the keratinocytes in the epidermal unit. Transfer of the melanin may be facilitated by the increased dendricity of the melanocytes after UVR treatment, which is a consequence of increased p16 expression and cell cycle arrest (34).

The possibility of an association between UVR, MC1R function, and p16 expression is intriguing. There is genetic evidence that *MC1R* variants are associated with an increased risk of melanoma and nonmelanoma skin cancers (13–15). In melanoma-prone families, the presence of *MC1R* variants has a modifier effect on *CDKN2A* mutations, increasing the penetrance and decreasing the mean age of onset of melanoma (35). The demonstration that α-MSH, acting through the MC1R is directly involved in the UV-induced p16 expression may provide a biological basis for increased skin cancer risk, and the genetic association between *MC1R* variants and *CDKN2A* mutation in melanoma. It may be that these *MC1R* variants have a reduced ability to increase p16 expression, thus disrupting the UV-induced checkpoint arrest with potential effects on genomic integrity and/or the tanning response.

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