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Production and release of proopiomelanocortin (POMC) derived peptides by human melanocytes and keratinocytes in culture: regulation by ultraviolet B

Ashok K. Chakraborty [a], Yoko Funasaka [b], Andrzej Slominski [c,*], Gennady Ermak [c], Jim Hwang [c], John M. Pawelek [a], Masamitsu Ichihashi [b]

[a] *Department of Dermatology, Yale University School of Medicine, New Haven, CT, USA*
[b] *Department of Dermatology, Kobe University School of Medicine, Kobe, Japan*
[c] *Department of Pathology and Laboratory Medicine, Albany Medical College, Albany, NY, USA*

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Abstract

It is demonstrated that ultraviolet B (UVB) radiation stimulates increased expression of the proopiomelanocortin (POMC) gene which is accompanied by production and release of α-melanocyte stimulating hormone (α-MSH) and adrenocorticotropin (ACTH) by both normal and malignant human melanocytes and keratinocytes. The production and release of both peptides are also stimulated by dibutyryl cyclic adenosine monophosphate (dbcAMP) and interleukin 1α (IL-1α) but not by endothelin-1 (ET-1) or tumor necrosis factor-α (TNF-α). *N*-acetyl-cysteine (NAC), a precursor of glutathione (GSH), an intracellular free radical scavenger, abolishes the UVB-stimulated POMC peptide production and secretion. Conclusions are as follows: (1) Cultured human cells of cutaneous origin, namely keratinocytes and melanocytes, can produce and express POMC; (2) POMC expression is enhanced by exposure to UVB, possibly through a cyclic AMP-dependent pathway; and (3) The action of UVB on POMC production may involve a cellular response to oxidative stress.

Keywords: RT-PCR; Radioimmunoassay; Melanocyte stimulating hormone; Adrenocorticotropin; Ultraviolet B

1. Introduction

Proopiomelanocortin (POMC) is a 31 kDa pro-hormone protein that is processed to various bioactive peptides including adrenocorticotropin (ACTH), melanotropins (α-, β-, γ-MSH), lipotropins, and endorphins [1]. POMC is expressed not only in the pituitary gland and in the central nervous system [2], but also in peripheral tissues [1–4], and in a variety of non-pituitary tumors including melanomas [5]. POMC expression and processing culminates in the synthesis and release of POMC-derived peptides, a process that is under multi-hormonal control and stimulated by increased intracellular cAMP concentrations [1,3,4,6]. Two of the POMC-peptides, ACTH and α-MSH are well recognized as regulators of melanogenesis [7]. Recent demonstrations of POMC expression in murine [8], and human skin in vivo [9,10] suggest that local production of POMC peptides might play a role in both cutaneous melanogenesis as well as in regulation of the skin immune system [7].

Melanocytes and keratinocytes are included as members of the neuroendocrine and immune systems of the skin [11,12]. Preliminary studies from different laboratories have shown that indeed all these cells were found to contain α-MSH [13], immuno-reactive ACTH [9,10] or β-endorphin [5,9]. Schauer et al. [14] reported that the normal human keratinocytes and epidermal cancer cells, A431, synthesize and release both ACTH and α-MSH to the culture medium, and that this process was enhanced by UVB irradiation. Recently Wintzen et al. [15] reported that normal human keratinocytes produce β-endorphin and β-lipotropic hormone after stimulation by UV, IL-1α or phorbol ester, and Kippenberger et al. [16] demonstrated that α-MSH is expressed in cultured human melanocytes and keratinocytes.

* Corresponding author: Fax: +1 (518) 2625927.

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The above results are consistent with our earlier proposal that the stimulatory effects of UVB on skin pigmentation may be mediated through the MSH/MSH receptor system [17–19]. In this regard we have recently shown that UVB stimulates the production of both POMC and MSH receptor mRNA by mouse melanoma cells and transformed keratinocytes in culture [20]. Here we extend these studies to the regulation of POMC peptide production by normal human melanocytes and keratinocytes as well as human transformed epidermal cells by UVB and other agents. We tested the effects of dbcAMP, a modifier of POMC expression in other cell systems [21–24]; IL-1α, tumor necrosis factor (TNFα) and endothelins, UV-sensitive regulators of melanocytic function [25–32]; and *N*-acetyl-cysteine (NAC), a precursor of glutathione (GSH) [33]. Results of these studies are discussed in the context of potential mechanisms regulating POMC expression in mammalian skin.

2. Materials and methods

2.1. Cell culture

Normal human melanocytes and keratinocytes were established from foreskins in culture and maintained as described previously [28,34]. For melanocytes, Ham's F10 medium supplemented with fetal calf serum, 5%; 12-*O*-tetradecanoyl phorbol-13 acetate (TPA), 85 nm; isobutylmethyl xanthine (IBMX), 0.1 mM; insulin, 1.0 mM; and bovine pituitary extract, 40 μg protein/ml, were used. Keratinocytes were cultured in keratinocyte-SFM (serum-free keratinocyte medium, Gibco, Life Technologies, NY) containing bovine pituitary extract, 25 μg/ml, and recombinant epidermal growth factor, 5 ng/ml. In both cases, streptomycin-penicillin (40 IU/ml), and fungizone (1.25 μg/ml) were added to the medium. Melanocytes at passages 2–6, and keratinocytes at passages 2 were used for these studies. Cells were deprived of TPA effect before any experimental treatment by culturing them in keratinocyte-SFM medium containing bovine pituitary extract (25 μg/ml) and recombinant epidermal growth factor (rEGF, 5 ng/ml) 7 days prior to the study of POMC expression [35,36].

The human melanoma cell line, SK-MEL188 (provided by Dr. A. Houghton, MD. Memorial Sloan-Kettering Cancer Center), and human squamous cell carcinoma, C4-1, (provided by Dr. M. Reiss, Yale University) were cultured in Dulbecco's modified Eagles medium (DMEM) with 1% non-essential amino acids, 2% fetal bovine serum, and 8% Serum Plus (JRH Biosciences, Lenexa, KS, USA).

2.2. Exposure to UV light

In the UVB irradiation experiments, cells were seeded at a density of 3×10^5 cells per well in a six-well Coster tissue culture plates in quadruplicate. UVB treatment was performed when the cultures became 70–80% confluent. During UVB irradiation, the culture medium was replaced with 0.3 ml phosphate-buffered physiologic saline (PBS). Dosages of 5–100 mJ/cm^2 UVB light were used (energy range of 260–380 nm with a peak at 302 nm; UVM-57 LAMP, UVP Inc, California). Immediately after phototreatment, PBS was removed and serum-free keratinocyte basal medium (which does not contain pituitary extract or rEGF) was added to the cells and incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. After 24 h, the media were collected from quadruplicate cultures, pooled separately for each conditions tested, and processed for extraction of POMC peptides. Cells were collected by minimal trypsin treatment, counted with a Fuchs-Rosenthal cytometer, before processing for extraction of POMC peptides. Cell viability was determined by trypan blue exclusion.

2.3. Treatment with cytokines, endothelin-1, N-acetyl cysteine (NAC), dbcAMP, and TPA

During the treatment the culture medium was replaced with keratinocyte-SFM basal media containing no further additions (control), recombinant human IL-1α (100 pg/ml, Genzyme, MA, USA), and human endothelin-1 (10 nM, Peptide Institute, Osaka), TPA (100 nm), or dbcAMP (1 mM). Purified natural human tumor necrosis factor-α (TNFα, provided by Hayashibara Biochemical Laboratories, Okayama, Japan), was also used in this experiment at the doses of 10 JRU/ml, i.e., 5 ng/ml [37]. To test the effects of protein kinase C (PKC) on POMC expression, some cells were treated with TPA (100 nm) for 3–4 h, after which the cells were cultured with TPA-free medium for an additional 20 h. Tyrphostin (30 μM), and genestein (1 μM), purchased from Gibco/BRL Co., were used as a tyrosine kinase inhibitors. NAC (500 μM), purchased from Sigma Chemical Co, was used as a precursor of GSH which acts as an intracellular free radical scavenger [33]. All the treatments mentioned above were continued for 24 h before extraction of POMC peptides or RNA.

2.4. Extraction of POMC peptides

For extraction of peptides, conditioned culture media were pooled separately for each condition tested, and protease inhibitors (aprotinin, 0.01%; PMSF, 1mM) were added, and the media were centrifuged at $16000 \times g$ for 30 min. The supernatants were collected, and according to the manufacturer's protocol (Peninsula) peptide extraction was carried out as follows. An equal volume of peptide extraction buffer A (provided with the kit, Peninsula, Cat. No. RIK-BA-1), containing 0.1% triflouroacetic acid (TFA), was added and centrifuged at $3000 \times g$ for 10 min at 4°C. The supernatants were then passed through a pre

equilibrated SEP-column containing 200 mg of C18 (Cat. No. RIK-SEPCOL 1, Peninsula). After slowly washing the column with buffer A, the peptides were eluted with 3 ml of buffer B (60% acetonitrile in 0.1% TFA, Cat. No. RIK-BB-1). The eluants were concentrated to dryness in a centrifugal concentrator. The residues were dissolved in 500 μl of RIA buffer (NaH_2PO_4, 19 mM; Na_2HPO_4, 81 mM; pH 7.4; NaCl, 0.05 M; BSA, 0.1%; Triton X-100, 0.1%; and NaN_3, 0.01%) for the assays of αMSH and ACTH by the kit method (Peninsula).

Cell pellets were dissolved in 1 ml RIA buffer supplemented with 1% TX-100 and PMSF (1 mM), and aprotinin (0.01%), and processed as above for peptide isolation through the SEP column.

For human melanoma cells (SK-MEL188) and transformed squamous cell carcinoma (C4-1), peptides from culture media and cell pellets were extracted as described previously [20].

2.5. Radioimmunoassay of αMSH and ACTH

α-MSH and ACTH in normal human epidermal cells were measured using a commercial radioimmunoassay (RIA) kit (Peninsula). For human melanoma and squamous cell carcinoma, the RIA were done using a kit from Incstar (Stillwater, MN). However both antisera are highly specific as the cross reactivities among the POMC peptides, such as desacetyl-α-MSH, diacetyl-α-MSH and deamido-α-MSH, β-MSH, γ-MSH, ACTH, α,β,γ-endorphin, were less than 0.01% for rabbit anti-αMSH according to the kit's specifications, and as measured directly with the peptide standards in our laboratory. Likewise rabbit anti-ACTH (1–24) serum does not cross react with other POMC peptides, such as ACTH (1–10), ACTH (1–13), and α-MSH (cross reaction were less than 0.01%), but shows 100% reactivity with human ACTH (1–24), ACTH (1–39). Assay procedures were followed according to the kit's instructions. Briefly, 100 μl of the dissolved material was incubated for 24 h with the antibody against α-MSH and ACTH followed by re-incubation with ^{125}I-α-MSH or -ACTH for next 24 h. The bound ^{125}I-peptide was precipitated with goat anti-rabbit IgG (GARGG) and normal rabbit serum. The tubes were centrifuged at $3000 \times g$ for 20 min, 4°C. The supernatants were discarded and pellets were counted in a gamma counter. The concentrations of the α-MSH and ACTH were estimated according to the standard curves prepared for both peptides, in which known quantities of purified α-MSH and ACTH were used to compete for ^{125}I-α-MSH and ^{125}I-ACTH binding to the antibodies. Values of α-MSH and ACTH content were normalized to cell number. Levels were expressed as pg/0.5×10^6 cells or pg/ml medium for intracellular and culture medium levels, respectively. The experiment was repeated three times in duplicate, and for statistical analysis the values were calculated as a percent of control.

2.6. Statistical analysis

Statistical analyses of the data pooled from three independent experiments were done using the two-tail paired *t*-test with the help of STATVIEW program (Abacus). The differences were considered significant if $P < 0.05$.

2.7. Reverse transcriptase polymerase chain reaction (RT-PCR)

For RT-PCR studies, cells were grown in 75-cm^2 tissue culture flasks (Corning) to approx. 80% confluence. At 20 h post-treatment of cells with TPA or UVB (10 mJ/cm^2 for melanocytes; 25 mJ/cm^2 for keratinocytes) as described above, all cells were harvested, washed, pelleted by centrifugation, and used to isolate total cellular RNA. Briefly, total RNA was extracted using the RNAzol B isolation kit following the manufacturer's protocol (Cinna/BioTecx Laboratories, Houston, TX). The synthesis of the first strand cDNA was performed using the Superscript pre-amplification system according to the manufacturer's protocol (Gibco-BRL, Gaithersburg, MD). The total cellular RNA (2 μg) isolated with the RNAzol B isolation kit was reverse transcribed using oligo(dT) as a primer. For quantitative PCR, a control gene, glyceraldehyde phosphate dehydrogenase (GAPDH), was amplified using primers and conditions described by Robbins and McKinney [38]. Only samples that were proven to be free from DNA contamination by running PCR amplification with GAPDH primers without prior RT were used for the experiments. The 260 bp fragment derived from the exon 3 of the human POMC, was amplified using primers designed and synthesized commercially (National Bioscience, Plymouth, MN) with the following sequence: 5′-GAG GGC AAG CGC TCC TAC TCC-3′ (upper primer) and 5′-GGG GCC CTC GTC CTT CTT CTC-3′ (lower primer). The reaction mixtures contained $(NH_4)_2SO_4$ buffer, 25 mM (pH 9.0); $MgCl_2$, 2 mM; dNTP, 0.4 mM; and upper and lower primers, 4 μM each. POMC cDNA was heated at 94°C for 3 min., and then amplified for 30 cycles of 45 sec. at 95°C; 1 min at 58°C; 2 min at 72°C with a final extension of 7 min at 72°C. As a negative control, RNA samples that were not reverse transcribed, were run in parallel. Reverse transcribed pituitary total RNA was used as a positive control. The PCR products were separated electrophoretically on 1.5% agarose gels, stained with ethidium bromide and photographed under UV.

To confirm specificity, the products of RT-PCR were transferred to nylon membranes (Oncor, Gaithersburg, MD) after denaturation in 0.5 M NaOH and UV crosslinked to the membranes. The membranes were prehybridized in Hybrizol at 42°C for 4 h and the ^{32}P-labeled human POMC cDNA (gift of Dr. J. Roberts, Mount Sinai Medical Center, NY) was added to fresh hybridization buffer and

hybridization was allowed to proceed for an additional 15 hours at 42°C. The membranes were then washed twice for 5 min with 2 × SSC plus 0.01% SDS at room temperature, followed by two 1-h washes with 0.1 × SSC plus 0.1% SDS at 60°C. After a final rinse, X-ray film (Kodak XAR) was exposed to the membranes at −70°C for 5–6 h.

3. Results and discussion

UVB stimulated both production and secretion of α-MSH and ACTH by normal human melanocytes and keratinocytes in culture (Table 1). Maximum UVB effects were found for melanocytes and keratinocytes at 10 mJ/cm^2 and 25 mJ/cm^2, respectively. This is in agreement with our previous observations that keratinocytes require higher dose of UVB irradiation than melanocytes to exhibit phenotypic changes [19,43]. Since UVB can induce production of various cytokines and growth factors by melanocytes and keratinocytes such as IL1-α, ET-1 and TNFα [25–29,31], which can affect melanocyte proliferation and pigmentation [30,32], we tested the possibility that UVB-induced auto- or paracrine interactions may regulate POMC expression. Table 1 shows that while IL-1α has small but significant ($P < 0.01$ or 0.05 for melanocytes; $P < 0.001$ or 0.01 for keratinocytes) stimulatory effect on POMC production, ET-1 and TNFα did not affect production of ACTH and α-MSH by either normal human melanocytes or keratinocytes. In addition, dbcAMP clearly stimulated α-MSH and ACTH production and secretion by both melanocytes and keratinocytes (Table 1). This finding is in agreement with other reports showing that dbcAMP stimulates POMC expression and secretion of its peptides in different cell lines including mouse melanoma cells [20–24]. It is also consistent with a signal transduction system through corticotropin releasing hormone receptor (CRH-R) that via production of cAMP stimulates POMC activity in brain and pituitary gland [39]. Recently, we detected expression of CRH-R mRNA in mammalian skin [40]. Therefore, we suggest that POMC expression in human melanocytes and keratinocytes is also affected by increases in intracellular cAMP content.

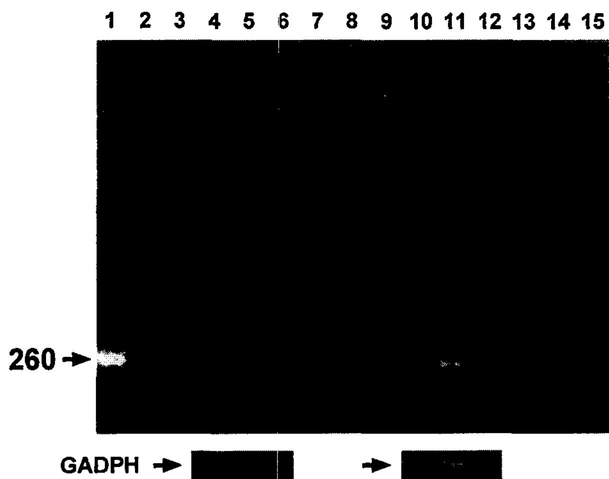

Fig. 1. Detection of POMC mRNA by semiquantitative RT-PCR in normal human melanocytes and keratinocytes treated with UVB and TPA. Upper panel: 260 bp POMC mRNA from exon 3 (arrow) was amplified (30 cycles) using primers described in Section 2. Lower panel: amplification of the glycerol phosphate dehydrogenase gene (GAPDH, arrow). Human pituitary (positive control, lane 1); buffer (lane 2); DNA size markers of 1000, 700, 525, 400, 300 and 100 bp (lane 3); untreated human keratinocytes (lane 4); human keratinocytes exposed to UVB, 25 mJ/cm^2 (lane 5); human keratinocytes treated with TPA, 100 nM (lane 6); negative control (lanes 7, 8, and 9; PCR amplification of RNA corresponding to samples in lanes 3, 4 and 5, respectively, without prior reverse transcription); untreated human melanocytes (lane 10); human melanocytes exposed to UVB, 10 mJ/cm^2 (lane 11); human melanocytes treated with TPA, 100 nM (lane 12); negative control (lanes 13, 14, and 15; PCR amplification of RNA corresponding samples in lanes 10, 11, and 12, respectively, without prior reverse transcription).

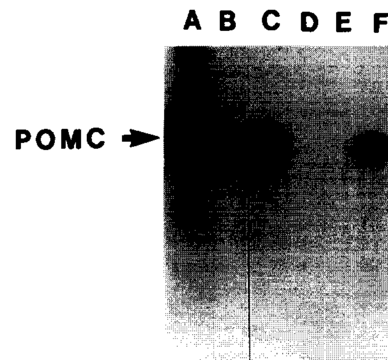

Fig. 2. RT-PCR Southern blot analysis of POMC mRNA expression in human melanoma cells, SK-MEL188, and human squamous cell carcinoma, C4-1. Arrow: POMC amplified mRNA hybridized to the human POMC exon 3 cDNA. Lane A, human pituitary (positive control); lane B, DNA size markers (as blank); lane C, human melanoma cells (SK-MEL188); lane D, negative control for SK-MEL188 (RNA amplified without prior reverse transcription); lane E, negative control for human squamous cell carcinoma, C4-1 (RNA amplified without prior reverse transcription); lane F, C4-1.

The RT-PCR assay (30 cycles) showed that both UVB and TPA stimulated the expression of a 260 bp product that migrated identically with a POMC transcript run as a positive control (Fig. 1, upper panel); the concentration of the amplified fragment of the GAPDH transcript, used as an internal control, remained unchanged during the treatment (Fig. 1, lower panel). We conclude that the amplified 260 bp product represents exon 3 of the POMC gene for the following reasons: (a) the size as predicted from primer sequences, (b) absence in control RNA samples amplified without previous reverse transcription (Fig. 1), and (c) hybridization to the human POMC cDNA (not shown). These results are in agreement with those of Schauer et al. [14] obtained with malignant human A431 keratinocytes demonstrating an increased expression of POMC gene and an increased production and secretion of α-MSH and ACTH after UVB and TPA treatment. During the revision

Table 1
α-MSH and ACTH synthesis and release by normal human melanocytes and keratinocytes

Treatment	Melanocytes				Keratinocytes			
	α-MSH		ACTH		α-MSH		ACTH	
	Intracellular (pg/0.5×10⁶ cells)	Medium (pg/ml)	Intracellular (pg/0.5×10⁶ cells)	Medium (pg/ml	Intracellular (pg/0.5×10⁶ cells)	Medium (pg/ml)	Intracellular (pg/0.5×10⁶ cells)	Medium (pg/ml)
Control	52±3 (100)	10±1 (100)	10±2 (100)	13±1 (100)	32±7 (100)	11±1 (100)	20±3 (100)	8±1 (100)
UVB								
5 mJ/cm²	69±5 (126±9) **	15±2 (146±24) **	15±1 (143±19) **	18±1 (130±8) **	ND	ND	ND	ND
10 mJ/cm²	80±12 (159±14) ***	18±1 (185±7) ***	16±1 (162±13) ***	22±1 (163±6) ***	57±1 (182±19) ***	18±7 (160±43) **	24±3 (122±10) *	16±4 (189±30) ***
25 mJ/cm²	66±6 (131±7) ***	16±2 (157±20) ***	14±1 (142±19) **	17±1 (127±5) **	75±9 (240±33) ***	32±2 (279±18) ***	52±3 (254±24) ***	21±2 (259±19) ***
50 mJ/cm²	ND	ND	ND	ND	36±11 (130±18) **	31±1 (269±10) ***	26±5 (133±15) *	34±6 (372±91) ***
dbcAMP	66±9 (129±8) **	30±1 (287±35) ***	18±4 (179±37) **	28±8 (209±43)***	50±9 (163±25) **	45±1 (388±17) ***	33±1 (160±14) ***	26±8 (314±69) ***
ET-1	48±6 (94±9) NS	12±1 (110±16) NS	11±3 (106±16) NS	12±3 (102±10) NS	40±1 (113±13) NS	10±1 (94±10) NS	16±2 (91±12) NS	7±3 (98±20) NS
IL-1α	72±3 (136±8) **	14±1 (136±14) **	16±3 (156±32) **	15±2 (116±13) *	57±2 (185±20) ***	16±1 (141±5) ***	34±7 (167±29) **	ND
TNFα	56±9 (112±18) NS	9±1 (95±3) NS	9±2 (103±12) NS	9±1 (80±8) *	ND	ND	ND	ND

Cell culture, treatment, and RIA of POMC peptides were done as described in Section 2. Values of α-MSH and ACTH content were normalized to cell number and expressed as: pg/0.5×10⁶ cells (intracellular level); pg/ml medium (released to the medium). Each data point represents the mean ± SD of two assays from a representative experiment. The experiments were repeated three times with similar results. In parentheses pooled data from three separate experiments done in duplicate were presented as a percent of control. The statistical analysis of these data was done using the two-tailed paired t-test (Abacus, STATVIEW program). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant; ND, not done.

Table 2
Effects of UVB on α-MSH and ACTH synthesis and release by human melanoma cells (SK-MEL188) and squamous cell carcinoma (C4-1)

Treatment	Melanoma				Squamous cell carcinoma			
	α-MSH		ACTH		α-MSH		ACTH	
	Intracellular ($pg/0.5\times10^6$ cells)	Medium (pg/ml)	Intracellular ($pg/0.5\times10^6$ cells)	Medium (pg/ml)	Intracellular ($pg/0.5\times10^6$ cells)	Medium (pg/ml)	Intracellular ($pg/0.5\times10^6$ cells)	Medium pg/ml
Control	21±4 (100)	12±6 (100)	10±1 (100)	15±2 (100)	26±2 (100)	14±4 (100)	9±1 (100)	11±1 (100)
UVB								
10 mJ/cm^2	36±1 (175±23) * * *	39±1 (246±85) * *	18±1 (180±19) * * *	23±1 (159±14) * *	ND	ND	ND	ND
20 mJ/cm^2	38±1 (180±24) * * *	40±2 (229±79) * *	20±1 (206±19) * * *	26±1 (177±15) * * *	40±5 (165±52) *	41±4 (294±48) * * *	27±3 (302±38) * * *	36±4 (342±28) * * *
40 mJ/cm^2	74±14 (357±71) * * *	68±3 (430±149) * *	28±1 (286±26) * * *	38±3 (260±26) * *	ND	ND	ND	ND
50 mJ/cm^2	ND	ND	ND	ND	39±1 (159±47) *	40±4 (283±48) * * *	24±5 (268±55) * * *	35±6 (330±42) * * *
100 mJ/cm^2	ND	ND	ND	ND	70±6 (287±88) * *	63±3 (446±178) * *	50±4 (554±63) * * *	63±1 (534±127) * * *

α-MSH and ACTH were measured using a commercial radioimmunoassay (RIA) kit (Incstar, Stillwater, MN) as described in Section 2. Values of α-MSH and ACTH content were normalized to cell number and expressed as: $pg/0.5\times10^6$ cells (intracellular level); pg/ml medium (released to the medium). Each data point represents the mean ± SD of two assays from a representative experiment. The experiments were repeated three times with similar results. In parentheses pooled data from three separate experiments done in duplicate were presented as a percent of control. The statistical analysis for this data was done using two-tail paired *t*-test (Abacus, STATVIEW program). * $P < 0.05$; * * $P < 0.01$; * * * $P < 0.001$; NS, not significant; ND, not done.

Table 3
Effects of *N*-acetyl cysteine (NAC) on UVB-induced α-MSH and ACTH production in normal human melanocytes and keratinocytes

Treatment	Melanocytes				Keratinocytes			
	α-MSH		ACTH		α-MSH		ACTH	
	Intracellular (pg/0.5×10⁶ cells)	Medium (pg/ml)	Intracellular (pg/0.5×10⁶ cells)	Medium (pg/ml)	Intracellular (pg/0.5×10⁶ cells)	Medium (pg/ml)	Intracellular pg/0.5×10⁶ cells	Medium pg/ml
Control	52±3	10±1	10±2	13±1	32±7	11±1	20±3	8±1
	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
UVB	80±12	19±1	16±1	22±1	75±9	33±2	52±3	22±2
	(159±14) ***	(185±7) ***	(162±13) ***	(163±6) ***	(240±33) ***	(279±18) ***	(254±24) ***	(259±19) ***
NAC	44±2	12±1	14±1	12±2	25±4	14±1	23±1	9±3
	(95±4) NS	(110±12) NS	(128±19) *	(97±11) NS	(91±7) NS	(107±5) NS	(106±9) NS	(110±20) NS
NAC+UVB	40±2	14±1	10±2	13±1	32±1	15±1	26±3	8±3
	(92±6) NS	(119±22) NS	(102±10) NS	(101±4) NS	(102±11) NS	(109±11) NS	(107±15) NS	(95±21) NS

Cells were incubated with or without NAC (500 μM) for 1 h before UVB irradiation in 6 well tissue culture dishes as described in Section 2. 10 and 25 mJ/cm^2 doses of UVB were used for melanocytes and keratinocytes, respectively. Immediatley after UVB irradiation cells were incubated with or without NAC-containing medium for an additional 23–24 h. At 24 h post treatment, culture media and cells were collected, processed for RIA of α-MSH and ACTH. Intracellular production of POMC peptides was expressed as $pg/0.5\times10^6$ cells, and secretion of POMC peptides was expressed as pg/ml culture medium. Each data point represents the mean ± SD of two assays from a representative experiment. The experiments were repeated three times with similar results. Pooled data from three separate experiments are given in parentheses which were done in duplicate and were presented as a percent of control. The statistical analysis for this data was done using two-tail paired *t*-test (Abacus, STATVIEW program). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant.

of this manuscript Wintzen et al [41] reported the similar observation of POMC gene products regulation in normal human keratinocytes by UV, TPA, and IL1-α.

We further extended our studies by testing established in vitro lines of human melanoma (Sk-MEL188) and human squamous cell carcinoma (C4-1). As with normal melanocytes and keratinocytes described above, UVB in a dose dependent way stimulated production and secretion of αMSH and ACTH in both cell types (Table 2). Furthermore both cell types expressed the POMC gene as shown by RT-PCR amplification followed by hybridization with exon 3 cDNA of the POMC gene (Fig. 2). Thus, these studies together with previous reports [8,14] strongly indicate that cultured epithelial and pigment cells of human epidermis have the capability to express POMC, and that UV irradiation stimulates the production and secretion of POMC peptides.

Since UVB, IL-1, dbcAMP, and MSH are known to enhance MSH receptor activity in mouse melanoma cells [18,42–44] or human melanocytes [45], it is expected that agents that stimulate MSH synthesis and its release may in turn result in homologous up-regulation of MSH receptor activity and ultimately lead to receptor mediated cascade of events for melanocyte activity, in particular melanin formation. This, in conjunction with our previous observations, supports the notion that at least one action of UVB may be expressed through the MSH/MSH receptor system [18,42,46].

What is the mechanism of UVB-induced POMC expression? Since it is reported that the mammalian ultraviolet response can involve activation of tyrosine kinases [47] we tested the effect of tyrosine kinase inhibitors on POMC expression. Neither tyrphostin (30 mM), nor genistein (1 mM) affected UVB induced MSH and ACTH production (data not shown). However, UV irradiation in addition to DNA damage, causes lipid peroxidation followed by generation of free radicals [48] and depletion of the intracellular pool of reduced glutathione (GSH), resulting in oxidative stress [49]. Evidences are there that active oxygen species (AOS) produced by UVB irradiation may play a role in melanogenesis [50]. In this context UV-induced AOS could regulate epidermal melanin unit by increased expression of melanogenic α-MSH and ACTH neuropeptides. To test this possibilities, we used *N*-acetylcysteine (NAC) which is readily taken up by cells and is rapidly converted to GSH that acts as an intracellular free radical scavenger [33]. As shown in the Table 3, the UVB-induced α-MSH and ACTH production were suppressed to the non-irradiated control level by NAC (500 μM) treatment. This result suggests that NAC may inhibit the production of α-MSH and ACTH possibly through attenuation of the oxidative stress triggerd by UVB. Interestingly, several other inducers of the UV response were also reported to cause oxidative stress [51–53], either through production of free hydroxyl radicals and H_2O_2 or by reacting with free SH groups (alkylating agents). In addition, it was reported that dipyrimidine dithymidylic acid (pTpT) which is generated by UV radiation, can stimulate melanogenesis in both cultured mouse melanoma cells, and in guinea pig skin [54]. Such a proposed initiating mechanism for mediation of the UV signal could be compatible with the above findings on UV and the expression of melanogenic POMC peptides.

In summary, our data provide evidence that cultured human melanocytes and keratinocytes produce POMC peptides and that such production can be stimulated by UVB or dbcAMP. The results further suggest that in human skin POMC peptide production might be regulated by cAMP and/or oxidative stress pathways.

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References

[1] Smith, A.I. and Funder, J.E. (1988) Endocrine Rev. 9, 159–179.
[2] Autelitano, D.J., Lundblad, J.R., Blum, M. and Roberts, J.L. (1989) Annu. Rev. Physiol. 51, 715–726.
[3] DeBold, C.R., Menefee, J.K., Nicholson, W.E. and Orth, D.N. (1988) Mol. Endocrinol. 2, 862–870.
[4] Drouin, J., Nemer, M., Charron, J., Gagner, J.P., Jeannotte, L., Sun, Y.L., Therrien, M. and Tremblay, Y. (1989) Genom. 31, 510–519.
[5] Slominski, A. (1991) FEBS Lett. 291, 165–168.
[6] Jones, M.T. and Gillham, B. (1988) Physiol. Rev. 68, 743–818.
[7] Slominski, A., Paus, R. and Wortsman, J. (1993) Mol. Cell Endocrinol. 93, C1–C6.
[8] Slominski, A., Paus R., Wortsman, J. and Mazurkiewicz, J.E. (1992) Experientia 48, 50–54.
[9] Slominski, A., Wortsman, J., Mazurkiewicz, J.E., Matsuoka, L., Dietrich, J., Lawrence, K., Gorbani, A. and Paus, R. (1993) J. Lab. Clin. Med. 122, 658–666.
[10] Farooqui, J.Z., Medrano, E.E., Abdel-Malek, Z. and Nordlund, J. (1993) Ann. N.Y. Acad. Sci. 680, 508–510.
[11] Cramer, S.F. (1991) Arch. Pathol. Lab. Med. 115, 115–119.
[12] Kupper, T.A., Lee, F., Birchall, N., Clark, S. and Dower, S. (1988) J. Clin. Invest. 82, 1787–1792.
[13] Lunec, J., Pieron, C., Sherbet, G.V. and Thody, A.J. (1990) Pathobiol. 58, 193–197.
[14] Schauer, E., Trautinger, F., Kock, A., Schwarz, A., Bhardwaj, R., Simon, M., Ansel, J.C., Schwarz, T. and Luger, T.A. (1994) J. Clin. Invest.93, 2258–2262.
[15] Wintzen, M., Yaar, M., Avilla, E.M., Vermeer, B.J. and Gilchrest, B.A. (1996) J. Invest. Dermatol. 106, 3–10.
[16] Kippenberger, S., Bernd, A., Loitsch, S., Ramirez-Bosca, A., Bereiter-Hahn, J., Holzmann, H. (1995) Eur. J. Dermatol. 5, 395–397.
[17] Bolognia, J., Murray, M., Pawelek, J. (1989) J. Invest. Dermatol. 92, 651–656.

[18] Chakraborty, A.K., Orlow, S.J., Bolognia, J. and Pawelek, J.M. (1991) J. Cell Physiol. 147, 1–6.
[19] Chakraborty, A.K. and Pawelek, J.M. (1993) J. Cell Physiol. 157, 344–350.
[20] Chakraborty, A.K., Slominski, A., Ermak, G., Hwang, J. and Pawelek, J.M. (1995) J. Invest Dermatol. 105, 655–659.
[21] Yang, Z., Huang, W., Lee, D., Copolov, L.D. and Lim, A.T. (1993) J. Neuroendocrinol. 5, 371–380.
[22] Knight, R.M., Farah, J.M., Bishop, J.F. and O'Donohue, T.L. (1987) Peptides 8, 927–934.
[23] Boutillier, A.L., Monnier, D., Koch, B. and Loeffler, J.P. (1994) Neuroendocrinology 60, 493–502.
[24] Yang, Z., Lee, D., Huang, W., Copolov, L.D. and Lim, A.T. (1994) Brain Res. 648, 99–108.
[25] Luger, T.A. (1989) Acta Derm. Venereol. (Stockh) 69, 61–76.
[26] Oxholm, A., Oxholm, P., Staberg, B. and Bendtzen, K. (1988) Br. J. Dermatol. 118, 369–376.
[27] Kupper, T.S., Chua, A.O., Flood, P., McGuire, J. and Gubler, U. (1987) J. Clin. Invest. 80, 430–436.
[28] Imokawa, G., Yada, Y. and Miyagishi, M. (1992) J. Biol. Chem. 267, 24675–24680.
[29] Kock, A., Schwarz, T., Urbanski, A., Peng, Z., Vetterlein, M., Micksche, M., Ansel, J.C., Kung, H.F., Kuger, T.A. (1989) J. Natl. Cancer Inst. 81, 36–42.
[30] Swope, V.B., Abdel-Malek, Z.A., Kasem, L.M. and Nordlund, J.J. (1991) J. Invest. Dermatol. 96, 180–185.
[31] Swope, V.B., Sauder, D.N., Mckenzie, R.C., Sramkoski, R.M., Krug, K.A., Babcock, G.F., Nordlund, J.J. and Abdel-Malek, Z.A. (1994) J. Invest. Dermatol. 102, 749–753.
[32] Yada, Y., Higuchi, K. and Imokawa, G. (1991) J. Biol. Chem. 266, 18352–18357.
[33] Meister, A. (1991) Pharmacol. Therap. 51, 155–194.
[34] Funasaka, Y., Boulton, T., Cobb, M., Yarden, Y., Fan, B., Lyman, S.D., Williams, D.E. Anderson, D.M., Zakut, R., Mishima, Y., Halaban, R. (1992) Mol. Biol. Cell 3, 197–209.
[35] Kirnbauer, R., Charvat, B., Schauer, E., Kock, A., Urbanski, A., Foster, E., Neuner, P., Assmann, I., Luger, T.A. and Schwarz, T (1992) J. Invest. Dermatol. 98, 320–326.
[36] Horikawa, T., Norris, D.A., Yohn, J.J., Zekman, T., Travers, J.B. and Morelli, J.G. (1995) J. Invest. Dermatol. 104, 256–259.
[37] Fukuda, S., Ando, S., Sanou, O., Taniai, M., Fuji, M., Masaki, N., Nakamura, K., Ando, O., Torigoe, K., Sugimoto, T. and Kurimoto, M. (1988) J. Lymphokine Res. 7, 175–185.
[38] Robbins, M. and Mckinney, M. (1992) Mol. Brain Res. 13, 83–92.
[39] Reisine, T., Rougon, G., Barbet, J., Affolter, H.V. (1985) Proc. Natl. Acad. Sci. USA. 82, 8261–8266.
[40] Slominski, A., Ermak, G., Hwang, J., Chakraborty, A.K., Mazurkiewicz, J.E. and Mihm, M. (1995) FEBS Lett. 374, 113–116.
[41] Wintzen, M., Yaar, M., Burbach, J.P.H. and Gilchrest, B.A. (1996) J. Invest. Dermatol. 106, 673–678.
[42] Chakraborty, A.K. and Pawelek, J.M. (1991) Biochem. Biophys. Res. Commun. 175, 839–845.
[43] Birchall, N., Orlow, S.J., Kupper, T. and Pawelek, J. (1991) Biochem. Biophys. Res. Commun. 175, 839–845.
[44] Siegrist, W. and Eberle, A.N. (1993) J. Receptor Res. 13, 263–281.
[45] Siegrist, W., Stutz, S. and Eberle, A.N. (1994) Cancer Res. 54, 2604–2610.
[46] Pawelek, J.M., Chakraborty, A.K., Osber, M.P., Orlow, S.J., Min, K.K., Rosenzweig, K.E. and Bolognia, J.L. (1992) Pigment Cell Res. 5, 348–356.
[47] Devary, Y., Gottlieb, R.A., Smeal, T. and Karin, M. (1992) Cell 71, 1081–1091.
[48] Black, H.S. (1987) Photochem. Photobiol. 46, 213–221.
[49] Lautier, D., Luscher, P. and Tyrell, R.M. (1992) Carcinogenesis 13, 227–232.
[50] Tobin, D., Thody, A.J. (1994) Exp. Dermatol. 3, 99–105.
[51] Cerruti, P. (1985) Science 227, 375–381.
[52] Cerruti, P., Trump, B.F. (1991) Cancer Cells 3, 1–7.
[53] Trush, M.A. and Kensler, T.W. (1991) Free Radic. Biol. Med. 10, 201–209.
[54] Eller, M.S., Yaar, M. and Gilchrest, B.A. (1992) Nature 372, 413–414.