**α Melanocyte stimulating hormone (αMSH) stimulates normal human melanocyte growth by binding to high-affinity receptors**

Michele De Luca¹⁺, Walter Siegrist², Sergio Bondanza¹, Monica Mathor¹⁺, Ranieri Cancedda¹ and Alex N. Eberle²

¹Laboratorio di Differenziamento Cellulare, Istituto Nazionale per la Ricerca sul Cancro, 16132 Genoa, Italy
²Laboratory of Endocrinology, Department of Research (ZLF), University Hospital and University Children’s Hospital, CH-4031, Basel, Switzerland

*Author for correspondence
†On leave of absence from: Department of Application of Nuclear Techniques in Biological Sciences, National Nuclear Energy Commission (IPEN-CNEN), São Paulo, Brazil

**SUMMARY**

The combined action of cholera toxin (CT)-dependent activation of the adenylate cyclase signaling pathway, stimulation of protein kinase C, and activation of the tyrosine kinase activity of cell surface receptors and proto-oncogene products, have been shown to stimulate melanocyte proliferation. However, natural factors responsible for the optimal stimulation of normal human melanocyte growth, either isolated or co-cultured with keratinocytes, remain largely unknown. αMSH (α melanocyte stimulating hormone) has previously been shown to bind to murine and human melanoma cells and to stimulate their adenylate cyclase and tyrosinase activity. In contrast, very little is known about the presence and function of αMSH receptors in normal human melanocytes. We now report that αMSH: (i) binds to normal human melanocytes through a single class of high-affinity receptors; (ii) does not induce per se melanocytes to enter the S-phase of the cell cycle; (iii) does indeed stimulate melanocyte proliferation in a dose-dependent fashion; but its stimulatory effect requires bFGF and/or the activation of protein kinase C.

Key words: αMSH, melanocytes, melanoma, epidermis

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**INTRODUCTION**

Normal human melanocyte growth is mediated by three of the major intracellular messengers involved in the transduction mechanism by cell surface receptor proteins: namely, the activation of adenylate cyclase, the diacylglycerol (DAG)-dependent stimulation of protein kinase C and the tyrosine kinase activity of cell surface receptors and proto-oncogene products (for recent reviews see Yaar and Gilchrest, 1991; Halaban, 1991). Cholera toxin (CT) is known to increase the intracellular levels of cyclic adenosine monophosphate (cAMP), and phorbol-12-myristate-13-acetate (PMA) is a potent stimulator of protein kinase C. Both substances have been widely used to sustain human melanocyte proliferation. However, most of the known hormones and growth factors that stimulate receptors evoking the phosphoinositol cascade or the adenylate cyclase activation fail to replace PMA and CT (or dibutyryl-cAMP and isobutylmethylxanthine; Halaban, 1988), the only exception being the endothelins (a family of vasoconstrictive polypeptides), which are secreted from human keratinocytes, activate melanocyte protein kinase C and stimulate their growth (Yada et al., 1991; Imokawa et al., 1992). Basic fibroblast growth factor (bFGF), which acts through a receptor endowed with tyrosine kinase activity, is synthesized by keratinocytes as well, and induces human melanocyte proliferation (Halaban et al., 1988). Still, bFGF and PMA (or endothelins) do not fully account for the melanocyte growth rate attained in vivo, during the healing of wounds or in vitro, when melanocyte proliferation is potently stimulated by co-cultured keratinocytes (De Luca et al., 1988a,b, 1993). Thus, natural factors responsible for the optimal stimulation of normal human melanocyte growth, either isolated in culture or co-cultured with keratinocytes, remain largely unknown.

One of the most obvious candidates for the adenylate cyclase-dependent melanocyte growth stimulation is α melanocyte stimulating hormone (αMSH), which has been shown to increase intracellular levels of cAMP in target cells (Ranson et al., 1988). However, despite the activation of the adenylate cyclase pathway observed in human melanocytes after αMSH stimulation (Ranson et al., 1988), biological effects have not been convincingly demonstrated in these cells (Yaar and Gilchrest, 1991). Moreover, very little data exist about the presence of specific high-affinity MSH receptors in normal human melanocytes.

We now report that: (i) αMSH binds to normal human melanocytes through a single class of high-affinity recep-
tors; (ii) αMSH does not induce melanocytes, per se, to enter the S-phase of the cell cycle; (iii) αMSH does indeed stimulate melanocyte proliferation in a dose-dependent fashion, but its mitogenic effect requires bFGF and/or the activation of protein kinase C.

MATERIALS AND METHODS

Keratinocyte culture

Human epidermal keratinocytes were obtained from skin biopsies of healthy volunteers and cultured on a feeder-layer of lethally irradiated 3T3-J2 cells (a gift from Prof. Howard Green, Harvard Medical School, Boston, MA) as described (Green et al., 1979; De Luca et al., 1988a). In brief, skin biopsies were minced and trypsinized (0.05% trypsin/0.01% EDTA) at 37°C for 3 h. Cells were collected every 30 min, plated (2.5×10^4/cm^2) on lethally irradiated 3T3-J2 cells (2.4×10^4/cm^2) and cultured in 5% CO_2 humidified atmosphere in keratinocyte growth medium: Dulbecco-Vogt Eagle’s (DMEM) and Ham’s F12 media (3:1 mixture) containing fetal calf serum (FCS, 10%), insulin (5 µg/ml), transferrin (5 µg/ml), adenine (0.18 mM), hydrocortisone (0.4 µg/ml), CT (0.1 nM), tri-iodothyronine (2 nM), epidermal growth factor (EGF, 10 ng/ml), glutamine (4 mM) and penicillin-streptomycin (50 i.u./ml). Sub-confluent primary cultures were passaged in secondary cultures as described (De Luca et al., 1988a). In these culture conditions, keratinocyte colonies, in both primary and secondary culture, reconstitute a stratified squamous epithelium closely resembling the epidermis in vivo (Green, 1980; Compton et al., 1989; De Luca et al., 1990; Pellegrini et al., 1992), physiologically populated by melanocytes (De Luca et al., 1988a,b), and suitable for autologous and permanent grafting onto patients (Gallico et al., 1984; De Luca et al., 1989; Romagnoli et al., 1990). 3T3-J2 cells were cultured in DMEM containing calf serum (10%), glutamine (4 mM) and penicillin-streptomycin (50 i.u./ml).

Melanocyte culture

Normal human melanocytes were isolated from in vitro reconstituted epidermis. Confluent keratinocytes (from primary or secondary cultures) were trypsinized (2-3 d after confluence) and the cell suspension was plated in the absence of feeder-layer, at a cell density of 2.5×10^4/cm^2 in melanocyte growth medium (MGM-1): E-199 containing fetal calf serum (FCS, 5%), insulin (5 µg/ml), transferrin (5 µg/ml), adenine (0.18 mM), hydrocortisone (0.4 µg/ml), CT (0.1 nM), tri-iodothyronine (20 nM), epidermal growth factor (EGF, 10 ng/ml), bFGF (1 ng/ml), glutamine (4 mM), penicillin-streptomycin (50 i.u./ml). Genetin (100 to 250 µg/ml) was added for 2-4 d to avoid human fibroblast overgrowth. At 24 h after seeding, the medium was changed and free-floating cells were removed. After 2-4 d, melanocytes, identified by their morphology and reactivity to 1,3,4-dihydroxyphenylalanine (DOPA), were further purified by differential trypsinization: melanocytes, preferentially detached after short periods of trypsinization (2-3 min), were collected in growth medium and plated at a cell density of approximately 5×10^3/cm^2. Sub-confluent cultures were passaged 1:3 and cultured as above. Some strains of melanocytes were isolated and cultured in MGM-2, which, basically, is MGM-1 containing PMA (1 ng/ml). After their isolation (100% positivity to DOPA reaction, usually obtained after 2-4 passages), melanocytes were used in experiments. DOPA reaction was performed as described (De Luca et al., 1988a).

[^3]H]thymidine incorporation, growth assays and melanin contents

αMSH used in these experiments was purchased from Sigma. Isolated melanocytes (15 different strains) were plated in basal medium (MGM depleted of CT, PMA and bFGF) onto 96-well plates (2×10^4 cells/well). Factors were added 12 h later.[^3]H]thymidine (5 nCi/ml) was then added every 12 h. After an additional 12 h of incubation in medium containing[^3]H]thymidine, cells were trypsinized, collected with the aid of a cell-harvester and incorporated radioactivity was β-counted. In experiments shown in Figs 3 and 4 (below) cells were exposed for 36 h to indicated factors before[^3]H]thymidine addition. In selected experiments cells were starved from CT for 8 d before addition of factors. For growth assays, melanocytes (1.5×10^5) were plated in basal medium onto 6-well plates. Factors were added 12 h after plating. Cells were counted 10 d later. For each cell strain experiments were performed at least twice in triplicate.

To measure melanin content, melanocytes (5×10^5) were cultured in the presence of bFGF (1 ng/ml) and in the presence (10 ng/ml) or in the absence of αMSH. After 1-10 d, melanin was measured as described (Horikoshi et al., 1991). Briefly, cells were lysed in 0.1 M NaOH for 2 h at 90°C and centrifuged at 16,000 g for 20 min. The melanin content in the supernatant was measured as the absorbance at 475 nm of a cell lysate containing 1 mg of protein per ml. Values were compared with a standard curve for synthetic melanin obtained in 0.5 M KOH.

αMSH binding assay

[Nle4, D-Phe7]-αMSH was enzymatically iodinated using Enzymobeads (Bio-Rad, Richmond, CA) as previously described (Siegrist et al., 1989). The radioiodination product was purified on a 1 ml syringe packed with 0.4 ml Spherisorb ODS/10 mm silica using a stepwise increasing gradient of methanol in 1% TFA. In addition, the tracer was purified by reversed-phase HPLC and lyophilized before each experiment.

Melanocytes were plated into 24-well Costar dishes at a density of 2.5×10^3 cells per and allowed to attach for 2 d in 1 ml of MGM-1. The plates were washed once in MEM with 25 mM HEPES containing 0.2% bovine serum albumin (BSA) (washing buffer). Incubation with various concentrations of radioligand was performed in 0.5 ml MEM-HEPES containing 0.2% BSA and 0.3 mM 1,10-phenanthroline at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 2 h. Non-specific binding was assessed by addition of an excess of unlabeled αMSH (3 µM). After incubation, the plates were put on ice and washed three times with ice-cold washing buffer. Cells were detached with 2×0.5 ml of 1 M NaOH and γ-counted. Binding data were analyzed with the Ligand program (Munson and Rodbard, 1980).

RESULTS

αMSH binding

Human melanocytes were isolated and cultured from in vitro reconstituted epidermis in primary or secondary culture. Binding assays were performed as described in Materials and Methods. Fig. 1 shows the saturation binding data (A) and the corresponding Scatchard transformation (B) from human melanocytes (strain MP30) using the superpotent MSH agonist [Nle4, D-Phe7]-αMSH as a radioligand. Scatchard analysis pointed to a single class of binding sites with a dissociation constant (K_d) of 54.4 pM (± 20%) and a B_max of 280 receptors per cell (± 13%). Binding assays performed with other strains of melanocytes gave comparable results in terms of K_d, but variable results in terms of receptor number per cell. For example, strain MP27 showed a K_d of 35.7 pM (± 26%) with a markedly higher B_max of 831 receptors per cell (± 5%). Both the
receptor affinity and the number of binding sites per cell were comparable to values found in a panel of human melanoma cell lines, where the receptor number varied between 391 and 1590 sites per cell (Siegrist et al., 1989).

**αMSH stimulation of melanocyte growth**

In preliminary experiments we found that optimal CT and bFGF concentrations inducing melanocyte growth, were 10-10 M and 1 ng/ml, respectively. [3H]thymidine incorporation assays were performed on different strains of isolated normal human melanocytes. As shown in Fig. 2 (three different cell strains), cells exposed to optimal concentrations of CT or αMSH (10 ng/ml, see also Fig. 3) did not enter the S-phase of the cell cycle (open and filled squares). Basic FGF alone induced [3H]thymidine incorporation (filled circles). However, the simultaneous addition of bFGF and αMSH or CT gave the maximal growth stimulation (open circles and filled rhombi). αMSH was able to substitute for CT (Fig. 2) and the combined addition of CT and αMSH did not produce further [3H]thymidine incorporation (see also Fig. 4). Each point was averaged from triplicates. Variation among triplicates was <4%. Comparable results were obtained in 15 different melanocyte strains. To rule out the possibility of an influence of residual CT effects on the αMSH mitogenic activity, [3H]thymidine incorporation assays were performed on three different melanocyte strains, cultured for 8 days in the absence of CT prior to factor addition. The mitogenic effect of αMSH on melanocytes was comparable to the effect shown in Fig. 2 (not shown). We also performed an αMSH dose-response curve on melanocyte strain MP184. As shown in Fig. 3, αMSH was ineffective in the absence of bFGF, even at concentrations up to 100 ng/ml. In the presence of 1 ng/ml of bFGF the growth-stimulatory effect of αMSH was readily observed with hormone concentrations as low as 1 ng/ml, and maximal values were already reached at 10 ng/ml. Similar results were obtained from three different melanocyte strains. The αMSH-induced [3H]thymidine incorporation, although less pronounced, was observed also when melanocytes were exposed to the combined action of bFGF and PMA (Fig. 4, filled bars), but only in the absence of exogeneously added CT (Fig. 4, open bars). Each point was the average of triplicates. Variation among triplicates was <6%. These data were reproduced in 15 different cell strains. To investigate whether melanocytes proceed through mitosis under the influence of αMSH, cells were cultured in the presence of bFGF, PMA and αMSH, and counted 10 days later as described in Materials and Methods. As shown in Table 1, the melanocyte number increased when αMSH was added either to bFGF or to bFGF and PMA, but only in the absence of CT.

We then tested the effect of αMSH on melanogenesis as described under Materials and Methods. In agreement with previous reports showing no αMSH melanogenic effect on cultured human melanocytes (Ranson et al., 1988; Friedmann et al., 1990), we could not demonstrate any αMSH-dependent increase of melanin content in these cells.
All experiments described above were duplicated in cells isolated and cultured in the presence of PMA (see Materials and Methods) and comparable results were obtained.

**DISCUSSION**

The pro-opiomelanocortin gene product is a 31-kDa pro-hormone that is post-translationally cleaved to generate the opiomelanocortin peptides: ACTH, β-endorphin, β-lipotropin (β-LPH) and several isotypes (α, β, γ) of MSH; αMSH being the biologically most active form for melanocytes (Eberle, 1988a).

αMSH has been previously shown to bind murine and human melanoma cells through a single class of high-affinity receptors (Siegrist et al., 1989; Solca et al., 1989), to stimulate adenylate cyclase activity in target cells (Ranson et al., 1988) and to regulate tyrosinase and melanogenesis in murine melanoma cells (Fuller et al., 1987; Jimenez et al., 1988; Hirobe, 1992). Nevertheless, direct interaction of αMSH with normal human melanocytes has never been clearly demonstrated. We took advantage of the possibility to isolate and culture large quantities of melanocytes from in vitro reconstituted human epidermis (De Luca et al., 1988a,b), to demonstrate that these cells do indeed possess high-affinity αMSH receptors. Binding assays performed on different cell strains gave comparable dissociation constants but variable numbers of binding sites per cell. The very low abundance of receptors per cell may also explain why some previous attempts at binding studies have been unsuccessful. While this manuscript was under revision, αMSH high-affinity binding sites (with similar

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**Fig. 2.** [³H]thymidine incorporation. Isolated melanocytes (3 different strains) were plated in basal medium (MGM depleted of CT, PMA and bFGF) onto 96-well plates. CT (10-10 M, filled squares), αMSH (10 ng/ml, open squares), bFGF (1 ng/ml filled circles), bFGF + CT (open circles) and bFGF + αMSH (filled rhombi) were added 12 h later. [³H]thymidine assays were performed as described in Materials and Methods. Comparable results were obtained in 15 different cell strains. For each cell strain experiments were performed at least twice in triplicate. Each point was averaged from triplicates. Variation among triplicates was <4%.

**Fig. 3.** [³H]thymidine incorporation. Isolated melanocytes (strain MP184) were plated in basal medium (MGM depleted of CT, PMA and bFGF) onto 96-well plates. Increasing concentrations of αMSH were added 12 h later in the absence (left panel) or in the presence (right panel) of 1 ng/ml of bFGF. [³H]thymidine assays were performed as described under Materials and Methods. The experiment was performed twice in triplicate. Each point was averaged from triplicates. Variation among triplicates was <6%. Comparable results were obtained in 3 different cell strains.
Fig. 4. [3H]thymidine incorporation. Isolated melanocytes (strain MP46) were plated in basal medium (MGM depleted of CT, PMA and bFGF) onto 96-well plates. Indicated factors were added 12 h later in the presence (open bars) or in the absence (stippled bars) of CT (10−10 M). [3H]thymidine assays were performed as described in Materials and Methods. The experiment was performed three times in triplicate. Each point was averaged from triplicates. Variation among triplicates was <6%. Comparable results were obtained in 15 different cell strains.

values in terms of both $K_d$ and $B_{max}$) were also demonstrated in normal human melanocytes by Donatien et al. (1992).

Even the role of αMSH in eliciting defined biological responses in normal human melanocytes has been doubted (Yaar and Gilchrest, 1991). In particular, αMSH has been reported by Herlyn et al. (1988) to induce melanocyte growth, whereas several other laboratories could not find any αMSH mitogenic activity (see Yaar and Gilchrest, 1991, for a recent review). Here we show that αMSH does indeed stimulate normal human melanocyte proliferation. Even though αMSH (and CT), per se, does not induce melanocytes to enter the S-phase of their cell cycle, it elicits its mitogenic effect in the presence of other growth stimuli such as bFGF and/or activators of the protein kinase C. This observation agrees well with the complex and multifactorial mechanism regulating normal human melanocyte growth (Yaar and Gilchrest, 1991; Halaban, 1991; De Luca et al., 1988b, 1993). Interestingly, it has been recently shown that both bFGF and αMSH might have an autocrine role in stimulating melanoma cell growth and tumor progression (Halaban, 1988; Lunec et al., 1990). The αMSH mitogenic activity on melanocytes seems to be mediated by the activation of the adenylylate cyclase signaling pathway. Indeed, αMSH signaling has been reported to be mediated by activation of adenylylate cyclase in target cells (Ranson et al., 1988). This seems to be the case also in normal human melanocytes, since the effects of CT and αMSH were not additive, both in [3H]thymidine incorporation assays (Figs 2 and 4) and in growth assays (Table 1), and αMSH has been shown to increase intracellular levels of cAMP directly in these cells (Ranson et al., 1988). However, from our data, a different signaling pathway for the αMSH mitogenic activity in these cells cannot be ruled out.

It is worth noting that, within the epidermal/melanin unit, most of the melanocyte functions are specifically and directly regulated by surrounding keratinocytes (De Luca et al., 1988a,b), through a very complex paracrine mechanism involving both positive and negative modulators. Indeed, keratinocyte-derived bFGF (Halaban, 1988), and a novel factor endowed with protein kinase C-stimulatory properties (De Luca et al., in preparation), cooperate in inducing melanocyte growth, while keratinocyte-derived interleukins 1 and 6, and tumor necrosis factor α, inhibit melanocyte proliferation (Swope et al., 1991). In addition, it has been shown that keratinocyte-derived NGF regulates the migratory properties and dendriticity of surrounding melanocytes (Di Marco et al., 1991; Yaar et al., 1991). Thus, it is tempting to speculate that the combined action of keratinocyte-derived cytokines and the regulation of their micro-environmental concentrations, contribute to the proper melanocyte spatial distribution during embryogenesis and wound healing as well as to repigmentation of wounds. Based on these considerations, and since little is known on αMSH gene expression in peripheral tissues (Siegrist et al., 1988; Eberle, 1988b), it will be of great interest to investigate whether keratinocytes regulate melanocyte growth also through the paracrine synthesis and secretion of αMSH.

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REFERENCES


