Actions of Melanotropins on Mouse Melanoma Cell Growth In Vitro [1,2]

Zalfa A. Abdel Malek,[3] Mac E. Hadley,[3,4] Marvin D. Bregman,[5] Frank L. Meyskens, Jr.,[5] *and* Victor J. Hruby[6,7]

ABSTRACT—Melanotropins induce melanogenesis in mouse Cloudman S91 melanoma cells by stimulating the activity of tyrosinase. In monolayer culture, α-melanocyte-stimulating hormone and the superpotent analogue 4-norleucine, 7-D-phenylalanine-α-melanocyte, which had prolonged effects on tyrosinase activity, did not inhibit the proliferation of melanoma cells even at concentrations that elicited maximal tyrosinase stimulation. In soft agar the melanotropins stimulated the formation of melanized colonies and increased the cloning and proliferative potentials of melanoma cells. Both melanotropins increased the number of small (42–104 μm in diameter) colonies at initial plating densities ranging from 625 to 7,500 cells/dish. The number of larger (>104 μm in diameter) colonies was also increased except at densities 5,000 cells or more/dish, wherein the proliferative capacity was inhibited; yet the cloning efficiency was still increased. Therefore, in bilayer soft agar cultures, melanotropins stimulate the growth of the clonogenic S91 melanoma cell population under conditions that allow for optimal expression of the cloning and proliferative potentials of these cells.—JNCI 1986; 76:857–863.

Melanotropic peptides such as α-MSH are known to induce melanogenesis within integumental melanocytes (*1, 2*). Likewise, in Cloudman S91 murine melanoma, α-MSH has been shown to stimulate melanin synthesis, an effect that can be monitored by measuring the increase in the activity of tyrosinase, the rate-limiting enzyme of the melanin biosynthetic pathway (*3*).

The mechanism of action of α-MSH involves binding of the hormone to specific membrane receptors. This initial event results in stimulation of adenylate cyclase activity and in increased production of intracellular cAMP. The elevated cAMP levels trigger a cascade of phosphorylation events that lead to stimulation of transcriptional and translational processes that result in increased tyrosinase activity (*3*). The effect of α-MSH on the growth (proliferation) of melanoma cells has been a controversial issue. Numerous reports exist on the inhibitory effects of α-MSH on the proliferation of melanoma cells (*3–8*). However, this inhibition has been observed in vitro, not in vivo (*3, 4, 8*), and has been attributed to several factors, the most important of which are cell density, infrequent change of the culture medium (feeding), and accumulation of by-products of melanin synthesis (*9*). Evidence for the toxicity of melanin by-products comes from the observation that some of these intermediates, like L-DOPA and 5,6-dihydroxyindole, retard the growth of S91 melanoma cells (*10*). It has been shown that α-MSH can either stimulate or inhibit the proliferation of S91 melanoma cells depending on the culture conditions under which the cells are maintained, such as the frequency of refeeding and the level of tyrosine, the substrate for melanin formation, in the culture medium (*9*).

To further investigate these problems, we have examined the relative effects of α-MSH and the superpotent analogue [Nle^4, D-Phe^7]-α-MSH on the growth of S91 (CCL 53.1) cells in monolayer culture as well as in bilayer (soft agar) culture. [Nle^4, D-Phe^7]-α-MSH has been shown to be more potent than the native hormone α-MSH in several melanocyte bioassays (*11, 12*). This peptide is at least one hundredfold-more effective than α-MSH in stimulating S91 melanoma cell tyrosinase activity (*13*). This hormone analogue also exhibits ultraprolonged effects (also called "residual activity") on tyrosinase activity. [Nle^4, D-Phe^7]-α-MSH is therefore considered a potentially important investigative probe for studies on the factors affecting melanoma cell proliferation in two diverse culture systems: monolayer and bilayer soft agar cultures. The results shed new light on the proliferative response of melanoma cells exposed to melanotropins as affected by culture conditions.

MATERIALS AND METHODS

Materials.—Ham's medium F10 and penicillin and streptomycin solution (100 U/ml and 100 μg/ml, respectively) were purchased from GIBCO (Santa Clara, CA). Fetal bovine and horse sera were either obtained from GIBCO or from KC Biologicals (Lenexa, KS). [3′,5′-^{3}H]Tyrosine (sp act, 53.8 Ci/mmol) was obtained from

ABBREVIATIONS USED: CCL = certified cell line(s); L-DOPA = L-dihydroxyphenylalanine; α-MSH = α-melanocyte-stimulating hormone; [Nle^4, D-Phe^7]-α-MSH = 4-norleucine, 7-D-phenylalanine-α-MSH.

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[3] Department of Anatomy, Arizona Health Sciences Center, University of Arizona, Tucson, AZ 85724.

[4] *Address reprint requests to* Dr. Hadley.

[5] Department of Internal Medicine and Cancer Center, Arizona Health Sciences Center, University of Arizona.

[6] Department of Chemistry, University of Arizona, Tucson, AZ 85721.

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New England Nuclear (Boston, MA), and α-MSH was purchased from Sigma Chemical Co. (St. Louis, MO). [Nle^4, D-Phe^7]-α-MSH was synthesized as described previously (*11, 12*).

Stock cultures.—Cloudman S91 3960, CCL 53.1, is a moderately melanotic murine melanoma cell line that was obtained from the American Type Culture Collection Repository (Rockville, MD) and has been alternately maintained by limited subculturing as monolayers or as tumors in syngeneic DBA/2J mice. All experiments were performed on early passage (<10) cells to minimize phenotypic drift that is often observed in long-term cultures. Cells were grown in Ham's medium F10 supplemented with 10% horse serum and 2% fetal calf serum, both heat inactivated at 56°C for 30 minutes to prevent the degradation of α-MSH by serum enzymes. The medium was also supplemented with 100 U penicillin/ml and 100 μg streptomycin/ml. Cells were incubated at 37°C in a humid atmosphere of 5% CO_2 and 95% air.

Effects of melanotropins on monolayer cultures of S91 CCL cells.—The prolonged effects of α-MSH and [Nle^4, D-Phe^7]-α-MSH on tyrosinase activity were determined by seeding cells at 2×10^5 cells/25-cm^2 flask in 4 ml medium and treating them the next day with the melanotropins (10^{-7} *M*) for 24 hours. The melanotropins were removed from the culture flasks prior to determining their prolonged effects. The flasks were rinsed with melanotropin-free media several times until no melanotropic activity could be detected in the incubation media by the frog skin bioassay. This assay is sensitive to melanotropin concentrations at least tenfold lower than the minimal effective dose required to stimulate tyrosinase activity. The medium in all flasks was changed daily, and medium containing [^{3}H]tyrosine at 1 μCi/ml (i.e., 4 μCi/flask) was added to a set of control and experimental flasks 24 hours prior to data collection.

For experiments involving 6-day exposure to α-MSH or [Nle^4, D-Phe^7]-α-MSH, cells were seeded at 2×10^5 cells/25-cm^2 flask and were allowed to attach overnight. Then the medium was removed and replaced with fresh medium with the appropriate melanotropin added at 10^{-7} *M each day* thereafter. Twenty-four hours before data collection, the medium was replaced with medium containing [^{3}H]tyrosine (1 μCi/ml) as well as the melanotropin. At the end of each exposure period, the labeled medium from each flask was collected for assay of tyrosinase activity and the cells were harvested with EDTA-containing Tyrode's solution and counted with the aid of a hemacytometer.

Tyrosinase assay.—Tyrosinase activity was determined by using a modification of the charcoal absorption method of Pomerantz (*14*), which measures the amount of ^3H_2O released during the conversion of [^{3}H]tyrosine to L-DOPA, a reaction catalyzed by tyrosinase. Duplicate 1-ml samples were taken from each flask, and each sample was treated with 1 ml of activated charcoal (10% wt/vol in 0.2 *N* citric acid) and centrifuged. From each sample, 1 ml of the supernatant was passed through a Dowex 50 W column. The columns were then rinsed with 1 ml of 0.1 *N* citric acid, and the eluents were collected into scintillation vials that received 12 ml of scintillation fluid [toluene: Triton X-100, 2:1 vol/vol, plus 5.5 g 2,5-diphenyl-1,3-oxazole/liter (Beckman Instruments, Inc., Fullerton, CA)] and were counted in a Beckman LS-8000 scintillation spectrometer. Tyrosinase activity was expressed as cpm/10^6 cells and then as a percent of control enzyme activity.

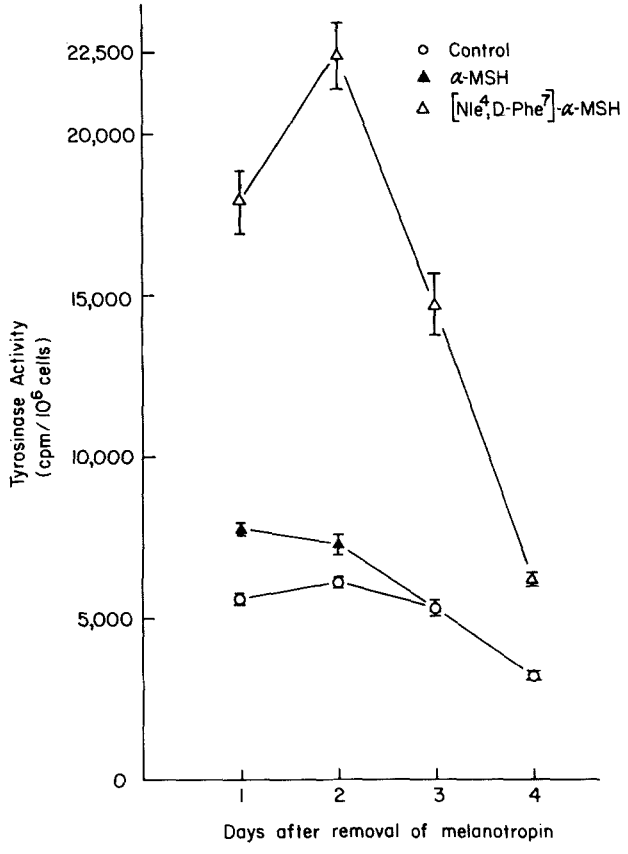
Effects of melanotropins on bilayer agar cultures.—The colony-forming assay may be the best in vitro test to determine cellular transformation (*15*). This assay has been used to study the chemosensitivity of human tumor cells to various drugs (*16, 17*) and to determine the effects of biologic modifiers on the clonogenic potential of melanoma cells (*18–20*). The effects of melanotropins on the ability of S91 melanoma cells to form colonies in soft agar were determined by plating a known number of cells into 30-mm diameter petri dishes in 1 ml of 0.3% agar in serum-supplemented Ham's medium F10 over an underlayer of 1 ml of the same medium with 0.5% agar. Cultures were continuously exposed to the appropriate melanotropin and were incubated for 10 days. It has been observed that the proliferation of S91 CCL cells is rapidly initiated and that about 40% of the cells plated divide once after 1 day in agar and continue to divide approximately every 32 hours (*21*). A sequential increase in the size of growth units is observed until proliferation stops 9 days after plating. After 10 days of incubation, colonies were counted and grouped into two categories according to diameter (42–104 μm and >104 μm) with the FAS II (Bausch and Lomb, Inc., Rochester, NY) optical image analyzer (*22*). By using a nomogram that we have established, ln (No. of cells/growth unit)=0.87–2.80 ln (cell diameter) + 2.38 ln (growth unit diameter), the number of cells per growth unit could be calculated (*23*). From this equation, it can be determined that CCL growth units greater in diameter than 42 μm are composed of at least 10 cells, which corresponds to three divisions, and that growth units greater than 104 μm in diameter contain at least 105 cells, the outcome of 6-cell divisions.

RESULTS

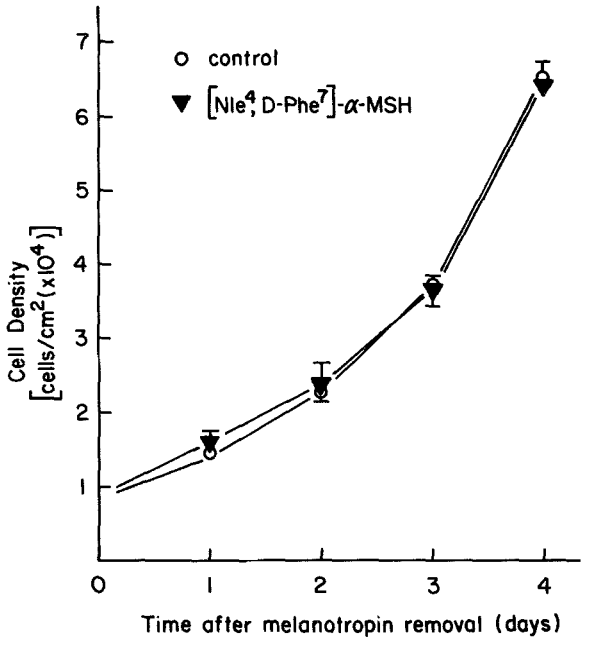
In the present study, the effects of α-MSH and the potent analogue [Nle^4, D-Phe^7]-α-MSH on melanogenesis and proliferation of Cloudman S91 cells were determined. [Nle^4, D-Phe^7]-α-MSH was shown to be about one hundredfold more potent than α-MSH in stimulating tyrosinase activity of CCL cells (*13*). This analogue also exhibited ultraprolonged activation of tyrosinase, as enzyme activity was maintained for days after initial contact of the cells with the peptide and after removal of the melanotropin from the culture medium. Upon treating CCL cells with 10^{-7} *M* [Nle^4, D-Phe^7]-α-MSH for 24 hours, we elicited a twofold increase in enzymatic activity above basal level 24 hours following removal of the melanotropin from the culture medium. Tyrosinase activity was further increased after 48 hours and remained remarkably elevated above control for 4 days in

the absence of [Nle^4, D-Phe^7]-α-MSH (text-fig. 1). Under these monolayer culture conditions and despite near-maximal enzymatic activation, the rate of proliferation of melanotropin-treated cells was not inhibited (or reduced) relative to the growth rate of untreated (control) cells (text-fig. 2).

The proliferation of CCL cells in monolayer culture was not retarded even with continuous and daily exposure to 10^{-7} *M* α-MSH or [Nle^4, D-Phe^7]-α-MSH. After 72 and 96 hours of exposure to either melanotropin, tyrosinase activity was maximally stimulated eightfold to ninefold above basal levels (text-fig. 3). At these exposure periods the melanotropins did not inhibit the proliferative rate of CCL cells. Even after 6 days of contact with either α-MSH or [Nle^4, D-Phe^7]-α-MSH, cellular growth in melanotropin-treated flasks was comparable to that observed in control cultures (text-fig. 4). As shown in text-figures 2 and 4, in the presence or absence of melanotropins these cells in monolayer have a doubling time of about 24 hours during log phase of growth (i.e., days 1–5).



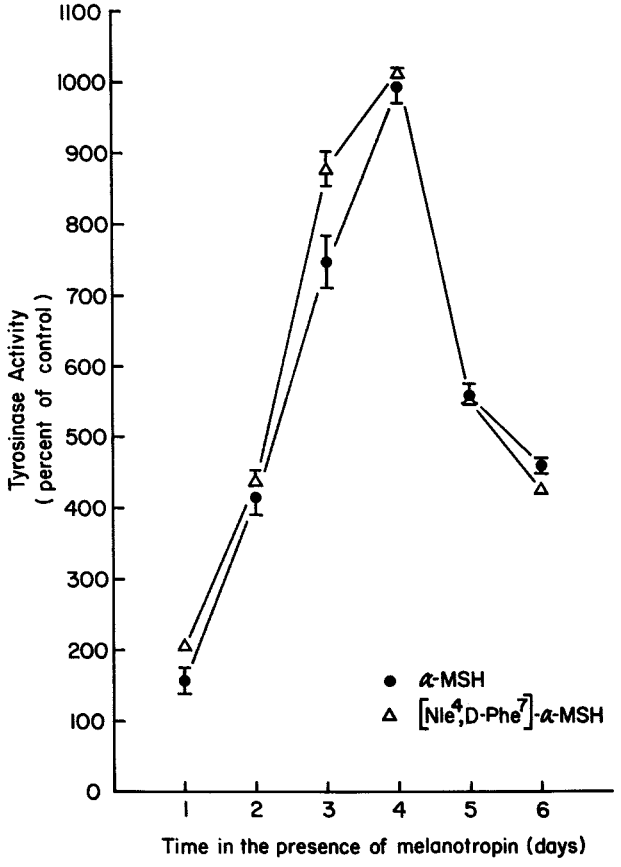
TEXT-FIGURE 1.—Prolonged tyrosinase stimulation after 24 hr of exposure to 10^{-7} *M* α-MSH or [Nle^4, D-Phe^7]-α-MSH. Cells were seeded into flasks and were treated the next day with 10^{-7} *M* α-MSH or [Nle^4, D-Phe^7]-α-MSH for 24 hr. Melanotropins were then removed by a series of rinses, and tyrosinase activity was determined 24 hr after melanotropin removal and daily thereafter for 3 more days. Tyrosinase activity is expressed as mean cpm/10^6 cells ± SE of 12 determinations for the control groups and of 6 determinations for the expt groups. *Vertical bar*, mean ± SE.



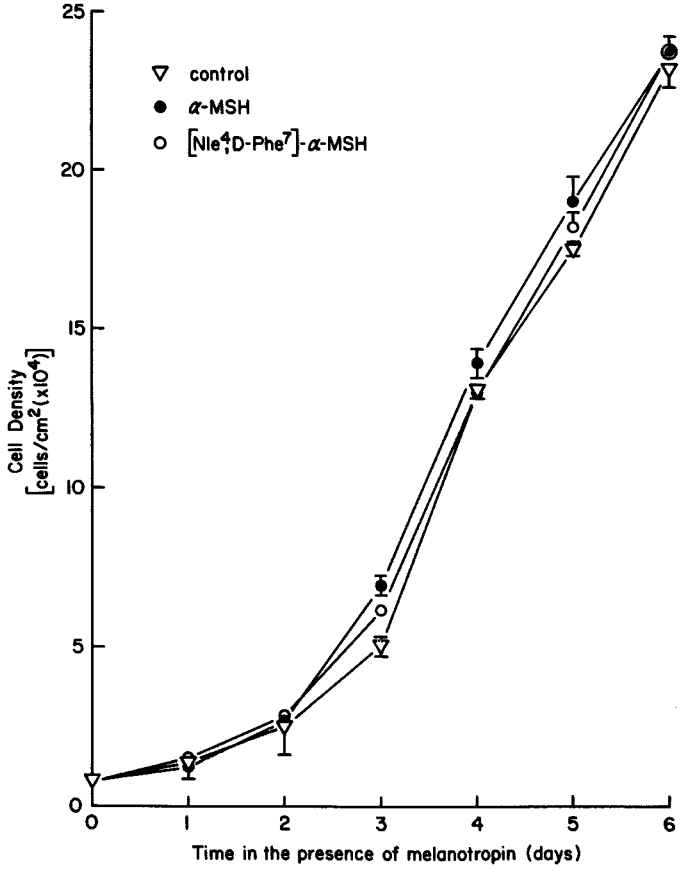
TEXT-FIGURE 2.—Cells were seeded at 0.8×10^4 cells/cm^2 and were treated the next day with 10^{-7} *M* [Nle^4, D-Phe^7]-α-MSH for 24 hr, after which time the melanotropin analogue was removed from the culture flasks. Cell density/cm^2 was determined by counting the number of cells/flask with the aid of a hemacytometer 24 hr after melanotropin removal and daily thereafter for 3 more days. Each value (*vertical bar*) represents the mean cell density/cm^2 ($\times10^4$) ± SE of 3 determinations (i.e., of triplicate flasks) for the control and the melanotropin-treated [Nle^4, D-Phe^7]-α-MSH flasks.

Further evidence for a lack of growth inhibition of S91 melanoma cells by melanotropins came from studies on the effects of α-MSH and [Nle^4, D-Phe^7]-α-MSH on the ability of CCL cells to clone and proliferate in soft agar. Cells were plated at 5,000 cells/dish and continuously treated with different concentrations of α-MSH and [Nle^4, D-Phe^7]-α-MSH. This resulted in the formation of extremely melanized colonies and in the stimulation of cloning efficiency as evidenced by an increase in the total number of colonies/dish (42–104 μm in diameter; table 1). The proliferative capacity of melanoma cells (i.e., formation of colonies with diameters greater than 104 μm) was not affected by α-MSH but was, however, inhibited by higher concentrations ($\geq10^{-8}$ *M*) of [Nle^4, D-Phe^7]-α-MSH (table 1).

Knowing that the size of growth units decreases with increased plating density (*17, 21*), we plated the cells at different cell densities and continuously treated them with 10^{-7} *M* α-MSH or [Nle^4, D-Phe^7]-α-MSH. At plating densities lower than 5,000 cells/dish, both melanotropins stimulated the cloning efficiency, as well as the proliferative capacity of the melanoma colony-forming cells (text-figs. 5, 6). The proliferative capacity of CCL cells was inhibited by melanotropins only when the initial plating density was equivalent to or exceeded 5,000 cells/dish, although at these densities the cloning efficiency was still increased (text-figs. 5, 6). These



TEXT-FIGURE 3.—Stimulation of tyrosinase activity by continuous exposure for 6 days to 10^{-7} *M* α-MSH or [Nle^4, D-Phe^7]-α-MSH. Cells were seeded into flasks and were treated the next day with 10^{-7} *M* α-MSH or [Nle^4, D-Phe^7]-α-MSH for 24 hr and daily thereafter for 6 days. Tyrosinase activity was determined daily and was first expressed as cpm/10^6 cells and then as percent of control (basal) activity. *Each value (vertical bar)* represents the mean percent of control ± SE of 6 determinations.



TEXT-FIGURE 4.—Cells were seeded at 0.8×10^4 cells/cm^2 and were treated the next day with 10^{-7} *M* α-MSH or [Nle^4, D-Phe^7]-α-MSH. Cells were maintained in the presence of the melanotropins for 6 consecutive days, with medium in each flask changed and with fresh melanotropins added daily. Number of cells/flask was determined daily with the aid of a hemacytometer. *Each value (vertical bar)* represents the mean cell density/cm^2 ± SE of 3 determinations (i.e., triplicate flasks).

results indicate that melanotropins are indeed stimulatory to the growth of S91 melanoma cells in soft agar under conditions that allow for optimal expression of their cloning and proliferative potentials.

DISCUSSION

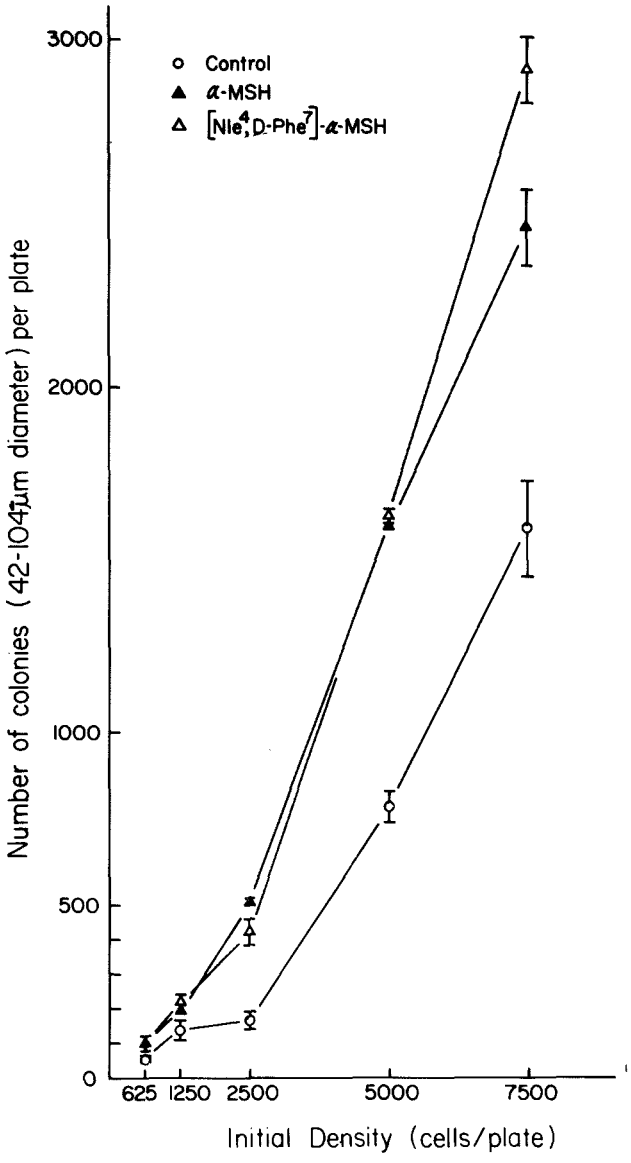
α-MSH-induced stimulation of tyrosinase activity, and thus melanogenesis, in murine melanoma cells has

TABLE 1.—*Effects of melanotropins on the clonogenic potential and the proliferative capacity of melanoma cells plated in soft agar*[a]

Concentration, *M*	No. of colonies with diameter 42-104 μm/dish[b]	No. of colonies with diameter >104 μm/dish[b]
Control	684±28	652±46
α-MSH, 10^{-9}	756±72	880±126
α-MSH, 10^{-8}	1,042±42	600±16
α-MSH, 10^{-7}	1,764±90	636±22
α-MSH, 10^{-6}	1,536±46	588±74
[Nle^4, D-Phe^7]-α-MSH, 10^{-11}	1,026±86	688±20
[Nle^4, D-Phe^7]-α-MSH, 10^{-9}	1,468±74	614±70
[Nle^4, D-Phe^7]-α-MSH, 10^{-8}	1,292±146	468±36
[Nle^4, D-Phe^7]-α-MSH, 10^{-7}	1,286±64	576±24
[Nle^4, D-Phe^7]-α-MSH, 10^{-6}	1,610±38	468±8

[a] Cells were plated at 5,000 cells/dish and were continuously exposed to the above concentrations of α-MSH or [Nle^4, D-Phe^7]-α-MSH. After 10 days of incubation, the colonies were counted and grouped into two categories according to diameter (42-104 and ≥104 μm).

[b] Each value represents the mean number of colonies/dish ± SE of 6 determinations for the control group and of 3 determinations for the melanotropin-treated groups.

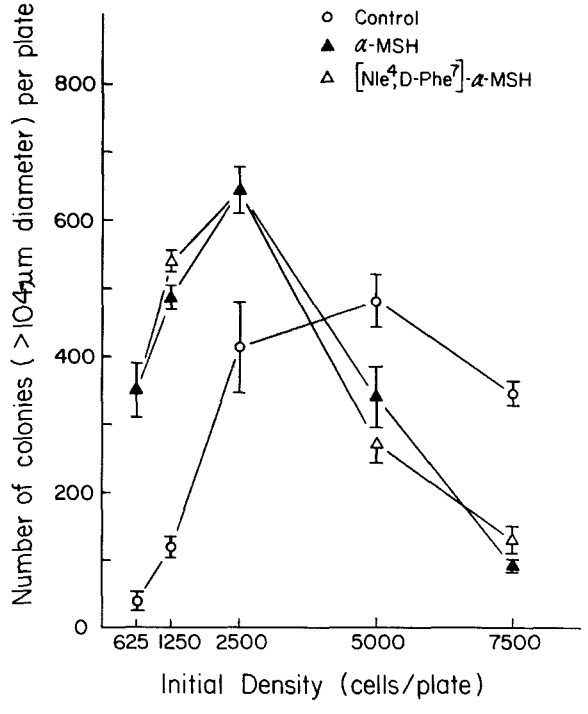


TEXT-FIGURE 5.—Number of colonies with diameters of 42-104 μm. Response of cells plated at different densities in soft agar to continuous exposure to melanotropins. Cells were plated in soft agar at 625, 1,250, 2,500, 5,000, and 7,500 cells/dish and were continuously exposed to 10^{-7} *M* α-MSH or [Nle^4, D-Phe^7]-α-MSH. After 10 days of incubation, the colonies in each dish were counted and grouped into two categories according to diameter. *Vertical bar*, mean ± SE.

often been correlated with growth inhibition (decreased proliferative capacity). However, in both Cloudman S91 and B16 melanoma, the inhibitory effect of α-MSH on proliferation has been observed in vitro, not in vivo (*3, 4, 8*). In S91 melanoma, α-MSH did not affect the rate of tumor growth in vivo (*3, 4*). In this tumor type, however, growth was reported to be inhibited when α-MSH treatment was accompanied by feeding tumor-bearing mice a tyrosine-rich diet, while melanoma tumors in animals maintained on a regular diet were not reduced in size with α-MSH treatment.

In the present experiments, the S91 melanoma CCL, which has a moderate basal tyrosinase activity and a doubling time of about 24 hours, responded to treatment with [Nle^4, D-Phe^7]-α-MSH with about a fourfold increase in tyrosinase activity, which was maintained for days after the melanotropin analogue was removed from the culture flasks (text-fig. 1). However, contrary to many previous reports, these effects were not accompanied by retardation of proliferation in monolayer culture (text-fig. 2). With continuous exposure to melanotropins, the rate of cell growth was not reduced even when cultures approached confluency (text-fig. 4), a result that contrasts with other investigators' findings relative to retardation of S91 melanoma cell proliferation by prolonged (≥3 days) melanotropin treatment (*5, 24*). Fuller and Lebowitz also noted that α-MSH failed to affect proliferation in this melanoma cell line (*25*). The lack of inhibition of cellular proliferation in our experiments might be attributed to daily supplementation of the cells with fresh media, thus replenishing essential nutrients and removing any accumulated metabolic by-products. Actually, it is possible that, under the present monolayer culture conditions, cell division is proceeding at such a rapid rate (once every 24 hr) that any positive effects that the melanotropins might have on growth go unnoticed.

In soft agar, both α-MSH and [Nle^4, D-Phe^7]-α-MSH stimulated colony formation and proliferation of cells plated at low densities (text-figs. 5, 6). Inhibition of proliferation of melanotropin-treated cells was only evident when cells were plated at very high densities (≥5,000 cells/dish) and treated with high concentrations of



TEXT-FIGURE 6.—Number of colonies with diameters >104 μm. *See* text-fig. 5 legend for test and methods used.

melanotropins ($\geq 10^{-8}$ *M;* text-figs. 5, 6; table 1), a condition that may result in cell crowding, depletion of nutrients, and accumulation of toxic metabolic byproducts. It has been frequently observed that when the initial plating density is very high, the number of cells per growth unit is decreased, probably because nutrients are exhausted by the large number of single cells plated, even before small growth units begin to form. We have shown this phenomenon to occur in several cell lines of human origin, including, among others, ovarian and multiple myeloma, as well as human and murine S91 melanoma (*17, 21*).

The nutritional requirements of CCL cells to clone in soft agar are more stringent than their requirements to grow in monolayer. In serum-free media, CCL cells grow in monolayer and yet fail to clone in agar; however, the addition of α-MSH allows the colony-forming cells to express their cloning and proliferative potential in the absence of serum (*26*).

Wick has reported that α-MSH does not retard the proliferation of S91 melanoma cells plated in soft agar and that growth inhibition was only evident when α-MSH and theophylline, an agent that mimics or augments α-MSH effects on melanogenesis, were concomitantly added (*27*). This observation supports the conclusion that α-MSH does not exert a direct inhibitory effect on the proliferative potential of melanoma cells.

It has been suggested that α-MSH stimulates the growth of S91 melanoma cells that have a low basal tyrosinase level but inhibits the growth of cells with a high basal tyrosinase activity (*9*). However, we did not find such a correlation since α-MSH stimulated the growth of CCL cells that have a moderate-to-low basal tyrosinase activity (text-fig. 1) as well as the growth of a highly melanotic S91 cell line that has a high basal tyrosinase level (*26*). It has also been reported that α-MSH stimulates the growth of slowly proliferating melanoma cells; yet it inhibits that of fast-growing cells (*9, 28*). Our data, however, indicate that melanotropins are stimulatory to the growth of CCL cells that divide rapidly in monolayer culture (once every 24 hr) and in soft agar (once every 32 hr) (*21*).

The effect of α-MSH on melanoma cell proliferation may be mediated through cAMP. Pawelek et al. have reported a biphasic effect of this cyclic nucleotide on S91 melanoma cells: stimulation of growth at low concentrations and inhibition at high concentrations (e.g., following treatment with α-MSH or prostaglandin E_1) (*28*). However, in this study, we have shown that neither α-MSH nor its potent analogue [Nle^4, D-Phe^7]-α-MSH, both of which increase adenylate cyclase activity (*29*) and thus intracellular cAMP levels, inhibited the growth of S91 CCL cells under conditions that allowed optimal phenotypic expression (stimulation of melanogenesis and colony formation). Also, melanotropins stimulate the proliferation of normal epidermal melanocytes in amphibians (*30*). If cAMP is indeed involved in melanotropin regulation of melanoma cell growth, then this cyclic nucleotide is a growth promoter for S91 melanoma cells, in particular, for the CCL utilized in this study. However, this does not seem to be the case, since it has previously been reported that prostaglandins, which also stimulate melanogenesis via a cAMP-mediated mechanism (*31*), inhibit the growth of S91 melanoma cells (*32*).

In a recent report, it was documented that B16 melanoma tumors enhance their own growth by secreting an insulin-like factor(s) (*33*). In another report, it was suggested that the ability of transformed cells to clone in agar was directly related to the amount of intercellular fibronectin (*34*). Therefore, melanotropins may induce colony formation by stimulating the secretion of melanoma growth-promoting factors, by increasing the production of fibronectin, or by some presently unknown mechanism. The present results might support a view that, except under conditions wherein nutritive substrates are limiting or where cytotoxic products accumulate, the normal physiologic action of melanotropins is to enhance melanogenesis, which may be coupled to cellular proliferation.

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