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α-Melanocyte stimulating hormone and its analogue Nle^{4}DPhe^{7}α-MSH affect morphology, tyrosinase activity and melanogenesis in cultured human melanocytes

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SUMMARY

Although melanocyte stimulating hormone (MSH) peptides are known to stimulate pigmentation in man, previous reports suggest that human melanocytes are relatively unresponsive to these peptides in vitro. This may be related to the conditions under which the melanocytes were cultured. Thus, we have re-investigated the in vitro effects of MSH peptides using human melanocytes cultured in the absence of artificial mitogens. Human melanocytes were incubated with α-MSH or its potent analogue Nle^{4}DPhe^{7}α-MSH for 3 days. After 18 hours, melanocyte morphology had evolved from mainly bipolar to dendritic in approximately 66% of cultures. Nle^{4}DPhe^{7}α-MSH produced dose-related increases in both tyrosinase activity and melanin content although the degree of response was variable and tyrosinase activity was the relatively more responsive to the peptide. Similar results were obtained with α-MSH, but, although the effect on melanin content was similar to that of Nle^{4}DPhe^{7}α-MSH, the effect on tyrosinase activity was less marked. The preliminary EC_{50} values for the actions of the MSH peptides suggest that they may be equipotent in their actions on human melanocytes. In addition, we have demonstrated that the common melanocyte mitogens 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and cholera toxin affect basal melanogenesis and modulate the effects of the MSH peptides. However, not all melanocyte cultures showed melanogenic responses to the MSH peptides. Ability to respond was unrelated to basal levels of tyrosinase activity or melanin content. In at least some cultures, morphological and melanogenic responses appear to be independent of one another. For reasons that are unknown, a small number of human melanocyte cultures show neither morphological nor melanogenic responses to the MSH peptides. Neither peptide had any effect on cell numbers, regardless of whether the cultures were able to respond morphologically or melanogenically. The present findings are particularly interesting in light of recent developments in the molecular biology of the MSH receptor.

Key words: melanocyte, MSH, morphology, tyrosinase, melanin

INTRODUCTION

Although it is recognised that melanocyte stimulating hormone (MSH) stimulates melanogenesis in murine melanocytes (Geschwind et al., 1972; Burchill et al., 1986) and melanoma cells (Wong and Pawelek, 1973; Hearing and Jimenez, 1987), there has been much debate as to whether the peptide has a pigmentary function in man. Lerner and McGuire (1961, 1964) were the first to show that MSH peptides increase skin darkening in human subjects and this has been confirmed more recently with Nle^{4}DPhe^{7}α-MSH, a potent analogue of α-MSH (Levine et al., 1991). There are, however, several reports that MSH peptides fail to stimulate tyrosinase activity, the rate-limiting enzyme in melanogenesis, in cultured human melanocytes (Halaban et al., 1983; Ranson et al., 1988) and, accordingly, have little or no effect on melanin content (Wilkins et al., 1982; Friedmann et al., 1990).

The reason for this lack of responsiveness of human melanocytes to MSH in vitro is not clear but it cannot be explained by a failure to express MSH receptors, since we have recently demonstrated the presence of specific α-MSH binding sites on cultured human melanocytes (Donatien et al., 1992) while Halaban et al. (1983) have demonstrated the specific binding of β-MSH to these cells. The MSH receptor has recently been cloned and has been shown to be expressed in human melanoma and melanocytes (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992).

The poor response to MSH in vitro could be a consequence of the way in which the melanocytes were cultured and, in particular, the use of artificial mitogens that are included to promote melanocyte growth. There is evidence that one of these mitogens, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), reduces the melanogenic effect of MSH on B16 murine melanoma cells (Mufson et al., 1979). Cholera toxin has a similar effect on the responsiveness of S91 Cloudman melanoma cells to α-MSH (O'Keefe and Cuatrecasas, 1974). We have shown that TPA and cholera toxin affect the binding of Nle^{4}DPhe^{7}α-MSH to human melanocytes (Donatien et al.,

1992). As a consequence, we routinely culture human melanocytes in the absence of TPA, cholera toxin and other artificial mitogens (Donatien et al., 1993a; Hewitt et al., unpublished data). Human melanocytes grown in this way respond to MSH, showing changes in attachment to extracellular matrix proteins (Hunt et al., 1991, 1993a).

In this study, we have examined the question of whether MSH peptides can stimulate tyrosinase activity and melanogenesis in human melanocytes cultured in the absence of artificial mitogens.

MATERIALS AND METHODS

Human melanocytes were obtained from 21 foreskins, three samples of breast skin and one sample of bat ear correction skin. All skin samples, with the possible exception of one foreskin and one sample of breast skin (Asian donors) were from Caucasian subjects. Donor age ranged from 1-25 years. The method of culturing melanocytes in the absence of artificial mitogens was based on that previously described by Donatien et al. (1993a). The melanocytes were initially grown in co-culture with keratinocytes from the same donor in MCDB 153 medium (Sigma) adjusted to 0.3 mM tyrosine and 0.5 mM Ca^{2+} and supplemented with bovine hypothalamus extract, selected amino acids and 0.5-1% foetal calf serum (Hewitt et al., unpublished data). Once the majority of the cells had adhered to the flask, the serum was omitted to reduce the possibility of fibroblast contamination. After 1-2 weeks, the melanocytes were detached and separated from the keratinocytes by differential trypsinisation and grown as pure cultures in MCDB 153 medium as above. Serum (1%) was included in the medium for 24 hours after subculturing to promote attachment to the substratum.

To investigate the effects of the MSH peptides, 2.5×10^5 melanocytes were seeded into each well of a 6-well tissue culture plate and allowed to attach overnight in the presence of 1% serum. Fresh medium (without serum) containing Nle^{4}DPhe^{7}α-MSH (10^{-12}-10^{-7} M; a gift from Professor M. Hadley, University of Arizona), or α-MSH (10^{-10} to 10^{-7} M) (Ciba-Geigy) was added every 2-3 days.

Tyrosinase activity was estimated by measuring the rate of oxidation of L-DOPA (Takahashi and Parsons, 1992). Cells (2×10^5) were suspended in 50 μl cold M/15 phosphate buffer, pH 6.8, containing 1% (w/v) Triton X-100. After pipetting and vortexing to lyse the cells, the extract was clarified by centrifugation at 10000 rpm for 5 minutes. L-DOPA (2 mg/ml) was prepared in phosphate buffer as above without Triton X-100 (assay buffer). Samples (40 μl) of cell lysate were added to the wells of a 96-well plate and the assay was started by the addition of 100 μl L-DOPA solution at 37°C. Control wells contained 40 μl lysis buffer or boiled cell lysate. Absorbance at 490 nm was read every minute for at least 20 minutes at 37°C on a microplate reader fitted with a temperature control mechanism (Dynatech Laboratories). One unit of tyrosinase activity was arbitarily defined as a rate of increase of 1 absorbance unit per h per 10^6 cells in the initial linear region of a plot of absorbance against time. There was no increase in absorbance in the control wells.

For melanin determination, 2×10^5 cells were solubilised in 100 μl 1 M NaOH and diluted with 400 μl distilled water. Absorbance at 475 nm was compared with a standard curve of synthetic melanin (Sigma) prepared in a final NaOH concentration of 0.2 M.

To investigate the effects of TPA and cholera toxin, cells were cultured as described previously but, 24 hours after seeding into wells, TPA (1.6×10^{-8} M) or cholera toxin (1×10^{-9} M) was added in fresh medium, control wells receiving fresh medium only. Culture was continued in the presence of these mitogens for a further 5 days, Nle^{4}DPhe^{7}α-MSH (10^{-9} M) being added on day 3. Cells were then harvested and assayed for melanin content and tyrosinase activity as described previously.

RESULTS

The effects of MSH peptides on morphology

Before the addition of the MSH peptides, human melanocytes cultured in our system exhibited a mainly bipolar morphology with occasional tripolar cells. Nle^{4}DPhe^{7}α-MSH induced melanocyte dendricity, this effect occurring at a concentration of 10^{-11} M, with more marked effects at concentrations of 10^{-10} M and 10^{-9} M (Fig. 1). α-MSH had a similar effect on morphology (data not shown). However, 9 out of a total of 25 cultures (36%), all from foreskins from individuals aged 2-9 years, failed to show any morphological responses to the MSH peptides.

The effects of MSH peptides on tyrosinase activity and melanin content

As expected, there was wide variation in the basal levels of melanin in the cultured human melanocytes, ranging from 2.3 to 44.8 μg/10^6 cells with a mean and s.e.m. of 16.3 ± 2.55 μg/10^6 cells (21 determinations from 20 different cultures). Likewise, the basal levels of tyrosinase activity varied between cultures, ranging from 0.16 to 7.67 units with a mean and s.e.m. of 2.13 ± 0.52 units (16 determinations from 11 different cultures). To allow comparisons between cultures, tyrosinase activity and melanin content after MSH were expressed as percentages of the basal levels for that culture.

Like the effects on morphology, the effects of the MSH peptides on melanogenesis were extremely variable in that while some cultures were very responsive, we were unable to demonstrate any response in others. Cultures segregated into two groups, responders and non-responders: those showing less than a projected arbitary 20% increase in tyrosinase activity or melanin content at MSH peptide concentrations of 10^{-9} M were deemed to be non-responders.

The pooled data for responses to Nle^{4}DPhe^{7}α-MSH and α-MSH are shown in Tables 1 and 2. The apparent discrepancies in culture numbers are due to the fact that, because of the difficulty in obtaining large numbers of melanocytes from all the skin samples, it was not possible to measure both tyrosinase activity and melanin content, or to perform full dose-response experiments, on every culture.

Nle^{4}DPhe^{7}α-MSH had little effect on final cell numbers after a 3 day exposure to the peptide. This lack of effect was consistent regardless of whether the cultures were able to respond morphologically or melanogenically: thus, pooled cell number data for all the cultures are shown in Table 1. However, Nle^{4}DPhe^{7}α-MSH produced a dose-related increase in tyrosinase activity (Table 1 and Fig. 2) and, at 10^{-9} M, the concentration studied most frequently, tyrosinase activity was significantly greater than basal levels: $P<0.01$ by paired *t*-test on raw data. No culture failed to show an increase in tyrosinase activity in response to Nle^{4}DPhe^{7}α-MSH. There were also dose-related increases in melanin content in the majority of cultures (Table 1 and Fig. 2). Combining all the cultures, melanin content at 10^{-9} M was significantly greater than control levels: $P<0.001$ by paired *t*-test on raw data. While the increases in tyrosinase activity and melanin content were comparable at low concentrations of Nle^{4}DPhe^{7}α-MSH, above a concentration of 10^{-10} M tyrosinase activity was stimulated relatively more than melanin content. This effect was particularly marked in the culture for which data are shown in Fig. 2.

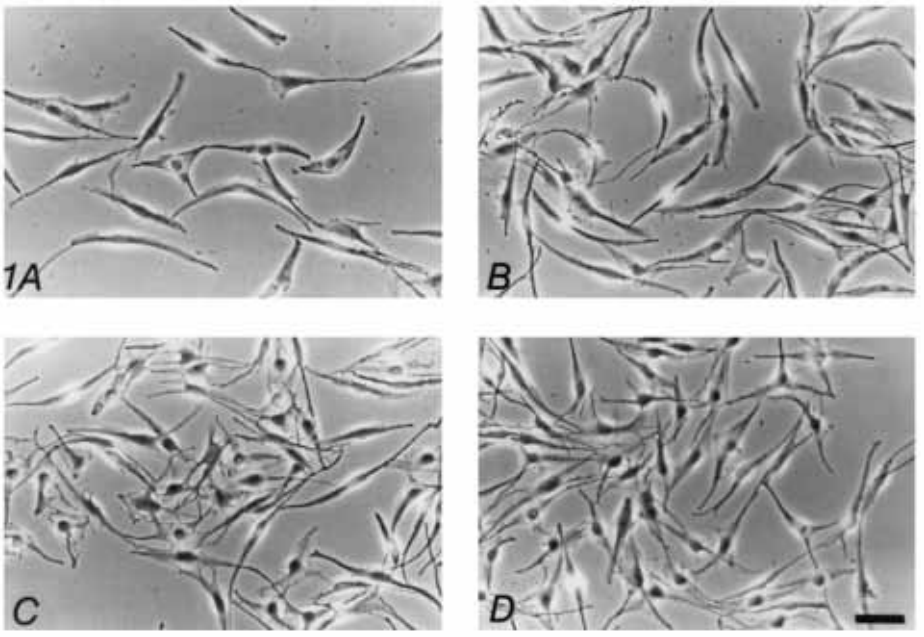

Fig. 1. The morphology of human melanocyte cultures 24 hours after the addition of Nle^{4}DPhe^{7}α-MSH. Bar, 20 μm. (A) Untreated control, (B) 10^{-11} M, (C) 10^{-10} M, (D) 10^{-9} M. There was no effect on cell numbers after a 72 hour exposure to Nle^{4}DPhe^{7}α-MSH.

Table 1. The effect of Nle^{4}DPhe^{7}α-MSH on cell number, tyrosinase activity and melanin content of cultured human melanocytes

Concn (log M)	Cell number	Tyrosinase activity		Melanin content	
		R	NR	R	NR
−12	105 (2)	89 (2)	nf	99 (2)	nf
	N=1	*N*=1		*N*=1	
−11	96±8 (2)	98±4 (4)	nf	107±6 (4)	113 (1)
	N=4	*N*=3		*N*=2	*N*=1
−10	101±6 (9)	131±6 (4)	nf	136±7 (5)	107 (1)
	N=7	*N*=4		*N*=3	*N*=1
−9	106±3 (30)	197±20 (12)	nf	154±7 (16)	104±5 (8)
	N=20	*N*=9		*N*=11	*N*=6
−8	93±5 (8)	278±43 (3)	nf	163±6 (6)	nf
	N=7	*N*=2		*N*=5	
−7	116±10 (4)	232 (2)	nf	122±2 (6)	nf
	N=3	*N*=1		*N*=3	

Values represent pooled data with number of determinations in parenthesis. *N*, number of different cultures. Results are given as percentages of untreated controls and expressed as mean ± s.e.m. R, responders; NR, non-responders; nf, none found.

Table 2. The effects of α-MSH on cell number, tyrosinase activity and melanin content of cultured human melanocytes

Concn (log M)	Cell number	Tyrosinase activity		Melanin content	
		R	NR	R	NR
−11	82 (2)	98 (1)	nf	95 (1)	nf
	N=1	*N*=1		*N*=1	
−10	109±8 (7)	133 (1)	102 (2)	123 (2)	84 (2)
	N=5	*N*=1	*N*=1	*N*=1	*N*=2
−9	104±5 (10)	155±2 (3)	124 (1)	136±14 (5)	119 (2)
	N=10	*N*=3	*N*=1	*N*=4	*N*=2
−8	111±7 (8)	168 (1)	100 (2)	138±9 (2)	100 (2)
	N=5	*N*=1	*N*=1	*N*=1	*N*=2
−7	104±9 (6)	130 (1)	81 (2)	139±9 (4)	nf
	N=3	*N*=1	*N*=1	*N*=3	

Values represented pooled data with number of determinations in parentheses. *N*, number of different cultures. Results are given as percentages of untreated controls and expressed as mean ± s.e.m. R, responders; NR, non-responders; nf, none found.

However, in some cultures, Nle^{4}DPhe^{7}α-MSH had no effect on melanin content. All six cultures that were unresponsive were derived from foreskin, age range 1-9 years. Basal melanin levels were not significantly different between the two groups, by assessing foreskin cultures only.

Preliminary data from similar experiments with α-MSH are shown in Table 2. α-MSH had little effect on final cell numbers after 3 days. Again, the lack of effect of α-MSH on cell number was consistent regardless of ability to respond to the peptide in other ways, so pooled data for cell number are shown in Table 2. There were dose-related increases in tyrosinase activity in the majority of cultures although the increases were relatively smaller than those seen with Nle^{4}DPhe^{7}α-MSH. However, there was no significant difference in basal tyrosinase activity between the cultures treated with Nle^{4}DPhe^{7}α-MSH and those treated with α-MSH. There were also dose-related increases in melanin content in the majority of cultures although, in contrast to tyrosinase activity, the increases were similar to those seen with Nle^{4}DPhe^{7}α-MSH. There was no significant difference in basal melanin levels between the cultures treated with Nle^{4}DPhe^{7}α-MSH and those treated with α-MSH. All of the cultures that failed to respond to α-MSH were derived from foreskin, donor age range 3-9 years. Although basal melanin levels were equal in responders and non-responders, the single culture that failed to show an increase in tyrosinase activity had the highest basal tyrosinase level of all the cultures in which activity was measured.

From the dose-response data, we have made estimations of the concentrations of Nle^{4}DPhe^{7}α-MSH that induce 50% of maximal responses (EC_{50} values). For the stimulation of melanin content, this was 0.08 nM and for tyrosinase activity 0.8 nM. These estimates were based on pooled data; however,

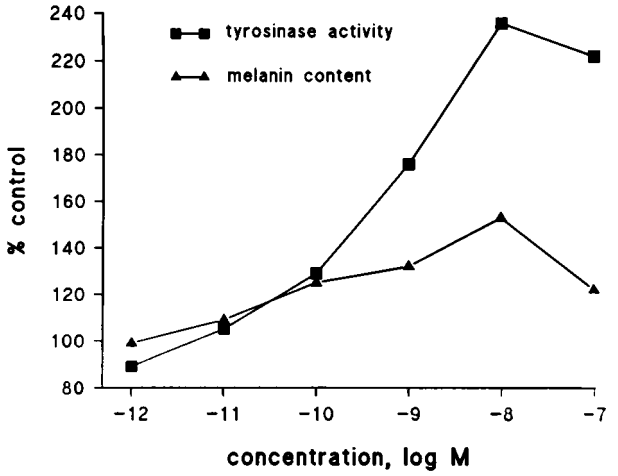

Fig. 2. Dose-related increases in tyrosinase activity and melanin content of human melanocytes in response to Nle^{4}DPhe^{7}α-MSH. Melanocytes derived from foreskin of a 2-year-old child. Data are means of duplicate determinations.

the values for pooled data were very similar to those calculated from the individual culture data shown in Fig. 2. Our preliminary estimates of the EC_{50} values for the responses to α-MSH suggest a value of 0.08 nM for the stimulation of melanin content and of <0.3 nM for tyrosinase activity.

Because not all cultures underwent morphological changes or increases in tyrosinase activity or melanin content in response to the MSH peptides, we investigated the possibility that the responses may be linked. Data are summarised in Table 3. Thus, in some cultures, morphological changes and increases in melanogenesis appear to be independent of one another. Furthermore, a small number of cultures are completely unresponsive to the MSH peptides.

The effects of TPA and cholera toxin on responses to the MSH peptides

The effects of TPA and cholera toxin are shown in Table 4.

As already demonstrated, Nle^{4}DPhe^{7}α-MSH increased both melanin content and tyrosinase activity although the effects in cultures 2 and 3 were relatively small. Prolonged treatment with 10^{-7} M α-MSH also increased melanin content.

TPA altered melanocyte morphology, causing the cells to become elongated, and also increased both melanin content and tyrosinase activity. However, when the cells were cultured in the presence of both TPA and the MSH peptides, the stimulatory effects of each on melanin content and tyrosinase activity were blocked although the MSH peptides still induced some degree of dendricity in the presence of TPA (data not shown).

Cholera toxin produced mainly dendritic melanocytes with occasional large, flattened cells. Melanin content was increased in one of the cultures and there was a corresponding increase in tyrosinase activity. In the presence of cholera toxin, Nle^{4}DPhe^{7}α-MSH stimulated both melanin content and tyrosinase activity although the increases were relatively smaller than those seen in the absence of cholera toxin. Because cholera toxin itself induced dendricity, it was difficult to assess the effect of Nle^{4}DPhe^{7}α-MSH on morphology in the presence of the artificial mitogen. However, in the presence of both compounds, melanocyte dendricity was maintained and perhaps slightly enhanced (data not shown).

Table 3. The relationship between morphological and melanogenic response to the MSH peptides

	Number of cultures		
	Total	Morphological responders	Morphological non-responders
Nle^{4}DPhe^{7}α-MSH			
Tyrosinase activity			
Responders	10	7	3
Non-responders	0	0	0
Melanin content			
Responders	12	10	2
Non-responders	6	4	2
α-MSH			
Tyrosinase activity			
Responders	4	3	1
Non-responders	1	0	1
Melanin content			
Responders	4	4	0
Non-responders	2	0	2

DISCUSSION

We have previously demonstrated the presence of MSH receptors on human melanocytes cultured without artificial mitogens (Donatien et al., 1992). The present results show that MSH peptides affect melanocyte morphology and increase tyrosinase activity and melanin content, confirming that these receptors are functional and showing, for the first time, that their activation induces melanogenesis.

In contrast to previous studies in which human melanocytes were obtained from neonates (Wilkins et al., 1982; Halaban et al., 1983; Ranson et al., 1988), we have used melanocytes from children and young adults. Our ability to demonstrate responsiveness to MSH peptides is probably not related to the use of melanocytes from older donors, since Friedmann et al. (1990) have reported that melanocytes obtained from the foreskins of children aged up to 7 years are relatively unresponsive. We suggest that the increased responsiveness observed in the present study is related to the conditions under which the melanocytes were cultured. In agreement with the results for B16 cells (Mufson et al., 1979), we have demonstrated that TPA blocks the effects of MSH peptides on melanogenesis in human melanocytes. This effect appears to be a negation of the stimulation of tyrosinase activity and may be mediated at the MSH receptor level, since we have shown that TPA reduces the binding of Nle^{4}DPhe^{7}α-MSH to human melanocytes (Donatien et al., 1992). However, this does not explain why the stimulatory effects of TPA itself on melanogenesis appear to be decreased in the presence of MSH peptides. Our observation that TPA increases basal tyrosinase activity and melanin content is supported by previous data. Halaban et al. (1983) have also demonstrated that long-term culture in the presence of TPA causes a significant increase in basal tyrosinase activity in human melanocytes and, furthermore, they have found that α-MSH has no effect on tyrosinase activity under these conditions. Our finding that cholera toxin increases both tyrosinase activity and melanin content may explain why other workers

Table 4. The effects of TPA and cholera toxin on the response to MSH peptides

	Melanin content (%)				Tyrosinase activity (%)
Culture no.:	1	2	3	4	5
Control	100	100	100	100	100
+ Nle^{4}DPhe^{7}α-MSH*	nd	117	117	171	183
+ α-MSH†	238	nd	nd	nd	nd
TPA‡	227	126	132	152	201
+ Nle^{4}DPhe^{7}α-MSH*	nd	102	91	114	127
+ α-MSH†	11	nd	nd	nd	nd
Cholera toxin§	nd	105	nd	126	259
+ Nle^{4}DPhe^{7}α-MSH*	nd	nd	nd	138	302

Values represent single determinations. Results are given as percentages of untreated controls. α-MSH and artificial mitogens added on day 1, melanin content determined on day 7. Experiments performed as described in Materials and Methods. *1×10^{-9} M, †1×10^{-7} M, ‡1.6×10^{-8} M, §1×10^{-9} M. nd, not determined.

who routinely culture human melanocytes in the presence of this artificial mitogen have demonstrated only small responses to MSH (Ranson et al., 1988; Friedmann et al., 1990). Since basal levels of tyrosinase activity and melanin are increased by cholera toxin, the additional effect of MSH appears to be restricted. However, in contrast, Halaban et al. (1983) have reported that the failure of human melanocytes to respond to MSH is unrelated to the presence of TPA and cholera toxin in the medium.

The MSH peptides had no effect on final cell numbers in this study. This confirms previous reports (Halaban et al., 1983; Eisinger et al., 1985; Ranson et al., 1988) that α-MSH is not a mitogen for cultured human melanocytes, but contrasts with the work of Herlyn et al. (1988), which showed that α-MSH has a significant effect on melanocyte number.

Nle^{4}DPhe^{7}α-MSH, in addition to increasing skin pigmentation in man (Levine et al., 1991), has been reported to be more potent than α-MSH in stimulating tyrosinase activity in murine melanoma cells (Hadley et al., 1985). In contrast, our data suggest that the two peptides may be equipotent in their effects on human melanocytes. Our preliminary EC_{50} values for the effects of the peptides on melanin content are not dissimilar to that for the α-MSH-induced increase in adenylyl cyclase activity in transfected cells expressing the human MSH receptor, a system in which α-MSH and Nle^{4}DPhe^{7}α-MSH are equipotent (K. G. Mountjoy and R. D. Cone, personal communication).

In terms of maximum response, Nle^{4}DPhe^{7}α-MSH appears to be more effective than α-MSH in stimulating tyrosinase activity. In addition, at the higher concentrations of both peptides, tyrosinase activity was increased relatively more than was melanin content. Naeyaert et al. (1991) have reported a lack of correlation between tyrosinase mRNA and pigment levels in cultured human melanocytes. Donatien et al. (1993b), while showing that Nle^{4}DPhe^{7}α-MSH increases tyrosinase mRNA, noted that the associated increase in melanin content of the cells was less than would be predicted from the tyrosinase mRNA levels. The apparent discrepancy between the increases in melanin content and tyrosinase activity seen in this study may be a consequence of the method of assay of the latter. Iozumi et al. (1993) have reported that tyrosinase activity in human melanocytes measured in situ is less than that measured in cell homogenates. While there was close agreement between in situ tyrosinase activity and melanin content, the correlation when tyrosinase activity was measured in cell homogenates was less good. These workers suggest that this may be related to the presence of a catalytically less active form of the enzyme that is released from the melanosome in the homogenate assay and then becomes activated. In addition, Nle^{4}DPhe^{7}α-MSH has been shown to induce prolonged stimulation of tyrosinase activity in murine melanoma cells (Hadley et al., 1985). These findings may account for the pattern of tyrosinase activity observed in the present study. While the MSH peptides have been shown to act at the transcriptional level (Donatien et al., 1993b), there is also the possibility that they may increase de novo synthesis of tyrosinase and, in addition, act at the post-translational level to activate the enzyme as demonstrated in the hair follicular melanocytes of mice (Burchill et al., 1993).

While the projected EC_{50} values for the effects of α-MSH on melanin content and tyrosinase activity may be similar, those for the effects of Nle^{4}DPhe^{7}α-MSH show some discrepancy. The reason for this is unclear but may be related to the tyrosinase assay method as discussed previously. Furthermore, the stimulation of melanin content is likely to be the net result of effects on other enzymes in addition to tyrosinase. However, the increased stimulation of tyrosinase activity relative to melanin content is more marked for Nle^{4}DPhe^{7}α-MSH than α-MSH and may represent different effects of the two peptides on the enzyme.

Although tyrosinase is the rate-limiting enzyme, tyrosinase-related proteins (TRPs) may also be important in regulating melanogenesis. We have recently demonstrated that α-MSH and Nle^{4}DPhe^{7}α-MSH increase the expression of TRP-1 in cultured human melanocytes. However, while some cultures also responded to the MSH peptides with increases in TRP-2 expression, others were unaffected (Tobin et al., 1993; Hewitt et al., unpublished data).

Within the cultures that showed increased melanin content after treatment with MSH peptides, the degree of response found in the present study was only slightly greater than that reported by Friedmann et al. (1990). However, the latter workers used higher concentrations of α-MSH for a longer period. In the experiment in which we used a comparable concentration of α-MSH for a similar length of time, we observed an increase in melanin content approximately fourfold greater than that described by these workers. While Nle^{4}DPhe^{7}α-MSH and α-MSH at best cause a mean increase in melanin content and tyrosinase activity of 50-180%, these effects are much less than those previously reported for murine melanoma cells (Hadley et al., 1985). Although murine melanoma cells have approximately 10-fold more α-MSH binding sites (Donatien et al., 1992), the EC_{50} values for the effects of the MSH peptides on melanin content and tyrosinase actvity are severalfold higher than those estimated for human melanocytes (Hunt, unpublished data). However, it is of interest that while ACTH 1-39 is less effective than α-MSH in stimulating melanogenesis in B16 murine melanoma cells (Lunec et al., 1992) we have recently shown it to be 10 times more potent in human melanocytes (Hunt et al., 1993b). Thus melanogenesis may be controlled differently in the two cell types.

In the hair follicular melanocytes of viable yellow mice, α-MSH increases the levels of the black-brown eumelanin

relative to the levels of the red-yellow phaeomelanin (Burchill et al., 1986). We have recently shown that both eumelanin and phaeomelanin are present in human epidermis (Thody et al., 1991). If MSH acts in a similar manner in human skin, it could explain why we have not observed large increases in the melanin content of cultured human melanocytes in response to MSH peptides. The main action of MSH may be to induce subtle changes in the ratio of the two types of melanin rather than increase total melanin content.

In addition to influencing melanogenesis, the MSH peptides also induced a more dendritic morphology in the majority of human melanocyte cultures. While other workers have demonstrated the effects of MSH peptides on the morphology of murine melanocytes and melanoma cells (Hirobe, 1978; Preston et al., 1987), we believe that this is the first time such an effect has been observed in cultured human melanocytes (see Yaar and Gilchrest, 1991, for review). A fundamental difference between the effects of Nle^{4}DPhe^{7}α-MSH on the morphology of murine melanoma cells and human melanocytes is the time taken to elicit a response. Preston et al. (1987) observed a response within 10 minutes of the addition of the peptide to Cloudman S91 melanoma cells while we have noted the induction of dendricity within 1 hour in B16 F1 cells (G. Hunt, unpublished observation). Human melanocytes, on the other hand, did not show an immediate response: morphological changes were only noticeable after overnight incubation with the peptides. Furthermore, in contrast to Cloudman S91 cells, dendricity in human melanocytes and B16 cells was stable until the termination of the experiment 3 days later.

It is well accepted that MSH acts via the adenylate cyclase/cAMP second messenger system and we have shown that cholera toxin, which acts via this system, also increases melanocyte dendricity. Although cAMP itself induces human melanocyte dendricity (Hewitt et al., unpublished data), this effect is not specific to melanocytes. Indeed, the addition of cAMP to a variety of cultured cell types induces similar morphological responses (see Edwards et al., 1993, for review). Keratinocyte-conditioned medium has been reported to induce dendricity in human melanocytes and B16 melanoma cells (Gordon et al., 1989; Lacour et al., 1992). All these changes in cell shape have been shown to involve cytoskeletal responses and we speculate that the effect of MSH on human melanocyte morphology may be via a similar mechanism (Hunt et al., 1993a). Although increases in intracellular cAMP are believed to be important in mediating melanogenesis, there may also be a role for protein kinase C (Gordon and Gilchrest, 1989, 1990; Pawelek, 1990; Buffey et al., 1992). Furthermore, protein kinase C may also be involved in regulating dendricity (Preston et al., 1987).

At present, there is no adequate explanation as to why a small number of our human melanocyte cultures fail to show any response to MSH peptides. It appears to be unrelated to basal levels of tyrosinase activity and melanin content. We have previously demonstrated that basal levels of tyrosinase synthesis in human skin vary according to skin type although α-MSH does not appear to stimulate tyrosinase synthesis in human skin (Burchill et al., 1990). Iwata et al. (1990), using human foreskins in organ culture, noted that Nle^{4}DPhe^{7}α-MSH only stimulated tyrosinase activity in 50% of cultures and the ability to respond was unrelated to whether the skin was from black or white donors. While Levine et al. (1991) have shown that the degree of skin darkening in response to Nle^{4}DPhe^{7}α-MSH varies with skin site, no conclusions regarding the influence of skin site or donor age on responsiveness of cultured melanocytes can be drawn from the present study. The observation that all non-responding cultures were derived from the foreskins of young children is more likely to be a consequence of the fact that over 80% of skin samples obtained in this study were from this site and age group rather than an effect of skin site or donor age on responsiveness.

The failure to respond to MSH peptides may be related to a lack of functional MSH receptors or insufficient numbers of receptors to elicit a measurable response, since we have shown that the average number of receptors/melanocyte is variable (Donatien et al., 1992). On the other hand, there may be a defect in the second messenger system. Recent work has shown that mutations in the mouse *extension* locus produce either a non-functional receptor, a receptor that is constitutively activated or a receptor that hyperactivates its effector (Robbins et al., 1993). The murine *agouti* locus appears to encode an antagonist that prevents α-MSH binding to its receptor (see Jackson, 1993, for review). Although these findings have not yet been applied to man, they may prove to be important in explaining human pigmentation and responsiveness to MSH.

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