Brazilian Journal of Medical and Biological Research (1998) 31: 1557-1564
ISSN 0100-879X

Topical application of a melanotropin analogue to vulgar vitiligo dermo-epidermal minigrafts

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Abstract

Human subjects with active vulgar vitiligo do not respond well to autologous dermo-epidermal minigrafting. Eighteen subjects were treated with the α-melanocyte-stimulating hormone (α-MSH) synthetic analogue [Nle^4, *D*-Phe^7]-α-MSH. The hormone (50 µl, 0.4 mM) was applied topically to 30-cm^2 lesions in which 29-48 minigrafts had been made. The hormone did not improve the success of the minigrafting and no differences were observed in local or distant repigmentation in treated subjects as compared to the placebo group. Aliquots of 24-h urine concentrated by lyophilization irreversibly darkened toad skins, demonstrating the presence of the analogue. This is the first report of the transdermal delivery of a topically applied melanotropin in living human subjects.

Key words
- Vulgar vitiligo
- α-MSH synthetic analogue
- Minigrafting

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Research supported by FINEP, CNPq, and FAPESP.

Received September 23, 1997
Accepted September 24, 1998

Introduction

Vitiligo is a pigmentation disorder that affects 0.5 to 4% of the world population. Its etiology is unknown, and the symptoms probably result from a variety of pathogenic alterations of the immune and neural systems, and of melanocyte structure and function (1-8).

The pigmentation of most vertebrates is regulated by α-melanocyte-stimulating hormone (α-MSH) secreted by the pars intermedia of the pituitary. The hormone is produced by the cleavage of proopiomelanocortin, which is also the precursor protein of ACTH, ß-endorphins and γ-MSH. In humans, the pars intermedia is non-functional, but α-MSH is produced locally in the skin by Langerhans cells, keratinocytes and melanocytes themselves (9,10). Five melanocortin receptors belonging to the family of G-protein-coupled receptors have been cloned (11,12). The MC1 receptor is specific for the integumental melanocytes and active macrophages, and is highly selective for α-MSH (13). It has recently been demonstrated that α-MSH stimulates melanin synthesis and proliferation in human cultured melanocytes (14,15).

Various α-MSH analogues have been synthesized during the last decade by Hadley and colleagues (16-18). The [Nle^4, *D*-Phe^7]-α-MSH analogue, a superpotent agonist of mammalian melanocytes, is 100-1000 times more potent than the native hormone. Unlike the native hormone, it is resistant to inactivation by serum and brain enzymes, and by purified proteolytic enzymes (16,17). The synthetic hormone exhibited no toxicologic

effects nor did it favor tumor growth, metastasis or invasion in mice (19,20) and therefore was tested in humans. When intravenously injected (0.16 mg/kg) daily for 10 days, the peptide enhanced the epidermal pigmentation of healthy male subjects, with no significant side effects (21).

The analogue is highly lipophilic, being able to pass through the skin of mice and human cadavers (18), and might therefore be transdermally delivered. Topical application might be more efficient than injection, with higher availability at the hair follicle level, while liver metabolism and excretion into urine should be reduced or avoided.

We and others have previously reported that dermo-epidermal minigrafts were very effective in repopulating hypopigmentary lesions in segmental vitiligo patients, but much less effective for the vulgar type (22,23).

Since transplants provide viable melanocytes, our objective was to investigate in vulgar vitiligo patients: 1) the efficacy of topical application of [Nle^4, *D*-Phe^7]-α-MSH in stimulating melanocyte proliferation and melanocyte or melanosome spread from the dermo-epidermal grafts, and 2) the efficiency of transdermal delivery of the MSH analogue topically applied *in vivo* on those lesions.

Material and Methods

Subjects

Eighteen volunteers (15 men and 3 women) with vulgar vitiligo were selected. The duration of the disease varied from 3 to 46 years, with an average of 20.5 years (Table 1). Among the patients, 12 exhibited active dysfunction, that is, newly arisen achromic lesions, and 3 presented the Koebner phenomenon (Table 1). Some patients exhibited variable degrees of leukotrichia. Since most areas chosen for the study were hairless or bore a small vellus, this characteristic was not used as a parameter. The patients were submitted to the following blood tests: complete blood counts, glutamic-oxaloacetic acid transaminase (GOT), glutamic-pyruvic acid transaminase (GPT), alkaline phosphatase, glycemia, T3, T4, TSH, and early morning (8 a.m.) cortisol, immediately before and 4 months after the beginning of the treatment.

Minigrafting

A 30-cm^2 skin area bearing no internal pigmented spots was chosen in each individual. In 13 subjects the areas were located on the upper limbs as follows: forearm (7 subjects), wrist (4 subjects), arm (1 subject), and hand (1 subject). In two subjects the areas were on the lower limbs, i.e., the leg (1 subject) and the foot (1 subject). Finally, in one subject the area was located on the flank, and in two subjects the areas were on the abdomen.

The number of explants ranged from 29 to 48 depending on the shape of the area.The explants (1 mm in diameter) were removed from the retroauricular region and grafted 1 cm apart under local anesthesia with 3% prilocaine and a vasoconstrictor. Hemostasis was achieved with 50% iron perchloride, and the graft was occluded with micropore tape.

Treatment with the melanotropin analogue

Two weeks later, 9 subjects started receiving topical applications of a 0.4 mM solution of [Nle^4, *D*-Phe^7]-α-MSH in polyethyleneglycol 400 (group I), and the other 9, the placebo control, only polyethyleneglycol 400 (group II). The compositions of both solutions were unknown to the subjects and to the therapist until the end of the experiment.

For 2 months, the volunteers received 50 µl of solution I or II, topically applied twice a week by the therapist to the selected depigmented area of 30 cm^2. During an addi-

tional 5-month period, the patients were instructed to self-apply the solution once a day, twice a week, with weekly visits to the therapist. The final dose per application was 36.5 µg/30 cm^2 (2 x 10^{-8} mol). The evolution of treatment was monitored with the aid of a Woods light.

Urinary melanotropin assay

In the last week of treatment, 24-h urine was collected from 4 patients, lyophilized and resuspended in 100 ml of distilled water. The precipitate was removed by centrifugation, the supernatant was lyophilized once more and resuspended in 5 ml of distilled water. The solution was assayed in the toad skin bioassay to detect the presence of the α-MSH analogue. The assay is very sensitive and able to detect picomolar concentrations of [Nle^4, *D*-Phe^7]-α-MSH (24). Three different controls were used: 1) 0.1 nM [Nle^4, *D*-Phe^7]-α-MSH was assayed as the positive control; 2) urine from an untreated healthy 34-year-old male volunteer was similarly lyophilized and resuspended as above and assayed as the negative control; 3) an 18-h urine sample from a healthy 46-year-old female volunteer was collected after one topical application of the analogue to 30 cm^2 of the forearm, lyophilized, resuspended in 100 ml, and assayed.

The thigh and dorsal body skins of male toads, *Bufo ictericus,* were removed and placed in Ringer solution containing: 11.2 mM NaCl, 1.9 mM KCl, 1.1 mM $CaCl_2$, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.30-7.45. Square skin pieces (2.5 x 2.5 cm) were mounted individually between two PVC rings and maintained in 10 ml of Ringer at 23°C (±1.0) for at least 60 min to provide maximal lightening conditions (maximally aggregated melanosomes). A Photovolt reflectometer was used to measure the reflectance of each skin and an initial mean value was obtained for each group of skins. The addition of increasing doses of a darkening agonist such as the α-MSH analogue [Nle^4, *D*-Phe^7]-α-MSH to each group for 60 min results in a dose-dependent darkening (melanosome dispersion) of the skins (24). The responses to 100 µl of the 4 urine samples from topically treated patients were compared to the maximal response evoked by 0.1 nM [Nle^4, *D*-Phe^7]-α-MSH and to 100 µl of control urine samples. The 60-min readings of reflectance (skin color) were recorded and are reported as percent change of the initial basal value.

The use of human subjects in this investigation was approved by the Ethics Committee of the University Hospital, University of São Paulo, and the patients gave their written informed consent.

Results

All patients showed good graft implantation, with no necrosis. Two patients abandoned treatment. Therefore, we ended the experimental treatment with 8 patients in each group. Among the 16 volunteers, 5 subjects had abnormal blood tests during evaluation. Subject 4 had hyperglycemia and high levels of serum GOT and GPT, and was hepatitis B anti-e and anti-c positive and HBSAg negative, hepatitis A IgG positive, IgM negative and anti-HCV negative, and had hepatic steatosis before treatment. Subject 5 had a history of a possible gestational diabetes. Subject 10 showed hyperthyroidism and hyperglycemia; subject 16 had diabetes for one year and a half, and subject 11 had hyperthyroidism just before the onset of vitiligo (Table 1). No alteration of laboratory tests during the application of [Nle^4, *D*-Phe^7]-α-MSH, or side effects such as those described after the administration of the analogue to healthy volunteers (21) were observed.

After 2 months, three subjects from group I exhibited a 1-3-mm pigmentation halo around the grafts, and one of them also presented pigmentation spots in distant lesions. The halo presented no further expansion

during treatment. Four individuals showed pigmentation only in the grafts and one had totally depigmented grafts (Table 2).

In the placebo group, 6 patients kept the pigmentation only in the grafts. In six individuals, pigmentation was observed in distant lesions. Only one patient exhibited a halo surrounding the grafts, but other spots of repigmentation distant from the grafts were also observed. The last subject showed depigmented grafts, but repigmented spots in distant sites (Table 2).

After the first phase, all volunteers started to apply the hormone daily. Twelve people completed the entire 5-month treatment, and 4 abandoned treatment after 3 months.

In the initial group I, two patients abandoned treatment after 3 months, one having depigmented grafts and no other sites of repigmentation, the other exhibiting a highly active expansion of the pigmentation halo, as already observed in phase I. The other 6 volunteers completed the 5-month period. Among them, two kept the same pigmentation rate at distant sites as before, one exhibited depigmentation of the grafts and pigmentation of distant lesions, one had only pigmented grafts, one exhibited a new achromic spot, and one presented a growing pigmentation halo around the grafts (Table 2).

In group II, two patients also abandoned treatment after 3 months, one exhibiting increasing repigmentation as in phase I, the other with unpigmented grafts and discrete pigmentation of distant spots. The other 6 volunteers completed the experiment. Among them, two had an increasing halo and pigmentation of distant lesions, three developed pigmentation at distant sites, which depigmented during the 4th month, and one exhibited a complete depigmentation of the grafts, but simultaneous appearance of pigmented and hypochromic maculae at various tegumental sites (Table 2).

In summary, during phase I, the control group exhibited a more intense repigmentation of distant lesions. None of the patients bearing halo around the grafts (3 of group I and 1 of group II) exhibited any increase in the rate of halo expansion.

Continuous application of the hormone-containing solution for the subsequent 5 months in all patients (phase II) did not

Table 1 – Clinical data of the subjects.

M = Male; F = female; spread = body, upper and lower limbs.

Subject	Sex/age (years)	Time of disease (years)	Related diseases	Affected areas	Koebner/ activity	Subject	Sex/age (years)	Time of disease (years)	Related diseases	Affected areas	Koebner/ activity
1	M/56	24		spread	-/+	9	M/51	46		limbs	-/+
2	M/55	21		spread	-/+	10	M/55	40	hyper-thyroidism + diabetes	spread	+/+
3	F/42	36		spread	-/+	11	M/32	5	hyper-thyroidism	spread	+/+
4	M/62	20	diabetes + hepatic steatosis	spread	-/+	12	M/49	15		spread	-/+
5	F/40	14	gestational diabetes	spread	-/+	13	M/46	4		spread	-/+
6	M/20	19		spread	-/-	14	M/63	25		spread	-/+
7	M/51	3		spread	-/-	15	M/29	14		spread	-/-
8	M/29	24		spread	-/-	16	F/35	20	diabetes	spread	+/+

result in any improvement of repigmentation in either group (Table 2).

Despite the lack of effect, the analogue was transdermally delivered, as indicated by the fact that the urine samples from 2 of the 4 sorted subjects and from the healthy volunteer exhibited an irreversible darkening activity on the toad skin assay (Figure 1). Compared to the maximal darkening response elicited by 0.1 nM [Nle^4, *D*-Phe^7]-α-MSH, the patient urines were able to promote 50% of the maximal response. Therefore, its concentration on the toad skins might be calculated as 0.03 nM, and in the final urine solution as 3 nM.

Discussion

Various treatments have been employed in an attempt to cure vitiligo, such as psoralens associated with ultraviolet light A (PUVA), corticoids, prostaglandin inhibitors, phenylalanine, di-hydroxyphenylalanine, 5-fluorouracil, melagenin and minigrafts (23,25).

Since [Nle^4, *D*-Phe^7]-α-MSH was effective in tanning healthy human subjects after daily injections with no toxic effects (21),

Table 2 - Summary of the responses of 16 subjects to topical application of [Nle^4, *D*-Phe^7]-α-MSH.

1st Phase: 2 months after the minigrafts

Homone analogue					Placebo				
Subject	Pigmentation halo (>1 mm)	Graft pigmentation only	Repigmentation at a distance	Graft depigmentation	Subject	Pigmentation halo (>1 mm)	Graft pigmentation only	Repigmentation at a distance	Graft depigmentation
1				+	9	+		+	
2		+			10		+	+	
3		+			11		+		
4	+				12		+	+	
5		+			13			+	+
6	+				14		+	+	
7	+		+		15		+	+	
8		+	+		16		+		

2nd Phase: 5 months after hormone application to both groups

Subject	Pigmentation halo (>1 mm)	Graft pigmentation only	Repigmentation at a distance	Graft depigmentation	Depigmentation at a distance	Subject	Pigmentation halo (>1 mm)	Graft pigmentation only	Repigmentation at a distance	Graft depigmentation	Depigmentation at a distance
1				+		9	+		+		
2		+				10		+	+		
3		+	+		+	11		+	+		
4	+				+	12				+	
5				+	+	13				+	+
6	+					14				+	+
7	+		+			15	+		+		
8		+	+			16				+	+

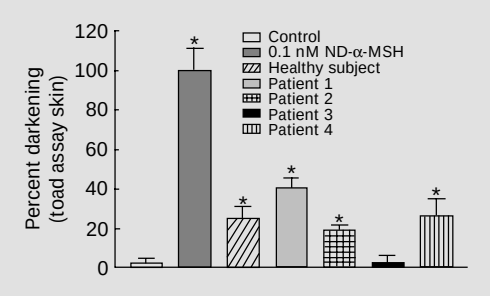

Figure 1 - Percent darkening of *Bufo ictericus* toad skins in response to 0.1 nM [NIe^4, *D*-Phe^7]-α-MSH (ND-α-MSH; considered as 100%) and to lyophilized urine samples from treated and untreated (control) human subjects. Each bar is the mean ± SEM (N = 6) value for skin darkening. *P<0.05 compared to control (ANOVA followed by Student-Newman-Keuls test).

we decided to investigate its effectiveness as a topical agent, on lesions with newly grafted melanocytes in vulgar vitiligo, and to determine if the analogue crossed the transdermal barrier *in vivo*.

However, our data demonstrated that the analogue did not improve the already expected poor repigmentation with minigrafts in unstable vulgar vitiligo lesions. To have a local effect, the peptide would have to reach the epidermal melanocytes, and the capillaries of the papillar dermis would account for the systemic distribution. Therefore, the reticular dermis would not constitute a barrier (18). It has been reported that facial and head skins were the most permeable regions for the absorption of peptides *in vitro*. The limbs appear to have a mild degree of permeability, whereas trunk skin appears to be the least permeable due to its thickness and fewer skin adnexa (18). The urine samples from the randomly selected subjects had a darkening effect on toad skin, demonstrating that the peptide had crossed the transdermal barrier and reached the systemic circulation, being therefore available to act on the entire tegument. This is the first report of transdermal delivery of a topically applied peptide hormone analogue in living human subjects.

Boissy and colleagues (6) reported that cultured melanocytes from vitiligo patients exhibited structural defects of the endoplasmic reticulum, such as dilations or circular shapes, in addition to alterations in melanosome compartmentalization. These morphological alterations could also be seen in pigmented and apparently healthy skin 90 cm from the achromic lesion. This might be one reason for the ineffectiveness of the hormone treatment. In addition, several authors (26-28) have pointed out that the stability of the segmental or vulgar vitiligo is a requirement for the success of treatments such as skin grafting. In fact, 66.7% of our patients had unstable vitiligo, which might also be the reason for the negative response to the peptide.

As previously reported (22,26,29), segmental vitiligo may be considered a more stable form than vulgar vitiligo, thus allowing a more successful melanocyte transplantation. Whether healthy skin melanocytes exhibit any metabolic or structural modifications in this type of hypopigmentation remains to be investigated. However, it seems that, regardless of vitiligo activity, once the lesion is superficially dermabrased, autologous cultured melanocytes or epidermal sheets grown *in vitro* are successfully implanted (30).

One should also bear in mind that the normal phenotypic expression of melanocytes, i.e., melanin synthesis, depends on the interaction of a variety of local peptide hormones, such as endothelins (ETs), basic fibroblast growth factor (bFGF), and agouti protein (31). Both ET-1 and bFGF potentiate the proliferative effect of α-MSH, but are antagonists of melanotropins concerning melanogenesis since they depress the activity and expression of tyrosinase, the key enzyme for melanin synthesis. The agouti protein has been considered to be the physiological regulator of pheomelanin (32), and the inverse agonist of the MCR1 α-MSH receptor (32). The 108-amino acid peptide is produced in the mammalian hair follicle and binds to the α-MSH receptor, depressing its activity and competing with the hormone (33). The agouti protein inhibits the melanogenic and the proliferative effects of α-MSH on normal human melanocytes (31). Therefore, agouti protein may also play a physi-

ological inhibitory role in human eumelanin production, and might be associated with hypopigmentary disorders such as vitiligo, thus preventing the action of the analogue.

Multiple genetic factors might cause melanocyte dysfunction, or render melanocytes susceptible to environmental, neural or immune stimuli (6). This would explain the diversity of etiologies for vitiligo, and the variable responses of the patients to currently available treatments.

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