Pharmacologic Response of a Controlled-Release PLGA Formulation for the Alpha-Melanocyte Stimulating Hormone Analog, Melanotan-I

Renu Bhardwaj,[1] Mac E. Hadley,[2] Robert T. Dorr,[3] Katarina Dvorakova,[3] Christine Brooks,[4] and James Blanchard[5,6]

Received December 1, 1999; accepted February 7, 2000

Purpose. The objective of this study was to evaluate in vitro and in vivo the melanogenic activity of one-month duration Melanotan-I (MT-I) implants prepared using poly (D,L lactide-co-glycolide) polymer.
Methods. The biological activity of the samples of MT-I released in vitro from the non-irradiated or gamma irradiated implants was measured using a frog skin bioassay. The effect of MT-I on skin pigmentation was measured using a Chroma meter (reflectometer) after subcutaneous administration of implants containing 4 mg MT-I to guinea pigs. Eumelanin, the black/brown melanin pigment, was quantified in skin biopsies as pyrrole-2, 3, 5-tricarboxylic acid using HPLC.
Results. The MT-I released in vitro from implants after 24 hours exhibited 100% melanotropic activity in frog skins compared to an identical concentration of a freshly prepared MT-I standard. The reflectance readings demonstrated a prolonged skin darkening for up to three months as evidenced by the decrease in the luminance values from 0 to −4.82. A 2.5-fold increase in eumelanin levels was observed after one month and the increased pigmentation lasted for 3 months.
Conclusions. The melanogenic response to MT-I implants persisted for three months and the increase in pigmentation, especially the increased eumelanin levels, could provide protection from ultraviolet radiation.

KEY WORDS: Melanotan-I; poly (D,L-Lactide-co-glycolide); pharmacologic response; controlled-release; melanogenesis.

INTRODUCTION

Ultraviolet radiation is one of the major contributing factors for skin cancer (1). It is a well known fact that dark-skinned people have a low risk of developing melanomas. Since melanin pigmentation is considered to be the most effective mechanism for skin protection against radiation-induced damage (2), Melanotan-I, an α-Melanocyte Stimulating Hormone (α-MSH) analog, was studied as a potential skin cancer protective agent. α-MSH is a tridecapeptide and plays an important role in the integumental color changes in hair and skin (3). The MT-I molecule differs from α-MSH in the substitution of norleucine (Nle) for methionine at position 4 and D-phenylalanine for L-phenylalanine at position 7, resulting in enhanced potency and prolonged biological properties (4).

MT-I is currently in Phase I trials at the Arizona Cancer Center (University of Arizona, Tucson) to evaluate its potential as a chemopreventive agent for sunlight-induced skin cancers. MT-I has a very short half-life of 1.07 (±0.88) hr in humans after intravenous administration (5) and, as a consequence, multiple injections are required to achieve and maintain the desired therapeutic activity. Hence, it seemed appropriate to develop a prolonged-action formulation for MT-I in order to provide extended skin protection. The biodegradable polymer, poly (D,L-Lactide-co-glycolide) (PLGA) with 50% lactide and 50% glycolide, was selected to develop an implant delivery system for this peptide (6).

The goal of this study was to evaluate the pharmacologic activity of an MT-I implant designed to provide a one-month duration of effect. Frog skin bioassays were performed to measure the bioactivity of the MT-I released from the implants in vitro with and without gamma irradiation sterilization. Melanogenic effects of MT-I were evaluated using a special breed of pigmented haired and hairless guinea pigs. Skin darkening potency of the MT-I implants was quantified in hairless guinea pig skin using non-invasive skin chromaticity (reflectance) measurements. The melanin pigment, eumelanin, was quantified in guinea pig skin biopsies using high-performance liquid chromatography.

MATERIALS AND METHODS

The research reported here was conducted in conformance with the "Principles of Laboratory Animal Care".

Preparation of MT-I Implants

The MT-I implants containing 1 and 4 mg peptide (1 mg for in vitro studies) were prepared using a hot melt-extrusion technique (6). The polymer used in this study was a lactide:glycolide polymer with a mole ratio of 50:50 and an intrinsic viscosity of 0.6 dL/gm. A coarse mixture of the peptide with fine granular PLGA was prepared manually in a mortar and fed into a melt extruder. The operating temperature was maintained at 45 to 70°C. The extrudate (implant) produced was cylindrical in shape and about 2 mm in diameter and 8–10 mm in length. The MT-I depots were packaged in sterilization pouches and sent to Isomedex (Chicago, IL) for ^{60}Co irradiation. The gamma radiation doses were 1.5, 2.5 and 3.5 Mrad for the frog skin bioassays and 2.5 Mrads for the in vivo studies.

Frog Skin Bioassay

The frogs (Rana Pipiens), were obtained from Kons Scientific Company, (Oshkosh, WI). In these assays, changes in skin color are monitored by a photovolt reflectometer (previously standardized against a white aluminum oxide plate) and recorded as differences from the initial baseline (zero) value (7,8). The back of the skin from each frog was fitted over an aluminum ring and held in place by an outer overlapping plastic

[1] Genzyme Corporation, 45 New York Avenue, Framingham, Massachusetts 01701.
[2] Department of Anatomy, University of Arizona, Tucson, Arizona 85721.
[3] Department of Pharmacology and Arizona Cancer Center, University of Arizona, Tucson, Arizona 85721.
[4] Dermatology Division, University of Arizona, Tucson, Arizona 85721.
[5] Department of Pharmacology and Toxicology, University of Arizona, Tucson, Arizona 85721.
[6] To whom correspondence should be addressed. (e-mail: blanchard@pharmacy.arizona.edu)

0724-8741/00/0500-0593$18.00/0

ring. Skins were placed in Ringer's solution (pH 7.4) for a two hour pre-experiment soak. After a number of changes of solution, the skin was placed (at room temperature) in a beaker containing sufficient solution (20 ml) to cover the skin. Each experimental group consisted of five skins and the initial reflectance value obtained from each skin was recorded as the baseline value. The mean initial reflectance value for each group of skins was assigned a value of 100% and the succeeding average values for each group of skins were recorded as the percentage change above or below that baseline value. The peptide solution was added to the solution bathing the skins and the reflectance reading recorded after 60 min. A decrease in the reflectance value occurred gradually due to darkening of the skin. One group of skins was maintained in Ringer's solution as a control. The in vitro release experiments were performed by incubating the non-irradiated and gamma irradiated implants in 1 ml of isotonic phosphate buffer medium maintained at pH 7.4 and 37°C. The MT-I released from the implants after 24 hours of incubation (i.e., about 3%) was compared with a reference MT-I solution using the frog skin bioassay. The concentration of MT-I released from the implants into the release media was measured using HPLC and the reference solutions were prepared based upon these assayed concentrations. This permitted the calculation of the percentage skin darkening response. A statistical analysis was performed on the standards and the samples using a paired t-test. A significance level of $p < 0.05$ was used for all statistical comparisons.

Pharmacologic Response Studies

The pharmacologic response studies were performed using a special breed of hairless and haired pigmented guinea pigs. Five hairless and five haired guinea pigs were used in this study. Each animal weighed about 500 g. All of the studies, including the skin reflectance, the histology, and the eumelanin measurements were conducted on the same group of animals. The guinea pigs were fasted for 24 hrs prior to the experiment and water was allowed *ad libitum*. Each animal was anesthetized using isoflurane (1–3%). An MT-I implant containing 4 mg peptide was administered subcutaneously in the abdominal region of each guinea pig. One implant was administered per animal. A placebo implant was used in the vehicle control group. Two circular punch biopsies (4 mm diameter) were taken from the flank region of each guinea pig prior to the implantation of the MT-I depot and once every month for 3 months following the implantation. One biopsy sample was used for histological examination and the other was used for the measurement of eumelanin concentration. The sections were fixed in formalin (10% v/v) and stained for melanin with the Fontana-Masson stain to highlight melanin positive cells.

Skin Reflectance Measurements

The melanotropic activity of the formulations was evaluated using five pigmented hairless guinea pigs. Skin darkening was measured by serial chromaticity measurements at four sites (head, shoulder, flank, abdomen) every week for three months, using a quantitative luminometer, the Minolta Chroma Meter Model CR-200b (Minolta Corporation, Osaka, Japan) (5). Six measurements were made at each site and the change in skin color in each animal was compared with its baseline reading. The baseline reflectance value was an average of data collected for three weeks at each site before the animals were implanted with the MT-I depot. Skin chromaticity is quantitated using three indices designated "L, a and b", in accordance with the Commission Internationale de l'Eclairage standard observer response. The luminance (L) value expresses relative brightness, varying from white to black. The "b" value represents color hues ranging from blue to yellow. The "a" value is a measure of yellow to red color and does not change proportionally with skin tanning. The instrument was calibrated prior to each reflectance measurement session using a zinc oxide plate of known color value as a standard. Statistical testing was performed on the "L" values for the four sites using an analysis of variance (ANOVA).

Measurement of Eumelanin

Concentrations of eumelanin in skin biopsies were measured by the method of Ito and Fujita (9), with minor modification. The method is based on the formation of pyrrole-2, 3, 5-tricarboxylic acid (PTCA) from eumelanin by permanganate oxidation and detection of PTCA by high-performance liquid chromatography (HPLC).

The PTCA standard was prepared by permanganate oxidation of 5-hydroxyindole-carboxylic acid (HICA) in 1 M sulfuric acid and crystallization from acetic acid. Briefly, HICA (150 mg) was dissolved in 50 ml of 1 M sulfuric acid and 25 ml of a 3% solution of potassium permanganate was added slowly to the mixture in 1 ml portions. The reaction was stopped with 5 ml of a 10% solution of sodium sulfate. The mixture was extracted three times with 70 ml of ether. The ether was evaporated and the residue was dissolved in 20 ml of glacial acetic acid, and then allowed to crystallize overnight at room temperature. The white crystals of PTCA were collected, dried under vacuum, and dissolved in water to a final concentration of 1 mg/ml. PTCA was subjected to mass spectral analysis to confirm its purity using fast atom bombardment detection (FAB-MS). A total of 10 scans were averaged to produce the spectrum. The PTCA standard was dissolved in a 1:1 water/glycerol mixture and this solution was applied to the tip of the FAB probe. The spectrum showed the presence of a molecular ion at m/z 200 which confirmed the identity of the MH^+ ion of PTCA (MW = 199). The PTCA generated using purified Sepia melanin and skin obtained from an African-American donor, which contains about 65% eumelanin, was used as a positive control. One ng of PTCA detected corresponds to approximately 50 ng of eumelanin in the original sample (10).

Samples from skin biopsies weighing 5 to 10 mg were homogenized with 100 μL of mouse liver homogenate and 900 μL of 1 M H_2SO_4, and gradually oxidized with 3% $KMnO_4$. Mouse liver homogenate was added to increase the stability of PTCA and to enhance the reproducibility of the assay (11). After 10 minutes the reaction was stopped with 100 μL of a 10% solution of Na_2SO_3. PTCA was extracted from the resulting solution with two 7 mL aliquots of diethyl ether. The ether was evaporated and the residue was dissolved in 120 μL of water.

The HPLC system consisted of a PE Biocompatible Binary Pump, a Hitachi AS-2000 autosampler, a Hewlett-Packard 1050 UV-VIS detector set at 269 nm and an Adsorbosphere HS C_{18} (4.6 × 150 mm, 5 μm particle size) column. The mobile phase consisted of 0.01 M potassium phosphate buffer, pH 2.1, and

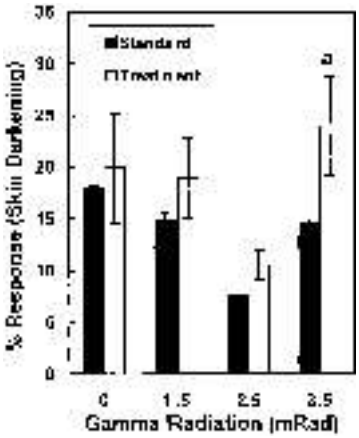

Fig. 1. The bioactivity of non-irradiated and irradiated samples of MT-I released in vitro from PLGA implants (designated as ™Treatment∫) containing 1 mg MT-I after 24 hours incubation time. Each point represents the mean 6 SEM of three replicates. [a]Significant increase in bioactivity compared to the MT-I reference standard (designated as ™Standard∫).

acetonitrile (96:4 v/v). The analysis was performed at room temperature at a flow rate of 1 mL/min. A statistical analysis was performed on the samples using a paired t-test. A significance level of $p < 0.05$ was used for all statistical comparisons.

RESULTS

Bioassays were performed on the MT-I released in vitro from the non-irradiated and irradiated PLGA implants after 24 hours of incubation. The standard curve was linear over the concentration range of 0.1 to 4 3 10^{23} ng/ml of MT-I. The maximum linear skin darkening response produced by MT-I in the frog skin bioassay was 50%. The interassay variability for the bioassay was about 23%, which is within the normal variation of this method (12). The percent response observed as frog skin darkening was compared with MT-I reference concentrations and the test samples. Figure 1 indicates that the non-irradiated MT-I samples as well as those exposed to 1.5 Mrad and 2.5 Mrad radiation doses had 100% bioactivity. This indicates that there was no degradation of MT-I during fabrication of the implants exposure to elevated temperature, and gamma irradiation. However, the samples irradiated with 3.5 Mrad showed significantly ($p < 0.05$) higher bioactivity, which may be due to the presence of some biologically active metabolites formed during gamma irradiation or possibly a change in the rate of release of MT-I from this formulation resulting from degradation of the polymer due to irradiation.

Figure 2 compares the cutaneous and follicular effect of implantation of the MT-I depot in hairless and haired guinea pigs. The melanotropic action of MT-I in hairless guinea pigs was observed as a cutaneous effect only. Skin darkening was more obvious around the torso. In the haired animals the effect was observed as darkening of the hair color from brown to black. Skin darkening also occurred, but to a lesser degree.

The histological sections on the biopsy samples show the epidermal and the dermal layers of the hairless and the haired guinea pig skin in Figs. 3 and 4, respectively. The epidermal layer of the hairless guinea pigs exhibits more dense melanin pigment within the melanocytes than does the epidermis of the haired animals. In the haired guinea pigs more melanin was contained within the melanocytes of the hair follicles. The increased pigmentation after implantation of the MT-I depot resulted in an increased number of melanin positive cells in the basal layers of the epidermis. Some granules of melanin

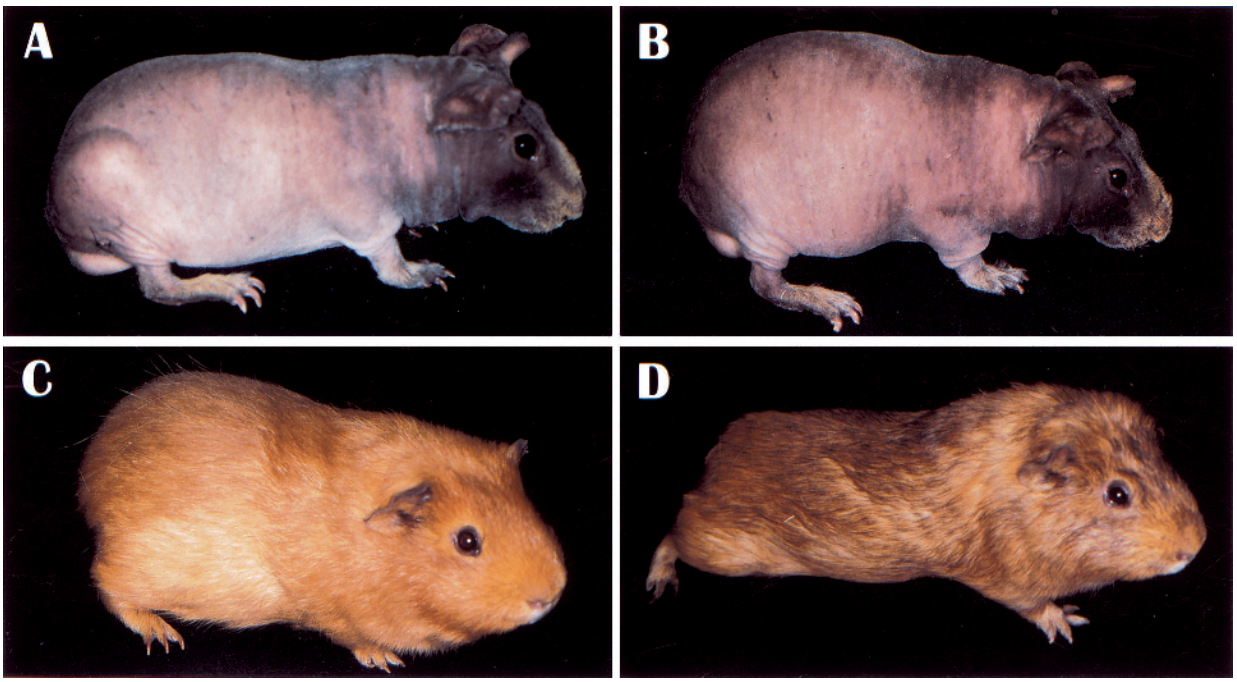

Fig. 2. Photographs of pigmented guinea pigs after subcutaneous implantation of depot containing 4 mg MT-I. Hairless guinea pig at zero time (A) and at three months (B). Haired guinea pig at zero time (C) and after three months (D).

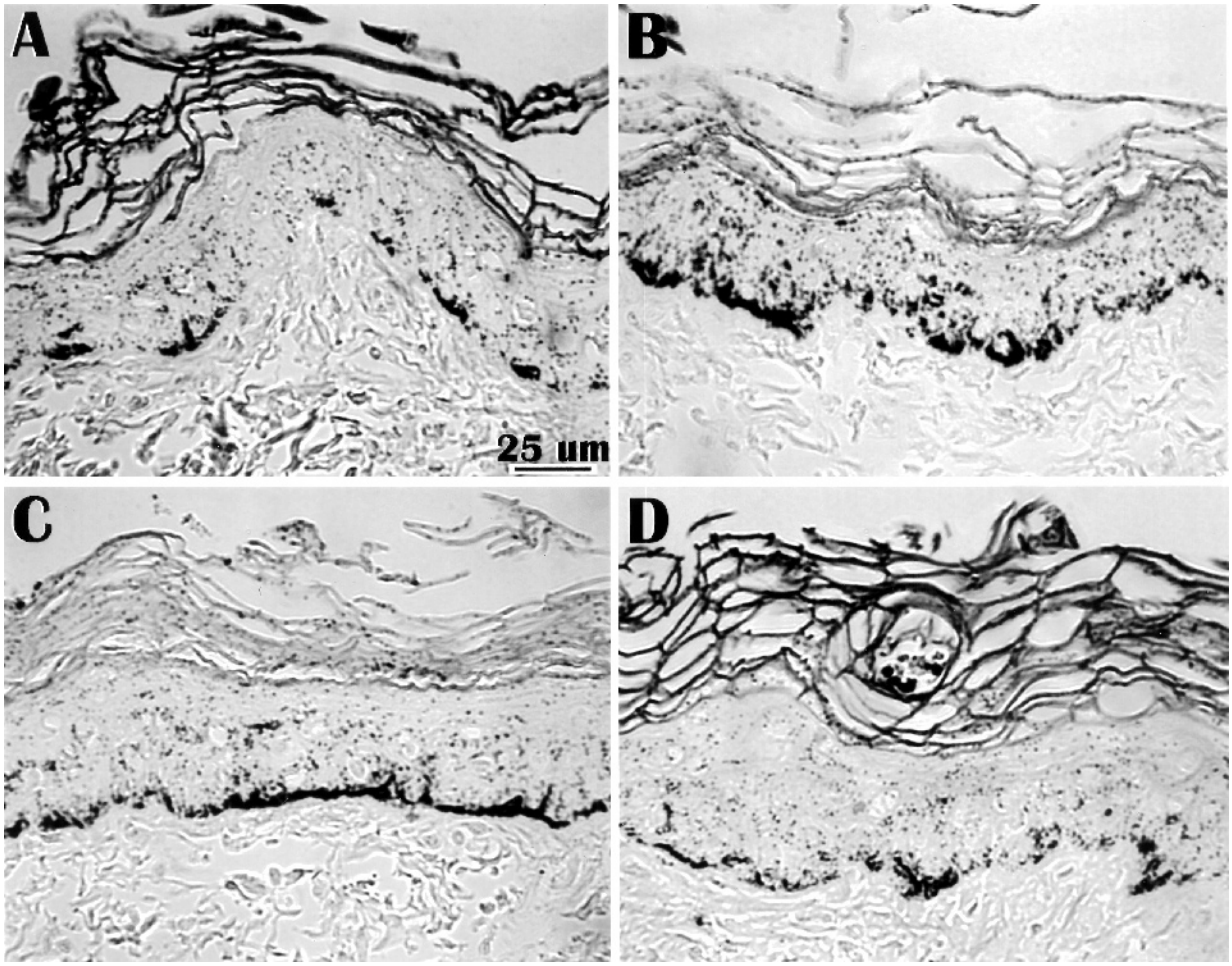

Fig. 3. Histological sections of hairless guinea pigs at baseline level (A), one month (B), two months (C) and three months (D) after the implantation of the 4 mg MT-I depot. The melanin pigment can be seen as dark granules at the junction of the basal epidermis (upper) and the dermis (lower).

were also found in the keratinocytes, indicating transport of pigments from melanocytes via the dendrites. The melanotropic effect in both types of animals peaked after one month and the melanin levels were maintained in the skin for 3 months. A qualitative evaluation of Figs. 3 and 4 indicated that similar melanin levels were present in the skin of the hairless guinea pigs and in the hair of the haired guinea pigs.

The reflectance readings showed a decrease in the Luminance (L) index value which measures the change in white to black hues. A negative "L" value signifies skin darkening. A decrease in the luminance value by one point indicates a visually perceptible skin darkening effect. There was no change in the "a" and the "b" indices of the reflectance values. The onset of skin darkening was observed within a week after implantation of the depot. The maximum effect was observed in the shoulder after one month and a maximum L value of -7.42 was produced. (Fig. 5). The four different anatomical sites of the guinea pigs showed different degrees of pigmentation, with the shoulder exhibiting the greatest effect. The intra-sample assay variability was less than 4% at all of the sites. The luminance value was significantly different at all four sites ($p < 0.001$). Elevated pigmentation continued for 3 months even after the degradation of the implants. This could be due to an increased affinity and activity of MT-I for the MSH receptors.

Eumelanin measurement in skin biopsies exhibited a concentration vs. time profile similar to the luminance values (Fig. 6). The eumelanin was quantified by measuring PTCA levels using HPLC. The retention time for PTCA was 5 min and the detector response was linear over the range of 2.5 ng to 50 ng of PTCA. The calibration curve run for nine different days on the cadaver skin showed a reproducibility of 0.923 ± 0.123 ng PTCA per mg of wet tissue weight. The skin from rats (2 day old) was used as a negative control. The positive control was human skin from African-American donor and sepia melanin which contained $65 \pm 10.01\%$ of eumelanin. The results found here were similar to those reported earlier (8). The extraction efficiency of PTCA from Sepia melanin was 70.02 % (SD = 17.68%) and with a % CV of 25.18%. The Sepia melanin was determined by HPLC as PTCA and then multiplied by the factor 50 to determine the amount of eumelanin in the skin sample. A 2.5-fold increase in eumelanin concentration was observed in about one month and the effect persisted for three months. The eumelanin concentrations over the three month time period were significantly different from the baseline values and from the average eumelanin (PTCA) concentration of 49.63 ± 5.21 ng/mg of skin observed in the vehicle control. These results illustrate that the pigmentation effects due to MT-I were reflected in a parallel fashion by both the luminance values and

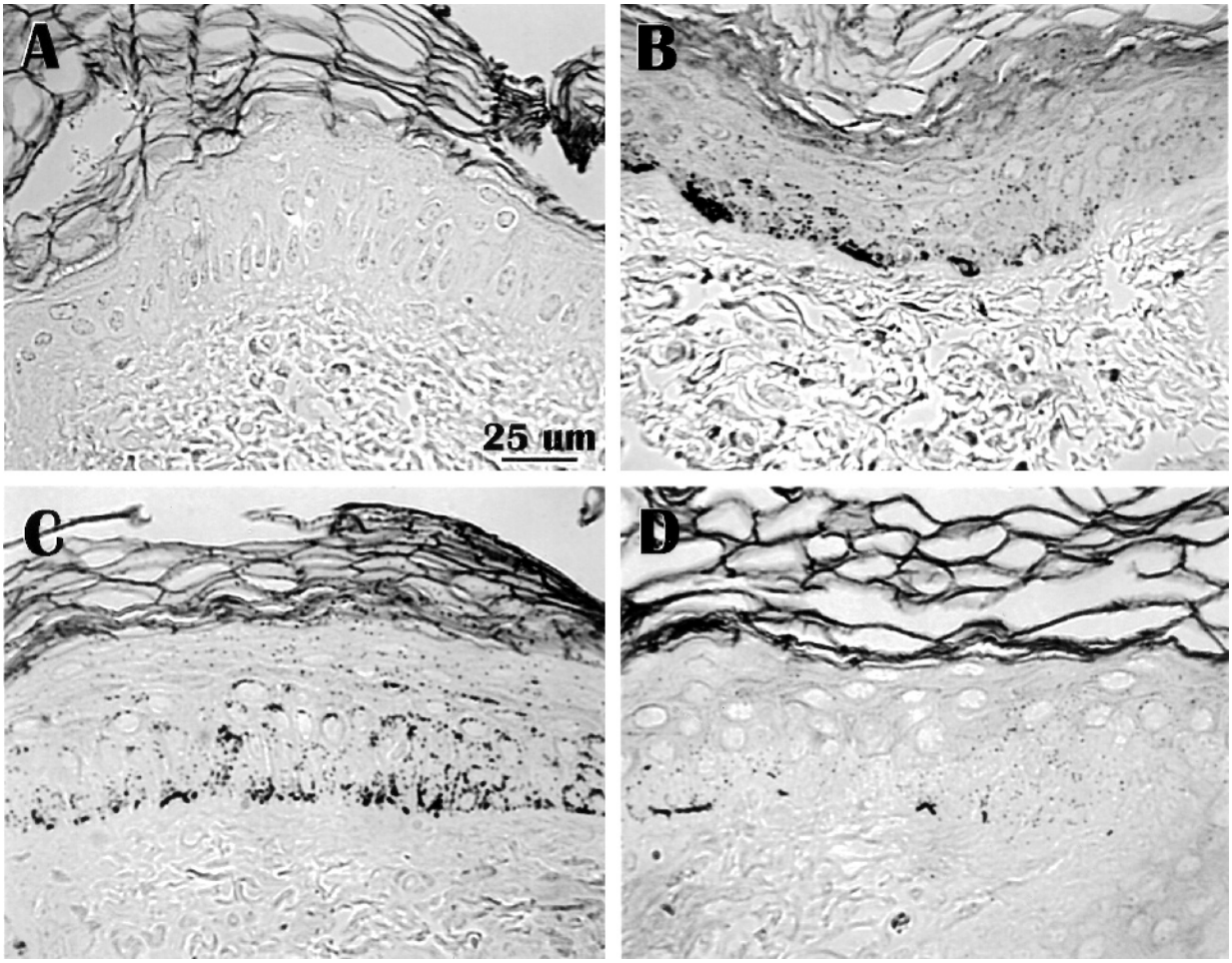

Fig. 4. Histological sections of haired guinea pigs at baseline level (A), one month (B), two months (C) and three months (D) after the implantation of the 4 mg MT-I depot. The dark-stained melanin granules in the basal epidermis are less dense in the haired animals due to the deposition of the melanin in the base of the hair follicles (not shown).

the eumelanin concentrations in the skin biopsies. No skin or hair darkening was observed for the placebo implant in any of the above evaluations.

DISCUSSION

The purpose of the controlled-release MT-I implant formulation based on PLGA polymer was to prolong the release of the peptide and thereby maintain enhanced skin pigmentation for at least one month. The MT-I released from the implants was bioactive and capable of producing melanotropic activity. In the frog skin bioassay, in response to MT-I, the melanosomes within the integumental melanophores migrate from a perinuclear position into the dendritic processes of the pigment cells. This centrifugal organellar movement results in skin darkening. The subsequent removal of the melanotropin from the frog skin by rinsing with Ringer's solution usually results in a rapid perinuclear (centripetal) re-aggregation of melanosomes, leading to a lightening of the frog skins back to their original baseline reflectance value (8).

The pharmacologic response evaluations were performed using a special breed of hairless and haired pigmented guinea pigs developed to study mammalian pigmentation. Hairless albino guinea pigs with an outbred Hartley background were bred with red-haired guinea pigs. Red-haired heterozygotes from the F_1 generation were then mated with each other or with hairless albino guinea pigs. The F_2 generation included hairless pigmented guinea pigs that retained their interfollicular epidermal melanocytes and whose skin was red brown in color. The pigmented guinea pigs were selected for this study because their skin contains active interfollicular epidermal melanocytes as well as active follicular melanocytes (13). The former are located in the basal layer in a pattern similar to that observed in humans (14). In addition, an increase in epidermal pigmentation and melanocyte number has been observed in guinea pigs following three different treatments: UVA and UVB irradiation (15), topical MSH plus UVB (16), and subcutaneous injections of MSH (17). The hairless pigmented guinea pigs also offer the advantage of a hairless cutaneous surface to permit noninvasive quantitation of skin pigmentation using skin reflectance measurements.

The prolonged decrease in the "L" values indicates an increase in the dark pigment, and the enhanced eumelanin concentrations confirm these findings. The two melanin pigments involved in skin pigmentation are eumelanin (black-brown) and pheomelanin (yellow-red). The cellular mechanisms regulating follicular and cutaneous melanogenesis in vertebrates are not

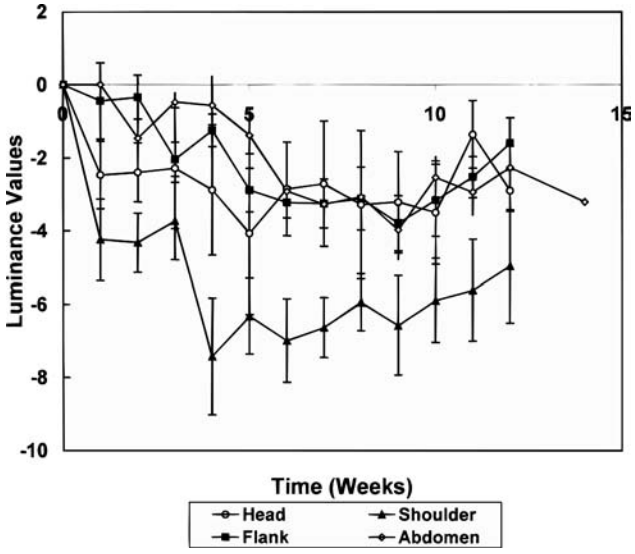

Fig. 5. Luminance value vs. time profile of the hairless guinea pigs measured using a Chroma meter after administration of a 4 mg MT-I implant. Each point represents the mean ± SEM of five replicates.

clearly defined but are certainly influenced by α-MSH, synthesized by the pituitary glands (18). Melanogenesis within the skin and hair follicles results from the synthesis of melanin within the melanosomes, which are the cytoplasmic organelles within the melanocytes (19). These melanosomes are delivered through dendritic processes of the cells to the adjacent keratinocytes of the epidermis wherein they enhance skin pigmentation and function in a photoprotective role. In the haired guinea pigs, the active growth of hair occurs during the anagen phase, which is followed by increased melanin production whose transfer by melanocytes to adjacent cells results in pigmentation of the hair shaft.

The mechanisms involved in the increased pigmentation after the implantation of the MT-I depot are unknown. MT-I might produce effects similar to α-MSH, which causes a cascade of biochemical events culminating in enhanced melanogenesis. These events include binding of α-MSH to the melanocyte receptors leading to activation of cyclic adenosine monophosphate (cAMP). This cyclic nucleotide then activates a cAMP-dependent protein kinase (20). This is followed by activation of nuclear transcriptional processes leading to increased tyrosinase activity. Tyrosine in the presence of tyrosinase is converted to DOPA which, in turn, is converted to DOPA quinone by the same enzyme. Through the formation of several indole intermediates, this process leads to the production of melanin and enhanced skin pigmentation.

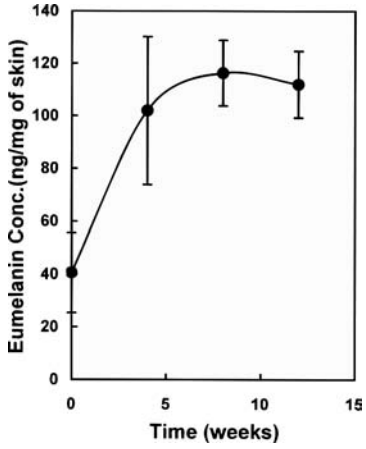

Fig. 6. Eumelanin concentration-time profile after the administration of 4 mg MT-I implants. Each data point represents the mean ± SEM of three replicates.

In addition, MT-I has proven to be 10–1000 times more active than α-MSH in bioassays and has demonstrated increased stability in tissue culture media (3). The most dramatic illustration of the superpotency of this peptide was observed on S91 melanoma cells where the activity of two key enzymes involved in the synthesis of melanin, i.e., tyrosinase and adenylate cyclase, were increased by fifty-fold and twenty-six fold, respectively (3). Previous studies using cultured melanoma cells have shown that MSH regulates melanocyte proliferation, transfer of melanin from the dendrites of melanocytes to keratinocytes, and pigmentation of the skin (21).

Eumelanin and its precursor, 5-6-dihyroxyindole, appear to possess potent photoprotective (antioxidant) properties (22), as opposed to the photodamaging effects of pheomelanin, upon exposure to UV radiation. Pheomelanin may form increased amounts of oxygen radicals upon irradiation with UV-visible light. None of these effects are observed with eumelanin. Hence, quantitation of eumelanin concentration in guinea pig skin after administration of the implant is a measure of the protective effect of MT-I from the ultraviolet rays of the sun.

CONCLUSIONS

This is the first attempt to formulate a prolonged-acting delivery system for this unique melanogenic peptide using a comprehensive approach to evaluate the suitability of an implant preparation. The approach included the determination of the in vitro release profiles of MT-I from the PLGA implants (6,23), a frog skin bioassay to ensure the retention of bioactivity following fabrication and gamma irradiation sterilization of the implants, and the pharmacologic response to the MT-I implants, using a unique inbred population of haired and hairless pigmented guinea pigs. Due to the prolonged biological activity of MT-I, the melanotropic action lasted for three months, thus reducing the need for implant administration from once-a-month to once every three months. In addition, the increase in melanin pigment, especially eumelanin, could provide protection against the photodamaging effects of UV radiation, thereby aiding in the prevention of skin cancers. Future studies could be performed to elucidate further the pharmacologic action of MT-I in order to understand the mechanism of prolonged melanogenesis in the skin. In addition, a study involving the combined effects of MT-I depot implantation with UV exposure to simulate the effect of sunlight on the skin would be an important step in the validation of this implant formulation as a useful therapeutic entity.

ACKNOWLEDGMENTS

We are grateful to Stephanie Cameron for her help with the animal work. We thank Frank T. Walmsley (Histology Service Core Laboratory, College of Medicine, University of

Arizona) and Douglas W. Cromey (Experimental Pathology Service Core, College of Pharmacy, University of Arizona) for their help in histological skin staining and digital imaging, respectively, which was supported by NIEHS grant # ESO6694. Support for this publication by grant # 2P01 CA27502 from the National Cancer Institute is gratefully acknowledged. Its contents are solely the responsibility of the authors and do not represent the official views of the National Cancer Institute. We are extremely grateful to Dr. John Pawelek of Yale University for his kindness in donating the haired and hairless pigmented guinea pigs.

REFERENCES

1. C. Frey and A. Hartman. U.S. trends in melanoma incidence and mortality. *J. Natl. Cancer Inst.* **83**:1705 (1991).
2. B. A. Gilchrest, H. Y. Park, M. S. Eller, and M. Yaar. Mechanisms of ultraviolet light-induced pigmentation. *Photochem. Photobiol.* **63**:1–10 (1996).
3. T. K. Sawyer, P. J. Sanfilippo, V. J. Hruby, M. H. Engel, C. B. Heward, J. B. Burnett, and M. E. Hadley. 4-Norleucine 7-D-phenylalanine-α-melanocyte-stimulating hormones, a highly potent α-melanotropin with ultralong biological activity. *Proc. Natl. Acad. Sci. USA* **77**:5754–5758 (1980).
4. M. E. Hadley, J. H. Mieyr, B. E. Martin, A. M. de La Castrucci, V. J. Hruby, T. K. Sawyer, E. A. Powers, and K. R. Rao. [Nle^4, D-Phe^7]-α-MSH: a superpotent melanotropin with prolonged action on vertebrate chromatophores. *Comp. Biochem. Physiol.* **81**:1–6 (1985).
5. S. O. Ugwu, J. Blanchard, R. T. Dorr, N. Levine, C. Brooks, M. E. Hadley, M. Aickin, and V. J. Hruby. Skin pigmentation and pharmacokinetics of melanotan-I in humans. *Biopharm. Drug Dispos.* **18**:259–269 (1997).
6. R. Bhardwaj and J. Blanchard. In vitro characterization and in vivo release profile of a poly (D, L-lactide-co-glycolide)-based implant delivery system for the α-MSH analog, Melanotan-I. *Int. J. Pharm.* **170**:109–117 (1998).
7. K. Shizume, A. B. Lerner, and T. B. Fitzpatrick. In vitro bioassay for the melanocyte stimulating hormone. *Endocrinology* **54**: 553–560 (1954).
8. A. M. de La Castrucci, M. E. Hadley, and V. J. Hruby. Melanotropin bioassays: in vitro and in vivo comparisons. *Gen. Comp. Endocrinol.* **55**:104–111 (1984).
9. S. Ito and K. Fujita. Microanalysis of eumelanin and pheomelanin in hair and melanomas by chemical degradation and liquid chromatography. *Anal. Biochem.* **144**:527–536 (1985).
10. A. J. Thody, E. M. Higgins, K. Wakamatsu, S. Ito, S. A. Burchill, and J. M. Marks. Pheomelanin as well as eumelanin are present in human epidermis. *J. Invest. Dermatol.* **97**:340–344 (1991).
11. S. Ito and K. Wakamatsu. An improved modification of permanganate oxidation of eumelanin that gives a constant yield of pyrrole-2, 3, 5-tricarboxylic acid. *Pigment Cell Res.* **7**:141–144 (1994).
12. S. O. Ugwu, J. Blanchard, L. D. Nguyen, M. E. Hadley, and R. T. Dorr. A comparison of HPLC and bioassay methods for plasma melanotan-II (MT-II) determination: application to a pharmacokinetic study in rats. *Biopharm. Drug Dispos.* **15**:383–390 (1994).
13. J. L. Bolognia, M. S. Murray, and J. M. Pawelek. Hairless pigmented guinea pigs: A new model for the study of mammalian pigmentation. *Pigment Cell Res.* **3**:150–156 (1990).
14. K. Jimbow, W. C. Quevedo, Jr., T. B. Fitzpatrick, and G. Szabo. Some aspects of melanin biology. *J. Invest. Dermatol.* **67**:72–89 (1976).
15. G. Imokawa, M. Kawai, Y. Mishima, and I. Motegi. Differential analysis of experimental hypermelanosis by UVB, PUVA and allergic contact dermatitis using a brownish guinea pig model. *Arch. Dermatol. Res.* **278**:352–362 (1986).
16. J. Bolognia, M. Murray, and J. Pawelek. UVB-induced melanogenesis may be mediated through the MSH-receptor system. *J. Invest. Dermatol.* **92**:651–656 (1989).
17. R. S. Snell. The effect of ultraviolet irradiation on melanogenesis. *J. Invest. Dermatol.* **40**:127–132 (1963).
18. A. B. Lerner and S. S. McGuire. Effect of alpha and beta melanocyte stimulating hormones on the skin colour of man. *Nature* **189**:176–179 (1961).
19. J. T. Bagnara, J. D. Taylor, and M. E. Hadley. The dermal chromatophore unit. *J. Cell. Biol.* **38**:67–79 (1968).
20. M. E. Hadley. *Endocrinology*, 4th ed., Prentice Hall, Upper Saddle, NJ, 1996, pp. 153–176.
21. J. Pawelek. Factors regulating the growth and pigmentation of melanoma cells. *J. Invest. Dermatol.* **66**:201–209 (1976).
22. K. Jimbow. Current update and trends in melanin pigmentation and melanin biology. *Keio J. Med.* **44**:9–18 (1995).
23. R. Bhardwaj and J. Blanchard. In vitro evaluation of Poly(D,L-lactide-co-glycolide) polymer-based implants containing the α-melanocyte stimulating hormone analog, Melanotan-I. *J. Contr. Rel.* **45**:49–55 (1997).