Report

A human placental extract: *in vivo* and *in vitro* assessments of its melanocyte growth and pigment-inducing activities

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Abstract

Background The authenticity of various prototype human placental extracts with biological activity, such as that inducing vitiligo repigmentation, is under serious criticism, mainly due to a lack of demonstration at the cellular level. Considering the present worldwide scenario with regard to the occurrence and treatment of vitiligo, a thorough scientific exploration of such extracts should be undertaken.

Method One such prototype placental preparation was prepared, and was evaluated with regard to its melanogenic action in C57BL/6J mice *in vivo* and its mitogenic and melanogenic activity on B16F10 mouse melanoma cells and normal human melanocytes *in vitro*. The extract was applied topically to mice with age-induced prolonged telogenic phase of hair growth (grey body coat hair). Standard ^{3}H-thymidine incorporation and spectrophotometric methods were followed to illustrate mitogenic and melanogenic effects at the cellular level.

Results The resurgence of blue skin, followed by shiny black hair, at the regions of application of the extract demonstrated the reversal of the age-induced prolonged telogenic phase of hair growth to the anagenic phase after topical application of the extract on C57BL/6J mice. Further support was obtained from histology where, at the extract-treated sites, the development of new melanogenic centers and hair follicles was observed. During *in vitro* studies, the vehicle-free extract constituents stimulated both mitogenesis and melanogenesis of B16F10 mouse melanoma cells in a concentration-dependent manner. The cell morphology and extent of melanogenesis also showed significant changes. In addition, two known melanocyte activity-modulating peptides, endothelin-1 (ET-1) and adrenocorticotropic hormone (ACTH), were determined in the extract, chiefly in the total lipid fraction, indicating their effective cutaneous permeation.

Conclusions The extract was found to be a potent mitogen in the *in vitro* condition and a potent melanogen in both the *in vitro* and *in vivo* situations. This strongly suggests its therapeutic potential for the repigmentation of vitiligo patches.

Introduction

Human placenta is an extremely rich reservoir of bioactive molecules. Some typical human placental extracts contain keratinocyte growth factor[1,2] or stimulators of endothelial cell growth.[3] In the human placenta, the occurrence of bioactive peptides, such as endothelin-1 (ET-1),[4,5] adrenocorticotropic hormone (ACTH),[6,7] and sphingolipids,[8,9] well-known modulators of different cellular responses, has already been documented. ET-1 is a versatile peptide, originally identified as a vasoactive component, demonstrating significant mitogenic,[10] dendricity-inducing,[11,12] and melanogenic[13] activities on melanocytes. Moreover, ACTH has been reported to play an important role in melanogenesis.[14] Sphingolipids and their metabolites act as crucial second messenger molecules controlling a delicate rheostatic switch to balance cell growth promotion and inhibition signals.[15–17]

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More interestingly, the physiologic growth and pigmentation of melanocytes show an inverse relationship; therefore, the switching actions of sphingolipid metabolites are of paramount importance in melanogenic induction. An alcoholic human placental extract ("Melagenina") has been claimed to be useful for pigment recovery in vitiligo, but did not stand up to this claim due to a lack of adequate scientific evidence. Thus, there was a need to provide authentic data with regard to its composition and the bioactive molecule(s) responsible for its therapeutic action. Extensive propositions were made for biological studies of the material at the cellular level using single cell cultures of melanocytes.[18] There are constant and serious demands, however, to have an extract of term human placenta containing important biomolecules, which would demonstrate the modulation of melanocyte activity, explicitly its proliferation, migration, and melanization. In vitiligo, melanocyte destruction at the sites of the lesions is immediately obvious; only the cells residing mainly at the outer root sheath of the hair follicles (in the vitiliginous areas) survive in the inactive (dopa-negative) form, together with a few live melanocytes in the epidermal regions and at the edge of vitiligo lesions. For the recovery of skin color, modulations of these cells in terms of their growth, migration, and melanogenesis are essential prerequisites.

In view of this, a prototype alcoholic placental extract was prepared[19] using human immunodeficiency virus (HIV)- and hepatitis B antigen-negative term human placenta, which was found to harbor a large number of biomolecules, including sphingolipids and small peptides.[20] During an early experiment with a young guinea pig, used as a preliminary animal model, the extract resulted in the darkening of the areola region around the nipples.[20] Two important bioactive peptides, ET-1 and ACTH, known to have a significant modulatory role on melanocyte activity, have already been identified in this extract.[21] In this report, we demonstrate the effectiveness of the extract in an *in vivo* experiment studying melanization and hair growth in C57BL/6J mice. Moreover, the (alcohol-free) extract constituents stimulated the growth and melanogenesis of melanocyte/melanoma cells, together with morphologic alterations, under *in vitro* culture conditions. Although the study itself is preclinical, it should be judged by its clinical insinuation. It is at the threshold of the clinical segment of the schedule, and has some clinical back-up in the form of a short-term randomized clinical trial.

Materials and methods

Preparation of the human term placental alcoholic extract and estimation of relevant biopeptides

We prepared an alcoholic extract of fresh, term, HIV- and hepatitis B-negative (both from mothers' venous and babies cord blood samples) human placenta, as mentioned previously.[19,20] HIV and hepatitis B were screened by enzyme-linked immunoabsorbent assay (ELISA) techniques using kits from Biochem Immunosystem, Canada, and Abott Diagnostics, USA, respectively. The tissue material was first washed, followed by trituration and temperature-controlled alcohol extraction.[19] Quantification of the extracted amounts of some specific lipid-associated and melanocyte response-modifying bioactive peptides was used to monitor the extraction parameters. Subsequently, both qualitative and quantitative assays of ET-1 and ACTH were carried out as described previously[21] in the extract or fractions thereof following standard ELISA (ELISA assay kit, Amersham Inc., USA) and radioimmunoassay (RIA)[22] (RIA assay kit, Immunotech, France) techniques (Table 1).

Application of the extract on telogenic C57BL/6J mouse skin

C57BL/6J black mice with age-induced early gray body coat hair, pinkish skin, and histologic "melanocyte disappearance,"[23,24] indicating the initiation of a prolonged telogenic phase of hair growth, were used as *in vivo* animal models to assess the pigment-inducing activity of the placental extract. In this condition, pigment recovery in the skin, together with fresh hair growth, could be considered synonymous with the induced growth and melanogenesis of melanocytes, concurring with repigmentation in

Table 1 Quantification of (lipid-associated) endothelin-1 (ET-1) and adrenocorticotropic hormone (ACTH) in placental extract batches

ET-1			ACTH		
Amounts in different batches of whole extract (pg/mL)	Amounts in $CHCl_3$–CH_3OH–H_2O (10 : 10 : 3 v/v) lipid fraction (pg/mL)	% content in the lipid fraction	Amounts in different batches of whole extract (pg/mL)	Amounts in $CHCl_3$–CH_3OH–H_2O (10 : 10 : 3 v/v) lipid fraction (pg/mL)	% content in the lipid fraction
95	74	77.8	3.9	3.1	79.4
119	88	73.9	4.7	3.8	80.0
123	97	78.8	4.1	3.2	78.0
110	85	77.2	3.3	2.7	81.8

vitiligo.[23,24] Any agent capable of reverting a prolonged telogenic phase of hair growth to the anagenic phase (visualized by a change in skin color with the growth of black hair) should have the potential for repigmentation in vitiligo patches,[25] except where leukotrichia is present. As hair follicles in vitiliginous regions are the prime reservoir of live melanocytes, which are, in turn, the primary source of pigment recovery, leukotrichia or pigment loss of hairs disastrously affects the repigmentation phenomenon.

The selected portion of C57BL/6J mouse skin was first clipped to remove early gray body coat hair and, on the telogenic pinkish skin, alcoholic extract was applied dropwise (0.2 mL/cm^2) with gentle rubbing (using the finger tips). The control set was maintained in parallel, with application of an equivalent amount of 60% aqueous alcohol vehicle on the opposite side of the same mouse or on the skin of another group of mice with clipped hair and of similar age. Both application sites were irradiated with IR exposure (IR lamp, Infraphil, 230 V/150 W, Philips) from a distance of approximately 45 cm for 5 min. The process was repeated 4–5 times during each application, and the whole process was carried out twice a day for 4 months without intermission. A. group of five or six mice was included in each experimental set. The animals were fed *ad libitum* in the animal house facility. Changes in skin color and associated hair growth were monitored on a regular basis.

Histologic examination of C57BL/6J mice

After local anesthesia with lignocaine hydrochloride, skin specimens were dissected out by incisional biopsy (4–6 mm and full depth including subcutaneous tissue) from both the extract- and vehicle-treated sites. Standard procedures were followed for histologic examination. Paraffin sections (thickness, 5 μm) were made and stained by the Mason–Fontana staining technique described for melanin.[26]

Cell culture

B16F10 mouse melanoma cells (obtained from the National Centre for Cell Science, Pune, India) were maintained in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (100 U/mL and 100 μg/mL, respectively) and used throughout the studies.

^{3}H-Thymidine incorporation

For mitogenesis studies on B16F10 mouse melanomas, cells were grown in medium supplemented with 2% fetal calf serum (FCS) in a humidified atmosphere of 5% CO_2 in an incubator at 37 °C. Cell proliferation assay was performed as reported previously.[27,28] Briefly, 3×10^4 cells/cm^2 were seeded in 96-well plates. After cell adherence by overnight incubation, the stimulants in the form of extract constituents in alcohol-free phosphate-buffered saline (PBS) emulsion were added in triplicate for each dose.

Subsequently, the culture was incubated for 24 h and harvested thereafter. ^{3}H-Thymidine (1 μC$_i$/mL) was added 6 h prior to the end of the total 24 h time period. The cells were separated, washed, and used to measure the incorporation of radioactivity by a liquid scintillation counter (Model-LKB Wallac 1209, Rackbeta).

Melanin assay

Melanin assay was performed according to the procedure of Naeyaert *et al.*[29] In short, 4×10^5 cells/cm^2 were seeded in six-well plates and incubated overnight for attachment, followed by the addition of the stimulants in duplicate and subsequent incubation for 48 h. The cells were then sequentially washed with PBS, trypsinized, harvested, pelleted, and dissolved in 1 mL of 1 N NaOH to measure the absorbance at 475 nm (λ_{max} = 475 nm). Commercially available synthetic melanin (Sigma) was used as standard.

Results

Application of the extract on C57BL/6J mice

On completion of the extract application course of 4 weeks, the skin color of the telogenic pinkish skin of aged C57BL/6J mice reverted to its anagen-like condition only at the placental extract-treated site and not at the vehicle-treated site (Fig. 1). The skin color changed to light bluish at first with further progressive darkening. Simultaneously, shiny black hair appeared in the extract-treated region with an appearance similar to that found in young animals. This growth was steadily maintained throughout the whole application period. Figures 2a and 2b show the results of application of the 60% alcohol vehicle and the extract, respectively, on separate sets of mice in a parallel experiment The results were in agreement with those shown in Fig. 1.

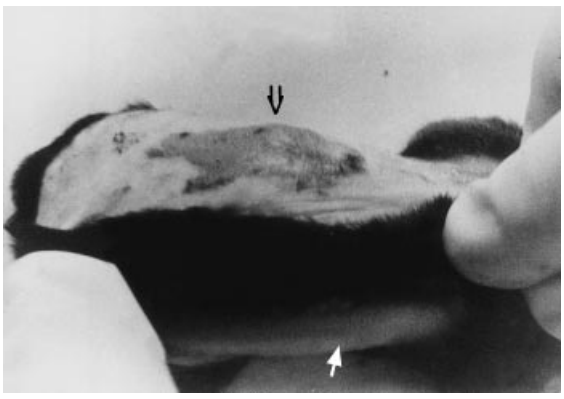

Figure 1 Effect of placental extract application on C57BL/6J mice at the age-induced telogenic stage of hair growth, marked by pinkish skin and graying of hair. ⇓ denotes the extract-treated region with the recurrence of the anagenic stage, as manifested by the growth of black hair with bluish skin. Other arrow denotes control (vehicle)-treated zone with no such growth cycle reversal.

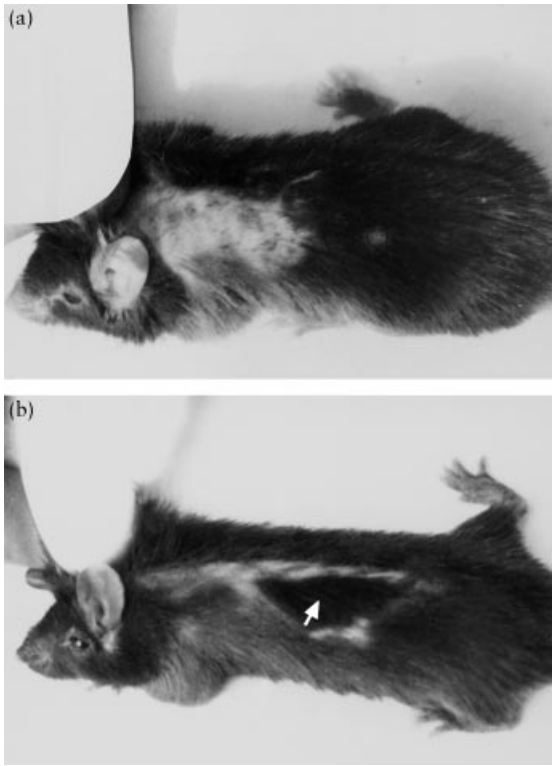

Figure 2 Effect of placental extract application on C57BL/6J mice at the age-induced telogenic stage of hair growth, marked by pinkish skin and graying of hair, when vehicle (control) and extract were applied on separate sets of animals of comparable age. (a) Vehicle-treated mouse with no sign of anagenic reversal. (b) Extract-treated mouse with distinct indications of growth cycle reversal to the anagenic stage, as shown by the growth of deep black shiny hair.

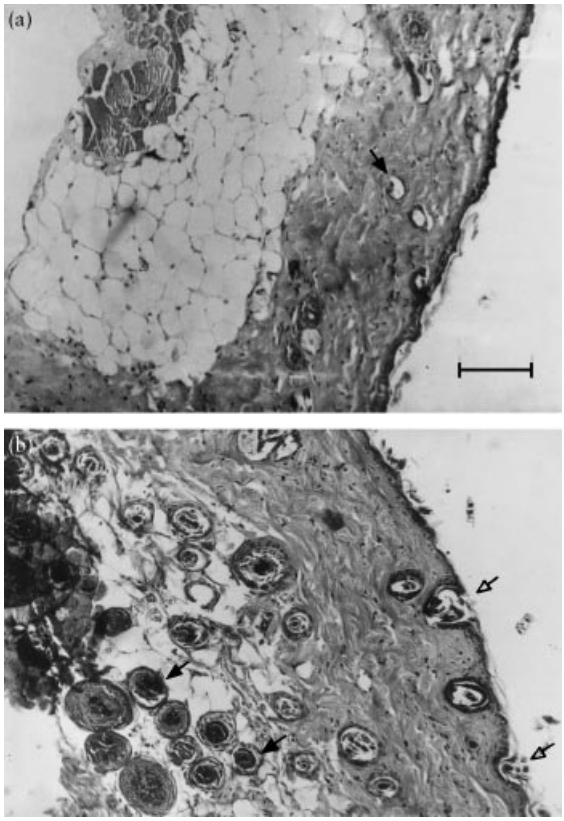

Figure 3 Cross-sectional views of the skin specimens. (a) Vehicle-treated mouse skin with a few melanizing centers (arrow) in the dermis layer. (b) Extract-treated mouse skin with a large number of melanizing centers (solid arrow), together with invagination at the top of the epidermis (open arrow) extending inside the adipocyte layer (magnification, × 400; scale bar, 60 μm).

Histologic examination

Skin specimens from both the extract- and vehicle-treated sites were examined histologically on both cross-sectional and longitudinal views. The alterations in skin organization, melanization, and hair follicle formation were remarkable at the extract-treated site only. Figure 3a and 3b show the cross-sectional views of the vehicle- and extract-treated skin specimens, respectively, whereas Fig. 4a and 4b show the corresponding longitudinal views. Both control slides (Figs 3a and 4a) are mostly hypotrophic. In the cross-sectional view of the experimental specimen, a significant enhancement in the number of melanizing centers, together with invagination at the top of the upper epidermis resembling the initiation of hair follicle growth, was observed in agreement with earlier reports[30,31] (Fig. 3b). In the longitudinal view, new hair follicle development was vividly distinct (Fig. 4b). Along the wall of the new hair follicle well, cells similar in appearance to migratory melanocytes were observed (identified by Mason–Fontana staining for melanin), with fairly large hair bulbs at the root (the size of the adipocyte layers, photographed under the same magnification, can be compared between the control and experimental sets). In the extract-treated skin section, the increase in the number of hair follicles was reflected by an enhancement in the number of melanizing centers. The roots of the newly developed hair bulbs passed beyond the dermis and embedded into the adipocyte layer (Fig. 4b, marked as arrow).

In vitro effect of extract on B16F10 mouse melanoma cells

The mitogenic and melanogenic effects of the placental extract constituents (in the alcohol-free condition) were assessed on the fast growing mouse melanoma cells, B16F10. Figure 5 demonstrates the mitogenic effect of the alcohol-free extract constituents, as measured by ^{3}H-thymidine incorporation. It is clear that the promotion of cell growth can be attributed to the low concentration of extract constituents (maximum at a dose of 50 μg/mL), whereas a high concentration induces growth inhibition. The effect was so prominent

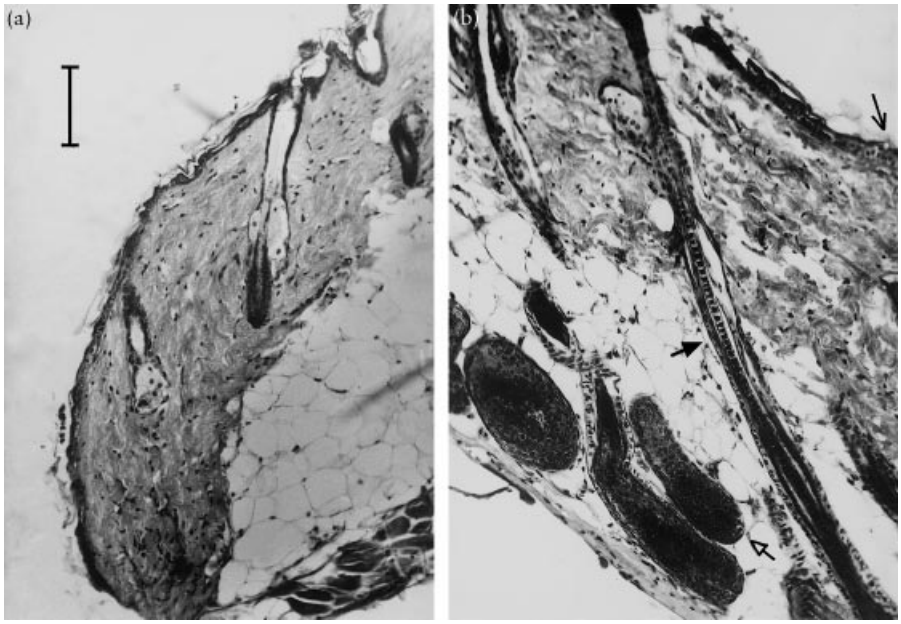

Figure 4 Longitudinal views of the skin specimens. (a) Vehicle-treated mouse skin where the hair follicles are short, thin, few in number, and within the dermal layer, which is dense or hypotrophic as in the cross-sectional sample. (b) Extract-treated mouse skin where a hypertrophic structure and an ample number of new hair follicles can be seen (open arrow) in the adipocyte layer, together with migratory (solid arrow) and repopulating (thin arrow) melanocytes in the epidermis (magnification, × 400; scale bar, 60 μm).

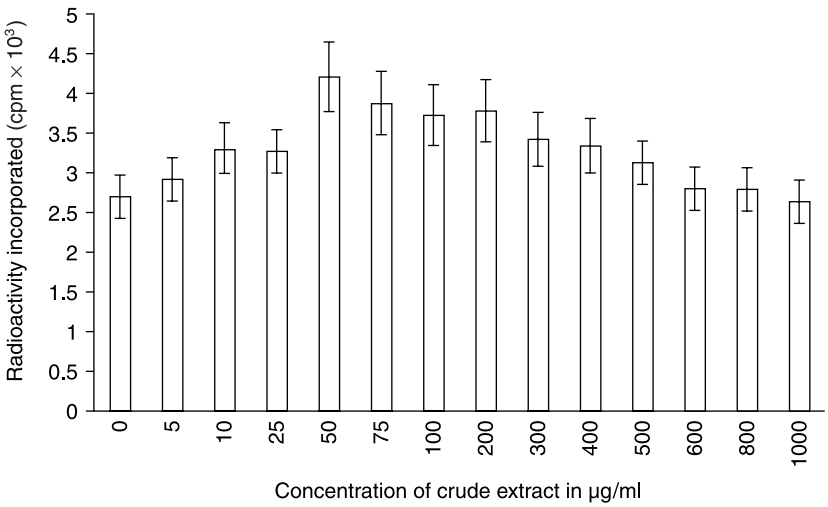

Figure 5 Effect of alcohol-free placental extract constituents on B16F10 mouse melanoma mitogenesis (measured in terms of ^{3}H-thymidine incorporation)

that, at a dose of 1000 μg/mL, ^{3}H-thymidine incorporation was less than half of that found for the control. More significantly, Fig. 6 illustrates that the growth inhibitory concentrations of the extract constituents promote melanization, with an optimum at a dose of 500 μg/mL. Thus, the extract is enriched with biomolecules capable of modulating both mitogenesis and melanogenesis of melanoma cells (B16F10) in a concentration-dependent manner.

Determinations of ET-1 and ACTH in the extract

The two significant melanocyte response-modifying bioactive peptides, ET-1 and ACTH, were assayed[21] in the extract concerned (Table 1). The quantities found were, on average, 112.0 pg/mL and 4.0 pg/mL, respectively. Interestingly, the majority of the two peptides were found in the lipid fractions, thus indicating a plausible lipid–peptide association with wide significance.

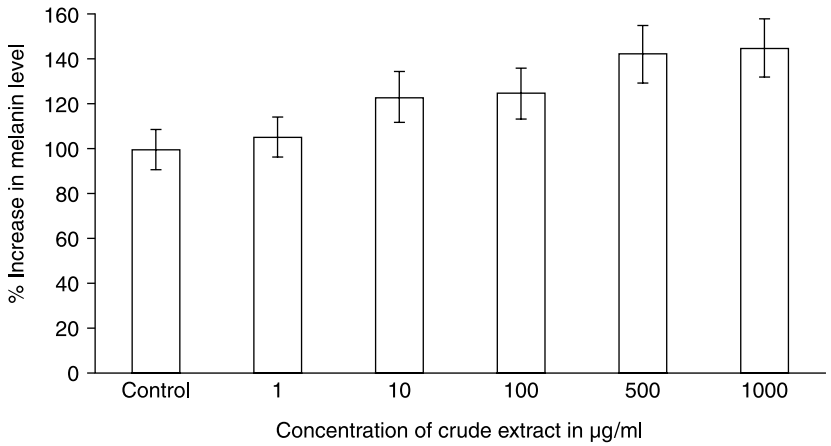

Figure 6 Effect of alcohol-free placental extract constituents on B16F10 mouse melanoma melanogenesis (measured in terms of spectrophotometric absorbance at λ_{max} = 475 nm)

Discussion

In this study, we have demonstrated that an alcoholic human placental extract induces melanogenesis in both an *in vivo* mouse model and an *in vitro* cell culture system. Age-induced prolonged telogenic phase of hair growth with the histologic disappearance of melanocytes in C57BL/6J mice[24] is a condition comparable to the disappearance of melanocytes in vitiligo depigmentation. The appearance of fresh black hair demonstrates the reversal of the telogenic phase to the anagenic phase of the hair growth cycle, thus illustrating the melanogenic action of the extract. In the surrounding ventral region of the extract-treated zone, graying of the hair continued, resembling the vehicle-treated area. Previously, it has been reported that an extract composed of peptides, amino acids, and different classes of lipid, including sphingolipids, causes skin darkening in a guinea pig model.[20] Histologic examination explicitly illustrated an enhancement in the number of melanin-forming centers and the development of new hair follicles, together with invagination at the top of the epidermis. This is conspicuously similar to the proposed model for the development of new hair follicles.[32] The new follicles were not only larger in size, but also passed into the adipocyte layer; therefore, they were distinct and proved to be the product of the action of placental extract constituents. The dermis region was hypertrophic compared to that of the control or vehicle-treated skin, which was hypotrophic. Hence, the structural organization of the skin undergoes some remarkable changes. Along the wall of the hair follicle well, upward migratory cells, resembling migratory melanocytes (identified by Mason–Fontana staining for melanin), appeared and were found to be distributed in the upper epidermis (Fig. 4b). The results in this study are similar to those found by Cui *et al.*,[33] and are significant in that this phenomenon of differentiation of the hair follicle premelanocytes into active forms and their subsequent migration to the epidermis are the key events for repigmentation in vitiligo lesions.

The use of IR light during *in vivo* topical application is beneficial and supportive in two ways. First, it effectively induces vasodilation, causing a significant increase in blood flow.[34] This enhanced blood flow distributes the active constituent(s) more effectively, thus facilitating its action. The interaction of IR light with the vehicle of the extract, alcohol, has some additional importance. Solvents such as alcohol can successfully induce transitory barrier perturbation,[35] facilitating the permeation of active lipid component(s). Penetration and vasodilation in combination efficiently boost the overall action of the extract.[34] IR irradiation is equivalent to heat treatment and can promote the dopa-oxidase activity of melanocytes, together with an enhancement in the number of dopa-positive and tyrosinase-related protein-1-positive melanocytes, in the *in vitro* condition.[36] In this study, we exposed the experimental animals to irradiation from a distance of 45 cm for a duration of only 5 min at a time, when the temperature did not in any circumstances exceed 38 °C. Moreover, the time interval between two successive irradiations was a minimum of 5 min and, by this time, the temperature had decreased and was expected to be lower than 38 °C. Hence, our experimental conditions were substantially less favorable than those reported previously[36] for the promotion of melanocytic activity. Therefore, we can conclude that the IR exposure in this study produced no side-effects equivalent to those of UVB or heat (42 °C),[36] such as sunburn, tanning, hyperplasia, immunologic changes, photoaging, and even photocarcinogenesis.[37] Lastly, IR use is more cost-effective from the patients' viewpoint.

The effect of the placental extract was also examined in *in vitro* culture conditions with B16F10 mouse melanoma cells. The results found were quite encouraging from the viewpoint of repigmentation in vitiligo.[38,39] Low concentrations of the extract constituents promoted a concentration-dependent mitogenesis, which attained a maximum value; with a further increase in concentration, inhibition of growth was induced, and melanogenesis was observed. Moreover, with a further

increase in concentration, melanogenesis reached an optimum when radioactive incorporation (i.e. mitogenesis) was almost comparable with the control value. This type of rheostatic duality in mitogenic action may be due to the sphingolipid component(s) of the extract.[16,20] This viewpoint is further supported by an ongoing experiment, in which we have been able to purify a sphingolipid component in a single spot by high-performance thin layer chromatography (HPTLC) from the extract preparation, and observed analogous results (Pal *et al*., in preparation). Possibly, some sort of stress is generated at high concentrations of the extract constituents, where cell growth is inhibited, and this stress condition may, in turn, lead to the induction of melanogenesis, which is a stress-induced phenomenon.[40] Moreover, the extract constituents induce growth suppression only; the cells remain fully viable and growth is resumed as normal after the addition of fresh growth medium (data not shown) devoid of any experimental growth modulator. The growth and melanogenic effects of the extract constituents on B16F10 mouse melanoma cells are confirmed by similar findings with normal human melanocytes (Pal *et al*., in preparation).

The presence of ET-1 and ACTH in the extract[21] is of crucial importance considering their significance in promoting melanocyte activity, such as mitogenesis, migration, and melanogenesis. Moreover, they remain in a highly lipid-associated form: more than 80% of the total in the lipid-containing organic phase and the remainder in the aqueous fraction (Table 1). For topical application, the therapeutic peptides do not penetrate the skin effectively, but their penetration can be enhanced by solubilization in alcohol,[41] and/or when they are strongly lipid associated.[35,42] Thus, the extract, which contains the peptides (together with certain lipid constituent(s)) in their lipid-associated form, is vital in promoting cellular growth and melanization, thereby producing repigmentation.

Therefore, the findings of both *in vivo* (C57BL/6J mice) and *in vitro* (B16F10 mouse melanoma cells) studies indicate the presence of activity modulators of melanocyte cells in the extract. Toxicity-related experiments with the extract preparation showed it to be nonmutagenic, noncytotoxic, and totally safe and skin-friendly in nature (Pal *et al*., in preparation). Hence, the inherent potency of the alcoholic human placental extract for repigmentation in vitiligo cases should be asserted. This assertion may gain further momentum when the results of a short-term randomized clinical trial on a small number of voluntary vitiligo vulgaris patients,[20,43] in which highly or moderately satisfactory pigment recovery was documented in more than 50% of cases, are considered. The proposed active components, i.e. bioactive peptides and some lipids, are highly physiologically compatible, so to bear therapeutic integrity. Further characterization of these active components is essential and is part of our future schedule.

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