Abstract. Melagenine, extracted from human placenta, has been shown to be effective in treating patients with vitiligo, yet the mechanisms of melagenine in inducing the repigmentation of vitiligo patients have not been fully investigated. Recent studies have suggested that melagenine stimulates melanocyte proliferation and melanogenesis. In this study, we utilized the NCCmelb4M5 melanoblast cell line to investigate the effects of melagenine on proliferation and differentiation of immature melanocytes or melanoblasts. NCCmelb4M5 cells were treated with different concentrations of melagenine (50-400 μg/ml), and MTT assay was performed to evaluate the effects of melagenine on proliferation of melanoblasts. RT-PCR and Western blotting were used to determine the expression of c-KIT and tyrosinase (TYR). Our results show that melagenine stimulates proliferation of NCCmelb4M5 cells in a dose-dependent manner with an optimal concentration of 100 μg/ml. Multipolar and highly branched dendritic network, as well as cluster-like growing cell assembly were visible in melagenine-treated NCCmelb4M5 cells. Melagenine induced expression of c-KIT, TYR and MITF. Our results provide insights into the molecular mechanism of the beneficial effect of melagenine in the treatment of vitiligo.

Introduction

Vitiligo is an acquired pigmented disorder characterized by depigmentation of skin and hair. Approximately 0-5% of the world's population is affected by this disease (1). Due to the poor understanding of the mechanism by which melanocytes (MCs) disappear from involved skin, treatment of vitiligo has generally been unsatisfactory. Traditional therapies that induce varying degrees of repigmentation in vitiligo patients include topical corticosteroids (2,3), phototherapy (4), photothermolysis (4), and helium-neon laser treatment (5).

It has been reported that MCs can be recruited from the outer root sheath of the hair follicle to repigment the vitiliginous skin through various forms of therapy (6,7). Recovery of vitiligo was also initiated by proliferation and differentiation of the immature melanocytes (melanocyte precursors or melanoblasts), followed by their upward migration to the nearby epidermis to differentiate into MCs and form perifollicular pigment islands (4).

Melanocyte precursor, NCCmeb4M5, has recently been established (8), and was derived from NCCmelb4 cells. These cells were positive for melanocyte markers such as tyrosinase-related protein 1 (TRP-1) and DOPAchrome tautomerase, but were negative for tyrosinase (TYR) and KIT. These characteristics made this cell line an ideal model to study the effects of many factors related to melanocyte development.

Recently, it was demonstrated that 12-O-tetradecanoyl phorbol 13-acetate (TPA) and cholera toxin (CT) induced KIT expression in NCCmelb4M5 cells which have the potential to differentiate into KIT-positive melanocyte precursors, and this may be useful in studying the mechanisms of development and differentiation of melanocytes in mouse neural crest cells (8).

Melagenine, an extract from human placenta, was first used for the treatment of vitiligo in 1991 (9). We previously reported that melagenine had therapeutic efficacy for child vitiligo patients by inducing repigmentation (10). Other studies have suggested that melagenine is an extremely rich reservoir of bioactive molecules, since some typical human placental extracts contain keratinocyte growth factor (11) or stimulators of endothelial cell growth (12).

It has been well documented that in the human placenta there are bioactive peptides such as endothelin-1 (ET-1) (13), adrenocorticotropic hormone (ACTH) (14) and sphingolipids (15), well-known modulators of different cellular responses. Our previous studies demonstrated that melagenine stimulates MC proliferation and melanin synthesis (10). Other researchers reported that human placental lipid induced melanogenesis in melanoma cells by increasing the expression of tyrosinase.
NCCmelb4M5 cells (1x10^5) were seeded in a MTT assay. Confluent for experiments. Culture medium was changed at a humidified atmosphere of 5% CO2 and 95% air at 37˚C in EMEM (School of Medicine, Japan). Cells were grown in a precursor derived from NCCmelb4 cells and was kindly provided by Professor Mizoguchi (St Marianna University School of Medicine, Japan). Cells were grown in a humidified atmosphere of 5% CO2 and 95% air at 37˚C in EMEM supplemented with 5% FBS. Culture medium was changed every 3 days, and cells were subcultured when they became confluent for experiments.

**Materials and methods**

**Preparation of melagenine.** Melagenine was prepared according to the method described previously (10), and the protocol was approved for use in our hospital by local authorities. Briefly, the total constituents of hydroalcoholic placentals extracts were treated with ethanol and filtered on a sephadex column. The resulting colorless and lucent liquid examined was approved for use in our hospital by local authorities.

**Cell culture.** The cell line NCCmelb4M5, is a melanocyte precursor derived from NCCmelb4 cells and was kindly provided by Professor Mizoguchi (St Marianna University School of Medicine, Japan). Cells were grown in a humidified atmosphere of 5% CO2 and 95% air at 37˚C in EMEM supplemented with 5% FBS. Culture medium was changed every 3 days, and cells were subcultured when they became confluent for experiments.

**MTT assay.** NCCmelb4M5 cells (1x10^5) were seeded in a 96-well tissue culture plate. One day after seeding, cells were treated with melagenine at various concentrations (0, 50, 100, 200, 400 μg/ml). After 72 h of incubation, proliferation rate of NCCmelb4M5 cells was measured with MTT proliferation assay kit (ATCC, USA) according to the manufacturer’s instructions.

**RNA isolation and semi-quantitative RT-PCR.** NCCmelb4M5 cells (1x10^6) were seeded onto a 6-well cell culture plate and incubated for 24 h. The cells were then treated with melagenine at a concentration of 100 μg/ml. After 72 h of incubation, total RNA was extracted from melagenine-treated NCCmelb4M5 cells by Trizol Reagent (Bio Basic Inc., Canada) and reverse-transcribed using a Reverse Transcription System Kit (MIB Fermentas, Vilnius, Lithuania) following the manufacturer's instructions. The cDNA as readout of mRNA was amplified by PCR using specific primers for c-KIT, TYR, MITF, and β-actin. Primers for c-KIT were, 5'- CGA CTG CCC GTG AAG TGG A-3' (sense) and 5'-GCA AGA AGG AGG GGG TCG G-3' (anti-sense). Primers for TYR were, 5'-TTC AAA GGG GTG GAT GAC-3' (sense) and 5'-GAC ACA TAG TAA TGC ATC-3' (anti-sense). Primers for MITF were, 5'-AGT CAC TAC CAG GTG CAG AC-3' (sense) and 5'-CTT GCT TCA GAC TCT GTG GG-3' (anti-sense). Primers for β-actin were, 5'-AGT GAC TGG GCT TCA GAC-3' (sense) and 5'-TTT AAA GGG GTG GAT GAC-3' (sense) and 5'-GAC ACA TAG TAA TGC ATC-3' (anti-sense).

**Statistical analysis.** The results were expressed as mean ± SD. The Student's t-test was used for statistical evaluation between control and experimental groups in the study. The difference was considered statistically significant at P-value <0.05.

**Results**

**Effect of melagenine on the proliferation of NCCmelb4M5 cells.** We studied whether melagenine affects proliferation of NCCmelb4M5 cells. Cells were treated with various concentrations of melagenine, and MTT assay was performed to measure the proliferation rate of those cells. Our results show that melagenine induced proliferation of NCCmelb4M5 cells in a dose-dependent manner with an optimal concentration of 100 μg/ml (Table I).

**Effect of melagenine on differentiation of NCCmelb4M5 cells.** To further investigate whether melagenine induces NCCmelb4M5 cell differentiation, we treated the cells with 100 μg/ml of melagenine and cell morphology was observed under a microscope. We found that after treatment with melagenine, a remarkable phenotype change occurred in NCCmelb4M5 cells. As shown in Fig. 1, multipolar and highly branched dendritic network, as well as cluster-like growing cell assembly were visible in melagenine-treated NCCmelb4M5 cells. These data indicate that melagenine effectively induced the phenotype changes. As NCCmelb4M5 cells are immature melanocytes and were originally negative for melanocyte markers such as c-KIT and TYR, we consider that the growth promotion elicited by melagenine might be associated with their differentiation.

**Induction of c-KIT and TYR by treatment with melagenine.** We then examined the effects of melagenine on the expression of c-KIT and TYR. After 72 h of treatment with melagenine, semi-quantitative RT-PCR and Western blot analysis were carried out to measure the expression of c-KIT and TYR. Our results demonstrate that melagenine treatment induced c-KIT and TYR expression at both mRNA and protein levels in...
NCCmelb4M5 cells (Fig. 2). These results suggest that melagenine stimulated differentiation of immature melanocyte precursors.

Induction of MITF by treatment with melagenine. To further investigate the molecular basis of melanocyte precursor differentiation induced by melagenine, mRNA expression of MITF was tested. NCCmelb4M5 cells were incubated with melagenine at various concentrations (0, 50, 100 μg/ml) for 72 h and then were collected and subjected to extraction of total cellular mRNA. After reverse transcription, cDNA was amplified by semi-quantitative RT-PCR using specific primers described in Materials and methods. The data shown are a representative result from separate experiments (A) and the densitometric analyses (mean ± SD) were obtained from three independent experiments. M, marker; 1, melagenine (0 μg/ml); 2, melagenine (50 μg/ml); 3, melagenine (100 μg/ml).

Discussion

It is well documented that recovery of vitiligo is initiated by proliferation, migration and melanogenesis of melanocytes as well as the migration and differentiation of melanocyte precursors. Treatment of vitiligo with topical melagenine has been proven effective in a series of pre-clinical studies (3,10). However, the underlying mechanisms of repigmentation induced by melagenine have not been thoroughly clarified.

Table I. Effect of melagenine on the proliferation of NCCmelb4M5 cells.

<table>
<thead>
<tr>
<th>Groups</th>
<th>OD (570 nm)</th>
<th>Average OD (570 nm)</th>
<th>Average OD (570 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.221</td>
<td>0.219</td>
<td>0.252</td>
</tr>
<tr>
<td>Melagenine (50 μg/ml)</td>
<td>0.413</td>
<td>0.452</td>
<td>0.383</td>
</tr>
<tr>
<td>Melagenine (100 μg/ml)</td>
<td>0.856</td>
<td>0.756</td>
<td>0.814</td>
</tr>
<tr>
<td>Melagenine (200 μg/ml)</td>
<td>0.766</td>
<td>0.634</td>
<td>0.726</td>
</tr>
<tr>
<td>Melagenine (400 μg/ml)</td>
<td>0.710</td>
<td>0.843</td>
<td>0.719</td>
</tr>
</tbody>
</table>

Cells were treated with melagenine at various concentrations; MTT assay was carried out after 72 h of incubation. Data are shown as the mean ± SD. Similar results were obtained in three independent experiments. *P<0.05.
Previously we reported that the direct effects of melagenine on melanocytes may contribute to its therapeutic effect (10). It has also been reported that melagenine treatment induces changes in melanocyte growth and pigment-inducing activities in the skin of C57BL/6J mice (18), yet the effects of melagenine on melanocyte precursors have not been extensively studied. Therefore, in this study, we employed melagenine precursor NCCmelb4M5 and investigated the effects of melagenine on its proliferation and differentiation.

In this study, we demonstrated that melagenine, which is a component of many bioactive molecules, promoted the growth of NCCmelb4M5 cells. The growth promotion was accompanied by increased expression of tyrosinase and c-KIT and alteration in cell shape from polygonal to bipolar or dendritic. In NCCmelb4M5 cell cultures, melagenine treatment also induced MITF expression. Collectively, our data suggest that melagenine directly stimulates proliferation and differentiation of immature melanocyte precursors.

Reverse transcription-PCR and Western blot analysis used in this study reveal that melagenine treatment induced c-KIT and TYR at both mRNA and protein levels in NCCmelb4M5 cells. This finding suggests that melagenine acts to promote the expression of c-KIT and TYR in immature melanocyte precursors and may also play an important role in modulating melanocyte development in vivo.

To the best of our knowledge, this is the first study to report the effect of melagenine on c-KIT and TYR expression. Previous in vivo studies have suggested the importance of transient c-KIT expression in melanoblast migration into the developing murine hair follicle (19). Part of our study demonstrated that melagenine enhanced migration of NCCmelb4M5 cells (data not shown). Whether the melagenine-induced expression of c-KIT in NCCmelb4M5 cells mediates cell migration needs to be further investigated.

Accumulating data indicate that MITF plays a pivotal role in regulating melanocyte development, survival, and differentiation (20,21). MITF is the human homolog of the mouse microphthalmia (mi) gene product, which was identified as a novel transcription factor of the basic helix-loop-helix (bHLH) leucine zipper protein family. Studies on the cell-specific expression of tyrosinase have shown that the M-box in the upstream promoter region of the tyrosinase gene is required for its efficient expression in melanocytes. This motif contains a canonical CANNTG motif that is recognized and bound by MITF.

Since melagenine induced tyrosinase expression, it would be interesting to see whether melagenine alters MITF expression in NCCmelb4M5 cells. In this study, RT-PCR analysis revealed that MITF mRNA expression is markedly increased by melagenine treatment, suggesting that MITF may be the key factor in melagenine-induced melanocyte differentiation. This is the first study showing the induction of MITF by treatment with melagenine.

Human placenta is enriched with numerous bioactive components, among which are melanocyte activity modulators including pro-opiomelanocortin peptides and corticotrophin releasing factor and other growth factors such as ET-1, bFGF (22-25). ET-1 is a versatile peptide, originally identified as a vasoactive component, demonstrating significant mitogenic, dendritic inducing and melanogenic activities in melanocytes (26). bFGF stimulates proliferation of murine melanoblasts, as well as human melanocytes (27).

Furthermore, the sphingolipids have a highly bioactive back bone (sphingoid bases and ceramides) and have been implicated in the regulation of cell growth and differentiation (28), with their metabolites also being implicated in controlling a delicate rheostatic switch to balance cell growth promotion and inhibition signals (29). Recently, one novel sphingolipid or its metabolites, namely sphingosylphosphorylcholine (SPC) and sphingosine-1-phosphate (S1P) was reported to be mitogenic in human melanocyte cells (30). Most recent studies reported that sphingolipids upregulate tyrosinase gene expression at transcription level (31). Our previous studies demonstrated the presence of sphingolipids and several growth factors such as ET-1 and bFGF in melagenine. Thus, the promotion of proliferation and differentiation of NCCmelb4M5 cells by melagenine is very likely due to the presence of the sphingolipids and some growth factors which have been proven to be effective stimulators of melanocyte development.

In conclusion, we showed in vitro evidence demonstrating direct effects of melagenine on proliferation and differentiation of melanocyte precursor NCCmelb4M5 cells. We also provided data showing that melagenine induces c-KIT, TYR and MITF expression in NCCmelb4M5 cells. Our findings provide insights into the molecular mechanism of melagenine in the treatment of vitiligo.

Acknowledgments

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References


