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A stimulatory effect of Cassia occidentalis on melanoblast differentiation and migration

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Abstract In vitiligo, the active melanocytes in the epidermis are totally missing, whereas melanoblast cells in the outer root sheath of hair follicles are not affected. In an attempt to find potent repigmenting agents for vitiligo therapy, pod extracts of Cassia occidentalis was found to be effective in inducing differentiation and migration of mouse melanoblast cell line. Methanolic extract redisolved in DMSO at 12.5 μg/ml was found to cause 3.5- to 3.8-fold melanin induction in melb-a melanoblast cells after 4 days in treatment medium. In addition it induced the tyrosinase activity and altered melb-a cell morphology. Transwell migration assay showed the potential of this herbal candidate to induce direct migration of treated cells. To the best of our knowledge, this is the first report investigating the effect of Cassia occidentalis on the differentiation and migration of melanoblast cells. The findings of present study are significant in designing preclinical and clinical studies on the efficacy of C. occidentalis as a stimulant for skin repigmentation in vitiligo.

Keywords Vitiligo · Repigmentation · Migration · Melanoblast · Cassia occidentalis

Introduction

Vitiligo is defined as a 'circumscribed, acquired, idiopathic, progressive hypomelanotic skin disorder, which is characterized by the development of patchy depigmented macules due to progressive loss of melanocytes' [15]. The precise cause of the loss of these epidermal melanocytes is unknown. Autoimmune, genetic, neural, oxidative stress, autotoxic, viral, and melanocytes detachment mechanisms have been proposed to explain the pathogenesis of vitiligo. Since the exact etiology of vitiligo is unknown, current treatment modalities are directed towards stopping progression and restore pigmentation either by stimulating the division and movement of existing melanocytes into depigmented areas or by stopping the underlying mechanism causing depigmentation [11].

In the vitiligo lesion, the active melanocytes in the epidermis are totally missing, whereas melanocyte-stem cells called melanoblasts, located at the bulge region of the hair follicle are not affected [3]. Therefore, the existences of these inactive melanocytes precursors provide sources for repigmentation in vitiligo [5]. And these inactive melanoblasts should be triggered to migrate and differentiate to synthesis melanin. Ultraviolet radiation or various cytokines such as α-MSH and stem cell factor from the lesional keratinocytes may trigger them [13]. However, the treatment duration is very long by current modalities, we need a new therapeutic agent to stimulate melanoblast for its migration and differentiation.

The use of plants for their therapeutic value is a part of the human history. Therapeutic use of psoralen-containing plants such as Ammi majus [9] and Psoralea corylifolia [2] has been depicted in pre-Hindu Vedic and ancient Egyptian texts [10]. An Ayurvedic herb, Picrophiza kurroa, has been reported to potentiate the effects of psoralens in the
phototherapy of vitiligo [1]. In another finding Ginkgo biloba extracts tend to work on some vitiligo patients due to its antioxidant and immunomodulatory properties [12]. Recently, a stimulatory effect of piperine, the major alkaloid found in the fruit of black pepper (Piper nigrum L.; Piperaceae) on melanocyte proliferation and dendricity has been reported. Also, treatment with piperine and synthetic analogues induced a marked pigmentation response in sparsely pigmented mouse model and hence supports the potential use of these compounds in treating vitiligo [4]. From the literature it became evident that so far no herbal formulations were reported to induce differentiation of melanoblasts. To the best of our knowledge, this study represents the first investigation to evaluate the effect of an herbal extract on the differentiation and migration of melanoblast cells.

Materials and methods

Materials

Murine fibroblast growth factor (FGF)-basic was purchased from PeproTech (Rocky Hill, NJ, USA). Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640 medium, Phorbol 12, 13-dibutyrate (PDBu), 3,4-dihydroxy-l-phenylalanine (l-DOPA), Alpha-melanocyte stimulating hormone (α-MSH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, USA). RPMI-1640, fetal bovine serum (FBS), trypsin-EDTA, phosphate buffered saline (PBS) and penicillin/streptomycin were purchased from Invitrogen Corp (CA, USA). Cassia occidentalis pods were collected from the field of Baguio City, Philippines and were authenticated by Dr. G. A. Reyes, College of Natural Sciences, Saint Louis University, Philippines. Dried pod was extracted three times with 99.5% methanol for 4 h at 37°C. The resulting mixtures were filtered and concentrated to dryness at 40°C under vacuum to produce a methanol extract. Methanolic extract was redissolved in DMSO for sample preparation.

Cell and culture conditions

Melb-a cells Melb-a, was kindly obtained from Dr. Bennett, Welcome Trust, Functional Genomics Cell Bank (London, UK). They were grown in a humidified atmosphere with 10% CO₂ at 37°C. Cells were routinely passed in complete RPMI 1640 medium supplemented with PDBu (20 nM), bFGF (1 ng/ml), fetal calf serum (5%), and L-Glutamine (2 nM). Cells were harvested by brief treatment with trypsin/ethylenediamine tetraacetic acid (EDTA) and were sedimented and resuspended in complete RPMI 1640 medium. Viable cells, determined by trypan blue exclusion, were counted in a hemocytometer, resuspended in the appropriate volume of complete RPMI 1640 medium, and seeded at 2 × 10⁵ cells per cm² in 10 cm cell culture dishes.

Melanin assay

For melanin assay, melb-a cells were seeded at 6 × 10⁴ cells/plate in 12 well plates. After allowing 1 day for cell attachment, the medium was changed and dried methanolic extract of pod dissolved in DMSO at different concentrations (from 3.12 to 25 μg/ml) were added in fresh complete RPMI 1640 medium. Equivalent volumes of diluent (DMSO) were added to untreated control (negative control). Medium supplemented with α-MSH (10 nM) was kept as positive control. Culture media were replaced every 2 days for 4 days (a total of two treatments). After 96 h, the cells were harvested by trypsinization. After washing twice with PBS, samples are dissolved in 1 N NaOH containing 10% DMSO. The samples are then treated at 80°C for 1 h and cooled. The absorbance of extracted melanin was read at 405 nm using an ELISA microplate reader [8] and was expressed as n-fold compared with the control. The effect of treatment on melanoblast cell morphology was determined using Phase contrast microscopy.

Cell migration assay

Migratory activity of melb-a cells was determined by assessing the ability of the cells to cross the 8.0 μm of migration chambers under chemokinetic conditions. Cell migration was assessed in transwell cell culture chambers. In transwell cell culture chambers (Costar 3422; Cambridge, MA, USA), polyvinylpyrrolidone-free polycarbonate filters with 8.0 μm pore size were precoated with 1% gelatin. After incubating the cell insert for 1 h, melb-a melanoblasts (2 × 10⁶/100 μl of cell suspension in serum free RPMI medium) were added to the upper chamber of cell insert and 600 μl of RPMI serum free medium with treatment samples added in the lower chamber. Transwells were incubated for 24 h. After 24 h the filters with gelatin intact were fixed with methanol, and then stained with hematoxylin and eosin. Cells on the upper surface of the filters were removed by wiping with a cotton swab. Cells that had migrated to the lower surface of the filters were counted under a microscope and were photographed. Number of cells that migrated across the filters in 4 high power fields (40 × objective) per insert was counted and values averaged. For each migration condition, three identical replicates were performed.
Viability assay

Melb-a cells were cultured at 4.0 × 10³ cells/ml in 96 well plate. After 24 h, the cells were treated with methanolic extract of Cassia occidentalis at different concentrations (from 3.12 to 25 μg/ml). After 96 h of incubation with test compound (herbal extract), 100 μl of MTT (5 mg/ml in phosphate buffered saline) solution was added to the wells. After 4 h of incubation the medium was removed and 100 μl of DMSO was added to dissolve the formazan produced in the cells. The absorbance of each well was then read at 540 nm by using an ELISA microplate reader. The optical density of formazan formed by control cells was used as reference [8].

Cellular tyrosinase assay

Cellular tyrosinase activity was assayed as DOPA oxidase activity. Melb-a cells were seeded in 60r dishes at a density of 2 × 10⁴ cell/dish and cultured for 24 h. After being treated with samples for 96 h, the cells were harvested with cold PBS and lysed with 100 μl of lysis buffer (0.1 M phosphate buffer, pH 6.8, containing 1% (w/v) Triton X-100) supplemented with protease inhibitors. The cells were disrupted by sonication for 30 min and lysates were clarified by centrifugation at 13,000 rpm for 20 min. After determining the protein content with a Bio-Rad protein assay kit, lysates were adjusted with lysis buffer to contain equal amounts of protein. These lysates were then added to wells (96-well plates) containing 2.5 mM l-DOPA in 0.1 M phosphate buffer (pH 6.8). After incubation at 37°C for 1 h, the absorbance of samples was measured at 475 nm using an ELISA reader and was expressed as n-fold compared with the control.

Statistical analysis

The values are expressed as mean standard deviation (SD). Differences between results were assessed for significance using the Student’s t test and a p value of <0.05 was accepted as significant.

Result and discussion

In a screening experiment conducted for finding skin whitening agents from a total of 311 Philippine plants, Cassia occidentalis was selected as a hyperpigmenting candidate. Cassia occidentalis is a leguminous plant (Family: Fabaceae, Genus: Cassia, Species: occidentalis) found ubiquitously in Asia and Europe as a weed among various crops [7]. Based on its hyperpigmenting activity we decided to investigate the potential of the herb in mouse melanoblast cells. Sviderskaya et al. [14] have successfully produced a cloned line of immortal melanocyte precursors, termed melb-a melanoblasts. The characteristic phenotype of these cells includes a lack of expression of tyrosinase and no ability to oxidize DOPA under standard culture conditions. However, as melanocyte precursors, they do express other melanocyte specific proteins such as Tyrp1 and Dct. Thus, melb-a melanoblast provide a good model to study the differentiation and migration of melanoblasts and to characterize the mechanisms involved though it is murine cell. First, we examined whether exposure to our plant extract would affect the cell viability of melb-a

Fig. 1 Effect of Cassia occidentalis pod extract on cell viability in cultured melb-a melanoblast cells. Data are expressed as percent change of the cell viability level relative to untreated control. Each determination was made in triplicate and data shown are means ± SD. * p < 0.01: statistically significant from the control

Fig. 2 Effect of Cassia occidentalis pod extract on melanin content in cultured melb-a melanoblast cells. Data are reported as n-fold compared with the control. Each determination was made in triplicate and data shown are means ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001: statistically significant from control
Fig. 3 Effect of *Cassia occidentalis* pod extract on cellular tyrosinase activity compared to the negative control without treatment. Data are reported as n-fold compared with the controls. Each determination was made in triplicate and data shown are means ± SD. *p < 0.01, **p < 0.05: statistically significant from the control.

melanoblasts. As shown in Fig. 1, *Cassia occidentalis* pod extract exerted no cytotoxic effect on the melb-a cells up to 12.5 μg/ml, but there is a reduction in viability at 25 μg/ml. Cell differentiation was measured on the basis of melanin content in the treated cells. The untreated cells remained white and showed negligible melanin content; while the cells treated with α-MSH showed 6- to 8-fold melanin stimulation. The melanin content was found to be increased by *C. occidentalis* pod extract in a dose-dependent manner as shown in Fig. 2. Cell pellets also clearly demonstrated the effect of treatment on melb-a differentiation. Maximum stimulation of melanin synthesis (3.5- to 3.8-fold) was observed at 12.5 μg/ml. Hence this extract concentration was used for subsequent experiments. An apparent reduction in cell viability at 25 μg/ml as reflected by viability assay could be reason for reduced melanin content at that particular concentration. This result suggests that *C. occidentalis* can induce melanoblast differentiation in a medium that defines and maintains melanoblasts in normal circumstances. Care was taken to adjust the seeding concentration so as to maintain the cell confluency below $8 \times 10^5$ cells/ml to avoid spontaneous differentiation [14] by the end of incubation time. Therefore, the differentiation observed after the treatment with the sample, cannot be attributed to the spontaneous differentiation. To further investigate the effect of treatment on tyrosinase induction, cellular tyrosinase assay was carried out. Compared with the control, treatment with *C. occidentalis* pod extract significantly stimulated the cellular tyrosinase activity in melb-a cells (25- to 28-fold) whereas α-MSH stimulated cellular tyrosinase activity in melb-a cells 35- to 38-fold (Fig. 3). Stimulation of tyrosinase activity suggested the DOPA reactivity in melanoblast cells which might have occurred through protein activation or maturation. When compared to control, cell expansion, dendritogenesis and pigmentation were noted in cells treated with α-MSH as well as with the *C. occidentalis* pod extract. In the cultures we noticed dendritogenesis in cells treated with α-MSH as well as with *C. occidentalis* extract when compared to triangular or bipolar morphology of untreated melb-a cells. Stimulation of dendritogenesis by α-MSH is well established [6] and similar morphological alteration upon treatment with herbal extract clearly shows that the candidate can stimulate differentiation, dendritogenesis and pigmentation of melanoblasts in a way similar to α-MSH (Fig. 4). Further studies have to be carried out in this line to establish this observation.

Treatment with the plant extract for 24 h incubation resulted in twofold increase in the number of melanoblast migrating to the lower surface of filters while treatment with α-MSH resulted in eightfold increase compared to the untreated control (Fig. 5). This result shows that *C. occidentalis* has a chemo attractant factor, which appears to stimulate the melanoblast migration and differentiation. In this experiment, cell migration was determined at the end of 24 h whereas the cell proliferation assay was determined after 96 h of incubation with test compound. Therefore, the increase in number of migrated cells cannot be attributed to cell proliferation. It has been reported that migration and differentiation of melanoblasts within the hair follicle.

Fig. 4 Effect of treatment on melb-a morphology. a Untreated melanoblasts, b culture treated with α-MSH, c culture treated with *Cassia occidentalis* pod extract (12.5 μg/ml). Phase contrast micrograph showing dendritogenesis in culture treated with α-MSH and *C. occidentalis*.
epithelium appears to require the localization of membrane-bound SCF to the basolateral cell compartment [16]. At this stage we do not know the underlying mechanism by which the candidate stimulates these melanocyte precursors. Further studies are in progress to isolate the active compound from the plant extract and to characterize the mechanism involved in melanoblast activation. These studies would be in relevant designing preclinical and clinical studies on the efficacy of Cassia occidentalis as a stimulant for skin repigmentation in vitiligo.

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