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Original Article

Pimecrolimus increases the melanogenesis and migration of melanocytes *in vitro*

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ABSTRACT Vitiligo is an intriguing depigmentary disorder and is notoriously difficult to be treated. The ultimate goal of vitiligo treatment is to replenish the lost melanocytes by immigration from hair follicle and to restore the normal function of melanogenesis by residual melanocytes. There are two types of topical calcineurin inhibitors called tacrolimus and pimecrolimus, and are recommended as the first-line treatments in vitiligo. Although pimecrolimus is efficacious for the repigmentation of vitiligo, its intrinsic mechanisms have never been investigated in vitro. This research aimed to study the ability of pimecrolimus on stimulating melanogenesis, melanocyte migration and MITF (microphthalmia associated transcription factor) protein expression. Results showed that pimecrolimus at the dosages of 1, 10, 10^2 nM were neither mitogenic nor cytotoxic to melanocytes. The addition of pimecrolimus at 10, 10^2 and 10^3 nM significantly increased intracellular tyrosinase activity, which was consistent with the elevated content of melanin content at the same concentrations. The peak effect was seen at 72 h in response to 10^2 nM pimecrolimus. Results of the wound scratch assay and Transwell assays indicate that pimecrolimus is effective in facilitating melanocyte migration on a collagen IV-coated surface. In addition, MITF protein yield reached the highest by pimecrolimus at 10^2 nM. In brief, pimecrolimus enhances melanin synthesis as well as promotes migration of melanocytes directly, possibly via their effects on MITF protein expression.

INTRODUCTION

Vitiligo is an intriguing depigmentary disorder affecting approximately 0.5% to 1% of the world population [1]. It has significant effects on quality of life and remains a persistent burden on patients. Vitiligo develops due to the progressive loss of functional melanocytes. Thus, the ultimate goal of vitiligo treatment is to replenish those melanocytes by promoting the migration of melanocytes from the outer root sheath of hair follicles to the depigmented area and to restore the normal melanogenic function of residual melanocytes [2,3].

The quest for effective treatments for vitiligo still continues. Based on the best available evidence combined with expert opinions, a new guideline for vitiligo was developed by the *Vitiligo Guideline Subcommittee of the European Dermatology Forum* in 2013. In this guideline, topical corticosteroids and calcineurin inhibitors (CIs) are recommended as first-line treatments [4]. Clobetasol proprionate is one of the oldest potent corticosteroid agent applied in the treatment of vitiligo. However, many side effects have been reported in its usage. Khalid applied 0.05% clobetasol propionate to treat 23 patients, there were mild atrophy in four cases, telangiectasia in two case, and acneiform papules in two participants [5]. There is mounting evidence showed that atrophy,telangiectasia, hypertrichosis, and acneiform eruptions may develop when topical corticosteroids were used, and this side effect hampers their use, especially when the face

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is involved [6-11]. In this circumstance, topical CIs provide a favorable alternative. A recent meta-analysis on the effect of topical CIs as monotherapy or combined with phototherapy for vitiligo treatment showed that CIs resulted in cosmetically acceptable repigmentation, particularly on the face and neck [1,8,12,13]. Moreover, 1% pimecrolimus cream has even been shown to be of equal efficacy to 0.05% clobetasol propionate ointment [14].

Despite the clinical efficacy of CIs, the underlying mechanisms of how they induce repigmentation in vitiligo have not been well studied. While there is in vitro evidence demonstrating a direct stimulating effect of tacrolimus on melanogenesis and melanocyte migration [15,16], no investigation has yet elucidated the impact of pimecrolimus on melanocytic functions. However, encouraging effects of pimecrolimus for vitiligo treatment have been shown.

METHODS

Human melanocyte culture

Human foreskin specimens were obtained under written informed consent of the donors. Melanocytes were isolated from the foreskin and maintained in Medium 254 (Life Technologies, USA) supplemented with human melanocyte growth supplement (HMGS) (Life Technologies, USA). Cell cultures were incubated at 37°C in 5% CO_2. Melanocytes at the third or fourth passage were used in the experiments.

Melanocyte proliferation assay

The proliferation rate of melanocytes was determined using a colorimetric MTT assay. Melanocytes were plated in 96-well microplates, and each well was pretreated with 100 μl of different concentrations (1, 10, 100, 1,000 nM) of pimecrolimus (BioVision, USA) for 3 days. Then, 50 μL of 5 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (KeyGEN BioTECH, China) solution was added to each well. The resulting formazan was dissolved with 150 μL dimethylsulfoxide (Sigma, USA). The absorbance of the samples was measured at a wavelength of 490 nm with a Multimode Plate Reader (Perkin Elmer, USA).

Tyrosinase activity assay

Tyrosinase activity was determined by measuring the rate of oxidation of L-DOPA to dopachrome according to our previous research [17]. Cells were incubated with different concentrations of pimecrolimus in 96-well microplates for 72 h and then lysed with 1% Triton X-100 (Sigma, USA). Ten-microliters of L-DOPA solution was then added to the lysates. The absorbance of dopachrome was measured at 475 nm. Tyrosinase activity is expressed as a percentage of the control.

Melanin content determination

Equal numbers of melanocytes (2×10^5 cells/mL) were plated in 6-well plates. Pimecrolimus (2.5 mL) at different concentrations was added to the wells and incubated for three days. The cells were then dissolved in 300 μL of a mixture consisting of 1N NaOH and 10% dimethylsulfoxide. The absorbance of the mixture was measured at 475 nm, and the melanin content was determined by comparing these values to those of a commercially available synthetic melanin (Sigma, U.S.A.) standard curve.

Migration ability of melanocytes treated with pimecrolimus

We used a scratch assay to assess the in vitro migration ability of pimecrolimus-treated cells. Melanocytes were grown to confluence in 6-well plates overnight. After incubation with a deficient medium consisting of M254 and 0.1% bovine serum albumin (Sigma, USA) for 24 h, the cell monolayers were scratched using a 10 μL pipette tip. Pimecrolimus (10 nM) was then added. The scratch size was initially set to be 100%, and the width of the scratch was measured at day 3. Melanocytes in the deficient medium was used as a control, and the final results are expressed as a percentage of the control.

For the transwell assays, melanocytes were suspended in 100 μL (2×10^5 cells/mL) of the previously described deficient medium. The cells were seeded into the upper chamber of type-IV-collagen-coated Costar transwell inserts (8 μm pore filters) in 24-well plates (Corning, USA). Pimecrolimus (10 nM) was added to the lower chamber. After 72 h of incubation, the transwell inserts were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Cells on the bottom layer were imaged in six randomly selected fields at 200× magnification.

Western blot analysis

After pretreated with pimecrolimus, melanocytes were harvested, and equal amounts of soluble protein were loaded and electrophoresed on an SDS-PAGE gel. The proteins were then transferred to polyvinylidene fluoride membranes (Roche, U.S.A.) and incubated with an anti-MITF monoclonal antibody (1:1,000, Cell Signaling Technology, USA) at 4°C overnight, followed by incubation with a horseradish peroxidase-linked secondary antibody (1:4000, Cell Signaling Technology, USA) for 2 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) system (Thermo, USA). Densitometric analysis was performed using Image J software.

Statistical analysis

Results are presented as the mean±standard deviation (SD). Statistical analysis was performed using one-way ANOVA and *t*-test. $p<0.05$ was considered to be statistically significant.

RESULTS

Effects of pimecrolimus on the viability of melanocytes

Compared to the control, pimecrolimus did not affect the viability of human melanocytes at concentrations of 1, 10 and 100 nM. The melanocyte proliferation rate was slightly elevated by pimecrolimus at the concentration of 1,000 nM. Therefore, pimecrolimus at the dosages of 1~100 nM was neither mitogenic nor cytotoxic to melanocytes, whereas pimecrolimus at the concentration of 1,000 nM showed mitogenic effects (Fig. 1A).

Melanogenesis of melanocytes incubated with pimecrolimus

After incubation with pimecrolimus at 10, 100 and 1,000 nM, melanocytes showed an increase of 122%, 128% and 125% in tyrosinase activity relative to the control (100%) ($p<0.05$), respectively. However, no stimulatory effect was shown on the tyrosinase activity at the concentration of 1 nM (Fig. 1B). Consistent with this increase in tyrosinase activity, pimecrolimus (10, 100 and 1,000 nM) also increased the melanin content by 171%, 178% and 176%, respectively, relative to the control (100%) ($p<0.05$) (Fig. 1C).

Effects of pimecrolimus on the migration of melanocytes

Pimecrolimus at a concentration of 10 nM showed no significant effect on cell viability. This concentration was therefore used in the scratch and transwell assays. Pimecrolimus at 10 nM resulted in statistically significant changes in migration in these assays. Specifically, pimecrolimus induced a more rapid reduction in scratch size (67% wound gap size after 3 days) compared to the control (83% wound gap size after 3 days), indicative of enhanced migration ($p<0.05$) (Fig. 2A). Cell counts showed no significant increase in cell density, indicating that the scratch areas were repopulated by migration rather than by increased proliferation. Transwell assays also showed that pimecrolimus increased the number of migrated cells in the lower chamber by 183% compared with the control ($p<0.05$) (Fig. 2B). These results suggest that pimecrolimus is effective in facilitating melanocyte migration on a collagen IV-coated surface.

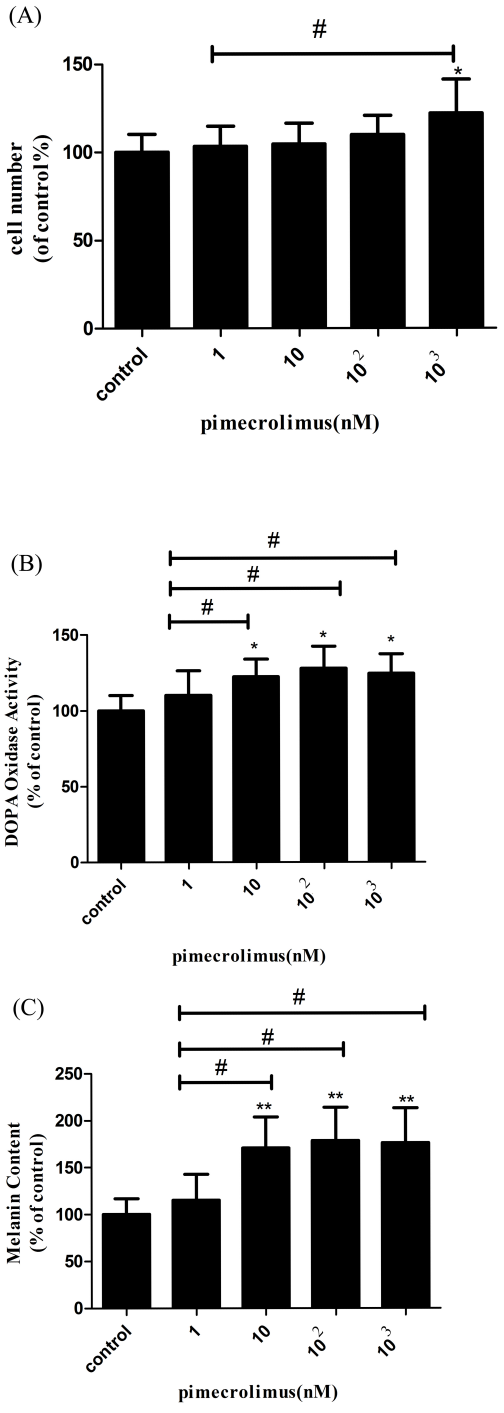

Fig. 1. Effects of pimecrolimus on the proliferation (A), tyrosinase activity (B) and melanin content (C) of human epidermal melanocytes. Cells were cultured for three days with pimecrolimus at various concentrations (1, 10, 100, and 1,000 nM) and compared with the control. Results represent the mean±SD of three independent experiments. *$p<0.05$ compared with the control.

Effects of pimecrolimus on MITF protein expression

To explore the mechanism by which pimecrolimus increases

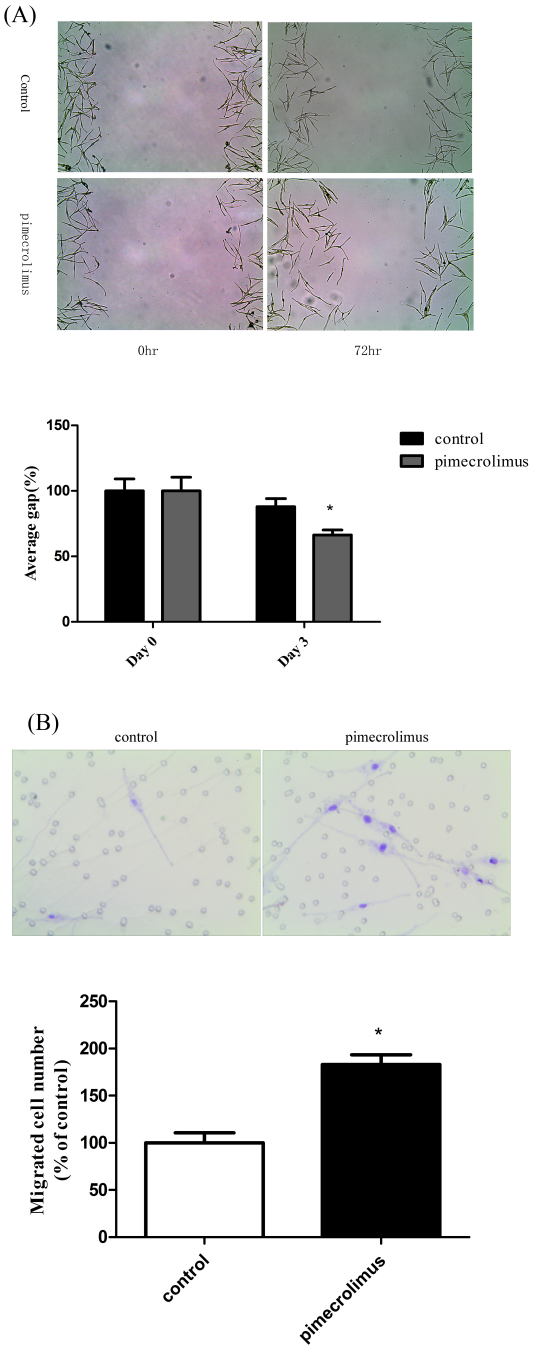

Fig. 2. Effects of pimecrolimus on melanocytic migration in vitro. (A) Migration was measured by scratch assay. The average gap was evaluated three days after incubation with pimecrolimus or the control. Results represent the mean±SD of three independent experiments. (B) Migration was measured by Transwell assay. Cells pretreated with pimecrolimus and the control were stained with crystal violet after they penetrated to the lower surface of the membrane. The immigrated cells were counted under a light microscope at ×200. Results represent the mean±SD of three independent experiments. *p<0.05 compared with the control.

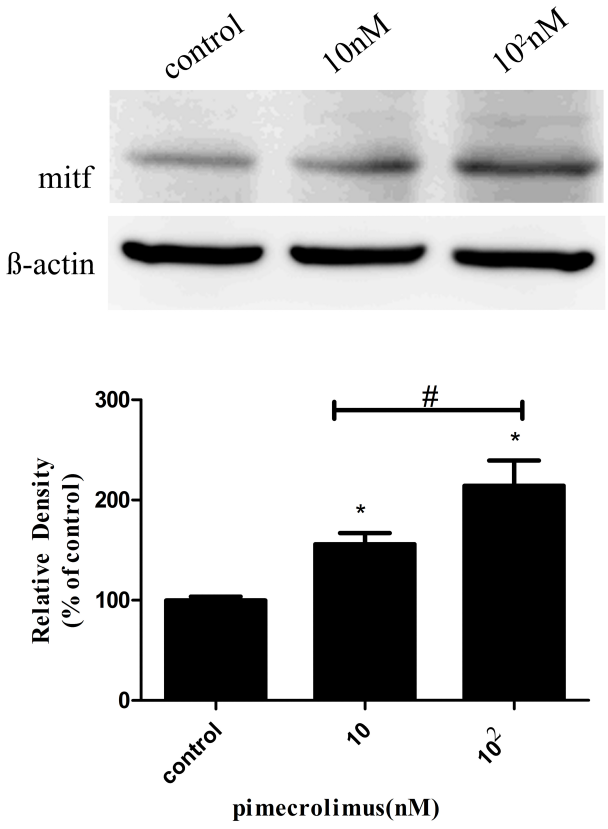

Fig. 3. Effect of pimecrolimus on the protein expression levels of MITF in melanocytes. The protein expression level of MITF was examined by Western blotting after a 3-day incubation with 10 or 100 nM pimecrolimus. Results represent the mean±SD of three independent experiments. *p<0.05 compared with the control.

melanogenesis and migration ability, we determined the effects of pimecrolimus on the protein expression of MITF using Western blotting. The results showed that treatment with pimecrolimus at 10 and 100 nM for 72 h significantly increased MITF expression levels by 156% and 214%, respectively, compared to the control (p<0.05) (Fig. 3).

DISCUSSION

Vitiligo is a common hypopigmentation disorder of the skin, with a prevalence of approximately 0.5~1% in the world population. Although it is not physically harmful, vitiligo is often psychologically devastating due to the depigmented macules on the skin that result from the selective loss of functional epidermal melanocytes [2,4,18]. Amelanotic melanocytes in the outer root sheath of hair follicles have been shown to migrate towards the achromic epidermis during the process of repigmentation and gradually restore the normal functions of melanin synthesis. In another aspect, the undestroyed melanocytes during depigmentation and at the border of depigmented lesions may also

reproduce and migrate to the depigmented patches upon external stimuli such as corticosteroids, UV irradiation, and keratinocyte-derived factors as basic fibroblast growth factor (bFGF), leukotrienes C4 (LTC4), endothelin 1 (ET-1) and stem cell factor (SCF) [19].

Topical corticosteroids have long remained the mainstay of vitiligo treatment. However, their use is limited by concerns about local and systemic adverse effects with extended use. The recent emergence of CIs provides us with a favorable alternative to corticosteroids. Calcineurin can dephosphorylate, and thus activate, nuclear factor of T cells (NFAT). The activated NFAT is then translocated into the nucleus, where it up-regulates the expression of interleukin 2 (IL-2), which, in turn, stimulates the growth and differentiation of T cells [20]. There are a variety of known CIs, including cyclosporine A, pimecrolimus and tacrolimus. Recently, calcineurin activity has been detected in keratinocytes, melanocytes and other tissues [16,21]. It is likely that the actions of CIs in the treatment of vitiligo are multi-fold, exhibiting not only the classical aspect of exerting immunosuppressive effects but also stimulating melanogenesis and melanocytic migration.

The influence on melanocytic functions and skin pigmentation varies by CI. Although skin hyperpigmentation develops in some patients after systemic cyclosporin A treatment, tyrosinase activity and melanin production have been shown to be inhibited in vitro by 10 μm cyclosporin A, with a consistent decrease of tyrosinase at both the protein and mRNA levels [22]. Tacrolimus, another CI, has been shown to promote migration and tyrosinase activity in melanocytes while suppressing proliferation. However, in one study, tacrolimus alone had no effect on migration in cultured melanoblasts, while demonstrating synergistic effects with ET-1 and ET-3 on the promotion of cell migration and melanogenesis [23,24]. In addition, tacrolimus increases UVB-mediated melanosome secretion and uptake and enhances the transfer of melanosomes from melanocytes to keratinocytes [24].

Similar to tacrolimus, topical pimecrolimus is widely applied in vitiligo treatment. Pimecrolimus cream (1%) has been shown to be as effective as 0.05% clobetasol propionate [25]. While narrow-band UVB irradiation is currently recommended as a first-line therapy in vitiligo, a double-blind study showed that 1% pimecrolimus cream is superior to narrow-band UVB [26]. Despite these documented therapeutic effects on vitiligo, there have no attempts to investigate the effect of pimecrolimus on skin melanocytes.

Tyrosinase is an enzyme that catalyzes the rate-limiting step of melanin biosynthesis in melanocytes. The expression levels of tyrosinase and its related proteins (tyrosinase-related protein 1 and tyrosinase-related protein 2) are mainly regulated by microphthalmia transcription factor (MITF), which is an important regulator of melanocyte differentiation, proliferation and survival and is highly coupled with melanogenesis [18,27]. Any therapy for vitiligo must promote not only the repopulation of melanocytes but also melanogenesis and melanocyte migration.

In general, pimecrolimus achieves its best response when used on neck, face and genital vitiligo. This may partially be due to the greater density of hair follicles in these areas, which may serve as a vital source for stimulating and replenishing these diminished melanocytes during the repigmentation of vitiligo. Therefore, in this study, we examined the direct effects of pimecrolimus on melanogenesis and migration in cultured human melanocytes. The results showed that pimecrolimus at the dosages of 1, 10, 100 nM was neither mitogenic nor cytotoxic to melanocytes. However, at a concentration of 1,000 nM, pimecrolimus was mitogenic. In addition, we studied the effect of pimecrolimus on tyrosinase synthesis to clarify its mechanism of action on melanocytes. The results indicated that the addition of pimecrolimus at 10, 100 and 1,000 nM significantly increased intracellular tyrosinase activity in human melanocytes, which is consistent with the elevation of the melanin content at the same concentrations. The peak effect was observed at 72 h in response to 100 nM pimecrolimus. The results of the wound scratch and transwell assays indicate that pimecrolimus is effective at facilitating melanocyte migration on a collagen IV-coated surface. These results reasonably explain the well-known perifollicular repigmentation pattern in vitiligo treated with pimecrolimus. Consistent with the stimulating effect of pimecrolimus on melanogenesis, the MITF protein yield was the highest at 100 nM pimecrolimus.

In conclusion, we provide in vitro evidence demonstrating a direct effect of pimecrolimus on melanogenesis and melanocytic migration. These findings may provide a theoretical basis and clinical evidence for the effectiveness of pimecrolimus in vitiligo treatment.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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