INVESTIGATIVE REPORT

Basic Fibroblast Growth Factor Promotes Melanocyte Migration via Increased Expression of p125^{FAK} on Melanocytes

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Vitiligo is an acquired pigmentary disorder characterized by depigmentation of skin and hair. Melanocyte migration is an important event in re-pigmentation of vitiligo. We have demonstrated that narrow-band ultraviolet B (UVB) irradiation stimulated cultured keratinocytes to release a significant amount of basic fibroblast growth factor (bFGF). Furthermore, narrow-band UVB enhanced migration of melanocytes via increased expression of phosphorylated focal adhesion kinase (p125^{FAK}) on melanocytes. The aim of this study was to investigate the effect of recombinant human bFGF (rhbFGF) on melanocyte migration. The relationship between the expression of p125^{FAK} and melanocyte migration induced by rhbFGF was also studied. Our results demonstrated that rhbFGF significantly enhanced migration of melanocytes and p125^{FAK} expression on melanocytes. Herbimycin A, a potent p125^{FAK} inhibitor, effectively abolished rhbFGF-induced melanocyte migration. The combined results indicated that p125^{FAK} plays an important role in the signal transduction pathway of melanocyte migration induced by bFGF. *Key words: migration; melanocyte; basic fibroblast growth factor; p125^{FAK}.*

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Approximately 0.5–1.5% of the world's population is affected by vitiligo (1). The underlying mechanism of how functional melanocytes (MCs) disappear from the involved skin is still unclear. Stress, accumulation of toxic compounds, infection, autoimmunity, mutations, altered cellular environment, and impaired MC migration and/or proliferation, have all been suggested to be involved in the pathogenesis of vitiligo (2–4). Recovery from vitiligo is initiated by the activation (5) and proliferation (6) of immature MCs from the outer root sheath of the hair follicle, followed by upward migration to the nearby epidermis to form perifollicular pigmentation islands and by subsequent downward migration to the hair matrices to produce melanin (7). Other mechanisms proposed to participate in the vitiligo recovery process include migration of normal MCs from normal skin juxtaposed to the affected site (8). Regardless of the intricate process involved, MC migration plays a crucial role in vitiligo re-pigmentation. Therefore, understanding of the mechanisms involved in MC movement will be invaluable for the development of effective treatment for vitiligo.

During migration, the interaction of cells with surrounding extracellular matrix (ECM) is mediated by integrins expressed on the cell surface (9). The binding of ECM to integrins resulted in biological signals being transmitted from ECM into the cell (9). Tyrosine phosphorylation of focal adhesion kinase (p125^{FAK}) appears to be an important component in the signal transduction pathway (10). Increased p125^{FAK} expression may modulate cytoskeletal proteins necessary for MC migration (11).

MC proliferation may be stimulated via the receptors for tyrosine kinase growth factors: basic fibroblast growth factor (bFGF), stem cell factor (SCF), hepatocyte growth factor (HGF) and nerve growth factor (NGF) (12, 13). These growth factors are also capable of inducing migration of MCs. Neighbouring keratinocytes (KC), may release these aforementioned growth factors and thereby influence the growth and physiological functions of MC *in vivo*.

The treatment of vitiligo has generally been unsatisfactory and disappointing. Conventional therapies that induced varying degrees of re-pigmentation in patients with vitiligo include topical steroids, phototherapy and photochemotherapy. Our previous study demonstrated that supernatants derived from narrow-band ultraviolet B (UVB) irradiation induced release of various MC growth factors from cultured KCs, among which the increas in bFGF is most significant (14). Moreover, narrow-band UVB could also directly enhance the migration of MCs via the increased expression of p125^{FAK} on cultured MCs. In this study, the effects of bFGF on MC migration were explored. NGF, another growth factor released by KCs demonstrating significant effect on MC migration (12, 15, 16), was used as a reference. In addition, the relationship between p125^{FAK} and MC migration induced by bFGF/NGF was investigated.

MATERIALS AND METHODS

Melanocyte culture and treatment

The MCs used in this study were derived from healthy human foreskins. Three samples from different adult donors undergoing routine circumcisions were cleaned of excess subcutaneous tissue, cut into small pieces (5×5 mm), and incubated with 0.25% trypsin (Gibco, Grand Island, New York, USA) at 4°C overnight. The epidermal sheets were separated from dermis by fine forceps, and the epidermal cell suspension was prepared as described previously (17). The cells were pelleted by centrifugation (500×g, 10 min) and resuspended in serum-free medium containing MC basal medium MCDB 153 (Sigma, St Louis, MO, USA), which contains epidermal growth factor (10 ng/ml), hydrocortisone (10^{-7} M), insulin (1.5 µg/ml), cholera toxin (2 ng/ml), transferrin (10 µg/ml), bovine pituitary extract (30 µg/ml), phorbol myristate acetate (10 ng/ml), penicillin (100 U/ml) (Gibco) and streptomycin (100 µg/ml) (Gibco). The epidermal growth factor, hydrocortisone, insulin, cholera toxin, transferrin, bovine pituitary extract and phorbol myristate acetate were purchased from Sigma. The epidermal cell suspension was plated onto 10 cm² Petri dishes, and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Twenty-four hours after primary seeding, the medium was changed, and it was subsequently changed every 2 days. About 7–10 days after primary seeding, the semiconfluent MCs were incubated with 0.25% trypsin in 0.01% ethylenediamine tetra-acetic acid solution (Gibco) at 37°C for 3–5 min, harvested with soybean solution (Gibco), centrifuged (500 × g, 10 min), and the cell pellet was resuspended in serum-free medium and re-incubated. The second- or third-passage cells were used in the following experiments. Cultured human MCs were stimulated with 0, 5, 10 and 20 ng/ml of recombinant human bFGF (rhbFGF) (R&D, Minneapolis, MN, USA) or recombinant human nerve growth factor (rhNGF) (R&D) and incubated for the following experiments. The concentrations of rhbFGF used in our study were chosen based on previous studies (14, 18).

Melanocyte migration assay

A time-lapse photography assay modified from Horikawa et al. (19) for measurement of cell migration was used. MCs are located in the basal lamina of the skin. Since type IV collagen is a major component of the basal lamina, type IV collagen was used as the substratum in the MC migration experiment. CELLocate slips (Eppendorf, Hamburg, Germany) were coated with 10 µg/ml type IV collagen, and dried at 37°C for 2 h. MCs were seeded on CELLocate cover slips in a 24-well culture plate ar a density of 1×10⁴ cells/well and incubated for 2 h until attachment. The cells were incubated with 0, 5, 10 and 20 ng/ml of rhbFGF or rhNGF. For measurement of migration distance, we first selected and photographed a field on the CELLocate slip. Then the photographs of the same field were taken at 0, 4, 8, 12, 16, 20 and 24 h after stimulation. Linear measurements of migration distance were made using cell nuclei as the reference points. Ten to 12 cells for each group were randomly selected. The migration distances of every selected MC at each time point (i.e. 0–4 h, 4–8 h, 8–12 h, 12–16 h, 16–20 h and 20–24 h) were measured with a ruler. The summation of distances of each indicated point represented the random migration distance of MC in 24 h. Since the photographs taken under the microscope were magnified, conversion from "measured" distance to "actual" distance was required. According to the manual, every side of the square on the CELLocate slip measures 175 µm (shown in Fig. 1). Therefore, a conversion ratio was established by measuring the side of square (175 µm) on the photograph and equating this distance to 175 µm, the actual distance on the CELLocate slip. The average migration distances of the randomly selected 10–12 cells were recorded.

Blocking test for melanocyte migration

For the migration-blocking test, MCs were pre-treated with 1 µg/ml herbimycin A (a potent inhibitor for p125^FAK) (Sigma) for 1 h, followed by various concentrations (0, 5, 10 and 20 ng/ml) of rhbFGF or rhNGF for 24 h. Microphotographs were then taken, at the times indicared over a period of 24 h, and 10–12 cells were selected for each group in order to measure the migration distances (19).

Immunofluorescent localization of p125^FAK

MCs were seeded on type IV collagen-precoated glass cover slips at a density of 2×10⁴ cells/well and incubated with the indicated concentrations of rhbFGF and rhNGF. After 2, 6, 18 and 24 h, they were washed with phosphate buffered saline (PBS) and then fixed with 100% methanol at –20°C for 20 min. Cells were blocked with 10% bovine serum albumin (Sigma) in PBS for 30 min at room temperature to eliminate non-specific binding. After washing briefly with PBS, the cells were incubated overnight at 4°C with 200 µl of anti-p125^FAK monoclonal antibody (1:100 dilution, BioSource, Camarillo, CA, USA). After three washings with PBS, the cells were incubated for 1 h at room temperature with 200 µl of biotin-conjugated goat anti-rabbit antibody (1:100 dilution, ImmunoResearch, Baltimore, PA, USA). After three washings with PBS, the cells were incubated for 1h at 4°C with FITC-conjugated streptavidin (1:100 dilution, ImmunoResearch, Baltimore, PA, USA). After washing with PBS, the cells on the glass cover slips were embedded in mounting medium (Aquamount; Lerner Labs, Pittsburgh, PA, USA), and then the cells were photographed with a Zeiss fluorescence microscope (Axioskop 2 plus) (11).

Statistical analysis

SPSS for Windows version 10.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The results were expressed
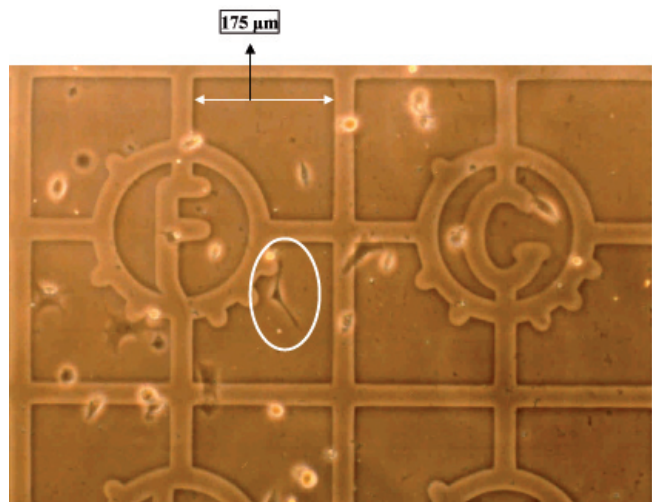

Fig. 1. A CELLocate cover slip under the microscope. The CELLocate microgrid cover slip locates a cell's position during migration. The letters of the alphabet and Arabic numerals are marked on the cover slip. The diameter of the slip is 12 mm and the side of each square measures 175 µm. Migration distances of MCs can be estimated by tracing the movement of selected cells (as shown inside the ellipse) over a period of 24 h.

as mean±standard deviation (mean±SD). Student's *t*-test was used for statistical evaluation between control and experimental groups in the study. A *p*-value of <0.05 is considered to be statistically significant.

RESULTS

Both bFGF and NGF significantly enhanced melanocyte migration

According to the studies of cell movement by Norris et al. (20), a number of characteristic types of movement, including random movement, chemotaxis, chemokinesis and haptotaxis, are seen. In this study we examined the influences of rhbFGF/rhNGF on MC migration; the type of migration seen in our experiments is classified as chemokinesis; activated random movement. A prominent increase in MC migration was noted 24h after rhbFGF and rhNGF stimulation, respectively (Fig. 2A). When the random movement of MCs cultured without rhbFGF or rhNGF stimulation was used as the control (100%), the migration of MC treated with 5, 10 and 20 ng/ml rhbFGF was 115.5±20.9%, 149.4±25.0% and 153.8±21.8%, respectively, whereas the migration of MC stimulated with 5, 10 and 20 ng/ml rhNGF was 122.4±36.9%, 142.3±36.6% and 96.6±39.5%, respectively. Thus both rhbFGF (10 and 20 ng/ml) and rhNGF (10 ng/ml) significantly enhanced MC migration ($p < 0.05$).

Melanocyte migration stimulated by bFGF, but not by NGF, was significantly abrogated by p125[FAK] inhibitor

Since 10 ng/ml of rhbFGF and 10 ng/ml of rhNGF exhibited a significant effect on MC migration, these concentrations were applied in the following migration-blocking experiment. Herbimycin A, a potent inhibitor of p125[FAK],

was used to clarify the role of p125[FAK] on rhbFGF and rhNGF-induced MC migration. Addition of 1 µg/ml of herbimycin A significantly abrogated the rhbFGF-induced MC migration (68.9±22.6% vs. 100.0±25.0%, $p < 0.05$), but showed no effect on rhNGF-induced MC migration (97.8±34.5% vs. 100.0±36.6%) (Fig. 2B).

Expression of p125[FAK] on MC was significantly stimulated by bFGF

The constitutive expression of p125[FAK] is known to be dot-like and confined to distal dendrites of MCs (21). Our results demonstrated that the expression of p125[FAK] on MCs was not obvious after 2 h of stimulation with 10 ng/ml rhbFGF. However, after 6 h of incubation with 10 ng/ml rhbFGF, the expression of p125[FAK] on MCs was marked (Fig. 3C) compared with the control group (Fig. 3A). On the other hand, the expression of p125[FAK] on MCs stimulated with 10 ng/ml rhNGF was scant at all time points (Fig. 3B). These results indicate that rhbFGF significantly stimulates the expression of p125[FAK] on MCs.

DISCUSSION

Cell motility is a critical event during foetal development, wound healing and tumour invasion (22). Cell migration involves a complex interplay between stimulatory factors and cell-to-cell or cell-to-matrix protein interactions. The classification of cell motility, based on the nature of stimulus, is into: random movement, chemokinesis (equivalent concentration of attractant), chemotaxis (concentration gradient of attractant) and haptotaxis (movement on extracellular matrix) (20).

Vitiligo is a skin disease caused by selective destruction of MCs, which is difficult to treat. One of the best
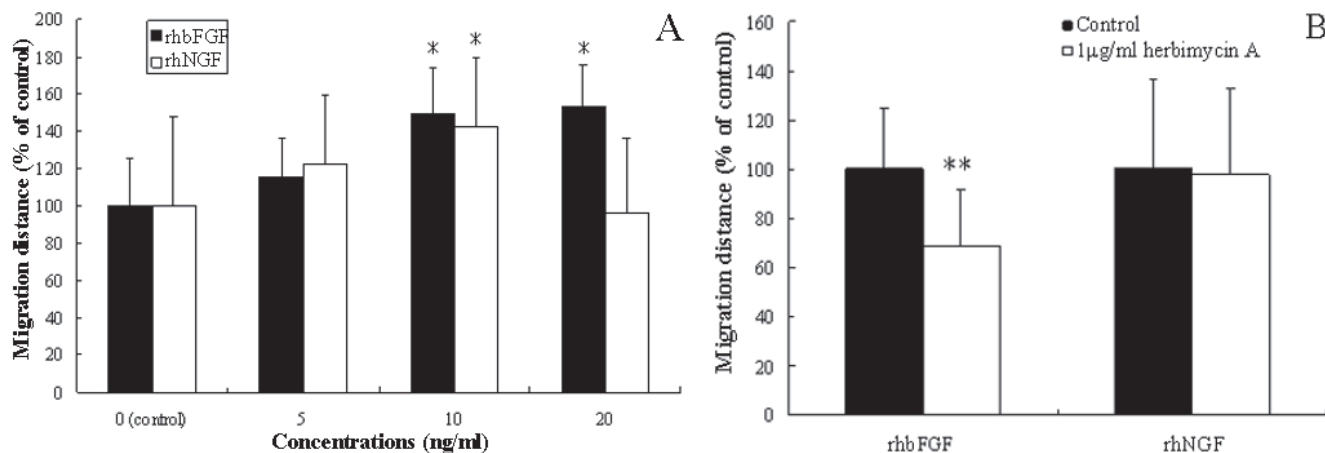

Fig. 2. Effects of rhbFGF and rhNGF on melanocyte (MC) migration and the effect of herbimycin A, an inhibitor of p125[FAK]. Cultured human MCs were seeded on type IV collagen pre-coated CELLocate cover slips at 2×10⁴ cells/well and incubated for 2 h. Time-lapse photographs were taken at the indicated time points (4 h) and the migration distance (µm) after 24 h was measured. The result was expressed as percentage compared with control group. (A) The migration of MCs stimulated with 0 (control), 5, 10 and 20 ng/ml rhbFGF or rhNGF. (B) MCs stimulated with 10 ng/ml rhbFGF or rhNGF with or without 1 µg/ml of herbimycin A. *$p < 0.05$ compared with control group; **$p < 0.05$ compared with 10 ng/ml rhbFGF treated group. Error bars, represent mean ± SD of 10~12 cells.
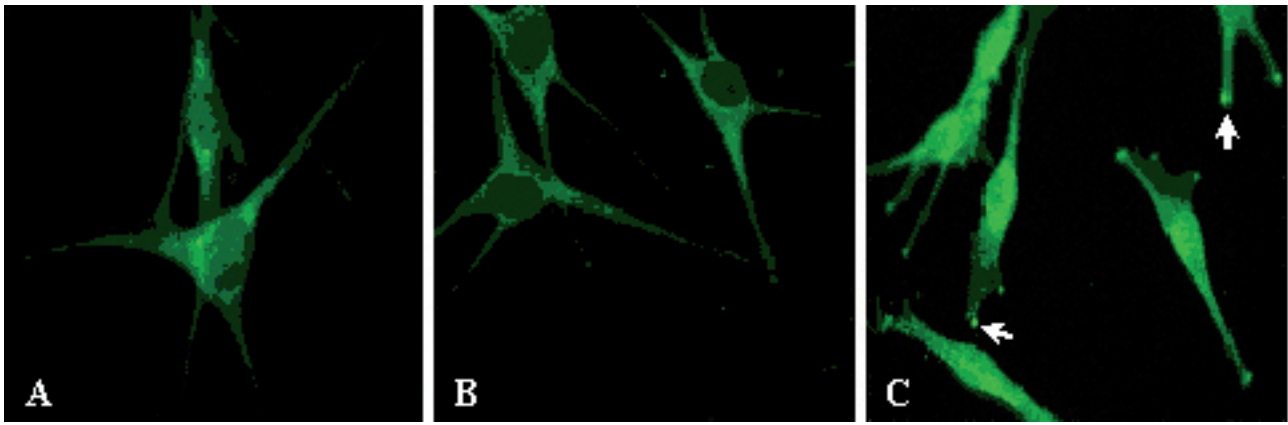
Fig. 3. Expression of p125^{FAK} on melanocytes determined by immunofluorescence (IF) microscopy. Compared with control (A) and 10 ng/ml rhNGF treated (B) cells a marked increase in expression of p125^{FAK} was observed (*arrows*) on melanocytes treated with 10 ng/ml recombinant human bFGF (rhbFGF) for 6h (C). Similar results were obtained in three independent experiments.

treatments is photo(chemo)therapy. It is known that the migration of inactivated MCs from the outer root sheath of hair follicles onto clinically depigmented epidermis after phototherapy is crucial to the vitiligo re-pigmentation process (5, 7, 20, 23). KCs are natural neighbours of MCs and have an intricate relationship to MCs (24) *in vivo*. Our previous studies demonstrated that helium-neon laser stimulated the release of bFGF and NGF by KCs. Furthermore, the supernatants derived from helium-neon laser irradiated KCs significantly enhanced MC migration (25). Recently, we reported that narrow-band UVB-irradiation stimulated cultured KCs to release various MC growth factors, among which the increase in bFGF was most significant (14). bFGF has been demonstrated as a natural mitogen for MC growth (26). In addition, bFGF is recognized as the crucial factor produced by KCs that enhances the growth and survival of MCs. Since both helium-neon laser and narrow-band UVB are well-established therapies for vitiligo, it is reasonable to speculate that bFGF has profound influences on MC migration.

Our results demonstrated that 10 and 20 ng/ml rhbFGF, as well as 10 ng/ml rhNGF, significantly enhanced MC migration (Fig. 2A). Since our previous studies showed that narrow-band UVB irradiation significantly stimulated MC migration through augmented expression of p125^{FAK} on MCs (14), we examined whether p125^{FAK} was involved in bFGF and NGF-induced MC migration. The results (Fig. 2B) revealed that addition of 1 µg/ml of herbimycin A significantly abolished 10 ng/ml rhbFGF-induced MC migration (p <0.05). By contrast, herbimycin A displayed no significant influence on 10 ng/ml rhNGF-induced MC migration. These results indicated that increased expression of p125^{FAK} on MCs is not always required to stimulate MC migration.

To corroborate our results obtained from migration studies, we further explored the expression of p125^{FAK} on MCs stimulated with 10 ng/ml rhbFGF or rhNGF. The results revealed a higher expression of p125^{FAK} on MCs stimulated with rhbFGF compared with controls.

Focal adhesion kinase is a non-receptor tyrosine kinase, which co-localizes with integrins to focal contacts (27). Accumulating evidence suggests that p125^{FAK} plays a pivotal role in transducing a variety of signals that modulate cell migration and cytoskeletal proteins necessary for cell migration (11, 28, 29). Numerous studies have verified a strong correlation between p125^{FAK} and cell motility, and increased expression of this kinase enhances cell migration (11, 30, 31). We conclude that bFGF stimulates MC migration via increased expression of p125^{FAK} on the cells.

On the other hand, the immunofluorescent (IF) staining assay for p125^{FAK} revealed that 10 ng/ml rhNGF did not significantly stimulate the expression of p125^{FAK} on MCs. Since 10 ng/ml rhNGF significantly enhanced MC migration, our combined results from the migration-blocking and IF staining assays indicate that signal transduction pathways other than p125^{FAK} are involved in NGF-induced MC migration. NGF was reported to exert its effect on MCs by binding to cognate NGF-R (p75) expressed on MCs (15, 32). In addition, activation of extracellular signal-regulated kinase, mitogen-activated protein kinase and phosphatidylinositol 3-kinase by NGF could result in increased migration of the cells (33–35). Further studies are required to elucidate the possible signalling pathways for NGF-induced MC migration.

ACKNOWLEDGEMENT

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