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Migration of human umbilical cord blood mesenchymal stem cells mediated by stromal cell-derived factor-1/CXCR4 axis via Akt, ERK, and p38 signal transduction pathways

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ABSTRACT

Human mesenchymal stem cells (hMSCs) have been used for cell-based therapies in degenerative disease and as vehicles for delivering therapeutic genes to sites of injury and tumors. Recently, umbilical cord blood (UCB) was identified as a source for MSCs, and human UCB-derived MSCs (hUCB-MSCs) can serve as an alternative source of bone marrow-derived mesenchymal stem cells (BM-MSCs). However, migration signaling pathways required for homing and recruitment of hUCB-MSCs are not fully understood. Stromal cell-derived factor-1 (SDF-1), a ligand for the CXCR4 chemokine receptor, plays a pivotal role in mobilization and homing of stem cells and modulates different biological responses in various stem cells. In this study, expression of CXCR4 in hUCB-MSCs was studied by western blot analysis and the functional role of SDF-1 was assessed. SDF-1 induced the migration of hUCB-MSCs in a dose-dependent manner. The induced migration was inhibited by the CXCR4-specific peptide antagonist (AMD3100) and by inhibitors of phosphoinositide 3-kinase (LY294002), mitogen-activated protein kinase/extracellular signal related kinase (PD98059) and p38MAPK inhibitor (SB203580). hUCB-MSCs treated with SDF-1 displayed increased phosphorylation of Akt, ERK and p38, which were inhibited by AMD3100. Small-interfering RNA-mediated knock-down of Akt, ERK and p38 blocked SDF-1 induced hUCB-MSC migration. In addition, SDF-1-induced actin polymerization was also blocked by these inhibitors. Taken together, these results demonstrate that Akt, ERK and p38 signal transduction pathways may be involved in SDF-1-mediated migration of hUCB-MSCs.

1. Introduction

Mesenchymal stem cells (MSCs) have generated much interest as a potential source of cells for cell-based therapeutic strategies [1]. Currently, bone marrow-derived MSCs (BM-MSCs) are investigated in preclinical and clinical settings because of their self- renewal and multipotent differentiative capacities and immunosuppressive activity [2]. However, for clinical use, BM may be detrimental because of the highly invasive donation procedure and the decline in MSC number, differentiation and proliferative potential with increasing age [3,4]. Therefore, MSCs derived from other sources could represent a better alternative. MSCs can be isolated from various other tissues, including umbilical cord blood (UCB), adipose tissue, and muscle [5,6]. Human UCB-derived MSCs (hUCB-MSCs) are good substitutes for BM-MSCs because of the immaturity of newborn cells. The hUCB-MSCs can migrate to areas of various pathology such as inflammatory lesions and brain tumors [7,8]. This pathotropism of hUCB-MSCs makes them useful for regeneration of damaged tissues, as well as for targeted delivery of therapeutic genes to sites of pathology.

Recently, many studies have proven that MSC migration was regulated by numerous cytokines, growth factors, and their receptors [9,10]. Stromal cell-derived factor-1 (SDF-1), which is also known as CXCL12, belongs to the CXC subfamily and was first cloned from bone marrow stromal cells and shown to induce proliferation and differentiation of B cell progenitors [11]. SDF-1 is expressed in a wide range of normal tissues, and is a potent chemoattractant for hematopoietic cells facilitating their transmigration through endothelial cell barriers [12]. Most importantly, SDF-1 is thought to regulate hematopoietic stem cell (HSC) migration into and out of the bone marrow [13]. Its receptor, CXCR4, a

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seven-transmembrane G protein-coupled receptor (GPCR) is highly conserved across species and expressed in numerous types of embryonic and adult stem cells. The SDF-1/CXCR4 interaction plays a crucial role in the homing and engraftment of HSC in bone marrow [14]. In addition, our previous study has shown that CXCR4-transfected human umbilical cord blood-derived mesenchymal stem cells exhibit enhanced migratory capacity toward gliomas. Despite the important role of CXCR4/SDF-1 in the regulation of the homing and trafficking of stem cells, relatively little is known about the signal transduction pathways that mediate these effects in hUCB-MSCs. Phosphoinositide 3-kinase (PI3K)/Akt, extracellular signal related kinase (ERK), and p38 mitogen-activated protein kinase (MAPK) signal transduction pathways are involved in the regulation of directional cell migration by chemokines in various cell types [15–17].

In this study, we aimed to investigate the effect of SDF-1 on migration of hUCB-MSCs and the functional role of Akt, ERK and p38 signal transduction pathways in SDF-1-induced migration of hUCB-MSCs. We demonstrated that SDF-1a increased the migration of hUCB-MSCs. Akt, ERK, p38 signaling pathways may be involved in hUCB-MSC migration by SDF-1. In addition, these signaling pathways plays an important role in SDF-1 induced actin polymerization of hUCB-MSCs.

2. Materials and methods

2.1. Stem cell culture and reagents

Human UCB harvest and expansion of MSCs isolated from UCB was conducted as previously reported [18]. The separated MSCs were subcultured at a concentration of 5×10^4 cells/cm^2 in MEM-α (Invitrogen, Carlsbad, CA) and used for experiments during passages 5–8. All media were supplemented with 2 mmol/L L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS) (all from Invitrogen). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. SDF-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CXCR4 inhibitor AMD3100 was purchased from Sigma–Aldrich (St. Louis, MO). The PI3K inhibitor LY294002 and ERK/MAPK inhibitor PD098059 were acquired from Cell Signaling Technology (Beverly, MA). The p38 MAPK inhibitor SB203580 was purchased from Calbiochem (La Jolla, CA).

2.2. Transwell migration assay

The migratory ability of hUCB-MSCs was determined using Transwell plates (Corning Costar, Cambridge, MA) that were 6.5 mm in diameter with 8 μm pore filters. In brief, cells were suspended in serum-free medium and seeded into the upper well, and 600 μl of SDF-1-containing medium was placed in the lower well of a Transwell plate. Following incubation for 5 h at 37 °C, cells that had not migrated from the upper side of the filter were scraped off with a cotton swab, and filters were stained with the Diff-Quik™ three-step stain set (Sysmex, Kobe, Japan). The number of cells that had migrated to the lower side of the filter was counted under a light microscope at ×200 magnification in five randomly-selected fields.

2.3. Western blotting

CXCR4 antibody (BD, Franklin Lakes, NJ), β-actin antibody (Sigma–Aldrich), and phospho-Akt, Akt and phospho-ERKs, ERKs, phospho-p38, and p38 antibodies (New England Biolabs, Waltham, MA) were used for the analyses. Cells were rinsed with phosphate-buffered saline (PBS) and lysed for 30 min on ice in RIPA-B buffer (0.5% Nonidet P-40, 20 mM Tris, pH 8.0, 50 mM NaCl, 50 mM NaF, 100 μM Na_3VO_4, 1 mM dithiothreitol, and 50 μg/ml phenylmethanesulfonylfluoride). Insoluble material was removed by centrifugation at 12000 rpm for 10 min at 4 °C. The proteins in the supernatant were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the resolved proteins were transferred to a nitrocellulose blot membrane. Each blot was blocked using PBS containing 5% skim milk and 0.05% Tween-20, incubated with the appropriate antibodies, and incubated with the secondary antibodies conjugated to horseradish peroxidase (HRP). The blots were subsequently assayed using the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

2.4. RNA interference experiments

Small-interfering RNA (siRNA) against human Akt, ERK, and p38, and a control siRNA were purchased from Cell Signaling Technology. hUCB-MSCs were cultured to 70% confluency, and then transfected with the siRNA duplex using Lipofectamine 2000 (Invitrogen), according to the manufacturer's recommended protocol. After 72 h of transfection, the cells were harvested to perform western blotting and migration assay.

2.5. F-actin polymerization assay

F-actin polymerization was measured as described previously [19]. Briefly, hUCB-MSCs were plated in wells of 96-well black microtiter plates with clear bottoms and stimulated with 100 ng/mL SDF-1. At indicated time points, cells were fixed and permeabilized for 15 min at room temperature with 0.3% Triton X-100 in PBS. Polymerized F-actin was stained with Alexa Fluor 568-Phalloidin (Invitrogen) for 30 min at room temperature. After washing, intracellular fluorescence was extracted in 0.1 ml methanol for 20 min at 4 °C. Quantification of F-actin was determined with a Spectra Max Gemini EM Spectrofluorometer (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 568 nm at room temperature.

2.6. Statistical analysis

All data are expressed as mean ± SE. Statistical differences between different test conditions were determined by using Student's *t* test. P values <0.05 were considered significant.

3. Results and discussion

3.1. Akt, ERK, and p38 pathways are involved in SDF-1-induced hUCB-MSC migration

The expression of CXCR4 has been observed on the surface of embryonic stem cells and several tissue committed stem/progenitor cells, such as HSCs, skeletal muscle satellite progenitor cells, and neural progenitor cells [20]. Recently, CXCR4 was reported to be expressed on the surface of MSCs and neural stem cells [21]. Here, CXCR4 was also expressed in hUCB-MSCs; its expression was not affected by SDF-1. To further investigate CXCR4 population of hUCB-MSCs, fluorescence-activated cell sorting was done, which revealed that 18% of hUCB-MSC populations were comprised of CXCR4 (data not shown). It has been reported that a small proportion of MSC express CXCR4, which contributes to their migration *in vitro* [22]. Based on the expression levels of CXCR4, the role of SDF-1 on hUCB-MSC migration was analyzed in a Transwell migration assay. SDF-1 induced hUCB-MSCs migration in a dose-dependent manner, with the maximum migration observed in 100 ng/ml

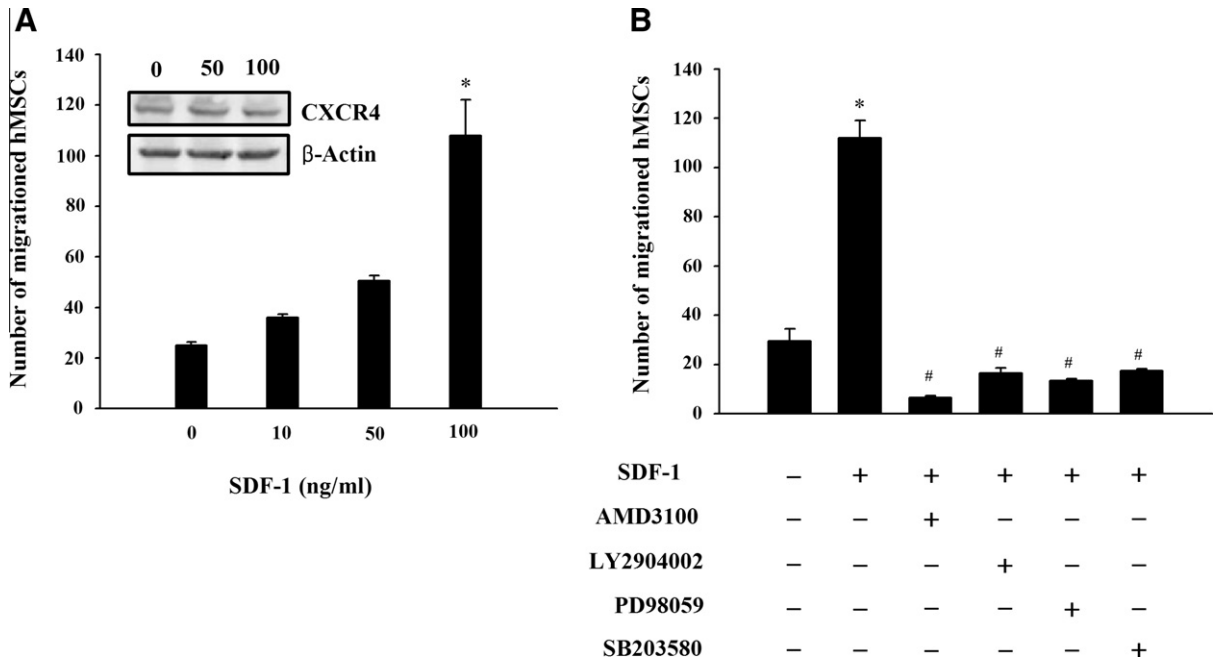

Fig. 1. Akt, ERK, and p38 pathways are involved in SDF-1-induced migration of hUCB-MSCs. (A) hUCB-MSCs were incubated with SDF-1 for various concentrations (0–100 ng/mL), and the surface expression of CXCR4 was determined using Western blot analysis. SDF-1 induced concentration-dependent hUCB-MSC migration. Transwell migration assay demonstrate the migration of UCB-MSCs in response to various concentrations (0–100 ng/mL) of SDF-1. A SDF-1 concentration of 100 ng/mL induced maximum migration. (B) SDF-1 (100 ng/mL) was used and hUCB-MSCs were not pretreated or pretreated with AMD3100 (5 mg/mL), PD98059 (20 mM), LY294002 (20 mM), or SB203580 (50 nM) for 30 min. SDF-1-induced UCB-MSC migration was significantly inhibited by AMD3100, LY294002, and SB203580. Serum-free medium was used as a experimental control. The experiments were performed three times in duplicate and the results are expressed as the mean ± SE, $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with non-pretreated group.

SDF-1 (Fig. 1A). This result indicated the relevance of SDF-1/CXCR4 in the migration of hUCB-MSCs. Similarly, it has also been reported that SDF-1 can induce the migration of human bone marrow stromal cells *in vitro*, and that CXCR4 might play a role in the engraftment of these cells in the brain tissue of immunodeficient mice [14].

Many studies have shown that SDF-1 activates diverse CXCR4-mediated signaling pathways [23], but no migration pathway of hUCB-MSCs has been identified. To investigate the functional role of Akt, ERK, and p38 signal pathways in SDF-1-induced migration, hUCB-MSCs were pretreated for 30 min with AMD3100 (a CXCR4-specific peptide antagonist), PI3K-specific inhibitor LY294002, MAPK/ERK (MEK)-specific inhibitor PD98059, and p38-specific inhibitor SB203580 before the migration assay. SDF-1-induced migration was significantly inhibited by AMD3100, suggesting that SDF-1-induced UCB-MSC migration was specifically due to its receptor CXCR4. In addition, treatment of UCB-MSCs with LY294002 (20 mM), PD98059 (20 mM), and SB203580 (50 mM) also significantly attenuated SDF-1-induced migration of hUCB-MSCs (Fig. 1B), indicating that the Akt, ERK, and p38 signal transduction pathways are required for SDF-1-induced migration of hUCB-MSCs. Taken together, these results demonstrate that Akt, ERK, and p38 transduction pathways play a crucial role in hUCB-MSC migration via SDF-1/CXCR4.

3.2. SDF-1 activates Akt, ERK, and p38 signal transduction pathways in hUCB-MSCs

Akt is a known downstream effector of the PI3K-dependent signaling cascade. MAPKs are a family of protein kinases that consist of ERKs, p38s, and c-Jun N-terminal kinases [24]. Both PI3K/Akt and MAPK/ERK signal transduction pathways mediate the cell migration induced by chemokines or cytokines [17]. However, the precise mechanism for how SDF/CXCR4 induces Akt, ERK, and p38 signaling in hUCB-MSCs is unclear. To further investigate the involvement of Akt, ERK, and p38 signal transduction pathways in migration, the effect of SDF-1 on Akt, ERK, and p38 phosphorylation was examined in hUCB-MSCs. Cells were stimulated with 100 ng/mL SDF-1 for different times. SDF-1 treatment of hUCB-MSCs stimulated time-dependent phosphorylation (Fig. 2A). The phospho-Akt level reached a maximum 10–30 min after treatment with 100 ng/mL SDF-1, and the increased phosphorylation diminished 60 min after SDF-1 treatment, whereas no significant change in total Akt expression was observed over the course of the experiment. SDF-1-induced ERK and p38 phosphorylation was similar to Akt phosphorylation. To further examine the effect of SDF-1 on Akt, ERK, and p38 phosphorylation, hUCB-MSCs were treated with several concentration of SDF-1 for 15 min with or without pretreatment with AMD3100. Stimulation with SDF-1 led to a concentration-dependent phosphorylation of Akt, ERK, and p38 (Fig. 2B). SDF-1-induced Akt, ERK, and p38 phosphorylation was inhibited by AMD3100, a CXCR4-specific peptide antagonist. This indicated the involvement in signaling of a CXCR4 dependent pathway. CXCR4 is a Gi-coupled chemokine receptor; SDF-1 binding binding to CXCR4 mobilizes calcium, decreases cyclic AMP within cells, and activates multiple signal transduction pathways, including PI3K, phospholipase C-γ/protein kinase C, and MAP kinases ERK1/2 [25]. Therefore, it is plausible that SDF-1 induces CXCR4-mediated migration of hUCB-MSCs by interfering with signaling pathways other than the Akt, ERK, and p38 pathways.

3.3. Knockdown of Akt, ERK, and p38 inhibits SDF-1-induced migration of hUCB-MSCs

To further investigate directly whether hUCB-MSC migration is mediated by SDF-1/CXCR4 signaling through the activation of the Akt, ERK, and p38 pathways, siRNA-mediated knockdown of Akt, ERK, and p38 protein was carried out. As expected, siRNAs caused

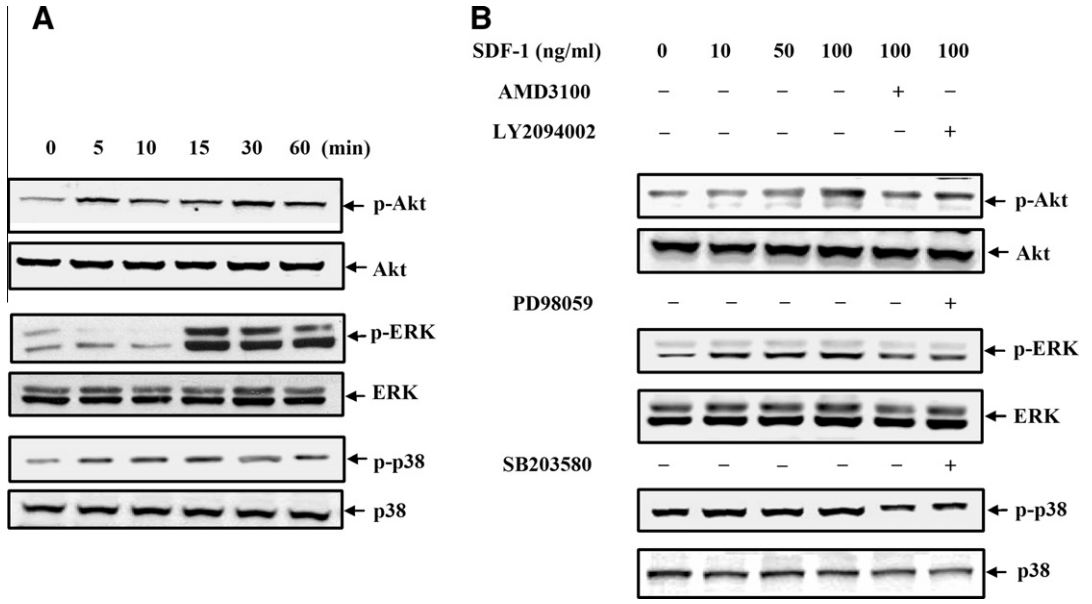

Fig. 2. SDF-1-induced Akt, ERK, and p38 phosphorylation in hUCB-MSCs. Cell lysates were prepared and used for Western blot with phospho-Akt, total Akt, phospho-ERK, total ERK, phospho-p38, or total p38 antibody. (A) hUCB-MSCs were stimulated with 100 ng/mL SDF-1 for 0, 5, 10, 15, 30, and 60 min. (B) hUCB-MSCs treated with several concentration of SDF-1 (0–100 ng/ml) for 15 min without pretreatment or following a 30 min pretreatment with 5 mg/mL AMD3100. Specific inhibitors (LY294002, PD98059, and SB203580) used as a control.

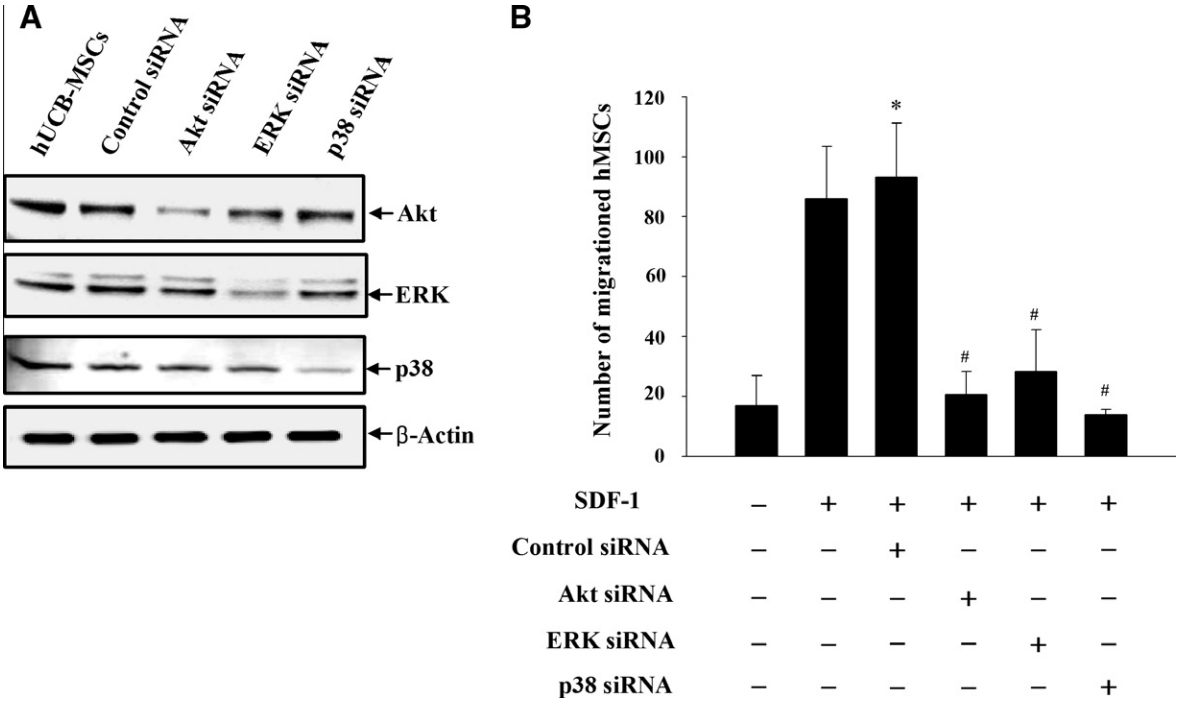

Fig. 3. Knockdown of Akt, ERK, and p38 blocks SDF-1-induced migration of hUCB-MSCs. hUCB-MSCs were transfected with control, Akt, ERK, and p38 siRNA for 72 h, followed by 24 h serum starvation. Cell lysates were prepared and used for Western blot and migration assay. (A) Representative results of nontransfected and Akt, ERK, and p38 siRNA transfected hUCB-MSCs probed with anti-Akt, anti-ERK, or anti-p38 antibody. Equal loading of proteins was confirmed by reprobing the same blot with anti-actin antibody. (B) Transwell migration assay demonstrating the migration of hUCB-MSCs in response to 100 nM SDF-1. SDF-1-induced hUCB-MSC migration was markedly inhibited by Akt, ERK, and p38 siRNA. The experiments were performed three times in duplicate and the results are expressed as the mean ± SE, $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with control siRNA-treated group.

down-regulation of Akt, ERK, and p38 protein within 3 days, and the affected hUCB-MSCs did not migrate when treated with SDF-1 (Fig. 3). These results indicated that the ERK, Akt, and p38 signaling pathways directly regulated SDF-1-induced migration of hUCB-MSCs. PI3K/Akt, but not MAPK/ERK, is also required for SDF-1α-mediated migration of progenitor cells [16]. However, some studies have shown that both the PI3K/Akt and MAPK/ERK signal transduction pathways are involved in the regulation of

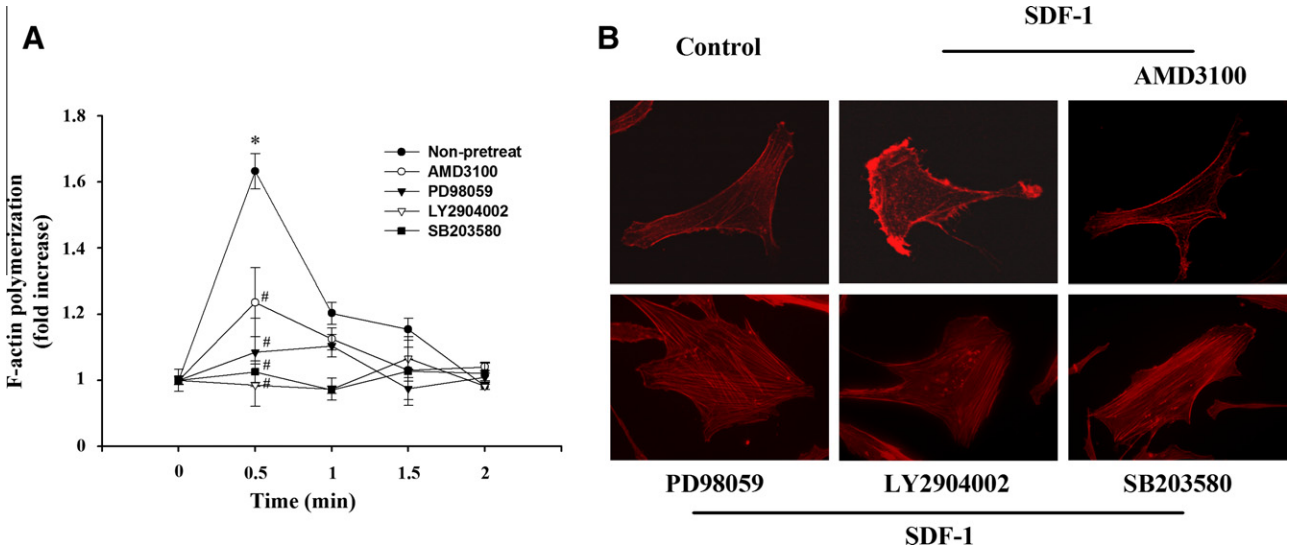

Fig. 4. CXCR4, PI3K, ERK, and P38 inhibitors reduce SDF-1-induced actin polymerization in hUCB-MSCs. hUCB-MSCs were serum-starved overnight and then were stimulated with 100 ng/mL SDF-1 for different times. (A) Quantification of F-actin cytoskeleton reorganization was performed by measuring red fluorescence intensity in each stage of cytoskeleton organization. hUCB-MSCS stimulated without or with SDF-1 for 0, 0.5, 1, 1.5, and 2 min. A representative graph for each stimulation time point is shown. The experiments were performed three times in duplicate and the results are expressed as the mean ± SE, $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with non-pretreated group. (B) F-actin was visualized by Alexa fluor 568-phalloidin labeling and viewed using fluorescence microscopy. Immunohistochemistry of unstimulated (Control) and SDF-1-stimulated hUCB-MSCs using phalloidin staining demonstrating actin cytoskeleton polymerization after 0.5 min of stimulation with SDF-1.

SDF-1-mediated migration [26,27]. Therefore, it seems that the signal transduction pathways involved in SDF-1/CXCR4-mediated cell migration are cell type-specific mechanism.

3.4. PI3K, ERK, and P38 inhibitors reduce SDF-1-induced actin polymerization in hUCB-MSCs

Migration is a complex phenomenon that includes polarization of cells toward a chemoattractant and cytoskeletal reorganization [28]. Actin-rich lamellopodia are formed at the leading edge of the cell, whereas a retracting uropod is present at the trailing edge. The rate of cell migration has been attributed to the capability of a cell to reorganize its cytoskeleton, more specifically, the rate of conversion of monomeric G-actin to filamentous actin (F-actin) [29]. F-actin polymerization, cell shape changes, and ensuing migration are under tight regulation of a number of signaling molecules.

Appropriately, the next experiment investigated whether these signaling pathways were also involved in the polymerization of F-actin. SDF-1 significantly induced F-actin polymerization within 30 s in hUCB-MSCs. However, pretreatment of hUCB-MSCs with the CXCR4, Akt, ERK, and p38 inhibitors significantly inhibited actin polymerization in response to SDF-1 (Fig. 4A). Immunocytochemical analysis of the distribution of F-actin in SDF-1-treated or untreated hUCB-MSCs revealed that F-actin was transiently concentrated in small spikes at the leading edge. However, polymerization of F-actin was not evident in inhibitor-pretreated hUCB-MSCs (Fig. 4B). These results indicate that Akt, ERK, and p38 pathways are also necessary for F-actin polymerization in response to SDF-1.

4. Conclusion

SDF-1 participates in the activation of Akt, ERK, and p38 signal pathways in hUCB-MSCs, resulting in induced migration and F-actin polymerization. Further studies are needed to identify the detailed molecular mechanisms of migration by SDF-1 in hUCB-MSCs.

Acknowledgments

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