

Sphingosine-1-Phosphate-Induced Migration and Differentiation of Human Mesenchymal Stem Cells to Smooth Muscle Cells

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Migration and differentiation of mesenchymal stem cells are crucial for tissue regeneration in response to injury. Sphingosine-1-phosphate (S1P) is a bioactive lipid that regulates a variety of biological processes, including proliferation, survival, differentiation and motility. In the present study, we determined the role of S1P in migration and differentiation of human bone marrow-derived mesenchymal stem cells (BMSCs). S1P stimulated migration of BMSCs in a dose- and time-dependent manner, and pre-incubation of the cells with pertussis toxin completely abrogated S1P-induced migration, suggesting involvement of G_i-coupled receptors in S1P-induced cell migration. S1P elicited elevation of intracellular concentration of Ca²⁺ ([Ca²⁺]_i) and pretreatment with VPC23019, an antagonist of S1P₁/S1P₃, blocked S1P-induced migration and increase of [Ca²⁺]_i. Small interfering RNA-mediated knockdown of endogenous S1P₁ attenuated S1P-induced migration of BMSCs. Furthermore, S1P treatment induced expression of α -smooth muscle actin (α -SMA), a smooth muscle marker, and pretreatment with VPC23019 abrogated S1P-induced α -SMA expression. S1P induced phosphorylation of p38 mitogen-activated protein kinase (MAPK), and pretreatment of cells with SB202190, an inhibitor of p38 MAPK, or adenoviral overexpression of a dominant-negative mutant of the p38 MAPK blocked S1P-induced cell migration and α -SMA expression. Taken together, these results suggest that S1P stimulates migration and smooth muscle differentiation of BMSCs through an S1P₁-p38 MAPK-dependent mechanism.

Key words : Sphingosine-1-phosphate, mesenchymal stem cells, migration, p38 MAPK, S1P receptor

Introduction

Mesenchymal stem cells (MSCs) possess self-renewal capacity, long-term viability, and differentiation potential toward diverse cell types, such as adipogenic, osteogenic, chondrogenic, and myogenic lineages. They can be isolated from a variety of tissues, including bone marrow and adipose tissues [2,31,34,36], suggesting that MSCs are highly useful for clinical applications in regenerative medicine. Intravenously-transplanted MSCs can home to injured and inflamed sites in animal models of myocardial infarction and cerebral ischemia [3]. Inflamed tissues produce a variety of inflammatory mediators, including chemokines, cytokines, and prostanoids [43], and several chemokines have been reported to attract MSCs *in vitro* [4,8,33,42]. MSCs have been shown to migrate in response to various growth factors, including platelet-derived growth factor, insulin-like growth factor, epidermal growth factor and hepatocyte

growth factor [6,7,17,32,40] and chemokines, such as stromal-derived factor-1, fractalkine and monocyte chemoattractant protein-1 [5,16,21]. However, the molecular mechanisms involved in migration of MSCs are still elusive.

Sphingosine-1-phosphate (S1P) is a bioactive lysophospholipid that is present in human plasma and serum [45] and released in large amounts (0.5 μ M in serum) from activated platelets [10]. As a factor released from activated platelets in inflamed or injured tissues, S1P regulates a variety of cellular responses, including cell growth, survival, differentiation and motility [14,35,37,39]. The effects of extracellular S1P are mediated by G protein-coupled receptors, termed S1P₁₋₅, which regulate diverse intracellular pathways [35,37,39]. Activation of S1P receptors has been shown to stimulate diverse signaling pathways involving ERK, JNK, p38 mitogen-activated protein kinase (MAPK), phospholipase C, phosphoinositide-3-kinase, and RhoA [11,39]. S1P has been reported to inhibit or stimulate cellular motility, depending on the cell type, differences in S1P receptor expression, and S1P concentration [35]. Activation of S1P₁ and S1P₃ stimulates migration through G_i-mediated activation

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of phosphatidylinositol-3-kinase, Akt and Rac [35,39], whereas S1P₂ signals through G_{12/13} for activation of RhoA and inhibition of Rac-dependent signaling, which is responsible for the inhibitory effect of S1P on cell migration [23,38,44]. S1P₄ is mainly expressed in immune cells and stimulates migration of transfected Chinese-hamster ovary cells by activation of cdc42 through a pertussis toxin (PTX)-sensitive manner [18]. S1P has been reported to stimulate migration of mouse bone marrow-derived MSCs (BMSCs) [27]. In addition, S1P mediated homing of BMSCs into injured liver and stimulated differentiation of cells into α -SMA-positive myofibroblasts [24]. However, S1P treatment has been shown to have no significant effect on migration of human BMSCs [15]. Therefore, it is still unclear whether S1P can regulate migration and differentiation of BMSCs.

In the present study, we explored the effects of S1P on migration and α -SMA expression of human BMSCs and characterized the signaling pathways associated with S1P-induced cellular responses.

Materials and Methods

Materials

Phosphate-buffered saline, α -minimum essential medium (α -MEM), trypsin, fetal bovine serum, and Lipofectamine^{plus} reagent were purchased from Invitrogen (Carlsbad, CA). S1P and PTX were purchased from BIOMOL (Plymouth Meeting, PA). VPC23019 was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). 1-Oleoyl-sn-glycero-3-phosphate (1-oleoyl-LPA), fatty acid-free bovine serum albumin, and Ki16425 were purchased from Sigma-Aldrich (St. Louis, MO). LY294002, Y27632, U0126, SP600125, and SB202190 were purchased from EMD Biosciences (San Diego, CA). Anti-phospho-p38 and anti-p38 MAPK antibodies were from Cell Signaling Technology (Beverly, MA). Fluo-4-AM was from Molecular Probes, Inc. (Eugene, OR). Culture plates were purchased from Nunc (Roskilde, Denmark). Horseradish peroxidase-labeled secondary antibodies and the enhanced chemiluminescence Western blotting system were from Amersham Biosciences (Pittsburgh, PA).

Cell culture

After informed consent, heparinized bone marrow was obtained from patients undergoing total hip arthroplasty, as approved by the Institutional Review Board of Pusan National University Hospital. BMSCs were isolated from

bone marrow as previously described [22]. For isolation of BMSCs, mononuclear cells from bone marrow were separated by centrifugation in a Ficoll-Hypaque gradient (density=1.077 g/cm³; Sigma, USA), and seeded at a density of 1×10^6 cells/cm². Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in growth medium (α -MEM, 10% fetal bovine serum, 100 units/ml of penicillin, 100 μ g/ml of streptomycin) until they reached confluence. Primary BMSCs were subcultured in tissue culture dishes at a density of 2,000 cells/cm². Cells expressed CD29, CD44, CD90, CD105 and did not express HLA-DR and c-kit²².

Cell migration assay

BMSCs migration was assayed using a Boyden chamber apparatus, as previously described [19]. Briefly, BMSCs were harvested with 0.05% trypsin containing 0.02% EDTA, washed once, and suspended in α -MEM at a concentration of 2×10^5 cells/ml. Membrane filters (8- μ m pore size) in disposable 96-well chemotaxis chambers (Neuro Probe, Inc.; Gaithersburg, MD) were precoated overnight with 20 μ g/ml rat-tail collagen at room temperature. Aliquots (50 μ l per well) of the cell suspension were loaded into the upper chambers, and test reagents were placed in the lower chamber, unless otherwise specified. To elucidate the signaling pathways involved in S1P-induced migration, cells were preincubated with pharmacological inhibitors for 15 min prior to loading. Following incubation of cells with S1P in the absence or presence of inhibitors for 12 hr at 37°C, filters were disassembled, and the upper surface of each filter was scraped free of cells by wiping with a cotton swab. The numbers of cells that had migrated to the lower surfaces of each filter were determined by microscopic counting of cells in four places ($\times 100$ magnification) after staining with hematoxylin and eosin.

Measurement of intracellular calcium concentration

Spatially averaged photometric [Ca²⁺] measurements from single cells were performed with the fluorescent Ca²⁺ indicator fluo-4-AM. In brief, BMSCs cells grown on 32 mm dish were incubated with serum-free α -MEM for 24 hr, loaded with 5 μ M fluo-4-AM for 40 min at 37°C in buffer A [135 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), pH 7.3], and washed twice with Hanks' balanced salt solution without phenol

red and Ca^{2+} . Fluo-4-AM-loaded BMSCs were treated with S1P. A Leica TCS-SP2 laser scanning confocal microscope (Leica Microsystems, Germany) was used for visualization of Ca^{2+} -mediated fluorescence in cells. Fluo-4 was excited with a 488-nm line of an argon laser, and fluo-4 fluorescence was collected between 510 and 525 nm. Scanning was performed every 1 sec for the indicated times, and the ratio of fluorescence intensity to initial fluorescence intensity (F/F_0) was calculated at each point for quantitative measurement.

Western blotting

Confluent, serum-starved BMSCs were treated with the indicated conditions, washed with ice-cold phosphate-buffered saline, and lysed in lysis buffer (20 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 10 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 , 30 mM sodium pyrophosphate, 25 mM β -glycerophosphate, 1% Triton X-100, pH 7.4). Lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, and stained with 0.1% Ponceau S solution. After blocking with 5% nonfat milk, membranes were immunoblotted with appropriate primary antibodies, and bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence system.

Reverse transcription-polymerase chain reaction analysis

Total cellular RNA was extracted by the Trizol method (Invitrogen, Carlsbad, CA). For reverse transcription-polymerase chain reaction (RT-PCR) analysis, aliquots of 2 μg each of RNA were subjected to cDNA synthesis with 200 U of M-MLV reverse transcriptase and 0.5 μg of oligo (dT) 15 primer (Promega, Madison, WI). cDNA in 2 μl of the reaction mixture was amplified with 0.5 U of GoTaq DNA polymerase (Promega, Madison, WI) and 10 pmol each of sense and antisense primers as follows: S1P₁ receptor (429-bp product), sense, 5'-TATCAGCGCGACAAGGA GAACAG-3' and antisense, 5'-ATAGGCAGGCCACCCAG GATGAG-3'; S1P₂ receptor (220-bp product), sense, 5'-TCGGCCTTCATCGTCATCCTCT-3' and antisense, 5'-CC TCCCGGGCAAACCACTG-3'; S1P₃ receptor (394-bp product), sense, 5'-CTGCCTGCACAATCTCCCTGACTG-3' and antisense, 5'-GGCCCCGCCGATCTCCT-3'; S1P₄ receptor (454-bp product), sense, 5'-GAGAGCGGGGCCACCA

AGAC-3' and antisense, 5'-GGTTGACCGCCGAGTTGAGG AC-3'; GAPDH 5'-TCCATGACAACCTTGGTATCG-3', 5'-TGTAGCCCAAATTCGTTGTCA-3'. The thermal cycle profile was as follows: denaturation at 95°C for 30 sec, annealing at 52-58°C for 30 sec, depending on the primers used, and extension at 72°C for 30 sec. Each PCR reaction was carried out for 30 cycles, and PCR products were size fractionated on 1.2% ethidium bromide/agarose gel and photographed under UV transillumination.

Transfection with small interfering RNA (siRNA)

siRNA duplexes were synthesized, desalted, and purified by Samchully Pharm. Co. Ltd. (Siheung, GyeongGi, Korea) as follows: S1P₁ 5'-GCU GCU CAA GAC CGU AAU UTT-3' (sense) and 5'-AAU UAC GGU CUU GAG CAG CTT-3' (antisense). Nonspecific control siRNA (D-001206-13-05) was purchased from Dharmacon, Inc. (Chicago, IL). For siRNA experiments, BMSCs were seeded on 60-mm dishes at 70% confluence, and were then transfected with siRNAs using the Lipofectamine plusTM reagent, according to manufacturer's instructions. Briefly, Lipofectamine plusTM reagent was incubated with serum-free medium for 15 min, and respective siRNAs were then added to the mixtures. Following incubation for 15 min at room temperature, the mixtures were diluted with serum free medium and added to each well. The final concentration of siRNAs in each well was 100 nM. Following incubation of BMSCs with serum-free medium containing siRNAs for 4 hr, cells were cultured in growth medium for 24 hr and the expression levels of S1P₁ and GAPDH were then determined by RT-PCR analysis.

Adenoviral expression of a dominant negative p38 MAPK mutant

The gene encoding the dominant-negative mutant of p38 MAPK (TY>AF) was kindly provided by Dr. J. Han [13] and used for production of recombinant adenoviruses using the AdEasy system (Stratagene, La Jolla, CA). Following the manufacturer's manual, recombinant adenoviruses were amplified and purified by density gradient ultracentrifugation according to manufacturer's manual. For adenoviral infection, exponentially growing BMSCs were infected with the appropriate amount of adenoviruses and incubated at 37°C for 2 hr with gentle shaking. Afterwards, fresh growth medium was added to each dish and further incubated for 48 hr. Expression levels of the dominant neg-

active mutant of p38 MAPK (DN-p38 MAPK) were determined by Western blot analysis.

Statistical analysis

Results of multiple observations are presented as means \pm SD. Statistical significance was assessed using Student's *t*-test or two-way ANOVA test, where indicated in the figure legends.

Results

S1P stimulates migration of human BMSCs through a Gi-dependent mechanism

In order to explore the question of whether S1P can regu-

late migration of human BMSCs, we examined the effect of S1P on migration of BMSCs using a Boyden chamber apparatus. S1P dose-dependently increased the migration of BMSCs with a maximal stimulation at 0.1 μ M (Fig. 1A) and S1P treatment stimulated migration of BMSCs in a time-dependent manner (Fig. 1B).

To examine the directional component of migration, we examined S1P-induced migration of BMSCs using a checkerboard analysis. As shown in Fig. 1C, addition of S1P in the bottom chamber induced migration of BMSCs into the lower surfaces of the filters. S1P enhanced migration in the absence of a gradient (equal concentrations in the top and bottom), suggesting that S1P stimulates random migration (chemokinesis) as well as directional migration (chemotaxis)

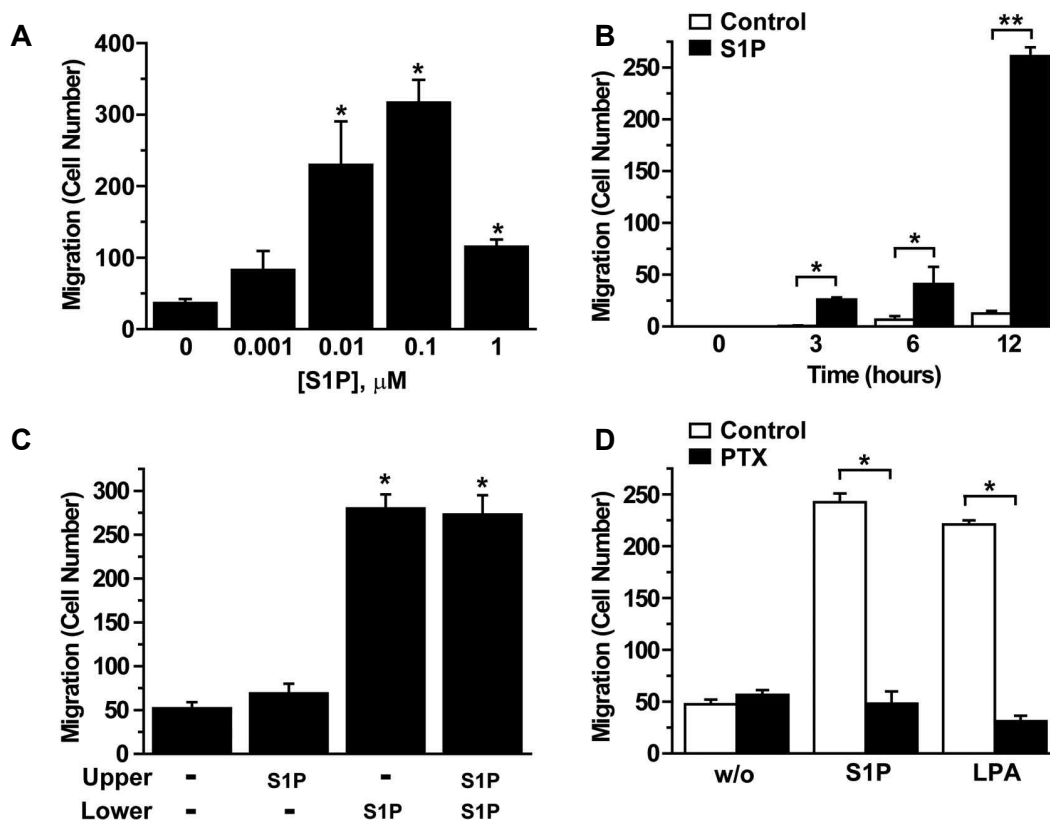


Fig. 1. Role of Gi in S1P-induced migration of BMSCs. (A) BMSCs were loaded onto the upper chambers of the Boyden apparatus and serum-free media containing the indicated concentrations of S1P were placed in the lower chambers. The numbers of migratory BMSCs were determined after 12 hr, as described under "Materials and Methods". (B) Serum-free media containing 0.1 μ M S1P or vehicles (control) were added to the lower chambers and the number of BMSCs that migrated to the lower surface of filters was determined after the indicated time periods. (C) Checkerboard analyses of S1P-induced migration. 0.1 μ M S1P or vehicles was placed in either the bottom, top, or both chambers of the Boyden apparatus, as noted, and cells were then allowed to migrate for 12 hr. Data represent average values \pm S.D. (n=4). (D) BMSCs were pretreated with serum-free medium containing vehicles or 100 ng/ml PTX for 24 hr, loaded into the upper chamber, and serum-free media containing vehicles (w/o), 0.5 μ M 1-oleoyl-LPA or 0.1 μ M S1P were added into the lower chambers. The number of cells migrated to lower surface of filters was determined after 12 hr. Data represent means \pm S.D. (n=4). *, $p<0.05$; **, $p<0.01$ by two-way ANOVA and Scheffe's *post hoc* test.

of BMSCs.

Involvement of G_i , a pertussis toxin-sensitive G protein, in S1P-induced migration has been reported in a variety of cell types [35,39]. We have demonstrated that LPA induced migration of human adipose tissue-derived MSCs through a G_i -dependent mechanism [20]. For assessment of whether the S1P-stimulated migration was mediated by G_i protein, we examined the effect of PTX on migration of human BMSCs. As shown in Fig. 1D, PTX treatment completely abrogated migration of BMSCs induced by not only LPA, but also S1P. These results indicate that S1P stimulates the migration of BMSCs through a G_i -dependent pathway.

Involvement of S1P₁ receptor in S1P-induced increase of $[Ca^{2+}]_i$

S1P has been reported to elevate intracellular concentration of calcium ($[Ca^{2+}]_i$) through activation of S1P receptors. Therefore, we next examined the effect of S1P on $[Ca^{2+}]_i$ of BMSCs. As shown in Fig. 2A, S1P rapidly increased $[Ca^{2+}]_i$ in BMSCs, and pretreatment of BMSCs with VPC23019, an antagonist specific for S1P₁ and S1P₃, completely inhibited S1P-induced elevation of $[Ca^{2+}]_i$ (Fig. 2B), suggesting that S1P receptors, S1P_{1/3}, play a key role in S1P-induced elevation of $[Ca^{2+}]_i$.

Involvement of S1P₁ receptor in S1P-induced migration

To further explore the question of whether S1P receptors are involved in S1P-induced migration, we examined the effects of VPC23019 on migration stimulated by S1P. As shown in Fig. 3A, VPC23019 completely inhibited S1P-in-

duced migration of BMSCs. In contrast, LPA-stimulated migration of BMSCs was not affected by pretreatment with VPC23019, suggesting that S1P_{1/3} receptors may play a key role in S1P-induced migration of BMSCs.

To clarify the molecular identities of S1P receptors involved in S1P-stimulated migration, we examined the expression levels of S1P receptors in BMSCs by RT-PCR analysis. As shown in Fig. 3B, S1P₁, S1P₂, and S1P₃, but not S1P₄, were expressed in BMSCs and S1P₁ is a S1P receptor isoform that is predominantly expressed in BMSCs. To explore involvement of S1P₁ in S1P-induced migration of BMSCs, we examined the effects of siRNA-mediated depletion of S1P₁ receptor on S1P-induced migration. As shown in Fig. 3C, the mRNA level of S1P₁, but neither S1P₂ nor S1P₃, in BMSCs was specifically down-regulated by transfection with siRNAs specific for S1P₁. We next examined the effects of knockdown of S1P₁ expression on S1P-stimulated migration. Depletion of endogenous S1P₁ completely abrogated S1P-induced migration, whereas LPA-induced migration was not affected by knockdown of S1P₁ expression (Fig. 3D). These results clearly indicate that S1P₁ plays a key role in S1P-stimulated migration of BMSCs.

S1P induces migration of BMSCs through multiple signaling pathways

S1P reportedly activates multiple signaling pathways, including ERK, p38 MAPK, RhoA, and phosphoinositide-3-kinase (PI3K) [35,39]. In order to explore the involvement of these signaling enzymes in S1P-induced migration of BMSCs, we examined the effects of pharmacological inhibitors of signaling enzymes on cell migration. As shown

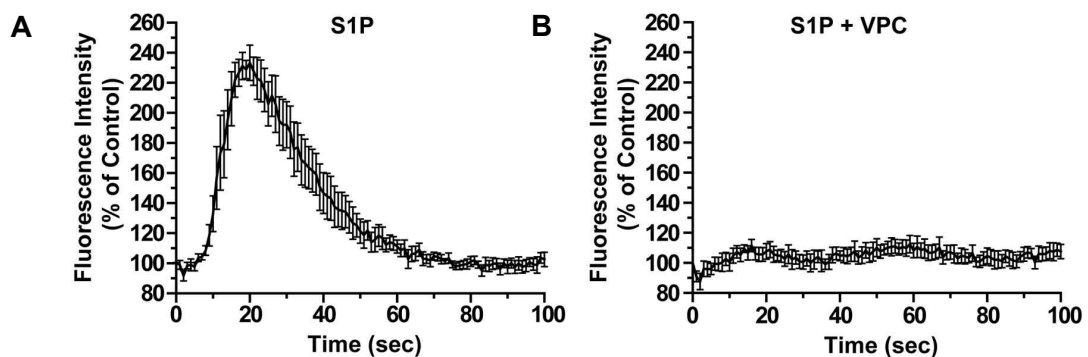


Fig. 2. S1P induces elevation of $[Ca^{2+}]_i$ in BMSCs. Serum-starved BMSCs were loaded with 5 μ M Fluo-4-AM for 40 min at 37°C in the absence or presence of 10 μ M of VPC23019 and then treated with serum-free medium containing 0.1 μ M S1P in the absence or presence of 10 μ M of VPC23019. Ca^{2+} -dependent fluorescence was measured every second for the indicated time periods, and fluorescence intensities of more than 20 different cells from time-lapse images were quantified over time. Results are expressed as percentage of the control (0 sec) and expressed as means \pm S.D.

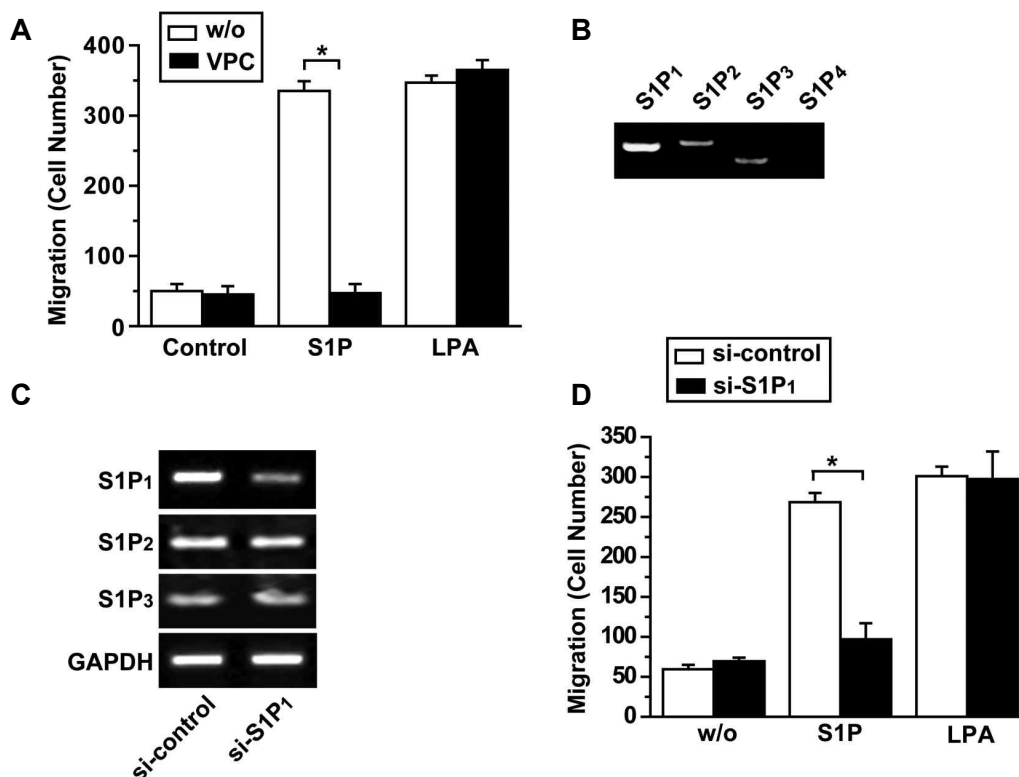


Fig. 3. S1P stimulates migration of BMSCs through an S1P₁-dependent mechanism. (A) BMSCs were pretreated with serum-free medium containing 10 μ M of VPC23019 or 1 μ M Ki16425 for 15 min, loaded into the upper chamber, and serum-free media containing vehicles (control), 0.5 μ M 1-oleoyl-LPA or 0.1 μ M S1P were added to the lower chambers. The number of cells that migrated to the lower surface of filters was determined after 12 hr. Data represent means \pm S.D. (n=4). *, $p < 0.01$ by two-way ANOVA and Scheffe's *post hoc* test. (B) mRNA levels of S1P₁, S1P₂, S1P₃ and S1P₄ in BMSCs were determined by RT-PCR. (C) BMSCs were transfected with either control siRNA (si-control) or S1P₁-specific siRNAs (si-S1P₁) and the mRNA levels of S1P₁, S1P₂, S1P₃, and GAPDH were determined by RT-PCR. (D) siRNA-transfected BMSCs were exposed to vehicles (w/o), 0.5 μ M 1-oleoyl-LPA or 0.1 μ M S1P, respectively, for 12 hr, and the number of migrated cells was determined. Data represent means \pm S.D. (n=4). * indicates $p < 0.01$ by two-way ANOVA and Scheffe's *post hoc* test.

in Fig. 4A, S1P-induced migration of BMSCs was markedly abrogated by pretreatment of cells with the PI3K inhibitor LY294002, the MEK/ERK pathway inhibitor U0126, the JNK inhibitor SP600125, or the p38 MAPK inhibitor SB202190. However, the Rho kinase inhibitor Y27632 had no significant impact on S1P-induced cell migration. While the role of the ERK and PI3K pathways in cell migration has been evidently reported in other cell types [35,39], involvement of p38 MAPK in S1P-induced cell migration is largely elusive. To explore the question of whether S1P can activate p38 MAPK in BMSCs, we examined the effects of S1P on phosphorylation of p38 MAPK in these cells. S1P treatment resulted in time-dependent increase of the phosphorylation levels of p38 MAPK with a maximal stimulation at 5 min (Fig. 4B). Pretreatment of cells with the p38 MAPK inhibitor SB202190 blocked S1P-induced phosphorylation of p38 MAPK (Fig. 4C), supporting an implication of p38 MAPK

in S1P-induced cell migration. S1P-induced phosphorylation of p38 MAPK was blocked by pretreatment of cells with VPC23019, suggesting involvement of S1P₁ in S1P-induced activation of p38 MAPK (Fig. 4D).

To confirm involvement of p38 MAPK in S1P-induced migration of BMSCs, cells were infected with an adenovirus bearing a kinase-deficient dominant negative mutant of p38 MAPK (DN-p38 MAPK). DN-p38 MAPK was highly expressed in DN-p38 MAPK adenovirus-infected cells (Fig. 4E) and overexpression of DN-p38 MAPK abrogated S1P-induced migration of BMSCs (Fig. 4F). Taken together, these results suggest that p38 MAPK plays a key role in S1P-induced migration of BMSCs.

S1P induces α -SMA expression in BMSCs

S1P has been reported to induce differentiation of mouse BMSCs to α -SMA-positive myofibroblasts *in vivo* which

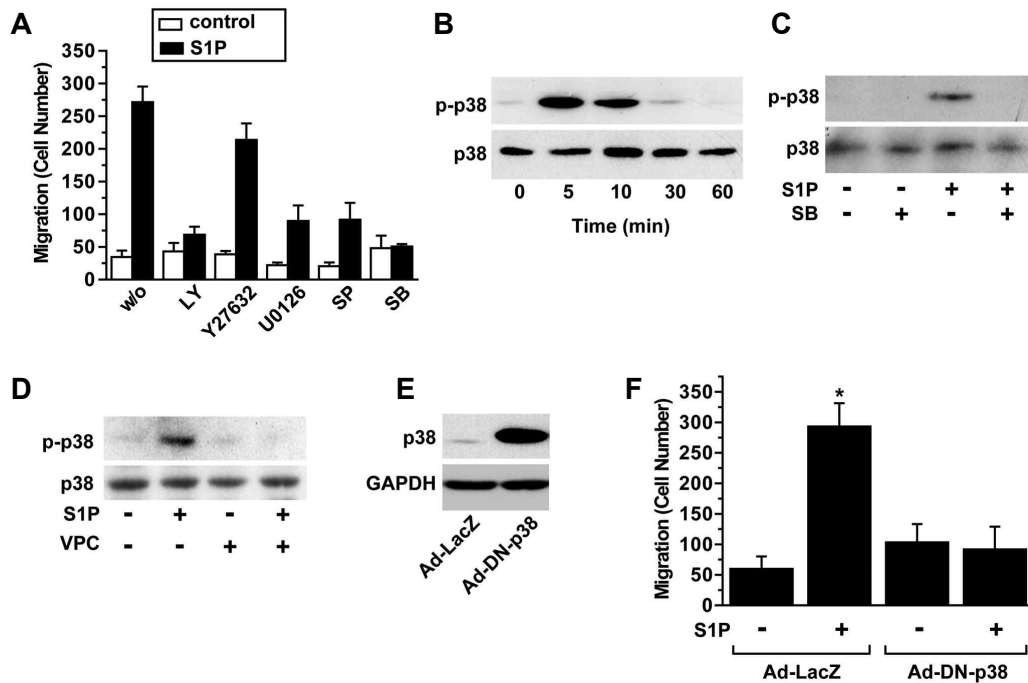


Fig. 4. Signaling pathways involved in S1P-induced migration of BMSCs. (A) BMSCs were pretreated with vehicles, 10 μ M LY294002, 10 μ M Y27632, 10 μ M SP600125 or 10 μ M SB202190 for 15 min, and then exposed to serum-free medium containing 0.1 μ M S1P or vehicles in the absence or presence of pharmacological inhibitors for 12 hr. The number of migrated cells was counted and data represent means \pm S.D. (n=4). (B) BMSCs were stimulated with 1 μ M of S1P for the indicated time periods. (C) BMSCs, which were preincubated in the absence or presence of 10 μ M of SB202190 for 15 min, were stimulated with 1 μ M of S1P or vehicles for 5 min. (D) BMSCs were pretreated with serum-free medium containing 10 μ M of VPC23019 or vehicles for 15 min and then treated with 1 μ M S1P or vehicles for 5 min. Phosphorylation and expression levels of p38 MAPK were analyzed by Western blotting with phospho-p38 and p38 MAPK antibodies, respectively. (E) BMSCs were infected with adenoviruses bearing DN-p38 MAPK or LacZ at MOI of 100 pfu for 48 h and then exposed to 1 μ M S1P or vehicles for 5 min. Expression levels of p38 MAPK were determined by Western blotting. To ensure equal loading of proteins, the expression levels of GAPDH were determined. (F) Adenovirus-infected cells were exposed to serum-free media containing 1 μ M S1P or vehicles for 12 hr. The number of migrated cells was counted and data represent means \pm S.D. (n=4). * indicates $p < 0.01$ by two-way ANOVA and Scheffé's *post hoc* test.

play a key role in tissue injury [24]. In order to explore the question of whether S1P treatment can directly induce differentiation of BMSCs to α -SMA-positive cells, we examined the effect of S1P on the expression level of α -SMA in human BMSCs. As shown in Fig. 5A, S1P treatment increased the expression levels of α -SMA at 0.1 μ M concentration, whereas the concentrations of S1P higher than 0.1 μ M inhibited α -SMA expression. S1P-induced α -SMA expression was abrogated by VPC23019, suggesting that S1P induced expression of α -SMA in human BMSCs through an S1P₁-dependent pathway (Fig. 5B). To elucidate the signaling pathways involved in S1P-induced α -SMA expression, we examined the effects of pharmacological inhibitors in S1P-induced α -SMA expression. As shown in Fig. 5C, S1P-induced α -SMA expression was largely attenuated by pretreatment of cells with U0126, SP600125, SB202190,

or Y27632, suggesting involvement of ERK, JNK, p38 MAPKs, and Rho kinase in S1P-stimulated α -SMA expression, whereas LY294002 had no significant impact on S1P-induced α -SMA expression, indicating that PI3K is not involved in the S1P induced α -SMA expression.

Discussion

S1P is released from activated platelets in inflamed or injured tissues and regulates a variety of cellular responses, including cell growth, survival, differentiation, and motility [14,35,37,39]. S1P has been shown to regulate diverse cellular responses by binding to its specific G protein-coupled receptors [35,37,39]. In the present study, we demonstrated that S1P induced migration of human BMSCs through an S1P₁-dependent mechanism. Accumulating evidence con-

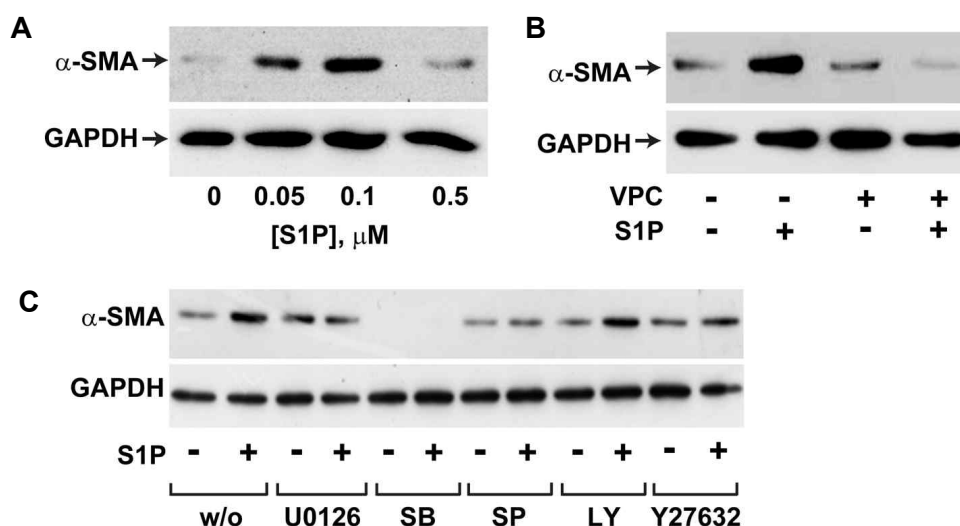


Fig. 5. S1P-induced α -SMA expression in human BMSCs. (A) BMSCs were treated with the indicated concentrations of S1P for 4 days. (B) BMSCs were treated with 0.1 μ M S1P or vehicles in the absence or presence of 10 μ M of VPC23019 for 4 days. (C) BMSCs were pretreated with vehicles, 10 μ M LY294002, 10 μ M Y27632, 10 μ M U0126, 10 μ M SP600125, or 10 μ M SB202190 for 15 min, and then exposed to serum-free medium containing 0.1 μ M S1P or vehicles in the absence or presence of pharmacological inhibitors for 4 days. Expression levels of α -SMA and GAPDH were determined by Western blotting.

sistently suggests a pivotal role of S1P₁ in S1P-induced migration of various cell types [35,37,39]. Furthermore, S1P stimulated migration of mouse BMSCs through an S1P₁-mediated mechanism [1,27]. These results support the notion that S1P₁ is involved in S1P-induced migration of human BMSCs.

In the present study, we demonstrated that S1P induced expression of α -SMA in human BMSCs through an S1P₁-dependent pathway. S1P₁ knockout mice exhibited embryonic hemorrhage, leading to intrauterine death owing to a defective migration of vascular smooth muscle cells/pericytes and incomplete vascular maturation [25]. S1P has been reported to induce expression of smooth muscle markers in cultured smooth muscle cells, suggesting a pivotal role of smooth muscle differentiation and vascular development [26]. α -SMA is expressed in not only contractile smooth muscle cells (SMCs) but also in myofibroblasts [9,29]. Myofibroblasts arise as a consequence of tissue injury, and they exhibit a contractile force that is required for wound closure [9,41]. S1P mediated homing of BMSCs in a mouse liver injury model and infiltrated BMSCs have recently been reported to contribute to formation of α -SMA-positive myofibroblasts within injured tissues [24]. Furthermore, S1P treatment induced expression of α -SMA in adipose tissue-derived MSCs [28]. These results suggest

that S1P plays a key role in differentiation of BMSCs to smooth muscle cells or myofibroblasts, which contribute to vascular development and regeneration of injured tissues.

Coupling of S1P₁ *via* G_i for activation of diverse signaling pathways, including Ras-MAP kinase, phosphoinositide-3-kinase and the phospholipase C pathway, as well as coupling of S1P₂ and S1P₃ to multiple G proteins, i.e. G_q, G_{12/13} and G_i for stimulation of the phospholipase C pathway and Rho pathway, as well as the G_i-dependent pathways, have been reported [11,14,37]. We demonstrated here that S1P-induced migration was prevented by pretreatment of cells with PTX, suggesting involvement of G_i in S1P-induced cell migration. An increasing body of evidence suggests involvement of p38 MAPK in migration of diverse cell types, including smooth muscle cells, endothelial cells, and epithelial cells [12]. However, the role of p38 MAPK in S1P-induced cell migration has not been clearly understood. We demonstrated that pharmacological inhibition of p38 MAPK or adenoviral expression of a dominant negative mutant of p38 MAPK abrogated S1P-stimulated cell migration. In support of the present study, the p38 MAPK inhibitor SB203580 completely abrogated the S1P-induced migration of OVCAR3 ovarian cancer cells [30]. Therefore, these results suggest that p38 MAPK plays a key role in S1P-induced cell migration. In addition, we demon-

strated here that S1P-induced migration of BMSCs was completely abrogated by either the MEK-ERK inhibitor U0126, JNK inhibitor, or the PI3K inhibitor LY294002, whereas the Rho kinase inhibitor Y27632 had only a marginal impact on S1P-induced cell migration. In addition, we showed that S1P induced α -SMA expression in BMSCs through mechanisms involving p38 MAPK, ERK, and JNK. In contrast to S1P-induced migration, S1P-induced α -SMA expression was abrogated by Y27632 but not by LY294002. These results led us to suggest that S1P-induced migration and differentiation of BMSCs to smooth muscle-like cells are differentially mediated by multiple signaling pathways involving p38 MAPK, JNK, ERK, Rho kinase and phosphoinositide-3-kinase.

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초록 : Sphingosine-1-phosphate에 의한 중간엽 줄기세포의 이동과 평활근세포로의 분화**송해영¹ · 신상훈^{1,2} · 김민영^{1,2} · 김재호^{1,2,*}**(부산대학교 ¹의학전문대학원 생리학교실, ²허혈조직재생연구센터)

중간엽 줄기세포의 이동과 분화는 손상된 조직의 재생을 위해 필수적이다. Sphingosine-1-phosphate (S1P)는 세포성장, 생존, 분화, 이동성 등 여러 가지 생명현상에 중요한 역할을 하는 생리활성 지질이다. 본 연구에서는 인체 골수유래 중간엽 줄기세포의 이동과 세포분화에 대한 S1P의 영향을 조사하였다. S1P는 중간엽 줄기세포의 이동을 증가시켰으며 pertussis toxin의 전처리는 S1P에 의한 세포이동을 억제하였다. 본 결과는 S1P에 의한 세포 이동과정에 Gi에 연결된 수용체가 관여함을 제시한다. S1P₁과 S1P₃ 수용체에 대한 길항제인 VPC23019의 전처리나 siRNA를 이용한 S1P₁ 수용체의 발현억제는 S1P에 의한 세포 내 칼슘 증가와 중간엽 줄기세포의 이동을 저해하였다. 또한, S1P의 처리는 중간엽 줄기세포에서 평활근세포의 표지유전자인 α -smooth muscle actin (α -SMA)의 발현을 증가시켰으며 VPC23019의 전처리는 S1P에 의한 α -SMA의 발현증가를 저해하였다. S1P는 중간엽 줄기세포에서 p38 mitogen-activated protein kinase (p38 MAPK)의 인산화를 촉진하였으며 p38 MAPK의 저해제인 SB202190의 전처리 또는 p38 MAPK의 dominant negative mutant의 과발현은 S1P에 의한 중간엽 줄기세포의 이동 α -SMA 발현증가를 억제하였다. 본 연구결과는 S1P가 S1P₁-p38 MAPK 신호전달기전을 통해 중간엽 줄기세포의 이동과 평활근세포로의 분화를 촉진함으로써 중간엽 줄기세포를 이용한 조직재생에의 활용 가능성을 제시한다.