

Thromboxane A₂ modulates migration, proliferation, and differentiation of adipose tissue-derived mesenchymal stem cells

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Abbreviations: α -SMA, α -smooth muscle actin; hADSCs, human adipose tissue-derived mesenchymal stem cells; MSCs, mesenchymal stem cells; SMCs, smooth muscle cells; TxA₂, thromboxane A₂

Abstract

Prostanoid metabolites are key mediators in inflammatory responses, and accumulating evidence suggests that mesenchymal stem cells (MSCs) can be recruited to injured or inflamed tissues. In the present study, we investigated whether prostanoid metabolites can regulate migration, proliferation, and differentiation potentials of MSCs. We demonstrated herein that the stable thromboxane A₂ (TxA₂) mimetic U46619 strongly stimulated migration and proliferation of human adipose tissue-derived MSCs (hADSCs). Furthermore, U46619 treatment increased expression of α -smooth muscle actin (α -SMA), a smooth muscle marker, in hADSCs, suggesting differentiation of hADSCs into smooth muscle-like cells. U46619 activated ERK and p38 MAPK, and pretreatment of the cells with the MEK inhibitor U0126 or the p38 MAPK inhibitor SB202190 abrogated the U46619-induced migration, proliferation, and α -SMA expression. These results suggest that TxA₂ plays a key role in the migration, pro-

liferation, and differentiation of hADSCs into smooth muscle-like cells through signaling mechanisms involving ERK and p38 MAPK.

Keywords: cell movement; cell proliferation; 15-hydroxy-11 α ,9 α -(epoxymethano)prosta-5,13-dienoic acid; mesenchymal stem cells; mitogen-activated protein kinase 1; mitogen-activated protein kinase 14; thromboxane A₂

Introduction

Mesenchymal stem cells or multipotent stromal cells (MSCs) can be isolated from a variety of tissues, including bone marrow and adipose tissues, and they possess self-renewal capacity, long-term viability, and differentiation potential toward diverse cell types, such as adipogenic, osteogenic, chondrogenic, and myogenic lineages (Prockop, 1997; Pittenger *et al.*, 1999; Short *et al.*, 2003; Barry and Murphy, 2004). In addition to their stem cell-like properties, MSCs exhibit homing ability to injured and inflamed sites in animal models of myocardial infarction and cerebral ischemia (Chamberlain *et al.*, 2007). Transplanted MSCs have been reported to differentiate into smooth muscle cells (SMCs) *in vivo* and contribute to the remodeling of vasculature (Davani *et al.*, 2003; Gojo *et al.*, 2003; Yoon *et al.*, 2005). We have previously demonstrated that lysophospholipids, such as sphingosylphosphorylcholine and lysophosphatidic acid, increased α -smooth muscle actin (α -SMA) expression in human adipose tissue-derived MSCs (hADSCs) (Jeon *et al.*, 2006, 2008), suggesting a potential role of MSCs as progenitor cells for SMCs.

Prostanoids, including prostaglandin D₂, prostaglandin E₂, prostaglandin F_{2 α} , prostaglandin I₂, and thromboxane A₂ (TxA₂), are produced from arachidonic acid by cyclooxygenase enzymes (Tilley *et al.*, 2001). TxA₂, a major prostanoid metabolite of arachidonic acid, is produced through isomerization of prostaglandin H₂ by TxA₂ synthase, predominantly in platelets and in response to a variety of physiological and pathological stimuli (Halushka *et al.*, 1989; Dogne *et al.*, 2005). TxA₂ mediates a broad range of cellular responses, including cell proliferation, migration, platelet aggre-

gation, and smooth muscle contraction (Bos *et al.*, 2004; Huang *et al.*, 2004b), and it plays a pivotal role in atherosclerotic vascular diseases, myocardial infarction, and bronchial asthma (Negishi *et al.*, 1995). Its actions are primarily mediated by the TP receptor (TxA₂ receptor), a G-protein-coupled receptor that triggers activation of MAPKs, including ERK and p38 MAPK (Bos *et al.*, 2004; Huang *et al.*, 2004b). However, functional involvement of

prostanoid metabolites on migration, proliferation, and smooth muscle differentiation of hADSCs has not been clarified.

In the present study, we sought to elucidate the role of TxA₂ and other prostanoids in the migration, proliferation, and smooth muscle differentiation of hADSCs. We demonstrate for the first time that TxA₂ stimulates migration, proliferation, and α -SMA expression in hADSCs through molecular

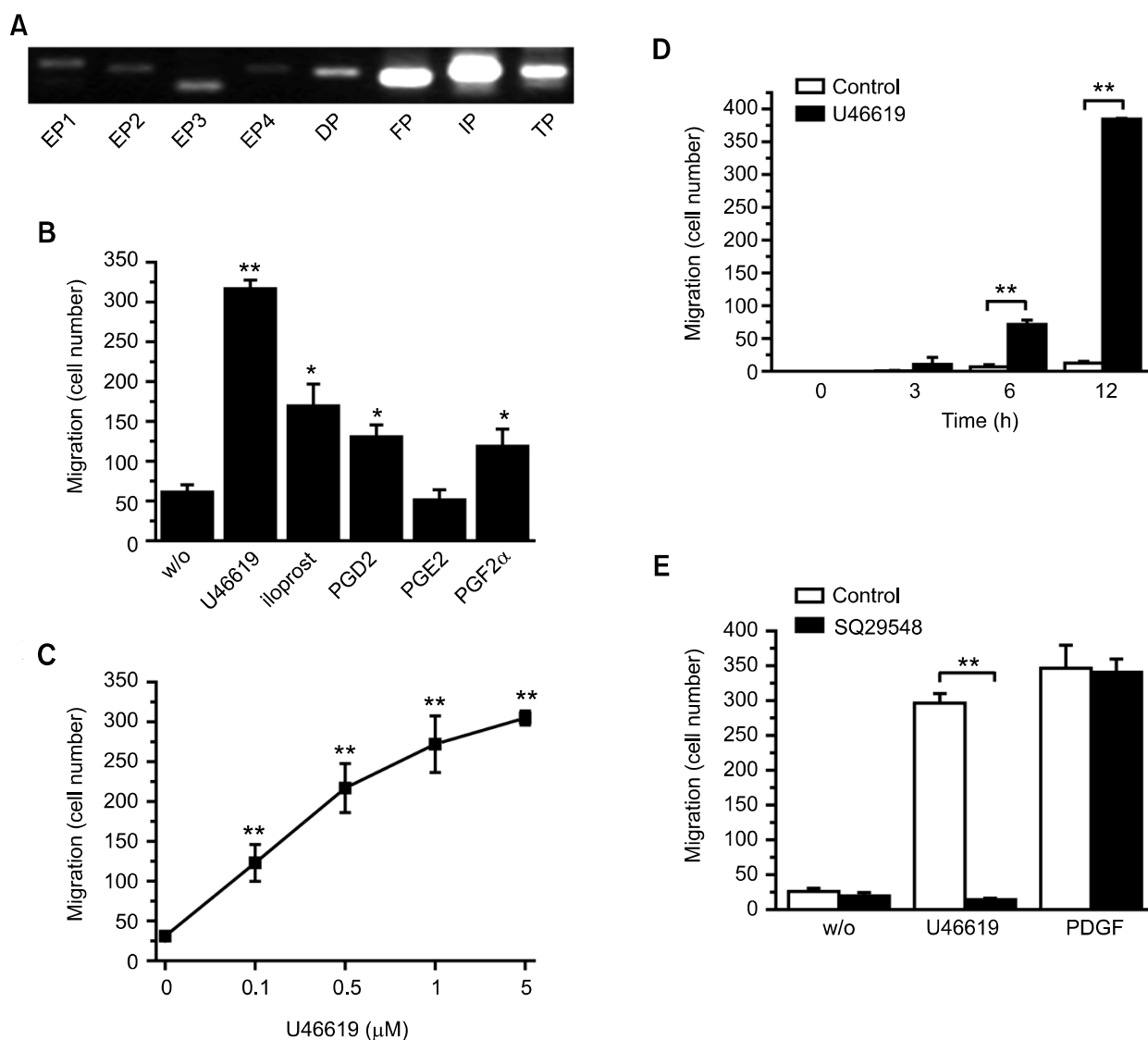


Figure 1. Effects of U46619 and prostanoid metabolites on the migration of hADSCs. (A) The mRNA levels of prostanoid receptors (EP1, EP2, EP3, EP4, DP, FP, IP, TP) in hADSCs were determined by RT-PCR analysis. Representative data from three independent experiments are shown. (B) Serum-free media containing 5 μ M U46619, iloprost, PGF2 α , PGD2, PGE2, or LTB4 were placed in the lower chambers of the Boyden apparatus, and the numbers of migratory hADSCs were determined after 12 h. (C) Indicated concentrations of U46619 were placed in the lower chambers, and the numbers of migratory hADSCs were determined after 12 h. (D) Serum-free media containing vehicles or 5 μ M U46619 were placed in the lower chambers, and the number of migrated hADSCs were determined after the indicated time periods. (E) Serum-free media containing 5 μ M U46619, 10 ng/ml PDGF-BB, and/or 10 μ M SQ29548 were placed into the lower chambers, and the numbers of migrating hADSCs were determined after 12 h. Data represent mean \pm SD ($n = 4$). *, $P < 0.05$; **, $P < 0.01$ versus control.

mechanisms involving ERK and p38 MAPK.

Results

U46619 stimulated migration of hADSCs through a TP receptor-dependent mechanism

In order to determine whether hADSCs express prostanoid receptors, we extracted total RNAs from hADSCs and performed RT-PCR with primers specific to each prostanoid receptor. As shown in Figure 1A, three prostanoid receptors, including FP, IP, and TP, were highly expressed in hADSCs. Compared to the expression levels of these prostanoid receptors, however, the DP and the EP receptors (EP1, EP2, EP3, and EP4) were expressed at relatively low levels. To explore the effects of prostanoid metabolites on hADSC migration, we measured the migration of the hADSCs toward prostanoid metabolites by using a Boyden appa-

ratus. As shown in Figure 1B, the stable TxA₂ analogue U46619 greatly stimulated hADSC migration. Iloprost (a stable analog of prostacyclin, an IP receptor agonist), prostaglandin D₂ (a DP receptor agonist), and prostaglandin F₂α (a FP receptor agonist) also increased migration of hADSCs, but their effects were less potent than those of U46619. In contrast, prostaglandin E₂ (an EP receptor agonist) had no significant impact on hADSC migration. U46619 dose-dependently stimulated hADSC migration with a maximal stimulation at 5 μM (Figure 1C), and the U46619-induced migration greatly increased after a 12 h exposure (Figure 1D). These results indicate that prostanoid metabolites derived from arachidonic acid stimulate migration of hADSCs, and that among these metabolites, TxA₂ is the most potent in stimulating hADSC migration.

In order to clarify whether the TP receptor mediated the U46619-induced migration, we examined the effects of SQ29548, a TP receptor-specific antagonist, on the U46619-induced migra-

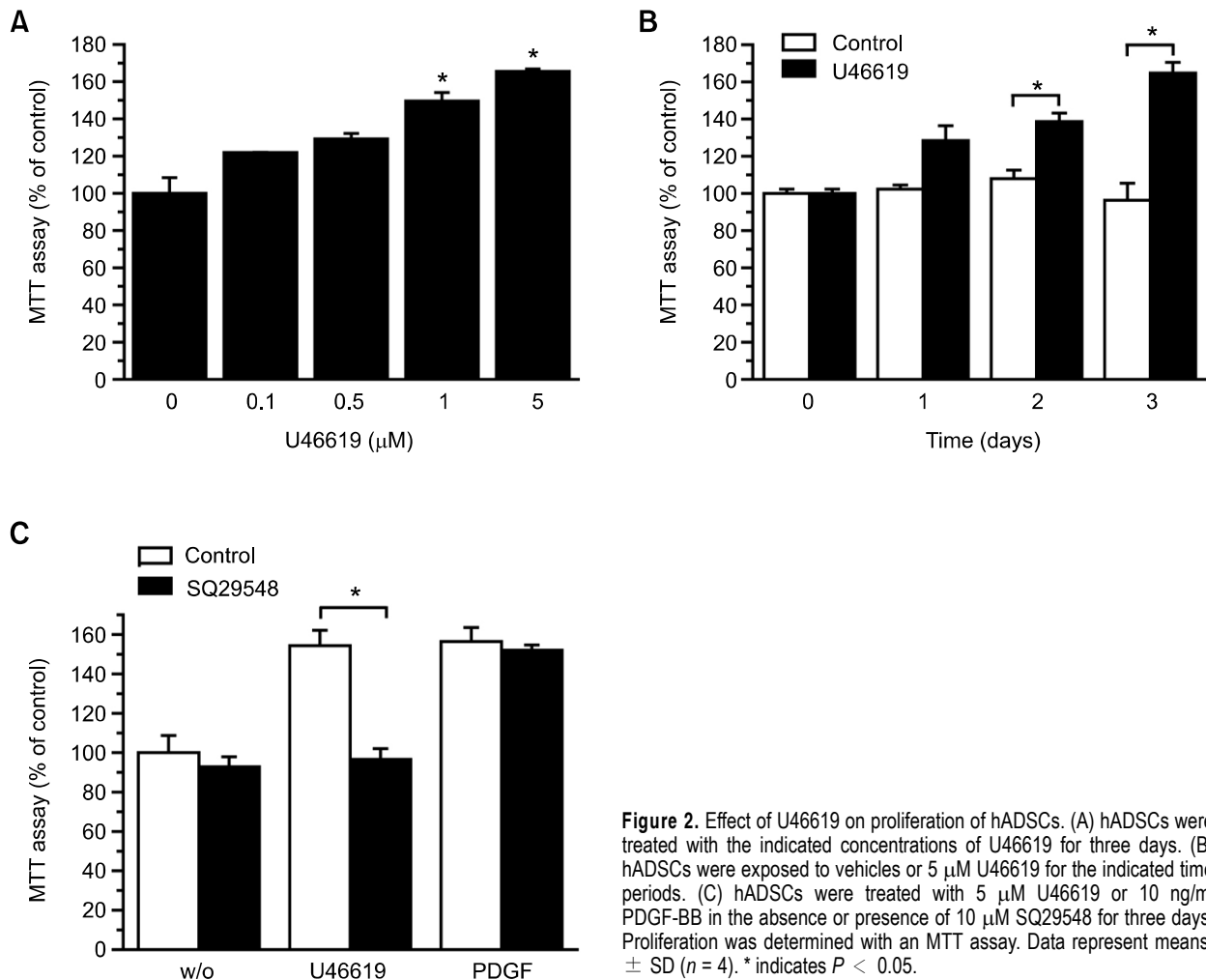


Figure 2. Effect of U46619 on proliferation of hADSCs. (A) hADSCs were treated with the indicated concentrations of U46619 for three days. (B) hADSCs were exposed to vehicles or 5 μM U46619 for the indicated time periods. (C) hADSCs were treated with 5 μM U46619 or 10 ng/ml PDGF-BB in the absence or presence of 10 μM SQ29548 for three days. Proliferation was determined with an MTT assay. Data represent means ± SD (n = 4). * indicates $P < 0.05$.

tion of hADSCs. As shown in Figure 1E, pretreatment of hADSCs with SQ29548 completely abrogated the migration of the cells stimulated by U46619, in contrast to the result that SQ29548 did not significantly impact PDGF-induced migration of hADSCs, suggesting that the TP receptor plays a specific role in U46619-induced migration of hADSC.

Activation of TP receptor induced proliferation of hADSCs

To investigate whether TxA₂ affected the proliferation of hADSCs, we examined the effect of U46619 on the proliferation of hADSCs using an MTT assay. U46619 treatment markedly stimulated hADSC proliferation in a dose-dependent (Figure 2A) and time-dependent (Figure 2B) manner. Pretreating the cells with SQ29548 completely abrogated the proliferation of hADSCs stimulated by U46619 but not PDGF-BB (Figure 2C), suggesting that TxA₂ stimulated not only migration but also proliferation of hADSCs through TP receptor-dependent mechanism.

U46619 induced migration and proliferation of hADSCs through ERK- and p38 MAPK-dependent pathways

U46619 reportedly activates MAPKs, including ERK and p38 MAPK (Bos *et al.*, 2004; Huang *et al.*, 2004b). To explore whether U46619 activated ERK and p38 MAPK as part of the mechanism underlying hADSC stimulation, we examined the effects of U46619 on the phosphorylation levels of ERK and p38 MAPK in these cells. As shown in Figure 3A, U46619 treatment dose-dependently increased phosphorylation levels of ERK and p38 MAPK. The phosphorylation levels of ERK and p38 MAPK observed after 5 min of U46619 stimulation (Figure 3B). Moreover, pretreating the cells with the MEK1/2 inhibitor U0126 or the p38 MAPK inhibitor SB202190 specifically inhibited the U46619-induced phosphorylation of ERK or p38 MAPK, respectively (Figure 3C). Pretreating the cells with either U0126 or SB202190 completely attenuated U46619-induced migration and proliferation of hADSCs (Figure 3D and E), suggesting

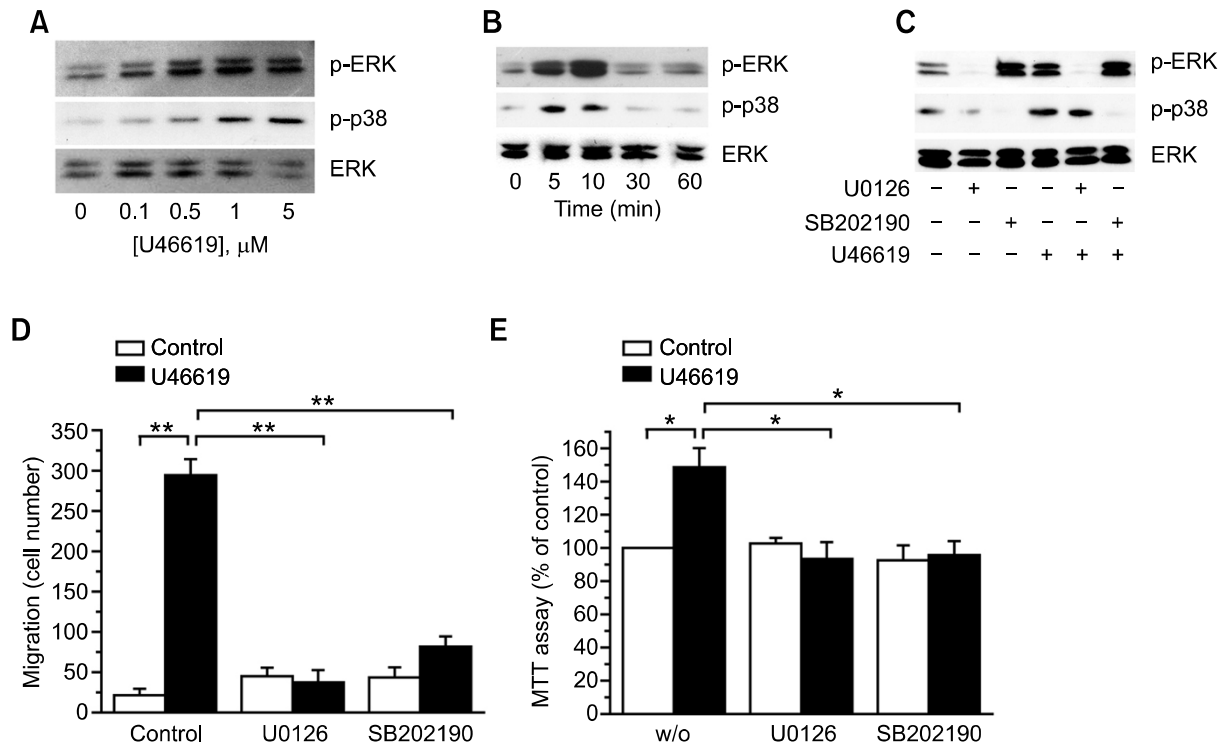


Figure 3. Roles of ERK and p38 MAPK in the U46619-induced migration and proliferation of hADSCs. (A) hADSCs were treated with the indicated concentrations of U46619 for 10 min. (B) hADSCs were treated with 5 μM U46619 for the indicated time periods. (C) hADSCs were pretreated with vehicles, 10 μM U0126, or 10 μM SB202190 for 15 min and treated with vehicles or 5 μM U46619 for 10 min. Phosphorylation levels of ERK and p38 MAPK and the expression levels of total ERK were determined by Western blotting. (D) Serum-free media containing vehicles or 5 μM U46619 in the absence or presence of 10 μM U0126 or 10 μM SB202190 were placed into the lower chambers, and the numbers of migrating hADSCs were determined after 12 h. (E) Serum-starved hADSCs were treated with 5 μM U46619 or vehicles (control) in the absence or presence of 10 μM U0126 or 10 μM SB202190 for three days, and proliferation was determined with an MTT assay. Data represent means ± SD (n = 4). *, P < 0.05; **, P < 0.01.

that both ERK and p38 MAPK are involved in the U46619-stimulated migration and proliferation of hADSCs.

U46619 induced differentiation of hADSCs into smooth muscle-like cells through ERK- and p38 MAPK-dependent pathways

To explore whether TxA₂ can induce differentiation of hADSCs to SMCs, hADSCs were treated with indicated concentrations of U46619 for four days, and then the expression levels of α -SMA, a SMC marker, were determined by Western blotting. As shown in Figure 4A, U46619 dose-dependently increased the expression of α -SMA. The expression of α -SMA was drastically increased after exposure of the cells to 5 μ M U46619 for four days (Figure 4B), suggesting U46619-induced differentiation of hADSCs to smooth muscle-like cells. The U46619-induced α -SMA expression was markedly abrogated by pretreating the cells with U0126 or SB202190 (Figure 4C), suggesting that ERK and p38 MAPK are involved in the U46619-stimulated differentiation of hADSCs into smooth muscle-like cells.

Discussion

In the present study, we demonstrated for the first time that TxA₂-induced activation of the TP receptor ultimately led to migration, proliferation, and smooth

muscle differentiation of hADSCs through mechanisms involving ERK and p38 MAPK. In support of these results, ERK and p38 MAPK reportedly play a key role in the migration of diverse cell types (Huang *et al.*, 2004a). Overexpression of a constitutively-active MEK1 mutant promoted the migration of FG carcinoma cells, whereas depletion of endogenous ERK by antisense oligonucleotides inhibited cell migration (Klemke *et al.*, 1997). In coronary artery smooth muscle cells, U46619 stimulated proliferation through an ERK-dependent mechanism (Morinelli *et al.*, 1994). Furthermore, p38 MAPK is involved in smooth muscle cell migration induced by PDGF, TGF- β , and IL-1 β (Hedges *et al.*, 1999), as well as PDGF-induced migration of endothelial cells (Matsumoto *et al.*, 1999). Furthermore, we have reported that sphingosylphosphorylcholine and lysophosphatidic acid induced α -SMA expression in hADSCs through ERK-dependent mechanism (Jeon *et al.*, 2006, 2008). Taken together, these results support the notion that ERK and p38 MAPK play a central part in the TxA₂-induced migration, proliferation, and smooth muscle differentiation of hADSCs.

TxA₂ is produced mainly in platelets, but it can also be synthesized by other cell types, including monocytes and vascular smooth muscle (Tilley *et al.*, 2001; Reiss and Edelman, 2006). While the levels of prostanoids like TxA₂ are generally very low in uninflamed tissues, they markedly increase as a part of the inflammatory response in injured tissues (Tilley *et al.*, 2001). The current study pro-

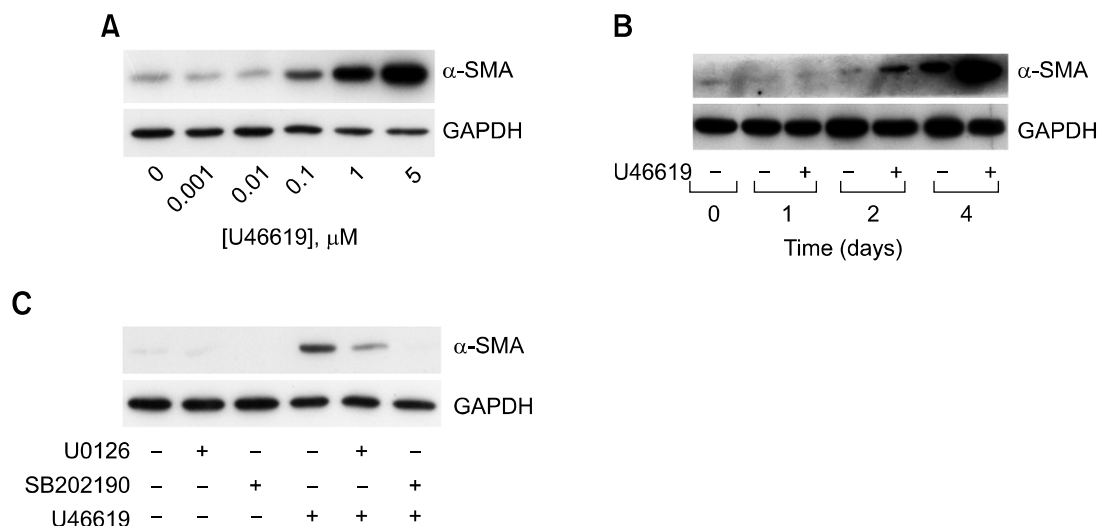


Figure 4. Effect of U46619 on the expression levels of α -SMA in hADSCs. (A) hADSCs were treated with indicated concentrations of U46619 for four days. (B) hADSCs were treated with vehicle or 1 μ M U46619 for indicated time periods. (C) hADSCs were treated with 5 μ M U46619 or vehicle in the absence or presence of 10 μ M U0126 or 10 μ M SB202190 for four days. The expression levels of α -SMA were determined by Western blotting with anti- α -SMA antibody. To ensure equal loading in each lane, the levels of GAPDH were determined by Western blotting. Representatives of three independent experiments are shown.

vides an unprecedented demonstration that TxA₂ potently activates migration and proliferation of MSCs and stimulates differentiation of the cells into smooth muscle-like cells. Taken together with the previous reports suggesting that MSCs can be recruited into injured and inflamed tissues (Wang *et al.*, 2002; Chamberlain *et al.*, 2007; Fox *et al.*, 2007; Ponte *et al.*, 2007), the results herein suggest that TxA₂ may participate in MSC-mediated tissue repair by stimulating homing of MSCs into injured tissues and inducing proliferation and differentiation of MSCs into smooth muscle-like cells. The implication of TxA₂-induced functional modulation of MSCs in the regeneration of injured tissues should be determined further.

Methods

Materials

PBS, α -minimum essential medium, trypsin, and FBS were purchased from Invitrogen (Carlsbad, CA). U46619, iloprost, prostaglandin F₂ α , prostaglandin D₂, prostaglandin E₂, and SQ29548 were purchased from Cayman Chemicals (Ann Arbor, MI). Human platelet-derived growth factor-BB (PDGF-BB) was purchased from R&D Systems, Inc. (Minneapolis, MN). U0126, and SB202190 were purchased from EMD Biosciences (San Diego, CA). Anti-phospho-ERK, anti-ERK, and anti-phospho-p38 MAPK were from Cell Signaling Technology (Beverly, MA). Anti-GAPDH antibody was purchased from Chemicon International Inc. (Temecula, CA). HRP-labeled secondary antibodies and the enhanced chemiluminescence Western blotting system were from Amersham Biosciences (Pittsburgh, PA). MTT, anti- α -SMA antibody, and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture

Subcutaneous adipose tissue was obtained from elective surgeries with the patients' consent, as approved by Institution Review Board of Busan National University Hospital. To isolate hADSCs, adipose tissues were washed at least three times with sterile PBS and treated with an equal volume of collagenase type I solution (0.1% collagenase type I and 1% BSA, buffered with Hank's balanced salt solution) for 60 min at 37°C with intermittent shaking. Floating adipocytes were separated from the stromal-vascular fraction by centrifugation at 300 *g* for 5 min. The cell pellet was resuspended in α -minimum essential medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin, and cells were plated in tissue culture dishes at 3,500 cells/cm². Primary hADSCs were cultured for 4-5 days until they reached confluence, at which point they were defined as passage "0". For experiments, hADSCs were used at passage 3-10.

Cell migration assay

hADSC migration was assayed using a Boyden chamber

apparatus, as previously described (Law *et al.*, 1996). Briefly, hADSCs were harvested with 0.05% trypsin containing 0.02% EDTA, washed once, and suspended in α -minimum essential medium 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 4°C. Aliquots (100 μ l per well) of the hADSC suspension were loaded into the upper chambers, and test reagents were placed in the lower chamber. After incubation for the indicated time periods, the filters were disassembled, and the upper surface of each filter was scraped free of cells by wiping it with a cotton swab. The numbers of cells that had migrated to the lower surfaces of each filter were determined by microscopically counting the cells in four places ($\times 100$ magnification) after staining with hematoxylin and eosin.

MTT assay

Proliferation was determined with a colorimetric MTT assay: MTT is metabolized by NAD-dependent dehydrogenase to form a colored reaction product (formazan), and the amount of dye formed directly correlates with the number of cells. To determine cell numbers, hADSCs were seeded in a 24-well culture plate at a density of 2×10^4 cells/well, cultured for 48 h in normal growth medium, serum-starved for 24 h, and treated with various reagents (or a vehicle control) for the indicated times. The cells were washed twice with PBS and incubated with 100 μ l of MTT (0.5 mg/ml) for 2 h at 37°C. The formazan granules generated by the cells were dissolved in 100 μ l of DMSO, and the absorbance of the solution at 562 nm was determined by using a PowerWave_x microplate spectrophotometer (Bio-Tek Instruments, Inc.; Winooski, VT) after dilution to a linear range.

Western blot analysis

Confluent, serum-starved hADSCs were treated with the indicated conditions, washed with ice-cold PBS, and lysed in lysis buffer (20 mM Tris-HCl, 1 mM ethylene glycol tetraacetic acid, 1 mM EDTA, 10 mM NaCl, 0.1 mM PMSF, 1 mM Na₃VO₄, 30 mM sodium pyrophosphate, 25 mM β -glycerophosphate, 1% Triton X-100, pH 7.4). Lysates were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and stained with 0.1% Ponceau S solution. After blocking with 5% nonfat milk, the membranes were immunoblotted with appropriate primary antibodies, and the bound antibodies were visualized with HRP-conjugated secondary antibodies and an enhanced chemiluminescence system.

RT-PCR analysis

Cells were treated as indicated, and total cellular RNA was extracted by the Trizol method (Invitrogen). For RT-PCR analysis, 2 μ g aliquots of RNA were subjected to cDNA synthesis with 200 U of M-MLV reverse transcriptase (Invitrogen) and 0.5 μ g of oligo (dT) 15 primer (Promega; Madison, WI). The cDNA in 2 μ l of the reaction mixture

was amplified with 0.5 U of GoTaq DNA polymerase (Promega) and 10 pmol each of sense and antisense primers, as follows: EP1 5'-GCTATGAGCTGCAGTACCC-3', 5'-CACGACACCACCATGATAC-3'; EP2 5'-CTCCTGAGA-AAGACAGTGCT-3', 5'-CTGATATTCGCAAAGTCCTC-3'; EP3 5'-AGCAGAAAGAATGCAACTTC-3', 5'-AGCTGGATGCATAGTTGTTT-3'; EP4 5'-CTCCTGAGAAAGACAGT-GCT-3', 5'-CTGATATTCGCAAAGTCCTC-3'; DP 5'-TTCTTCATGTCCTTCTTTGG-3', 5'-GCTGGAGTAGAGCACAG-AGT-3'; FP 5'-CTTCATGACAGTGGGAATCT-3', 5'-CCAA-AAATACTGCAAAGGAC-3'; IP 5'-GTGCAGGAACCTCA-CCTAC-3', 5'-AAAGAGGATGAGCATGGAC-3'; TP 5'-AA-CATTACCCTGGAGGAGAG-3', 5'-CGAAGAAGATCATGACGAC-3'. The thermal cycle profile was as follows: denaturation at 95°C for 30 s, annealing at 47-55°C for 45 s (depending on the primers used), and extension at 72°C for 45 s. Each PCR was carried out for 30 cycles, and PCR products were size-fractionated on 1.2% ethidium bromide/agarose gels and quantified under UV transillumination.

Statistical analysis

The results of multiple observations are presented as mean \pm SD. For multivariate data analysis, group differences were assessed with two-way ANOVA, followed by *post hoc* comparisons tested with Scheffe's method.

Acknowledgements

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