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Lysophosphatidic Acid in Malignant Ascites Stimulates Migration of Human Mesenchymal Stem Cells

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Abstract Lysophosphatidic acid (LPA) is elevated in ascites of ovarian cancer patients and is involved in growth and invasion of ovarian cancer cells. Accumulating evidence suggests a pivotal role of mesenchymal stem cells (MSCs) or stromal cells in tumorigenesis. In the present study, we demonstrated that ascites from ovarian cancer patients and LPA increased migration of human MSCs. The migration of MSCs induced by LPA and malignant ascites was completely abrogated by pretreatment with Ki16425, an antagonist of LPA receptors, and by silencing of endogenous LPA_1, but not LPA_2, with small interference RNA, suggesting a key role of LPA played in the malignant ascites-induced migration. LPA induced activation of ERK through pertussis toxin-sensitive manner, and pretreatment of MSCs with U0126, a MEK inhibitor, or pertussis toxin attenuated the LPA-induced migration. Moreover, LPA induced activation of RhoA in MSCs, and pretreatment of the cells with Y27632, a Rho kinase inhibitor, markedly inhibited the LPA-induced migration. In addition, LPA and malignant ascites increased intracellular concentration of calcium in MSCs, and Ki16425 completely inhibited the elevation of intracellular calcium. These results suggest that LPA is a crucial component of the malignant ascites which induce the migration of MSCs and elevation of intracellular calcium. J. Cell. Biochem. 104: 499–510, 2008.

Key words: lysophosphatidic acid; mesenchymal stem cells; migration; cancer; RhoA

Lysophosphatidic acid (LPA) is a small bioactive phospholipid produced by activated platelets, mesothelial cells, fibroblasts, adipocytes, and some cancer cells [Gaits et al., 1997]. It is involved in a variety of physiological and pathophysiological responses including wound healing, production of angiogenic factors, chemotaxis, neointima formation, tumor cell invasion, metastasis, and cell cycle progression [Mills and Moolenaar, 2003; Birgbauer and

Abbreviation used: LPA, lysophosphatidic acid; MSCs, mesenchymal stem cells; α-MEM, α-minimum essential medium; PBS, phosphate-buffered saline; PTX, pertussis toxin; PDGF-BB, platelet-derived growth factor-BB; 1-oleoyl-LPA, 1-oleoyl-*sn*-glycero-3-phosphate; BSA, bovine serum albumin; hADSCs, human adipose tissue-derived mesenchymal stem cells; hBMSCs, human bone marrow-derived mesenchymal stem cells; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interference RNA; $[Ca^{2+}]_i$, intracellular concentration of calcium.

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Chun, 2006]. Increasing body of evidence suggests that LPA is relevant to the pathogenesis of ovarian cancer: LPA levels are elevated in the blood and ascites of patients with ovarian cancer [Westermann et al., 1998; Xu et al., 1998; Xiao et al., 2001]. Furthermore, LPA was found to activate adhesion, migration, and invasion of several ovarian cancer cell lines [Xu et al., 1995; Bian et al., 2004]. Therefore, it is likely that LPA is not only a potential diagnostic marker, but also a crucial therapeutic target for the treatment of ovarian cancer.

The biological functions of LPA are mediated through several G protein-coupled receptors, that is, LPA_1/EDG-2, LPA_2/EDG-4, LPA_3/EDG-7, LPA_4/p2y9/GPR23, and LPA_5/GPR92 [Moolenaar et al., 2004]. LPA_1 and LPA_3 have been implicated in the LPA-induced cell migration of different types of cancer cells [Shida et al., 2003; Van Leeuwen et al., 2003; Yamada et al., 2004; Sengupta et al., 2007]. LPA receptors couple to at least three distinct G-protein subfamilies, notably $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ [Moolenaar et al., 2004; Birgbauer and Chun, 2006]. It has been reported that LPA-induced migration is regulated by several distinct signaling pathways, such as $G\alpha_{12/13}$-mediated activation of RhoA, G_i-mediated activation of phosphoinositide 3-kinase leading to activation of Rac, and G_i-dependent activation of ERK [Van Leeuwen et al., 2003]. It is well known that Rac is required at the front of the cell to regulate cell (re)spreading after initial rounding, lamellipodium formation and cell migration [Raftopoulou and Hall, 2004], and Rho activity in migrating cells is responsible for cell body contraction and rear end retraction, thereby promoting movement of the cell body and facilitating detachment of the rear end. In addition, G_i-ERK-dependent pathway has been shown to be involved in the LPA-stimulated migration of tumor cells [Stahle et al., 2003; Bian et al., 2004]. However, the involvement of RhoA and ERK in the LPA-induced migration has not yet been clearly defined.

Tumors are composed of neoplastic cells and stromal cell compartments, including fibroblasts, myofibroblasts, and pericytes. Cancer-stromal interaction is well known to play important roles during cancer progression. Stromal fibroblasts derived from human prostate carcinoma have been reported to exhibit tumor-promoting property, compared with control normal fibroblasts [Tlsty, 2001]. Moreover, carcinoma-associated fibroblasts which were isolated from invasive human breast carcinomas promoted the growth of mammary carcinoma cells and enhanced tumor angiogenesis [Orimo et al., 2005]. The functional role of stromal cells during tumorigenesis of ovarian cancer has been evaluated by several laboratories: Infiltration of endothelial cells into ovarian carcinoma tumors is dependent on the presence of myofibroblasts [Walter-Yohrling et al., 2003], and stromal infiltration and vascular maturation are an important checkpoint, linking the angiogenic switch with initiation of tumor progression [Gilead et al., 2004]. Therefore, it is likely that infiltration of stromal cells plays a pivotal role in carcinogenesis.

Mesenchymal stem cells (also called as stromal cells; MSCs) possess self-renewal capacity, long-term viability, and differentiation potential toward diverse cell types, such as adipogenic, osteogenic, chondrogenic, and myogenic lineages, and they can be isolated from a variety of tissues, such as bone marrow and adipose tissues [Prockop, 1997; Pittenger et al., 1999; Short et al., 2003; Barry and Murphy, 2004], suggesting clinical applications of MSCs for regenerative medicine. On the other hand, several recent studies indicate that MSCs could result in an adverse effect that is to favor tumor growth: Subcutaneous transplantation of tumor cells together with MSCs exhibited elevated capability of proliferation, rich angiogenesis in tumor tissues, and highly metastatic activity [Zhu et al., 2006]. Furthermore, MSCs have been shown to migrate to various in vivo sites of injury and tumors [Mills and Moolenaar, 2003; Ji et al., 2004; Satake et al., 2004; Studeny et al., 2004; Fukuda and Fujita, 2005; Nakamizo et al., 2005]. Interaction of stem cells with tumor environment is an emerging area of research, therefore, it would be of a great import to elucidate the factors that elicit migration of MSCs to tumors. In the present study, we demonstrated for the first time that LPA is involved in the malignant ascites-induced migration of human MSCs through activation of $G_{i/o}$-ERK- and RhoA-Rho kinase-dependent pathways.

MATERIALS AND METHODS

Materials

α-Minimum essential medium (α-MEM), phosphate-buffered saline (PBS), trypsin, fetal bovine

serum, and Lipofectamine™ 2000 reagent were purchased from Invitrogen (Carlsbad, CA). U0126, Y27632, and pertussis toxin (PTX) were from BIOMOL (Plymouth Meeting, PA). Anti-phospho-ERK and anti-ERK antibodies were from Cell Signaling Technology (Beverly, MA). Anti-RhoA antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Peroxidase-labeled secondary antibodies and the enhanced chemiluminescence kit were purchased from Amersham Biosciences. Fluo-4-AM was from Molecular Probes, Inc. (Eugene, OR). Human platelet-derived growth factor-BB (PDGF-BB) was purchased from R&D systems, Inc. (Minneapolis, MN). 1-Oleoyl-*sn*-glycero-3-phosphate (1-oleoyl-LPA), fatty acid-free bovine serum albumin (BSA), Ki16425, and 1-butanol were purchased from Sigma–Aldrich (St. Louis, MO). [Oleoyl-9,10-^{3}H]LPA (NEN; specific activity = 48 Ci/mmol) was from PerkinElmer Life Science (Waltham, MA).

Cell Culture

After informed consent, adipose tissues were obtained from patients undergoing elective abdominoplasty. For isolation of human adipose tissue-derived mesenchymal stem cells (hADSCs), adipose tissues were washed at least three times with sterile PBS and treated with an equal volume of 0.1% collagenase for 30 min at 37°C with intermittent shaking. The floating adipocytes were separated from the stromal-vascular fraction by centrifugation at 300*g* for 5 min. The cell pellet was filtered through a 100-μm nylon mesh to remove cellular debris and incubated overnight at 37°C/5% CO_2 in growth medium (α-MEM, 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin). Following incubation, the plates were washed extensively with PBS to remove residual nonadherent red blood cells. When the monolayer of adherent cells reached confluence, the primary hADSCs were subcultured at a concentration of 2,000 cells/cm^2. The primary hATSCs were cultured for 4–5 days until they reached confluence and were defined as passage "0." The passage number of hATSCs used in these experiments was 3–10.

Ascites From Patients With Ovarian Cancer or Liver Cirrhosis

Ascites were obtained from patients with ovarian cancer or liver cirrhosis with the patient's consent, as approved by the Institution Review Board. Approximately 10 ml of aspirations from ascites were collected, immediately centrifuged at 1,000*g* for 20 min to remove cells, and the supernatant was stored at −80°C until use.

Extraction of Lipid Components From Ascites and Denaturation of Ascites

To extract lipid components, an aliquot (200 μl) of ascites or 1-oleoyl-LPA was treated with 1 volume of 1-butanol. After vigorous shaking and centrifugation (5 min at 3,000*g*), the upper 1-butanol phase was collected and evaporated under nitrogen. The resultant lipids were solubilized in 200 μl of 1 mg/ml fatty acid-free BSA. To denature proteins in ascites, an aliquot (200 μl) of ascites or 1-oleoyl-LPA was heated at 100°C for 5 min, centrifuged at 15,000 rpm for 5 min to remove denatured proteins, and the supernatants were collected. To determine the recovery efficiency, [oleoyl-9,10-^{3}H]LPA (NEN; specific activity = 48 Ci/mmol) was added to the ascites and LPA, and the radioactivities of lipid fractions solubilized with BSA were measured by using a Tri-Carb liquid scintillation analyzer (PerkinElmer Life Sciences, Boston, MA).

Cell Migration Assay

Migration of the hADSCs 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-MEM at a concentration of 2×10^5 cells/ml. A membrane filter with 8-μm pores of the disposable 96 well chemotaxis chamber (Neuro Probe, Inc., Gaithersburg, MD) was precoated overnight with 20 μg/ml rat-tail collagen at 4°C, an aliquot (100 μl) of hADSCs suspension was loaded into the upper chamber, and test reagents were then placed in the lower chamber, unless otherwise specified. For elucidation of signaling pathways involved in the LPA-induced migration, the cells were preincubated with pharmacological inhibitors for 10 min before loading. After incubation of the cells with either LPA or ascites in the absence or presence of inhibitors for 12 h at 37°C, the filters were then disassembled, and the upper surface of each filter was scraped free of cells by wiping it with a cotton swab. The number of cells that had migrated to the lower surface of each filter was determined by counting the cells in four places

under microscopy at 100× magnification after staining with hematoxylin and eosin.

Reverse Transcription-Polymerase Chain Reaction Analysis

Cells were treated as indicated, and total cellular RNA was extracted by the Trizol method (Invitrogen). 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 (Invitrogen) and 0.5 μg of oligo (dT) 15 primer (Promega, Madison, WI). The cDNA in 2 μl 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: LPA_1 receptor 5′-TCTTCTGGGCCATTTTCAAC-3′, 5′-TGCCTRAAGGTGGCG CTCAT-3′, LPA_2 receptor 5′-CCTACCTCTTCCTCATGTTC-3′, 5′-TAAAGGGTGGAGTCCATCAG-3′; GAPDH 5′-TCCATGACAACTTTGGTATCG-3′, 5′-TGTAGCCAAATTCGTTGTCA-3′. The thermal cycle profile was as follows: denaturation at 95°C for 30 s, annealing at 52–58°C for 45 s depending on the primers used, and extension at 72°C for 45 s. Each PCR reaction was carried out for 30 cycles, and PCR products were size fractionated on 1.2% ethidium bromide/agarose gel and quantified under UV transillumination.

Transfection With Small Interference RNA

Small interference RNA (siRNA) duplexes were synthesized, desalted, and purified by Samchully Pharm. Co. Ltd. (Siheung, GyeongGi, Korea) as follows: LPA_1 5′-GGACUUGGAAUCACUGUUUUU-3′ (sense) and 5′-AAACAGUGAUUCCAAGUCCUU-3′ (antisense), LPA_2 5′-CCGCGAGUCUGUCCACUAUUU-3′ (sense) and 5′-AUAGUGGACAGACUCGCGGUU-3′ (antisense). Nonspecific control siRNA (D-001206-13-05) was purchased from Dharmacon, Inc. (Chicago, IL). For siRNA experiments, hADSCs were plated on 60-mm dishes at 70% confluence, and they were then transfected with siRNAs by using the Lipofectamine™ 2000 reagent according to manufacturer's instructions. Briefly, Lipofectamine™ 2000 reagent was incubated with serum-free medium for 10 min, and respective siRNAs were then added to the mixtures. After 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. After incubation for 6 h, the cells were cultured in growth medium for 24 h, and the expression levels of LPA_1, LPA_2, and GAPDH were then determined by RT-PCR analysis.

Western Blotting

hADSCs were incubated with serum-free α-MEM for 24 h, and the cells were treated with appropriate conditions, washed with ice-cold PBS, and then 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-glycerol phosphate, 1% Triton X-100, pH 7.4). Lysates were resolved by SDS–PAGE, transferred onto a nitrocellulose membrane, and then stained with 0.1% Ponceau S solution (Sigma–Aldrich) to ensure equal loading. After blocking with 5% nonfat milk, the membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence Western blotting system (ECL, Amersham Biosciences).

Rho Activation Assay

A commercially available pull-down assay kit (Rho activation assay kit, Upstate) was used to measure the effect of LPA on Rho activity in hADSCs. The cells were washed twice with α-MEM and incubated in fresh modified α-MEM without serum for 24 h. After treatment of the cells with LPA for the indicated times, the cells were lysed, and the activated Rho pull-down assay was performed according to the manufacturer's protocol. Protein concentration was determined prior to pull-down assay to equalize the total protein concentration of each treatment group.

Measurement of Intracellular Calcium Concentration

Spatially averaged photometric [Ca^{2+}] measurements from single cells were performed with the fluorescent Ca^{2+} indicator fluo-4-AM (Molecular Probes, Inc.). Thus, hADSCs cells grown on 32 mm dish were incubated with serum-free α-MEM for 24 h, loaded with 5 μM fluo-4-AM for 40 min at 37°C in buffer A [135 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 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+}. The fluo-4-AM-loaded hADSCs were treated with appropriate concentration of LPA or ascites. A Leica TCS-SP2 laser scanning confocal microscope (Leica Microsystems, Germany) was used to visualize Ca^{2+}-mediated fluorescence in the cells. Fluo-4 was excited with 488-nm line of an argon laser, and fluo-4 fluorescence was collected between 510 and 525 nm. Scanning was performed every 1 s 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.

RESULTS

Ascites Derived From Ovarian Cancer Patients Induce Migration of MSCs

To explore whether ascites from patients with ovarian cancer can induce migration of MSCs, malignant ascites were loaded into the lower chamber of the Boyden apparatus, and the migration of hADSCs from the upper chamber toward the lower chamber was measured. The malignant ascites dose-dependently increased the migration of hADSCs with a maximal stimulation at 1% concentration (Fig. 1). However, ascites from liver cirrhosis patients exhibited far less effects on the migration of hADSCs than the malignant ascites: The stimulatory effect of 1% ascites from liver cirrhosis patients on the migration was almost equivalent to that of 0.1% malignant ascites. These results indicate that malignant ascites from ovarian cancer patients potently stimulate the motility of hADSCs.

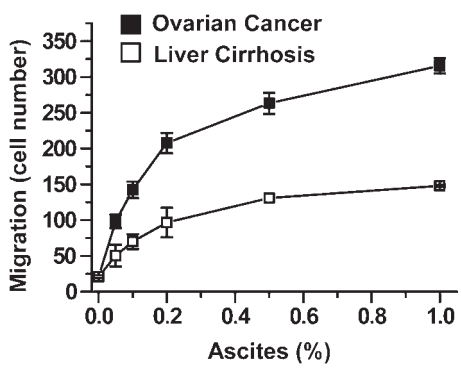

Fig. 1. Malignant ascites from ovarian cancer patients stimulate migration of hADSCs. hADSCs were treated for 12 h with indicated concentrations of ascites obtained from patients with ovarian cancer or liver cirrhosis, and the migration of the cells was determined by using a Boyden chamber apparatus. Data represent average values ± SD (n = 3).

LPA Induces Migration of MSCs

LPA has been suggested to be responsible for malignant ascites-stimulated motility of ovarian cancer cells [Bian et al., 2004; Ren et al., 2006]. To assess whether LPA is involved in the malignant ascites-induced migration of hADSCs, we examined the effect of LPA on the migration of hADSCs. As shown in Figure 2A, LPA dose-dependently increased the migration of hADSCs with a maximal stimulation at 1 μM concentration. Also, LPA time-dependently stimulated the migration of hADSCs, and the LPA-induced migration maximally occurred after exposure of hADSCs for 12 h (Fig. 2B).

To further explore the role of LPA in the malignant ascites-induced migration, we examined the effects of Ki16425, an antagonist specific for LPA_1 and LPA_3, on the migration stimulated by either malignant ascites or LPA. As shown in Figure 2C, Ki16425 completely inhibited the migration of hADSCs which was stimulated by either malignant ascites or LPA. In a previous study, we reported that PDGF-BB is a potent motility factor for hADSCs [Kang et al., 2005]. Therefore, we examined the effects of Ki16425 on the motility of hADSCs stimulated by PDGF-BB. Ki16425 had no significant impact on the PDGF-BB-induced migration of hADSCs, suggesting that LPA is a component responsible for the malignant ascites-induced migration of hADSCs.

To examine the directional component of migration, we examined LPA-induced migration of hADSCs by using a checkerboard analysis. As shown in Figure 2D, LPA was required in the bottom chamber for maximum migration of the cells to the bottom of the filter. The presence of LPA in the top, however, stimulated some cell migration to the bottom of the filter, albeit there was far less migration than with LPA in the bottom chamber. LPA enhanced migration in the absence of a gradient (equal concentrations in the top and bottom), suggesting that there is a large chemokinetic component for the migration. Not only LPA but also malignant ascites stimulated the migration even when same concentration of ascites was present in both chambers, suggesting that LPA in the malignant ascites stimulates random migration (chemokinesis) as well as directional migration (chemotaxis) of hADSCs.

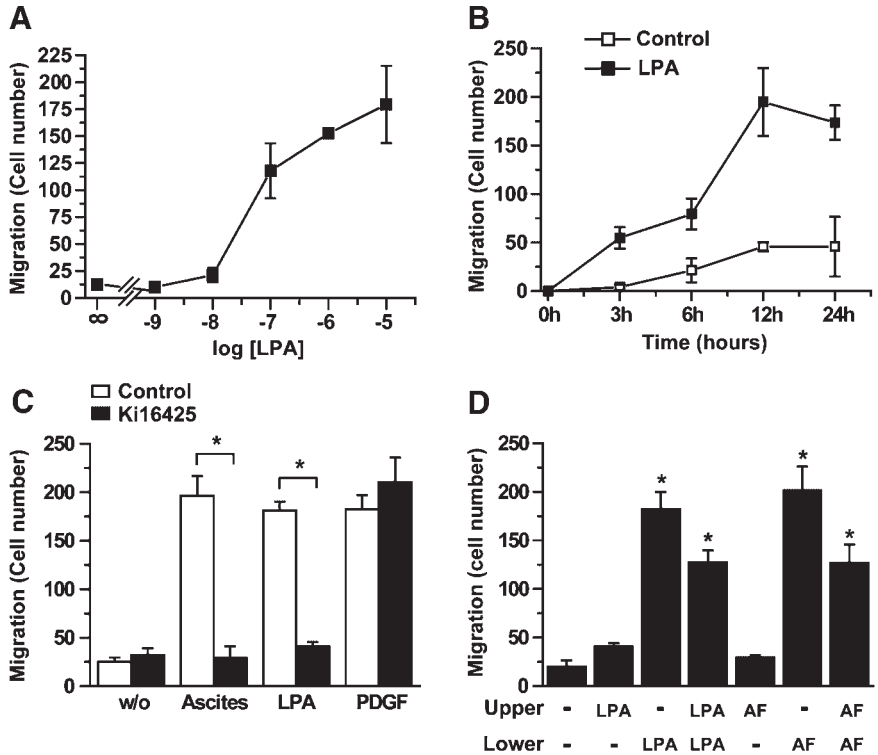

Fig. 2. LPA is responsible for the malignant ascites-induced migration of MSCs. **A**: hADSCs were treated with indicated concentrations of 1-oleoyl-LPA for 12 h. **B**: hADSCs were exposed to vehicles or 1 μM 1-oleoyl-LPA for indicated time periods. **C**: hADSCs were pretreated with vehicles or 10 μM Ki16425 for 15 min, and then treated with 1 μM 1-oleoyl-LPA, 1% malignant ascites, or 10 ng/ml PDGF-BB for 12 h. **D**: Checkerboard analyses of the cell migration induced by LPA and malignant ascites. 0.1 μM 1-oleoyl-LPA or 1% malignant ascites (AF) was placed in either the bottom, top, or both chambers of the Transwells as noted, and cells were then allowed to migrate for 12 h. The migration of the cells was determined by using a Boyden chamber apparatus. Data represent average values ± SD (n = 3). Asterisk (*) indicates $P < 0.05$ by Student's *t*-test.

LPA_1-Dependent Pathway is Involved in the LPA-Induced Migration of hADSCs

We recently reported that LPA_1 and LPA_2 are predominantly expressed isoforms of LPA receptor in hADSCs [Jeon et al., 2006]. To explore the involvement of LPA_1 and LPA_2 in the LPA-induced migration of hADSCs, we examined the effects of siRNA-mediated depletion of LPA receptors on the migration which was stimulated by either LPA or malignant ascites. As shown in Figure 3A,B, the mRNA levels of LPA_1 or LPA_2 in hADSCs were specifically down-regulated by transfection with siRNAs specific for LPA_1 or LPA_2, respectively. We next examined the effects of knockdown of LPA_1 or LPA_2 expression on the migration induced by LPA or malignant ascites. The motility stimulated by either LPA or malignant ascites was markedly attenuated by depletion of the endogenous LPA_1 (Fig. 3C), whereas depletion of the endogenous LPA_2 expression had no significant impacts on the migration of hADSCs. In contrast, PDGF-induced migration was not affected by depletion of endogenous LPA_1 or LPA_2. These results clearly indicate that LPA plays a key role in the malignant ascites-stimulated migration by activating LPA_1, but not LPA_2.

Lipid Components are Responsible for the Malignant Ascites-Induced Migration

Ascites from ovarian cancer patients have been shown to contain elevated level of lysophospholipase D, which is involved in the production of LPA from lysophosphatidylcholine [Tokumura et al., 2007]. Moreover, it has been reported that phospholipase A_2 is responsible for the production of LPA from peritoneal mesothelial cells [Ren et al., 2006]. To test whether the LPA-producing enzymes are responsible for the malignant ascites-induced migration of hADSCs, malignant ascites and

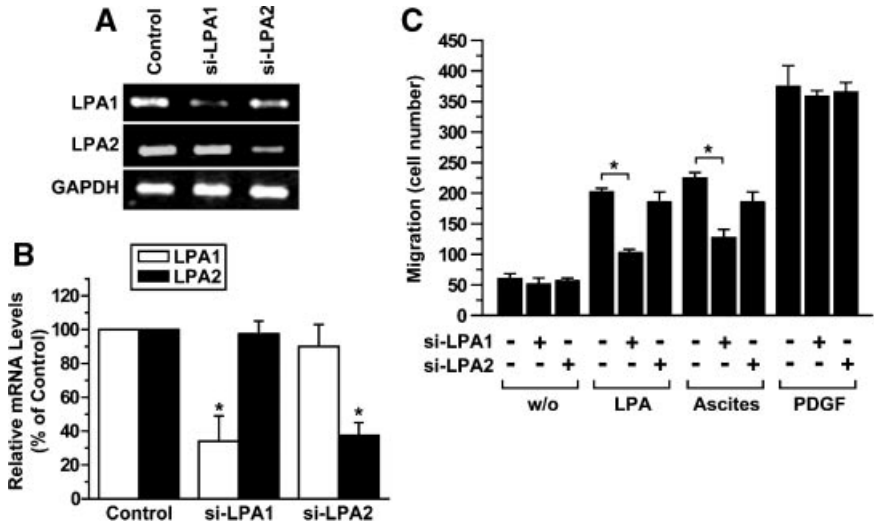

Fig. 3. Role of LPA_1 in the malignant ascites-induced migration of hADSCs. **A**: hADSCs were transfected with control siRNA or siRNAs specific for LPA_1 or LPA_2, respectively. The mRNA levels of LPA_1, LPA_2, and GAPDH were determined by semi-quantitative RT-PCR. **B**: The densities of LPA_1, LPA_2, and GAPDH were quantified from three independent experiments, and the expression levels of LPA_1 and LPA_2 were normalized to GAPDH levels. The data are presented as a percentage of control. Asterisk (*) indicates $P < 0.05$ versus control value by Student's *t*-test. **C**: The siRNA-transfected hADSCs were treated with vehicles, 1 μM 1-oleoyl-LPA, 1% malignant ascites, or 10 ng/ml PDGF-BB, respectively, for 12 h, and then the migration of the cells was determined. Data represent average values ± SD (n = 3). Asterisk (*) indicates $P < 0.05$ by Student's *t*-test.

LPA were heated at 100°C for 5 min, because many lipids, but not proteins, are thermostable. As shown in Figure 4A, the stimulatory effects of ascites and LPA on the migration of hADSCs were not affected by heating, suggesting that lipid components, but not protein factors, are involved in the ascites-stimulated migration. To support the involvement of lipid component in the ascites-induced migration, we next examined the effect of lipid fractions, which were extracted from malignant ascites with 1-butanol, on the migration. As shown in Figure 4B, the stimulatory effects of ascites and LPA on the migration were largely maintained in the 1-butanol fractions, although the malignant ascites-stimulated migration was not completely recovered by the lipid fractions derived from ascites. These results support the notion that lipid components are responsible for the malignant ascites-induced migration.

To determine the efficiency of LPA recovery during lipid extraction and heating, [^{3}H]1-oleoyl-LPA was added to malignant ascites or LPA, and the percentage of recovery of radiolabelled LPA was then measured after lipid extraction or heating. As shown in Figure 4C, most of the radioactivities spiked in ascites or LPA were recovered after lipid extraction or heat treatment, albeit the [^{3}H]1-oleoyl-LPA, which had been included in the malignant ascites, was not completely recovered after 1-butanol extraction. Taken together, these results suggest that LPA, but not LPA-producing enzymes, is responsible for the migration induced by malignant ascites.

LPA Induces Migration of hADSCs Through MEK-ERK- and RhoA-Rho Kinase-Dependent Pathways

It has been reported that G_i, a pertussis toxin-sensitive G protein, is involved in the LPA-induced migration of ovarian cancer cells [Stahle et al., 2003; Bian et al., 2004; Yamada et al., 2004]. To assess whether the LPA-stimulated migration was mediated by G_i protein, we examined the effect of PTX on the migration. As shown in Figure 5A, PTX treatment completely abrogated the LPA-induced migration of hADSCs. These results indicate that LPA stimulates the migration of hADSCs through LPA_1-G_i-dependent pathway.

To explore the involvement of G_i-ERK-dependent pathway in the LPA-stimulated migration of hADSCs, we examined the effects of the MEK inhibitor U0126 and PTX on the phosphorylation of ERK in response to LPA. As shown in Figure 5B, LPA induced phosphorylation of ERK in hADSCs, and pretreatment of the cells with U0126 abrogated the LPA-induced phosphorylation of ERK. Moreover,

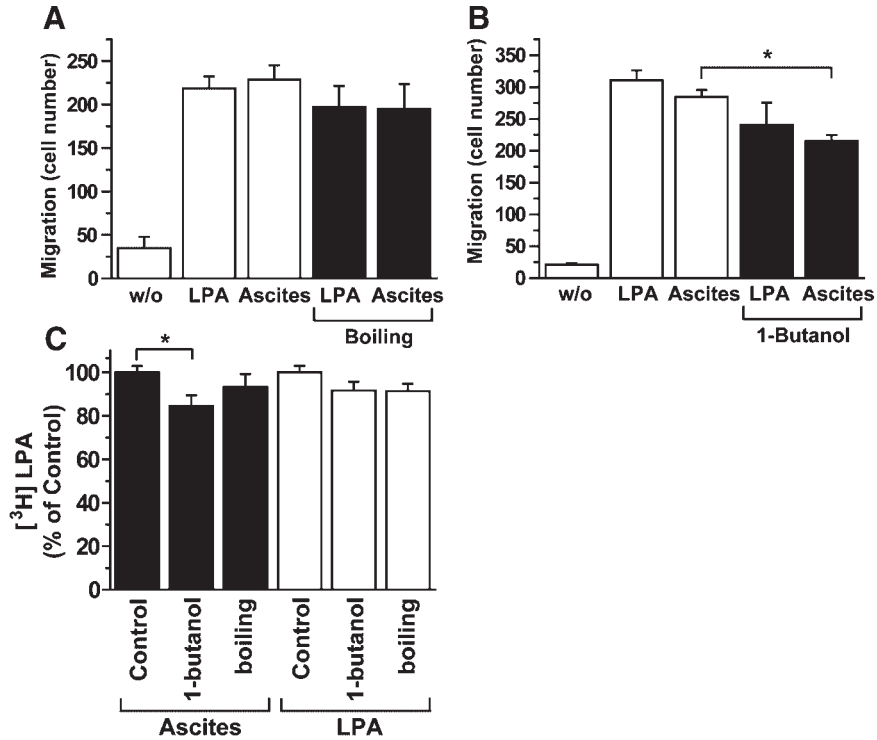

Fig. 4. Lipid components are involved in the malignant ascites-induced migration. **A**: To denature protein factors, the malignant ascites from ovarian cancer patients or 1-oleoyl-LPA were heated at 100°C for 5 min. hADSCs were then treated with vehicles, 1% ovarian cancer ascites, 1 μM 1-oleoyl-LPA, or heated fractions from ascites or 1-oleoyl-LPA for 12 h, and the migration of hADSCs was determined. **B**: Lipid components were extracted from ovarian cancer ascites or 1-oleoyl-LPA with 1-butanol as described under Materials and Methods Section. hADSCs were treated with vehicles, 1% ovarian cancer ascites, 1 μM 1-oleoyl-LPA, or 1-butanol fractions from ascites or 1-oleoyl-LPA for 12 h, and the migration of hADSCs was determined. **C**: 1×10^6 cpm [oleoyl-9,10-^{3}H]LPA was added into ascites and 1-oleoyl-LPA, and the radioactivities of the mock-treated controls, 1-butanol or heated fractions were determined by using a Tri-Carb liquid scintillation analyzer. Data represent average values ± SD ($n = 3$). Asterisk (*) indicates $P < 0.05$ by Student's *t*-test.

PTX attenuated the LPA-induced phosphorylation of ERK, suggesting an involvement of G_i-dependent pathway in the LPA-induced activation of ERK. To prove the involvement of ERK in the LPA-induced migration, we next examined the effect of LPA on the migration of hADSCs in the absence or presence of U0126. The LPA-induced migration of hADSCs was markedly suppressed by pretreatment of the cells with U0126 (Fig. 5C), suggesting that G_i-ERK pathway plays a pivotal role in the LPA-induced migration of hADSCs.

Activation of RhoA is associated with cell body contraction and rear end retraction [Raftopoulou and Hall, 2004]. To assess the involvement of RhoA-Rho kinase-dependent pathway in the LPA-stimulated migration, we determined the effects of LPA on the activities of RhoA by pull-down assays using RBD domain of Rhotekin, which selectively binds to GTP-bound active form of RhoA. LPA increased the amount of GTP-bound active form of RhoA in hADSCs (Fig. 5D). Therefore, we next examined the effect of Y27632, a Rho kinase inhibitor, on the migration induced by LPA. As shown in Figure 5E, Y27632 markedly attenuated the LPA-induced migration of hADSCs, suggesting that RhoA-Rho kinase-dependent pathway plays a pivotal role in the LPA-induced migration of hADSCs. Y27632 showed no significant impact on the LPA-induced activation of ERK, and U0126 did not affect the LPA-induced stimulation of RhoA (data not shown), implying that LPA induces migration through two independent pathways, that is, the MEK-ERK- and RhoA-Rho kinase-dependent pathways.

Ascites Derived From Ovarian Cancer Patients Increase Intracellular Calcium Concentration in Human Adipose Tissue-Derived Mesenchymal Stem Cells

To consolidate the notion that LPA is an active component of ascites, we examined the effects of malignant ascites and LPA on the

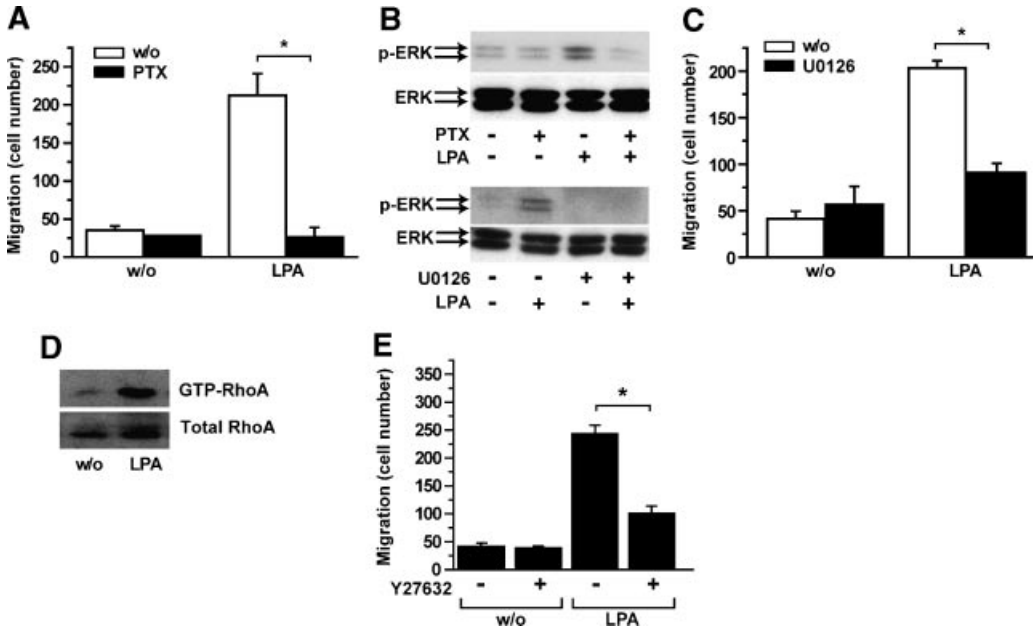

Fig. 5. Role of G_i-ERK- and RhoA-Rho kinase-dependent pathways in the LPA-induced migration. **A**: hADSCs were pretreated with serum-free medium, containing vehicles or 100 ng/ml PTX, for 24 h, and then treated with 1 μM 1-oleoyl-LPA for 12 h for determination of migration. **B**: hADSCs were pretreated with vehicles or 100 ng/ml PTX for 24 h, or 10 μM U0126 for 15 min as indicated, and then exposed to 1 μM 1-oleoyl-LPA for 10 min, and the phosphorylation and expression levels of ERK were determined by Western blotting. **C**: hADSCs were pretreated with 10 μM U0126 for 15 min, and then treated with 1 μM 1-oleoyl-LPA for 12 h for determination of migration. **D**: hADSCs were treated with vehicle or 1 μM LPA for 2 min at 37°C prior to assay for RhoA activation. Activated GTP-loaded RhoA was precipitated with a GST-fusion protein of the Rho binding domain of rhotekin. The amounts of RhoA in the precipitated fractions (**upper panel**) and whole cell lysates (**lower panel**) were determined by Western blotting with anti-RhoA antibody. **E**: hADSCs were pretreated with 10 μM Y27632 for 15 min, and then treated with 1 μM 1-oleoyl-LPA for 12 h for determination of migration. Data represent average values ± SD (n = 3). Asterisk (*) indicates $P < 0.05$ by Student's *t*-test.

intracellular concentration of calcium ($[Ca^{2+}]_i$). LPA rapidly increased $[Ca^{2+}]_i$ in hADSCs, and pretreatment of hADSCs with Ki16425 completely inhibited the LPA-induced elevation of $[Ca^{2+}]_i$ (Fig. 6A–C). Furthermore, malignant ascites also increased $[Ca^{2+}]_i$ and pretreatment with Ki16425 abrogated the increase of $[Ca^{2+}]_i$ stimulated by malignant ascites (Fig. 6D,E). These results indicate that LPA plays a key role in the malignant ascites-induced cellular responses, such as migration and elevation of $[Ca^{2+}]_i$.

DISCUSSION

Ovarian cancer is a highly metastatic tumor characterized by widespread intraperitoneal dissemination and ascites formation. In the current study, we demonstrated that LPA is responsible for the enhanced migration of hADSCs in response to malignant ascites from ovarian cancer patients through activation of LPA_1, as evidenced by the following findings: First, the migration induced by malignant ascites or LPA, but not by PDGF-BB, was completely abrogated by pretreatment of hADSCs with Ki16425, an antagonist for LPA_1 and LPA_3. Second, siRNA-mediated knockdown of endogenous LPA_1, but not LPA_2, attenuated the LPA-induced migration of hADSCs. Consistent with the present study, LPA_1 has been implicated in LPA-induced cell migration of different types of cancer cells [Shida et al., 2003; Van Leeuwen et al., 2003; Hama et al., 2004; Yamada et al., 2004]: Colon cancer cells that express LPA_1, but not LPA_2 or LPA_3, migrated after stimulation with LPA, whereas cells that express LPA_2 and LPA_3, but not LPA_1, did not migrate in response to LPA stimulation [Shida et al., 2003]. Furthermore, malignant ascites from pancreatic cancer patients induced migration of pancreatic cancer cells and pretreatment of the cells with Ki16425 or siRNA-mediated knockdown of endogenous LPA_1 abrogated the malignant ascites-stimulated migration. Together with the fact that LPA_1 is the most

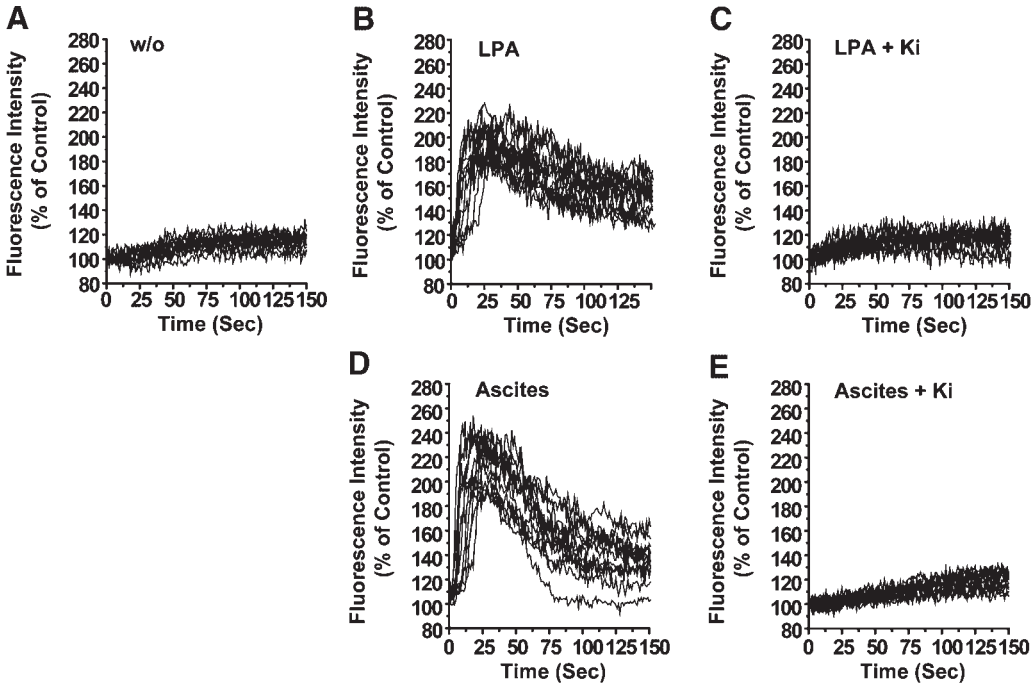

Fig. 6. LPA in malignant ascites induces elevation of $[Ca^{2+}]_i$ in hADSCs. Serum-starved hADSCs were loaded with 5 μM fluo-4-AM for 40 min at 37°C in the absence or presence of 10 μM Ki16425 (Ki), and then exposed to 1% malignant ascites or 1 μM 1-oleoyl-LPA. Ca^{2+}-dependent fluorescence was measured every second for indicated time periods, and fluorescence intensities of more than 20 different cells from time-lapse images were quantified over time. Representative data from three similar experiments are shown, and results are expressed as percentage of the control (0 s).

abundant isoform of LPA receptors expressed in hADSCs [Jeon et al., 2006], these results indicate that LPA in the malignant ascites stimulates migration of human MSCs through activation of LPA_1.

It has been reported that the LPA receptors are able to couple to many signaling pathways via G_i, G_q, and $G_{12/13}$ proteins [Mills and Moolenaar, 2003]. In the present study, we demonstrated that LPA induced activation of ERK through PTX-sensitive pathway, and the MEK inhibitor U0126 abrogated the LPA-induced migration. G_i protein has been shown to be involved in the LPA-induced migration of cancer cells through induction of ERK activation [Stahle et al., 2003; Bian et al., 2004]. Indeed, ERK has been implicated in the migration of many cell types: ERK pathway inhibitors, PD98059 and U0126, abrogated the migration of diverse cell types in response to extracellular matrix proteins, growth factors, and chemoattractants [Huang et al., 2004], and overexpression of constitutively active MEK1 mutant promoted the migration of FG carcinoma cells, whereas depletion of endogenous ERK by antisense oligonucleotides inhibited the cell migration [Klemke et al., 1997]. It has been demonstrated that ERK-mediated phosphorylation of various substrates, such as MLCK, paxillin, focal adhesion kinase, and calpain, is responsible for the ERK-dependent migration [Huang et al., 2004]. Taken together, these results suggest that LPA in the malignant ascites stimulates the migration of human MSCs through activation of LPA_1-$G_{i/o}$-ERK-dependent pathway.

Activation of RhoA-Rho kinase pathway has been shown to play a key role in the LPA-induced migration in a variety of cell types [Ai et al., 2001; Tangkijvanich et al., 2003; Bian et al., 2006]. RhoA-Rho kinase in migrating cells is associated with focal adhesion assembly and cell contractility, and is responsible for cell body contraction and rear end retraction [Raftopoulou and Hall, 2004]. In the present study, we showed that LPA induced the migration of hADSCs, and that Y27632-mediated inhibition of Rho kinase attenuated the LPA-induced migration. On the other hand, it has recently been reported that LPA itself had no significant impact on the migration of hBMSCs, and that pretreatment of the cells with LPA inhibited plasma-induced migration through activation of RhoA [Jaganathan et al., 2007],

whereas pharmacological inhibition of RhoA or Rho kinase potentiated the migration of hBMSCs toward plasma and LPA, suggesting that RhoA-Rho kinase pathway has an inhibitory effect on the migration of MSCs. A plausible explanation for the discrepancy could be that different sources of MSCs might be accountable for the contradictory effects of LPA. However, we found that LPA stimulated the migration of hBMSCs as well as hADSCs through Rho kinase-dependent mechanism (data not shown), suggesting that LPA stimulation of the migration is not restricted only to hADSCs. The underlying molecular mechanism associated with the differential effects of LPA and RhoA activation on cell migration should further be explored.

MSCs have been shown to populate into the sites of injury, inflammation, or tumor in vivo [Ji et al., 2004; Satake et al., 2004; Studeny et al., 2004; Fukuda and Fujita, 2005; Nakamizo et al., 2005]. We demonstrated in the present study that LPA in malignant ascites stimulated the migration of MSCs. Furthermore, we recently showed that LPA induced proliferation of hADSCs [Jeon et al., 2006], implying that LPA is a stimulatory factor for the mitosis and motility of MSCs. Indeed, LPA has been shown to play a key role in initiation or progression of a variety of cancers, including ovary, prostate, breast, and melanoma, by increasing motility and invasiveness of cancer cells [Mills and Moolenaar, 2003]. Furthermore, malignant ascites are a potent stimulator for mitosis and motility of ovarian cancer cells [Mills et al., 1990; Kim et al., 2006], thus suggesting that LPA may play a pivotal role in recruitment of MSCs to tumor microenvironment. Further studies are needed to demonstrate clearly whether LPA is involved in homing of MSCs into tumors in vivo.

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