Role of lipoprotein-associated lysophospholipids in migratory activity of coronary artery smooth muscle cells

Alatangaole Damirin,1,2 Hideaki Tomura,1 Mayumi Komachi,1 Jin-Peng Liu,1 Chihiro Mogi,1 Masayuki Toho,1 Ju-Qiang Wang,1 Takao Kimura,1 Atsushi Kuwabara,1 Yuji Yamazaki,3 Hideo Ohta,3 Doon-Soon Im,4 Koichi Sato,1 and Fumikazu Okajima1

1Laboratory of Signal Transduction, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan; 2Department of Biochemistry and Molecular Biology, College of Life Sciences, Inner Mongolia University, Huhhot, Inner Mongolia, China; 3Research Laboratory, Kirin Brewery, Miyahara, Takasaki, Japan; and 4Laboratory of Pharmacology, College of Pharmacy, Pusan National University, Busan, Republic of Korea

Submitted 10 August 2006; accepted in final form 17 January 2007

Atherosclerosis is a disease marked by the progressive accumulation of cholesterol and other substances in the arterial walls, eventually leading to vessel narrowing and obstruction. Low-density lipoprotein (LDL) plays a central role in this process, as it is the primary transporter of cholesterol from the liver to peripheral tissues. HDL, on the other hand, is involved in the reverse lipid transport, removing excess cholesterol from the arterial walls. This balance is critical for cardiovascular health, and disruptions in these processes can lead to the development of atherosclerosis.

In this study, the authors investigated the role of lipoprotein-associated lysophospholipids in the migratory activity of coronary artery smooth muscle cells (CASMCs). They found that lysophosphatidic acid (LPA), a component of LDL, can induce migration of CASMCs, mimicking the LDL action. This effect is mediated through LPA receptors, and the migration can be inhibited by LPA antagonists or S1P2 receptor-specific siRNA.

Moreover, the authors showed that the S1P component of HDL mediates the inhibition of LDL-induced migration, suggesting a role for HDL as a protective factor. These findings highlight the complex interactions between lipoproteins and their receptors in the regulation of vascular smooth muscle cell behavior, which is crucial for understanding the pathogenesis of atherosclerosis and the development of strategies to prevent or treat this disease.
intima formation was achieved by LPA as well as by oxLDL (54). Thus LPA is also listed as a potential mediator of LDL- and oxLDL-induced migration of SMCs.

In the present study, we examined the effects of plasma lipoproteins on the migration of human coronary artery SMC (CASMC), and we found that LPA mediates the LDL-stimulated migration of cells through LPA1 receptors. On the other hand, HDL alone hardly stimulated migration activity but markedly inhibited PDGF-induced migration through S1P and S1P2 receptors. Our results suggest that the balance of not only the content of LPA and S1P in lipoproteins but also the signaling pathway between LPA1 receptors and S1P2 receptors is an important determinant of whether the lipoprotein is a positive or negative regulator of SMC migration.

MATERIALS AND METHODS

**Materials.** 1-Oleoyl-α-glycero-3-phosphate (lysophosphatic acid; LPA), 1-α-lysophosphatidylcholine palmitoyl (LPC, C16:0), 1-α-lysophosphatidylcholine oleoyl (LPC, C18:1), and S1P were purchased from Cayman Chemical (Ann Arbor, MI); fatty acid-free bovine serum albumin (BSA) was from Calbiochem-Novabiochem (San Diego, CA); dioctyl glycerol pyrophosphate (DGPP 8:0), PGPC, and POVPC were from Avanti Polar-lipids (Alabaster, AL); PTX was from List Biological Laboratories (Campbell, CA); PAF was from Sigma-Aldrich (St. Louis, MO); and monoglyceride lipase (MG lipase) was from Asahi Kasei (Shizuoka, Japan). Ki-16425 [3-(4-[4-[(1-(2-chlorophenyl)ethoxy)carbonyl amino]-3-methyl-5-isoxazolyl] benzylsulfanyl) propanoic acid] was synthesized by Kirin Brewery (Takasaki, Japan), and VPC-12249 was a generous gift from Prof. Kevin R. Lynch (University of Virginia School of Medicine). Plasma lipoproteins were prepared by density gradient centrifugation; LDL (1.019–1.063 g/ml) and HDL (1.063–1.21 g/ml) were separated by sequential ultracentrifugation from freshly isolated plasma of normal healthy volunteers as described previously (13, 27). In the present study, we used plasma samples from eight healthy male volunteers at ages of 35 to 47 yr. Informed consent was obtained from each person for the use of samples. The lipoprotein fractions were then extensively dialyzed against mixture of 150 mM NaCl (9 volume) and PBS (1 volume) containing 0.1 mM EDTA and stored on ice. The lipoprotein preparations were used within 2 wk. This procedure is referred to LDL (or native LDL) in the present study. We performed oxidation by three different methods against extensively dialyzed LDL in the absence of EDTA; Cu2+-oxidized LDL was prepared by treatment with 10 μM Cu2+ at 37°C for 24 h (24), Fe2+-oxidized LDL was by dialysis with 1 μM Fe2+ at 4°C for 96 h (12), and spontaneously oxidized LDL was by incubation at 37°C for 48 h under gentle agitation (44). The extent of lipid peroxidation was measured as thiobarbituric acid-reacting substances (TBARS) (24).

**Cell culture.** Human coronary artery smooth muscle cells (CASMCs) were purchased from Cambrex (East Rutherford, NJ) and cultured as described previously (7). The cells with 7–10 passages were used. We confirmed that these cells were positive to the staining with α-actin, a smooth muscle cell marker. Unless otherwise specified, 24 h before experiments, the medium was replaced with serum-free Dulbecco’s modified Eagle’s medium (DMEM) containing 0.1% (wt/vol) BSA (fraction V). Where indicated, pertussis toxin (PTX) (100 ng/ml) was added to the culture medium 24 h before experiments.

**Cell migration assay.** The migration experiment was performed using a blind Boyden chamber apparatus, as previously described (18, 46). In brief, CASMCs were harvested with 0.05% trypsin containing 0.02% EDTA, washed once, and resuspended with DMEM containing 0.1% BSA. The cells (1 × 10⁶ cells in 100 μl) were loaded into the upper chamber, and test agents were placed in the lower chamber, unless otherwise specified. When the effects of LPA antagonists were examined, the cells were preincubated for 10 min with antagonists before being loaded. The number of cells that had migrated for 4 h to the lower surface was determined by counting the cells in four places under a microscope at ×400 magnification.

**Quantitative RT-PCR analysis.** Total RNA was isolated using Tri-Reagent (Sigma-Aldrich) according to the instructions from the manufacturer as described previously (7, 52). To evaluate the expression level of mRNAs for LPA receptor subtypes (LPA1, LPA2, LPA3, and LPA4/GPR23), S1P receptor subtypes (S1P1, S1P2, S1P3, and S1P3), and α-subunits of G proteins (Goα12, and Goα13), quantitative RT-PCR was performed using real-time TaqMan technology with a Sequence Detection System model 7700 (Applied Biosystems, Foster City, CA) as described previously (7). The specific probes for LPA receptors, S1P receptors, and G proteins were obtained from TaqMan Gene Expression Assays (Applied Biosystems). The ID number of the products is Hs00173500 for LPA1, Hs00173704 for LPA3, Hs00173857 for LPA1, Hs00271072 for LPA4/GPR23, Hs00173499 for S1P1, Hs00244677 for S1P2, Hs00245464 for S1P3, Hs00269446 for S1P2, Hs00258220 for S1P3, Hs00178099 for Goα12, Hs00183573 for Goα13, and Hs99999905 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression level of the target mRNA was normalized to the relative ratio of the expression of GAPDH mRNA. Each RT-PCR assay was performed at least three times, and the results are expressed as means ± SE.

**Transfection of small interfering RNA.** CASMCs were plated on 12 multiplates at 2 × 10⁶ cells/well. Sixteen hours later, small interfering RNAs (siRNAs, 50 nM) were introduced into cells using RNAiFect reagent (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. The cells were further cultured for 24 h. The mRNA levels for LPA receptor 1, S1P1 receptor, S1P2 receptor, Goα12, and Goα13 were measured using real-time TaqMan technology. Migration response was performed 24 h after serum starvation as described. The nonsilencing siRNA was obtained from Qiagen. The siRNAs targeted for S1P1 (M-003952-00), Goα12 (M-008435-00), and Goα13 (M-009484-00) were obtained from Dharmaco (Lafayette, CO). As for siRNA specific to LPA1 receptors, we used 21-mer oligonucleotide pairs (LPA1-228, 5’-r(CCGCCCGCUUCAUUUUCUdT)(dT)-3’ and 5’-r(AAGGAAAUAUAAGCCGCGGId)(dT)-3’), as described in the previous paper (24).

**Evaluation of contents of S1P and LPA.** S1P and LPA were selectively extracted as alkaline-soluble lipids as described previously (26). By this procedure, major lipid components, such as phosphatidylcholine, sphingomyelin, and other neutral lipids, can be removed. The S1P content was evaluated based on the ability of S1P to displace labeled S1P on S1P1 receptor (26) or to stimulate S1P3 receptor-mediated inositol phosphate production as described previously (27). A similar value was obtained by either method. Evaluation of LPA-equivalent activity was performed by a sensitive and specific bioassay based on the ability of LPA to inhibit CAMP accumulation in LPA1-expressing RHT7777 cells as described previously (52). The LPA-equivalent activity in the test sample was expressed as an LPA C18:1-equivalent level. Please note that this bioassay is unsuitable for a quantitative measurement of LPA; however, it excludes LPA species that cannot stimulate LPA1 receptors. Thus the bioassay is superior to know “active” LPA-equivalent content to stimulate LPA1 receptors.

**Data presentation.** All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as the means ± SE of more than six values of two or three different batches of cells unless otherwise stated. Statistical significance was assessed by the Student’s t-test; values were considered significant at P < 0.05.

**RESULTS**

LDL may stimulate migration of CASMCs through lipoprotein-associated LPA. We first examined the effect of oxidation on the migration activity of LDL in CASMCs. We prepared three kinds of oxLDL, i.e., Cu2+-oxidized LDL, Fe2+-oxidized LDL.
LDL, and spontaneously oxidized LDL, and we compared the migration activities of these oxLDLs with that of native LDL. As shown in Fig. 1A, native LDL induced a significant stimulation of migration. However, the activity of oxLDL depended on the preparation procedure; Fe^{2+}-oxidized LDL and spontaneously oxidized LDL showed slightly higher migration activity than native LDL did, but Cu^{2+}-oxidized LDL did not exert any significant activity. The lack of migration activity of Cu^{2+}-oxidized LDL was associated with a significant acceleration of lipid peroxidation, as suggested by the value of TBARS; the value was 33.3 nmol malondialdehyde (MDA)/mg protein. On the other hand, we could not detect any appreciable formation of TBARS for native LDL (2.26 nmol MDA/mg protein), Fe^{2+}-oxidized LDL (3.43 nmol MDA/mg protein), or spontaneously oxidized LDL (2.05 nmol MDA/mg protein), values of which were within the range of native LDL (<5 nmol MDA/mg protein), as defined by another group (24).

Since native LDL induced a significant migration response to an extent similar to that of minimally modified LDL, including Fe^{2+}-oxidized LDL and spontaneously oxidized LDL, we used native LDL (referred to as LDL) without further troublesome treatment in experiments.

As shown in Fig. 1B, LDL induced a bell-shaped migration response with a peak at 30 μg/ml. On the other hand, HDL only slightly stimulated cell migration. The migration response to LDL was completely inhibited by PTX treatment (Fig. 1B). LPA also stimulated migration (Fig. 1C), and PTX inhibited the migration response to LPA but not to PDGF (Fig. 1C). These results suggest that LDL induces cell migration through G protein-coupled receptors.

The effects on cell migration of bioactive lipids, which are supposed to be present in the lipoprotein, are shown in Fig. 1D. We employed LPA, S1P, PAF, PGPC, POVPC, and two kinds of LPC (C16:0 and C18:1) with different fatty acid substituents.
in the present study. CASMCs exhibited a clear bell-shaped migration response to LPA; however, other lipids only slightly affected the migration response compared with LPA. For example, 100 nM PGPC and LPC (C18:1) significantly, but slightly, stimulated migration (Fig. 1D), and PAF, P0VPC, and LPC (C16:0) were ineffective at concentrations from 1 nM to 10 μM (data not shown). S1P rather inhibited cell migration. The possible involvement of LPA in the LDL action was examined by treatment of an LDL preparation with MG lipase, which hydrolyzes monoglycerides such as LPA (52). The enzyme treatment markedly inhibited the migration response to LDL and LPA but not to PDGF (Fig. 1E). These results suggest that LDL-associated LPA may be involved in lipoprotein-induced cell migration.

LPA₁ receptors are involved in the LDL-induced migration of CASMCs. To characterize the receptors involved in the LDL action, we first used LPA receptor antagonists with different pharmacological specificities: Ki-16425 had a preference for LPA₁ and LPA₃ over LPA₂ (31), and VPC-12249 showed a preference for LPA₁ and LPA₃ but not for LPA₂ (10); however, DGPP 8:0 showed a preference only for LPA₃ (8). The migration response to LDL was inhibited by as low as 0.01 μM Ki-16425 and was almost completely inhibited by the LPA antagonist at 1 μM (Fig. 2A). The lipid molecules, including PGPC and LPC (C18:1), which exerted small but significant migration responses, as shown in Fig. 1D, were insensitive to even 10 μM Ki-16425 (data not shown), further excluding the possible involvement of these lipids in the LDL action. The LDL- and LPA-induced migration was inhibited by Ki-16425 and VPC-12249, antagonists for LPA₁ and LPA₃, but not by DGPP 8:0, an LPA₃-specific antagonist (Fig. 2B). None of these LPA receptor antagonists affected PDGF-induced migration (Fig. 2B), suggesting the specificity of the antagonists and the involvement of LPA₁ receptors in the LDL action.

As shown in Fig. 3A, we examined the expression patterns of LPA and S1P receptor mRNA in CASMCs. Among the LPA receptor subtypes, LPA₁ receptor mRNA is expressed at an extremely high level, supporting the possible role of LPA₁ receptors in the LDL action. In the second line of experiments, siRNAs against LPA₁ receptors were transfected into CASMCs to decrease the expression of the receptors. The siRNA transfection resulted in a marked reduction in the expression of LPA₁ receptor mRNA without a significant change in the expression of S1P₂ receptor mRNA (Fig. 3B), which was accompanied by a remarkable inhibition of migration response to LDL and LPA but not to PDGF (Fig. 3C). These results strongly suggest that LPA₁ receptors play a critical role in the migration response to LDL and LPA in CASMCs.

HDL inhibits the migration of CASMCs by the S1P component through S1P₂ receptors. As shown in Fig. 4, the LPA content is higher in LDL than in HDL, supporting the results of Fig. 1B in which LDL expressed higher activity on cell migration than HDL did. On the other hand, consistent with our recent study of rat aortic SMCs (48), HDL clearly inhibited the migration response to PDGF in human CASMCs (Fig. 5A). Under the experimental conditions, LDL hardly affected the response, probably due to the maximal activity of PDGF (Fig. 5A). Thus LDL exerts a stimulatory action, whereas HDL exerts an inhibitory action on the migration of CASMCs. The inhibitory activity of HDL may be explained by S1P; HDL contains roughly four times more S1P than LDL does (Fig. 4), and S1P has been shown to inhibit the migration of SMCs (5, 38, 46, 48). Indeed, S1P inhibited the LPA- and PDGF-induced migration of CASMCs (Fig. 5B).

The role of S1P in the HDL-induced action was further supported by the finding that siRNA specific to the S1P₂ receptor reversed the inhibitory action of HDL and S1P on the PDGF-induced migration (Fig. 6B), which was associated with a specific inhibition of the mRNA expression of S1P₂ receptors (Fig. 6A).
Role of G13 proteins in the S1P2 receptor-mediated inhibition of the migration response to S1P and HDL. The role of G13 proteins in the S1P2 receptor-mediated inhibition of cell migration has been characterized in S1P2 receptor-expressing Chinese hamster ovary cells in which both G12 and G13 proteins were suggested to be involved in the S1P2-mediated migration response (45). To verify the role of G12 and G13 proteins in vascular SMC migration, we used siRNAs specific to α-subunits of G12 and G13 proteins. As shown in Fig. 7A, Gα12 and Gα13 siRNA specifically and effectively inhibited the mRNA expression of the respective α-subunits of G proteins. Under this condition, the S1P- and HDL-induced inhibition of PDGF-induced migration was clearly reversed by Gα13 siRNA (Fig. 7B). However, we failed to prove the involvement of G12 proteins in S1P signaling. Thus S1P and HDL significantly inhibited PDGF-induced migration in the cells treated with Gα12 siRNA, although the PDGF-induced migration response was slightly attenuated by the treatment of the cells with Gα12 siRNA by itself (Fig. 7B).

LDL can exert inhibitory action on migration when LPA signaling is suppressed. As shown above, LPA is a major component of LDL for SMC migration, whereas S1P mediates the HDL-induced inhibition of cell migration. The lack of LDL to inhibit migration, however, might not be due to the absolutely low content of S1P because the degradation of LPA by MG lipase (Fig. 8A) or the antagonism of LPA receptors by Ki-16425 (Fig. 8B) resulted in the acquisition of the ability of LDL to inhibit PDGF-induced migration. The inhibitory action of LDL was reversed by siRNA specific to S1P2 receptors (Fig. 8C), suggesting the role of an S1P component of LDL as a mediator of the inhibitory action under the blockage of LPA signaling. Thus the balance of the content of LPA and S1P but not the absolute content of these lipids in LDL particles and/or the balance of the signaling of LPA1 and S1P2 receptors in the cells seems to be critical in determining whether the lipoprotein is a positive or negative regulator of migration.

DISCUSSION

The results of the present study indicate that LDL-associated LPA plays a critical role in lipoprotein-induced migration in CASMCs. This conclusion is consistent with recent studies in

---

Fig. 3. Small interfering RNA (siRNA) specific to LPA1 receptors inhibits migration response to LDL. A: expression of mRNAs for LPA and S1P receptor subtypes in CASMCs. The mRNA expression was assessed by real-time TaqMan PCR. Results are expressed as the relative ratios to GAPDH mRNA expression (B and C). CASMCs were transfected with nonsilencing RNA (control; open bar) or LPA1-specific siRNA (50 nM, closed bar). The mRNA expression of LPA1 receptor and S1P2 receptor (for evaluation of the specificity of siRNA) in B and cell migration response to LDL (30 μg/ml), LPA (100 nM), or PDGF (20 ng/ml) in C were measured. The results shown are means ± SE of three values of a representative experiment in A and of six values of two separate experiments in B and C. *Effect of siRNA was significant.

Role of G13 proteins in the S1P2 receptor-mediated inhibition of the migration response to S1P and HDL. The role of G13 proteins in the S1P-induced inhibition of cell migration has been characterized in S1P2 receptor-expressing Chinese hamster ovary cells in which both G12 and G13 proteins were suggested to be involved in the S1P2-mediated migration response (45). To verify the role of G12 and G13 proteins in vascular SMC migration, we used siRNAs specific to α-subunits of G12 and G13 proteins. As shown in Fig. 7A, Gα12 and Gα13 siRNA specifically and effectively inhibited the mRNA expression of the respective α-subunits of G proteins. Under this condition, the S1P- and HDL-induced inhibition of PDGF-induced migration was clearly reversed by Gα13 siRNA (Fig. 7B). However, we failed to prove the involvement of G12 proteins in S1P signaling. Thus S1P and HDL significantly inhibited PDGF-induced migration in the cells treated with Gα12 siRNA, although the PDGF-induced migration response was slightly attenuated by the treatment of the cells with Gα12 siRNA by itself (Fig. 7B).

LDL can exert inhibitory action on migration when LPA signaling is suppressed. As shown above, LPA is a major component of LDL for SMC migration, whereas S1P mediates the HDL-induced inhibition of cell migration. The lack of LDL to inhibit migration, however, might not be due to the absolutely low content of S1P because the degradation of LPA by MG lipase (Fig. 8A) or the antagonism of LPA receptors by Ki-16425 (Fig. 8B) resulted in the acquisition of the ability of LDL to inhibit PDGF-induced migration. The inhibitory action of LDL was reversed by siRNA specific to S1P2 receptors (Fig. 8C), suggesting the role of an S1P component of LDL as a mediator of the inhibitory action under the blockage of LPA signaling. Thus the balance of the content of LPA and S1P but not the absolute content of these lipids in LDL particles and/or the balance of the signaling of LPA1 and S1P2 receptors in the cells seems to be critical in determining whether the lipoprotein is a positive or negative regulator of migration.

DISCUSSION

The results of the present study indicate that LDL-associated LPA plays a critical role in lipoprotein-induced migration in CASMCs. This conclusion is consistent with recent studies in

---

Fig. 4. LPA and S1P contents in lipoprotein fractions. LPA and S1P contents in extensively dialyzed lipoproteins were evaluated. Note that LPA content is estimated as an LPA-equivalent activity. See MATERIALS AND METHODS in detail. The results shown are means ± SE of eight samples from eight healthy male volunteers at ages of 35 to 47. *Values between LDL and HDL are significant.

AJP-Heart Circ Physiol • VOL 292 • MAY 2007 • www.ajpheart.org
which LPA, especially unsaturated forms of LPA, was suggested to be involved in oxLDL-induced endothelial cell and platelet activation in vitro (37, 44) and neointima formation in vivo (53, 54). However, SMCs and a variety of other cell types, as well as cellular activities, including migration and proliferation, are involved in the in vivo formation of neointima. Previously, it was not known which cell types and cellular activities are practically regulated by LPA. LPA interacts with its cognate receptors, i.e., LPA1, LPA2, and LPA3, as well as LPA4/GPR23 receptors (11). Recent studies have suggested that, in addition to such LPA-specific G protein-coupled receptors, LPA has the potential to activate peroxisome proliferator-activated receptor γ (PPARγ) (54). LPA-induced neointima formation in rat carotid artery was inhibited by the PPARγ antagonist GW9662 (54). In the in vivo study, PTX was also effective in inhibiting neointima formation (54), suggesting that both intracellular PPARγ and extracellular G protein-coupled receptors may be involved in the processes.

LPA receptor subtypes involved in proatherogenic cellular activities, however, have not yet been characterized. The elucidation of such receptor subtypes is important not only in clarifying the mechanism of the remodeling of vascular walls but also in developing therapeutic drugs against vascular diseases. In the present study, we demonstrated for the first time that LPA plays a critical role, through LPA1 receptors, in the eases. In the present study, we demonstrated for the first time but also in developing therapeutic drugs against vascular dis-

Fig. 5. S1P is critical for inhibition by HDL of migration response to PDGF. A: inhibition by HDL but not by LDL of PDGF-induced migration. Migration response to the indicated concentrations of LDL or HDL was evaluated in the presence of PDGF (20 ng/ml). B: inhibition by S1P of migration response to PDGF (20 ng/ml) or LPA (100 nM). The results shown are means ± SE of six values of two separate experiments.

On the other hand, HDL alone stimulated migration only slightly, if at all. The difference in the magnitude of the response between LDL and HDL might simply show that the LDL samples might have been minimally oxidized during preparation because the preparation of LDL by the density-gradient ultracentrifugation method and subsequent dialysis takes about 2 days. Moreover, LDL may have been oxidized during incubation with SMCs for 4 h in the migration assay, resulting in LPA production. In fact, the oxidation of LDL during incubation with endothelial cells and SMCs (3, 24) and the formation of LPA in an LDL sample during oxidation in atherosclerotic lesions (44, 53, 54) have recently been reported.

Although the present results suggest that LPA is a critical component of LDL in the stimulation of SMC migration, other lipid components, including PAF, LPC, POVPC, and PGPC, may be involved in the development of atherosclerosis and restenosis. In fact, one species of LPC (C18:1) and PGPC significantly stimulated the migration of SMCs (Fig. 1D). In the present study, we mainly characterized native LDL. The lipid composition in LDL particles, however, is easily changed by the oxidative state. Moreover, in addition to SMC migration, these components have been shown to act on several cell types involved in vascular inflammation, resulting in the stimulation of the expression of a variety of genes, such as MCP-1, IL-8, tissue factor, and ICAM-1, and thereby stimulating the proliferation and apoptosis of SMCs and monocyte/endothelial interaction (20, 28). These early events finally lead to neointima formation and the development of atherosclerosis (29, 36).

On the other hand, HDL alone stimulated migration only slightly, if at all. The difference in the magnitude of the response between LDL and HDL might simply show that the LDL samples might have been minimally oxidized during preparation because the preparation of LDL by the density-gradient ultracentrifugation method and subsequent dialysis takes about 2 days. Moreover, LDL may have been oxidized during incubation with SMCs for 4 h in the migration assay, resulting in LPA production. In fact, the oxidation of LDL during incubation with endothelial cells and SMCs (3, 24) and the formation of LPA in an LDL sample during oxidation in atherosclerotic lesions (44, 53, 54) have recently been reported.

Although the present results suggest that LPA is a critical component of LDL in the stimulation of SMC migration, other lipid components, including PAF, LPC, POVPC, and PGPC, may be involved in the development of atherosclerosis and restenosis. In fact, one species of LPC (C18:1) and PGPC significantly stimulated the migration of SMCs (Fig. 1D). In the present study, we mainly characterized native LDL. The lipid composition in LDL particles, however, is easily changed by the oxidative state. Moreover, in addition to SMC migration, these components have been shown to act on several cell types involved in vascular inflammation, resulting in the stimulation of the expression of a variety of genes, such as MCP-1, IL-8, tissue factor, and ICAM-1, and thereby stimulating the proliferation and apoptosis of SMCs and monocyte/endothelial interaction (20, 28). These early events finally lead to neointima formation and the development of atherosclerosis (29, 36).

On the other hand, HDL alone stimulated migration only slightly, if at all. The difference in the magnitude of the response between LDL and HDL might simply show that the LDL samples might have been minimally oxidized during preparation because the preparation of LDL by the density-gradient ultracentrifugation method and subsequent dialysis takes about 2 days. Moreover, LDL may have been oxidized during incubation with SMCs for 4 h in the migration assay, resulting in LPA production. In fact, the oxidation of LDL during incubation with endothelial cells and SMCs (3, 24) and the formation of LPA in an LDL sample during oxidation in atherosclerotic lesions (44, 53, 54) have recently been reported.
48). In fact, S1P also inhibited the PDGF-induced migration of CASMCs, and the inhibitory action by HDL and S1P was suppressed by the siRNA specific to the S1P2 receptor (Fig. 6). Previous studies have suggested the role of S1P2 receptors in the inhibition of SMC migration by a specific molecular tool of siRNA.

Our results show the differential role of a lipid component in the lipoprotein particles: LPA is critical for LDL-induced stimulation, and S1P is essential for the HDL-induced inhibition of migration. However, the absolute concentration of each lipid molecule is not always an essential determinant of whether the lipoprotein is a positive or negative regulator of migration. Thus even LDL was able to inhibit PDGF-induced migration if LPA in the LDL fractions was degraded by MG lipase (Fig. 8) or LPA1 receptors were antagonized by

Fig. 6. siRNA specific to S1P2 receptors attenuates inhibitory migration response to S1P and HDL. A: expression of mRNAs for LPA and S1P receptor subtypes in CASMCs. The mRNA expression was assessed by real-time TaqMan PCR. Results are expressed as the relative ratios to GAPDH mRNA expression. B: CASMCs were transfected with nonsilencing RNA (control; open bar) or S1P2-specific siRNA (50 nM, closed bar). Cell migration response to LDL (30 μg/ml), LPA (100 nM), HDL (100 μg/ml), S1P (30 nM), PDGF (20 ng/ml), or their combination was measured. The results shown are means ± SE of six values of two separate experiments. *Effect of siRNA was significant.

48). In fact, S1P also inhibited the PDGF-induced migration of CASMCs, and the inhibitory action by HDL and S1P was suppressed by the siRNA specific to the S1P2 receptor (Fig. 6). Previous studies have suggested the role of S1P2 receptors in the inhibition of migration by means of a pharmacological tool with an S1P2 receptor antagonist JTE013 (34) and by the forced expression of S1P2 receptors (33, 48). The present study further supports the role of S1P2 receptors in the inhibition of SMC migration by a specific molecular tool of siRNA.

Our results show the differential role of a lipid component in the lipoprotein particles: LPA is critical for LDL-induced stimulation, and S1P is essential for the HDL-induced inhibition of migration. However, the absolute concentration of each lipid molecule is not always an essential determinant of whether the lipoprotein is a positive or negative regulator of migration. Thus even LDL was able to inhibit PDGF-induced migration if LPA in the LDL fractions was degraded by MG lipase (Fig. 8) or LPA1 receptors were antagonized by

Fig. 7. G13 proteins but not G12 proteins mediate S1P-induced inhibition of cell migration. A: specificity of Gα12 and Gα13 siRNA. CASMCs were transfected with nonsilencing RNA (Control) or siRNA against Gα12 or Gα13, and then their mRNA and LPA1 mRNA expression were measured. The mRNA expression pattern for Gα12, Gα13, and LPA1 shows the specific downregulation of the respective α-subunit mRNA of G12 protein or G13 protein under the conditions. B: cells were then assayed for migration responses to the indicated concentrations of S1P or HDL in the presence of PDGF (20 ng/ml). The results shown are means ± SE of three values in A and nine values in B of three separate experiments. *Effect of S1P or HDL was significant from PDGF alone.
Ki-16425 (Fig. 8). The inhibitory LDL effect on migration is mediated by S1P2 receptors, as evidenced by the complete reversal of the LDL effect by S1P2-specific siRNA (Fig. 8). The result with Ki-16425 is important, especially in terms of the therapeutic approach against vascular disease involving the aberrant migration of vascular SMCs, because this drug not only inhibits the migratory activity of LDL but also lets the lipoprotein acquire the ability to inhibit migration induced by agents other than LPA, such as PDGF. In other words, Ki-16425 can functionally change the proatherogenic LDL to an antiatherogenic lipoprotein such as HDL. The results suggest that the balance of signaling activity between LPA/LPA1 and S1P/S1P2 receptors may be important for lipoproteins to regulate the migration response. We have previously shown that S1P content was decreased while the content of LPC, a substrate of autotaxin or lysophospholipase D for LPA synthesis (50), was increased during the copper oxidation of LDL (13). Although it has not been confirmed whether such a drastic change in lipid mediator concentration occurs in vivo, as described above, LPA has been shown to be present in atherosclerotic lesions (44, 53). As for receptors, it is speculated that the expression of S1P receptor subtypes might be changed by vascular pathologies, such as atherosclerosis and restenosis after angioplasty (39).

The roles of the LPA1 receptor-Gi/o protein and its signaling pathways have been characterized in a variety of cell types (23, 25, 49). For example, phosphatidylinositol-3 kinase, small G proteins such as CDC42, Rac, and Rho, and several mitogen-activated protein (MAP) kinases, including p38MAPK and JNK, have been suggested as downstream signaling molecules of the LPA1 receptor-Gi/o protein leading to a migration response (23, 25, 49). On the other hand, downstream signaling pathways of S1P2 receptors have not been well characterized (39). In Chinese hamster ovary cells that are forced to express S1P2 receptors, G12 and G13 proteins have been reported to mediate the S1P2 receptor-induced inhibition of migration through the Rho signaling pathway (45). The present study clearly indicated the involvement of G13 proteins but not G12 proteins in the vascular SMC migration response. Thus G13 proteins are major G proteins to mediate signals arising from S1P2 receptors to the inhibitory migration machinery in vascular SMCs.

The involvement of a small G protein Rho in migratory activity has been extensively examined in the previous studies of vascular SMCs, but its role remains controversial (1, 22, 35, 38, 42). C3 toxin, by which ADP ribosylates and thereby inactivates Rho, and Y-27632, a ROCK inhibitor, have been shown to inhibit vascular SMC migration as induced by PDGF, thrombin, or LPA, suggesting the positive involvement of Rho or ROCK in SMC migration (1, 22, 35, 42). However, the results of experiments with the dominant negative Rho family of proteins suggest that Rac and CDC42 are critical for migration response as positive regulators but that Rho plays an inhibitory role in SMC migration (35, 38). The inhibitory role
of Rho in cell migration has also been reported in other cell systems (2, 21, 41). Further experiments are required to understand the role of intracellular signaling pathways, including the Rho signaling pathway, involved in the LPA1 and S1P2 receptor-mediated regulation of migration of SMCs.

In conclusion, the balance in the content of LPA and S1P in lipoproteins seems to be a critical determinant of whether the lipoprotein functions as a positive or negative regulator for cell migration and is a potentially useful biomarker for cardiovascular diseases. In addition, the balance of the signaling pathway between LPA1 receptors and S1P2 receptors in the cells may also be important to determine the direction of the lipoprotein actions on cell migration. Thus LPA1 and S1P2 receptors may be therapeutic targets for vascular diseases involving the aberrant migration of SMCs. LPA receptor antagonists such as Ki-16425 (31) are potential drugs for this purpose.

ACKNOWLEDGMENTS

We are grateful to Prof. Kevin R. Lynch of University of Virginia School of Medicine for generous gifts of LPA1-expressing RH7777 cells and VPC-162249 and to Chisuko Uchiyama for technical assistance.

REFERENCES


