Lysophosphatidic Acid and Autotaxin Stimulate Cell Motility of Neoplastic and Non-neoplastic Cells through LPA1*  

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Autotaxin (ATX) is a tumor cell motility-stimulating factor originally isolated from melanoma cell supernatant that has been implicated in regulation of invasive and metastatic properties of cancer cells. Recently, we showed that ATX is identical to lysophospholipase D, which converts lysophosphatidylcholine to a potent bioactive phospholipid mediator, lysophosphatidic acid (LPA), raising the possibility that autocrine or paracrine production of LPA by ATX contributes to tumor cell motility. Here we demonstrate that LPA and ATX mediate cell motility-stimulating activity through the LPA receptor, LPA1. In fibroblasts isolated from lpa1−/− mice, but not from wild-type or lpa2−/−, cell motility stimulated with LPA and ATX was completely absent. In the lpa1−/− cells, LPA-stimulated lamellipodia formation was markedly diminished with a concomitant decrease in Rac1 activation. LPA stimulated the motility of multiple human cancer cell lines expressing LPA1, and the motility was attenuated by an LPA1-selective antagonist, Ki16425. The present study suggests that ATX and LPA1 represent potential targets for cancer therapy.

Cell migration is an important cellular function for many physiological processes, such as embryonic morphogenesis, wound healing, immune-cell trafficking, and brain development (1). In addition to physiological functions, cancer cells use migration mechanisms that are similar to those that occur in non-neoplastic cells (2). The principles of cell migration were initially investigated in non-neoplastic fibroblasts, keratinocytes, and myoblasts, and additional studies on tumor cells identified the same basic mechanisms. Understanding more about the cellular and molecular basis of different cell migration/invasion mechanisms will help us to explain how cancer cells disseminate and should lead to new treatment strategies.

Lysophosphatidic acid (LPA)1 (1- or 2-acyl-sn-glycerol-3-phosphate) is a naturally occurring phospholipid. It evokes a variety of biological responses, including platelet aggregation, smooth-muscle contraction, neurite retraction, and cell proliferation (3, 4). LPA stimulates cell migration in many cell types in vitro, including fibroblasts, gliomas, T lymphomas, and colorectal cancer cells (5–7), indicating a potential role of LPA in cellular migration in both physiological and pathological conditions (8). A role for LPA signaling in cancer cell migration received further support from the identification of autotaxin (ATX), a protein previously implicated in neoplastic invasion and metastasis (9), as a major biosynthetic enzyme for LPA. ATX was found to be identical to lysophospholipase D, an LPA-producing enzyme in blood that converts lysophosphatidylcholine to LPA (10, 11). ATX also shows properties of a membrane pyrophospholipase D, which might also explain its bioactivities. It has been shown that LPA- and ATX-stimulated cell motility is attenuated by treating cells with pertussis toxin (PTX) (8, 12–13), suggesting that G protein-coupled receptors (GPCRs) coupled with Gi proteins are involved. LPA elicits most of the cellular events via signal transduction cascades downstream of its specific GPCRs, LPA1/Edg-2, LPA2/Edg-4, LPA1/Edg-4, LPA2/Edg-7, which belong to the Edg (endothelial cell differentiation gene) family, and LPA1/GPR23, a non-Edg family LPA receptor (4, 14–17). Non-GPCR pathways have also been proposed (18, 19). Several experiments have demonstrated that these GPCRs can mediate mitogen-activated protein kinase activation, phospholipase C activation, and calcium mobilization through PTX-sensitive (Gαi) and -insensitive G proteins (G12/13, and Gα13) (4). However, the LPA receptor subtype involved in LPA-induced cell motility remained to be identified.

In this study, we explored the role of each LPA receptor in LPA- and ATX-induced cell migration. Our results clearly indicate that LPA- and ATX-induced cell motility is driven by LPA1 activation. We also suggest a crucial role of Rac1 activation in LPA1-mediated cell migration.

EXPERIMENTAL PROCEDURES

Reagents—1-oleoyl-LPA (18:1) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Other chemicals were purchased from Sigma. Recombinant ATX/lysophospholipase D protein was prepared as described previously (10).

Cell Culture—Mouse skin fibroblast (MSF) cells were prepared from skin of newborn mice generated by wild-type or knock-out (lpa1−/−, single, lpa2−/− single, and lpa1−/− lpa2−/− double) intercrosses as described previously (20). MSF cells were cultured in minimum essential medium (Sigma) supplemented with 10% fetal bovine serum, and cells from the first to the fifth passages were used for all experiments. All
cancer cell lines used in this study were cultured in RPMI 1640 (Sigma) supplemented with 5% fetal bovine serum as described previously (21). Chemotaxis Assay—Cell migration was measured in a modified Boyden chamber as described previously (10). In brief, polycarbonate filters with 5-μm (MSF cells) or 8-μm pores (carcinoma cell lines) (Neuro Probe, Inc., Gaithersburg, MD) were coated with 0.001% of fibronectin (Sigma). Cells (1 × 10^6 cells in 200 μl/well) were loaded into upper chambers and incubated at 37 °C for 3 h to allow migration. The cell migration to the bottom side of the filter was evaluated by measuring optical densities at 590 nm. For PTX and Ki16425 treatment, cells were preincubated with 10 ng ml^{-1} of PTX for 24 h and 1 μM Ki16425 for 30 min, respectively.

Quantitative Real-time RT-PCR—Total RNA from cells was extracted using ISOGEN (Nippongene, Toyama, Japan) and reverse-transcribed using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Oligonucleotide primers for PCR were designed using Primer Express Software (Applied Biosystems, Foster City, CA). The sequences of the oligonucleotides used in PCR reaction were as follows. LPA1 (mouse)-forward gaccacactcagcctagtcaagac; LPA2 (mouse)-reverse ctta-cagtccaggccatcca; LPA 2 (human)-forward cgctcagcctggtcaagact; LPA 2 (human/-reverse tgcagaggctagcctaaa; LPA3 (mouse/-reverse gaggggccatccacagtctt. GAPDH (mouse/human)-forward gccaaggtcatccatgacaact; GAPDH (mouse/human/-reverse gaggggccatccacagtctt. GAPDH (human)-forward agacgccatcaagactagaa; GAPDH (human/-reverse gctctgagggcgacagaa. LPA1 (mouse)/forward caagaggctcatgacctgaac; LPA2 (mouse)-forward gccaaggtcatccatgacaact; LPA3 (mouse/human)-forward gccaaggtcatccatgacaact; LPA4 (mouse/human/-reverse gctctgagggcgacagaa. PCR reactions were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems). The transcript number of mouse GAPDH was quantified, and each sample was normalized on the basis of GAPDH content.

Intracellular Calcium Mobilization—A-2058 cells were incubated with 5 μM fura-2 acetoxymethyl ester (Dojin, Tokyo, Japan) in calcium ringer buffer (150 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, 0.1% bovine serum albumin, and 5 mM HEPES, pH 7.4) at 37 °C for 30 min. Following stimulation with LPA, cytosolic calcium was measured by monitoring fluorescence intensity at an emission wavelength of 590 nm and excitation wavelengths of 340 and 380 nm using a CAF-110 (JACS, Tokyo, Japan).

Fluorescence Microscopy—MSF cells were seeded onto glass coverslips, grown in the presence of serum to subconfluence, and starved for 24 h by replacing the medium with serum-free medium containing 0.1% bovine serum albumin. Then the cells were treated with 1 μM LPA in serum-free medium for 0 h and stained for F-actin with BODIPY FL phallacidin (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's protocol.

Rac1 and RhoA Activity Assays—Measurement of Rac1 and RhoA activities was performed as described previously (22). Cells starved for 24 h were stimulated with LPA (1 μM) and lysed for 5 min in ice-cold cell lysis buffer containing GST-pAK or GST-Rhotekin. The cell lysates were incubated with glutathione-Sepharose 4B (Amersham Biosciences) for 60 min at 4 °C. After the beads had been washed with the cell lysis buffer, the bound proteins were analyzed by Western blotting using anti-Rac1 antibody (BD Biosciences) or anti-RhoA antibody (Santa Cruz Biotechnology).

RESULTS

To determine whether LPA receptors are required for LPA-dependent cell motility and, if so, which receptor subtype and signaling cascade are utilized, we generated MSF cells isolated from newborn mice. The MSF cells expressed LPA1, LPA2, and LPA4 with an undetectable level of LPA1 as judged by quantitative real-time RT-PCR (Fig. 1A). LPA4 was knocked out in the MSF cells isolated from previously established LPA receptor knock-out mice (20, 23). The migratory response was completely abolished in MSF cells isolated from Lpa1−/− mice (Fig. 2A). MSF cells from Lpa2−/− mice migrated normally in response to LPA (Fig. 2A). The Lpa1−/− MSF cells migrated normally in response to platelet-derived growth factor, a potent inducer of migration for fibroblasts (Fig. 2B), indicating that the Lpa1−/− cells have defects in their response to LPA but not migration per se. We also found that ATX stimulated the migration of MSF cells (Fig. 3) in a PTX-sensitive manner (data not shown). The migratory response induced by ATX also disappeared in MSF cells from Lpa1−/− mice but not from wild-type or Lpa2−/− mice (Fig. 3). These data demonstrated that of the three LPA receptors expressed in the MSF cells, LPA1 is at least essential for LPA-stimulated cell migration. They also show that the motility effects of ATX are mediated by LPA signaling.

We next examined whether LPA1 is involved in LPA- or ATX-induced cell motility of carcinoma cells by using various carcinoma cell lines that differentially express LPA receptors. LPA stimulated cell migration of multiple carcinoma cell lines, including MDA-MB-231 (breast cancer), PC-3 (prostate cancer), A-2058 (melanoma), A549 (lung cancer), ACHN (renal cancer), SF295 (glioblastoma), and SF539 (glioblastoma) (Fig. 4). Interestingly, these cells were found to express LPA1 endogenously as judged by quantitative real-time RT-PCR (Fig. 4). LPA did not support the migration of MCF7 (breast cancer), HT-29 (colon cancer), KM-12 (colon cancer), OVCAR-4 (ovarian cancer), OVCAR-8 (ovarian cancer), NCI-H522 (lung
LPA<sub>1</sub>-dependent Cell Migration

Fig. 2. LPA<sub>1</sub> is essential for LPA-induced cell motility in mouse skin fibroblasts. LPA-induced (A) and platelet-derived growth factor-induced (B) migration of MSF cells isolated from lpa<sub>1</sub><sup>−/−</sup> (filled circles), lpa<sub>2</sub><sup>−/−</sup> (open circles), and wild-type (open triangles) mice. Results shown are representative of at least three independent experiments. Error bars indicate the S.D. of the mean.

Fig. 3. LPA<sub>1</sub> is essential for ATX-induced cell motility in mouse skin fibroblasts. ATX-induced migration of MSF cells isolated from lpa<sub>1</sub><sup>−/−</sup> (filled circles), lpa<sub>2</sub><sup>−/−</sup> (open circles), and wild-type (open triangles) mice. Results shown are representative of at least three independent experiments. Error bars indicate the S.D. of the mean.

Cancer, LNCaP (prostate cancer), and HeLa (cervical cancer) cells, and these cells did not appreciably express LPA<sub>1</sub> (data not shown). Recently, an LPA<sub>1</sub>-selective antagonist, Ki16425, (Ki values were 0.25 μM for LPA<sub>1</sub>, 5.60 μM for LPA<sub>2</sub>, and 0.36 μM for LPA<sub>3</sub>) were developed (24). It has not been tested whether Ki16425 affects the activation of LPA<sub>1</sub>. We then monitored intracellular calcium mobilization in HeLa cells transiently transfected with human and mouse LPA<sub>1</sub> cDNA and found that it was not inhibited by 1 μM Ki16425 (data not shown). Ki16425 inhibited the migratory response of LPA<sub>1</sub>-expressing cells to both LPA and ATX (Figs. 4 and 5). Because Ki16425 is also a weak antagonist for LPA<sub>3</sub>, it is possible that LPA<sub>3</sub> could be involved in the LPA- or ATX-stimulated cell motility of LPA<sub>3</sub>-expressing cells. However, carcinoma cell lines expressing LPA<sub>3</sub> but not LPA<sub>1</sub> (OVCAR-8 and LNCaP) did not migrate in response to LPA (Fig. 4). In addition, OMPT, an LPA<sub>1</sub>-selective agonist we recently developed (25), induced a smaller migratory response in A-2058 cells that were transfected with human and mouse LPA<sub>4</sub> cDNA and found that it was not inhibited by 1 μM Ki16425 (data not shown), an inhibitor of Rho kinase that inactivates the RhoA pathway. We therefore measured the LPA-induced activation of the two small GTPases, Rac1 and Cdc42 (28). Rac1 regulates lamellipodia, whereas RhoA regulates the formation of contractile actin-myosin filaments to form stress fibers (28). LPA-stimulated migration of MSF cells was efficiently blocked by pretreatment of the cells with Y-27632 (data not shown), an inhibitor of Rho kinase that inactivates the RhoA pathway. We therefore measured the LPA-induced activation of the two small GTPases, Rac1 and RhoA, in MSF cells. Rac1 and RhoA were measured with GST-Pak and GST-Rhotekin pull-down assays, respectively. When MSF cells from wild-type and lpa<sub>2</sub><sup>−/−</sup> mice were stimulated with 1 μM LPA, a GTP-bound form of Rac1 was dramatically increased (Fig. 8). By contrast, Rac1 activation was almost completely abolished in both lpa<sub>1</sub><sup>−/−</sup> and lpa<sub>1</sub><sup>−/−</sup>lpa<sub>2</sub><sup>−/−</sup> MSF cells (Fig. 8). Although RhoA activation in response to LPA was obvious in wild-type and lpa<sub>2</sub><sup>−/−</sup> MSF cells, it appeared to be
LPA₁-dependent Cell Migration

reduced in lpa₁⁻/⁻ MSF cells and markedly reduced in lpa₁⁻/-lpa₂⁻/⁻ MSF cells (Fig. 8). These results indicate that LPA₁ has a major role in both Rac1 and RhoA activation induced by LPA stimulation (Fig. 9). The Rac1 activation is predominantly dependent on LPA₁, whereas RhoA activation is less dependent on LPA₁. LPA₂ does contribute to the activation of RhoA in the absence of LPA₁ expression. In addition, RhoA can be activated to some extent (Fig. 8) in the absence of LPA₁ and LPA₂, indicating the presence of other LPA receptors and/or indirect mechanisms of RhoA activation (Fig. 9).

DISCUSSION

LPA is a multifunctional signaling molecule with diverse activities, including stimulation of cell motility. Recent identification of lysophospholipase D, an LPA-producing enzyme, as ATX, a cell-motility stimulating factor of cancer cells (10, 11), indicated that the activity is one of the intrinsic functions of LPA. In this study we showed that among the four LPA receptors identified so far, LPA₁ has a crucial role in LPA-induced cell migration of fibroblast cells (Fig. 2) and multiple cancer cells (Fig. 4), based on the observation that inactivation of LPA₁, either by gene-targeting technique or by a receptor-selective antagonist resulted in loss of migratory response. In addition, we observed that the migratory response induced by ATX again disappeared in these cells (Figs. 3 and 5). These results clearly show that ATX exerts its function through LPA₁ activation. Both LPA₁ and ATX are highly expressed in brain (14, 29). Recently it was reported that oligodendrocytes express LPA₁ and ATX during myelination (30, 31). We also found that LPA₁ and ATX are highly enriched within certain regions of developing mouse brain, such as olfactory bulb.² The colocalization of the two genes suggests that they also function co-operatively in physiological condition.

The migratory responses were found to be PTX-sensitive (Fig. 1A). By contrast, in a previous report LPA was found to stimulate cell motility of other cell types, such as lymphoma

cells, in a PTX-insensitive manner (6). Because lymphocytes express LPA2 predominantly with no detectable expression of LPA1 (data not shown), it is possible that LPA2 is involved in LPA-induced migratory response of non-carcinoma neoplasms, such as lymphoma cells. Splenocytes and thymocytes isolated from wild-type, $lpa_1^{-/-}$, $lpa_2^{-/-}$, and $lpa_1^{-/-}lpa_2^{-/-}$ mice did not show a migratory response to LPA in our system (data not shown). In addition, in the absence of selective agonists or antagonists for LPA2, we could not test the migratory effect of LPA2 on lymphoma cell migration. Further study is necessary to show the role of LPA2 in migratory response of lymphoma cells.

We previously showed that LPA1 and LPA2 had redundant functions in mediating multiple endogenous LPA responses, including phospholipase C activation, Ca\(^{2+}\) mobilization, cell proliferation, and stress fiber formation in mouse embryonic fibroblasts (20). In this study, we demonstrated that LPA-induced lamellipodia formation was severely affected in $lpa_1^{-/-}$ MSF cells (Fig. 7, A and B). In addition, we showed that LPA activates Rac1 in an LPA1-dependent manner, whereas it activates RhoA either in LPA1-, LPA2-dependent or LPA1-, LPA2-independent pathway (Fig. 8). Thus, LPA1 is able to activate both Rac1 and RhoA regardless of LPA2 expression (Fig. 9). Because the activation of both RhoA and Rac1 is essential for LPA-stimulated cell migration (12), the lack of Rac1 activation in $lpa_1^{-/-}$ MSF cells explains why the cells could not migrate in response to LPA. Many reports have

Fig. 6. LPA\(_3\) is not involved in LPA-induced cell motility. A, OMPT did not stimulate cell motility of LPA\(_3\)-expressing cell, A-2058. B, OMPT induced an intracellular calcium mobilization of the cells more efficiently than LPA.

Fig. 7. LPA induces lamellipodia formation through LPA\(_1\). A, fluorescence microscopy of BODIPY FL phallacidin-stained MSF cells from wild-type, $lpa_1^{-/-}$, and $lpa_2^{-/-}$ mice before (upper panels) or after (lower panels) LPA stimulation (1 \(\mu\)M, 3 h). The lamellipodia formation (arrows) was observed in wild-type and $lpa_2^{-/-}$ MSF cells but rarely observed in $lpa_1^{-/-}$ MSF cells. Bar, 20 \(\mu\)m. B, percentage of wild-type, $lpa_1^{-/-}$, $lpa_2^{-/-}$, and $lpa_1^{-/-}lpa_2^{-/-}$ MSF cells with lamellipodia after stimulation with 1 \(\mu\)M LPA.

Fig. 8. LPA-induced activation of Rac1 and RhoA in MSF cells. MSF cells were serum-starved for 24 h and stimulated with 1 \(\mu\)M LPA. Activated RhoA and Rac1 were isolated using GST-PAK (Rac1, upper panel) or GST-Rhotekin (RhoA, lower panel) coupled to glutathione-Sepharose beads. Rac1 and RhoA bound to the beads were detected by Western blotting using specific antibodies.
RhoA activation.
and stress fiber formation, which finally lead to activation of cell mo-
lism for their LPA-induced cell motility (Fig. 4). It is well
shown that Gi/o and G12/13 regulate the activation of Rac1 and
shown that G1i/o and G12/13 regulate the activation of Rac1 and
shown that G1i/o and G12/13 regulate the activation of Rac1 and
LPA, respectively (12, 32). Thus, our results again suggest that

tumor invasion and metastasis (34, 35). Furthermore, there is
accumulating evidence that elevated expression of ATX is ob-
served in various cancer tissues (36, 37) and that the expres-
sion is closely linked to the invasive and metastatic potency of
cancer cells (38). We therefore propose that both ATX and LPA1
are the potential targets for cancer therapy.

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Fig. 9. Model for LPA- or ATX-induced cell motility. ATX activ-
ates Rac1 and RhoA through Gi and G12/13, respectively, by producing
LPA. The activation of Rac1 and RhoA results in membrane ruffling
and stress fiber formation, which finally lead to activation of cell mo-
tility. The Rac1 activation is predominantly dependent on LPA1,
whereas RhoA activation is induced by either LPA1 or LPA2 activation.
LPA1- and LPA2-independent pathways also contribute partially to
RhoA activation.