Regulatory Effect of Lysophosphatidic Acid on Lymphocyte Migration

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Lysophosphatidic acid (LPA) is a lipid mediator that is known to exhibit chemotactic activity toward a variety of cancer cells. However, its effect on the immune system has not been studied extensively. Another lipid mediator, sphingosine-1-phosphate (S1P), has been shown to influence lymphocyte recirculation by regulating lymphocyte egress from lymphoid organs. In this study, we found that LPA inhibits spontaneous migration of mouse splenic lymphocytes through a chemorepulsive effect. We also demonstrated that LPA inhibits chemokine CCL21-induced lymphocyte migration. This inhibitory effect on CCL21-induced migration was confirmed with a synthetic agonist, oleyl thiophosphate. Considering that the signaling by CCL21 through cognate receptor CCR7 contributes to lymphocyte homing and dendritic cell trafficking to lymph nodes, LPA may play a role as a key regulator of these processes. The inhibitory effect of LPA is in remarkable contrast to the effect of S1P receptor signaling, which is known to potentiate lymphocyte chemotaxis involving CCR7.

Key words lysophosphatidic acid; chemorepulsive effect; lymphocyte migration; chemokine

Lymphocyte recirculation is known to be regulated by adhesion molecules and signaling by chemokines. CCL21 (also known as SLC or 6Ckine) is a chemokine constitutively expressed in endothelial cells of the high endothelial venules (HEV) of lymph nodes. CCR7, the corresponding receptor for CCL21, is known to be expressed on T and B cells. CCR7 is also expressed on dendritic cells (DC), and CCL21 has been shown to be involved in DC trafficking to draining lymph nodes.

Studies on lipid mediators such as sphingosine-1-phosphate (S1P) have demonstrated a significant effect of S1P on the process of lymphocyte recirculation. Signaling by S1P has been demonstrated to be involved in the lymphocyte egress from lymphoid organs. An immunosuppressant drug, FTY720, has been shown to bind and activate S1P receptors including S1P1 (also known as Edg1), and to inhibit lymphocyte egress. S1P exhibits chemotactic activity toward lymphocytes in a standard in vitro chemotaxis assay. Interestingly, S1P and FTY720 enhance the migration of splenic T cells toward CCL19 (another ligand for CCR7). Another study has also demonstrated that FTY720 enhanced CCL21-induced chemotaxis of T cells. S1P receptors and chemokine receptors are G protein-coupled receptors (GPCRs). These results suggested the presence of crosstalk between S1P and chemokine signaling pathways.

Recent studies suggested that chemorepulsive signals play a role in the regulation of chemotaxis. For example, apoptotic cells have been demonstrated to release chemotactic factors, such as ATP and uridine 5′-triphosphate (UTP), for macrophages that clear apoptotic cells. These factors, which are sensed through P2Y2 receptors, induce macrophage chemotaxis toward apoptotic cells. Although neutrophils also express P2Y2 receptors, neutrophil migration toward apoptotic cells is inhibited by chemorepulsive factors such as lactoferrin, which is released from apoptotic cells. Through this mechanism, excessive inflammation is avoided in tissues in which apoptosis occurs.

Lysophosphatidic acid (LPA) is a lysophospholipid mediator. The S1P and LPA receptors belong to the same family among GPCRs. LPA is generated through hydrolysis of lysophosphatidylcholine (LPC) by lysophospholipase D (lysoPLD), or by an enzyme exhibiting similar activity called autotaxin (ATX). Another pathway is the hydrolysis of phosphatidic acid (PA) by phospholipase A1 or A2. LPA can be generated outside of cells upon stimulation of some types of cell lines and blood platelets. LPA signals are transmitted through cell surface-specific GPCRs or cytosolic/nuclear peroxisome proliferator-activated receptor gamma (PPARγ). Cell surface LPA receptors of the endothelial differentiation gene (EDG) family comprise LPA1/Edg-2, LPA4/Edg-4, and LPA6/Edg-7. Other are receptors, LPA5/GPR23, LPA7, and LPA3, which are structurally distinct from receptors of the EDG family.

Human blood lymphocytes and leukocytes, human natural killer cells, and T-helper 1 (Th1) and T-helper 2 (Th2) cells have been shown to express LPA receptors. However, unlike the case of S1P, little is known as to whether LPA causes chemotaxis of lymphocytes or whether it can modulate chemotaxis induced by chemokines such as CCL21. In this study, we investigated whether LPA acts as a chemotactic or chemorepulsive factor for mouse lymphocytes. We also examined whether LPA has an effect on lymphocyte chemotaxis induced by CCL21.

MATERIALS AND METHODS

Mice Specific pathogen-free female BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and used at 6 weeks of age. Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka.

Reagents 1-Oleoyl lysophosphatidic acid and oleyl thiophosphate were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). RPMI 1640 was purchased from Nissui Pharmaceuticals (Tokyo, Japan). Bovine serum albumin (BSA) of fatty acid-free/low-endotoxin grade, and BSA fraction V were purchased from Sigma (St. Louis, MO, U.S.A.). Kanamycin sulfate and dimethylsulfoxide (DMSO) were pur-
chased from Wako Pure Chemicals (Osaka, Japan), and N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) from Nacalai Tesque (Kyoto, Japan). Mouse CCL21 was purchased from R&D Systems (Abingdon, U.K.). Lympholyte-M was purchased from Cedarslane (Burlington, Ontario, Canada). R-Phycocerythrin (PE)-conjugated rat anti-mouse B220 (RA3.6B2) was purchased from Immunotech (Mar- seille, France), and fluorescein isothiocyanate (FITC)-conju- gated hamster anti-mouse CD3ε (145-2C11) from eBio- science, Inc. (San Diego, CA, U.S.A.).

Cell Migration Assays Mice were killed by cervical dis- location. Spleen cells were released by gentle teasing with needles in RPMI 1640 medium and then passed through a stainless steel screen. Spleen cell suspensions were overlaid on a Lympholyte-M solution and then centrifuged (1000×g for 20 min at 20°C) to obtain a lymphocyte fraction. Cell migration assays were performed using 24-well transwell inserts of 6.5 mm diameter (Corning Costar, Amsterdam, Netherlands) with a pore size of 5.0 μm. For cell migration assays, 1×10^6 lymphocytes suspended in 100 μl of RPMI 1640 containing 0.5% BSA (fatty acid-free/low-endotoxin) were added to the top well of each transwell insert. In the bottom chamber, 600 μl of RPMI 1640 containing 0.5% BSA was added. Samples were added to the bottom chambers dissolved in phosphate buffered saline (PBS), 0.1% BSA-PBS or DMSO, as specified in the figure legends. After 4 h incubation at 37°C under 5% CO_2/95% air, the numbers of cells recovered in the bottom chambers were determined.

Flow Cytometry The cell surface phenotypes of lym- phocytes that migrated into the bottom chambers were determined by flow cytometry. PE-anti-mouse B220 and FITC- anti-mouse CD3ε were used for two-color immunofluorescence. Cells (1.6×10^9) were incubated in 100 μl of PBS containing 0.1% BSA (fraction V)–0.1% NaN_3 (PBS–BSA) with antibodies (1 μg/ml each) for 30 min at 0°C. After washing in PBS–BSA, the cells were analyzed with a flow cytometer (EPICS XL; Beckman-Coulter, Fullerton, CA, U.S.A.). The proportions of T cells (CD3-positive) and B cells (B220-positive) were determined by setting appropriate gates, and then the T and B cell numbers were determined.

Statistical Analyses Student’s t-test was performed for comparison of two samples. For multiple comparisons, ANOVA followed by Dunnett’s test was used.

RESULTS

Lysophosphatidic Acid Inhibits Lymphocyte Migration LPA receptors have been reported to be expressed on human blood lymphocytes and leukocytes.26,32 Because many types of G protein-coupled receptors are involved in cell migration, we examined the effect of 1-oleoyl lysophosphatidic acid (LPA) on lymphocyte migration by means of transwell assays. When LPA was added to the bottom chambers at a concentration between 0.001 and 1 μM, lymphocyte migration was inhibited as compared with that in the absence of LPA. A higher concentration of LPA (10 μM) did not inhibit migration (Figs. 1A, B). When LPA (1 μM) was added to the upper wells and bottom chambers, cell migration was also inhibited (Fig. 1B). However, when LPA (1 μM) was only added to the upper wells, no inhibition was observed (Fig. 1B). To determine whether the inhibition of cell migration was due to the cytotoxicity of LPA, lymphocytes were incubated with 1 μM LPA for 4 h at 37°C. The cell viability was 93.9±0.7% (with LPA) and 92.8±0.3% (without LPA), as determined with the trypan blue dye exclusion test.

Lysophosphatidic Acid Inhibits CCL21-Induced Chemotaxis of Lymphocytes We examined the effect of LPA on CCL21-induced lymphocyte chemotaxis. Substantial lymphocyte migration (20% of input cells) was observed when CCL21 was present in the bottom chambers (Fig. 2). When LPA was added together with CCL21 to the bottom chambers, chemotaxis was significantly inhibited with between 0.1 and 1 μM LPA. A higher concentration of LPA (10 μM) did not inhibit chemotaxis.

LPA Inhibits CCL21-Induced Chemotaxis of Both T
**Table 1.** LPA Inhibits CCL21-Induced Chemotaxis of Both T and B Cells

<table>
<thead>
<tr>
<th>Cell population</th>
<th>B220⁺</th>
<th>CD3⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input cell population</td>
<td>37.0 ± 5.0³</td>
<td>37.8 ± 5.7³</td>
</tr>
<tr>
<td>Migrated cells</td>
<td>2.6 ± 0.5¹</td>
<td>9.6 ± 0.7³</td>
</tr>
<tr>
<td>CCL21</td>
<td>1.1 ± 0.2³</td>
<td>5.7 ± 0.3³</td>
</tr>
<tr>
<td>CCL21 + LPA</td>
<td>1.1 ± 0.2³</td>
<td>5.7 ± 0.3³</td>
</tr>
<tr>
<td>LPA-induced inhibition</td>
<td>57.7 ± 2.9%⁹</td>
<td>40.6 ± 2.1%⁹</td>
</tr>
</tbody>
</table>

a) Number of B cells (B220⁺) and T cells (CD3⁺) were calculated from cell counting and flow cytometric analyses. b) 1×10⁵ cells (lymphocyte fraction of mouse spleen) were added to the top well. c) Data are expressed by cell number (×10⁴ cells) as mean ± S.D. (triplicate). d) Cells migrated into the bottom chamber in the presence of CCL21 (250 ng/ml) with or without LPA (1 μM). e) Data are expressed by percent inhibition as compared with the condition of CCL21 alone (mean ± S.D.). LPA affected migration of B cells more than that of T cells (p < 0.01, t-test).

**Fig. 3.** Inhibition of CCL21-Induced Lymphocyte Chemotaxis by Oleyl Thiophosphate

Lymphocytes (1×10⁶ cells) were added to the top well. The stock solution of oleyl thiophosphate (10 mM) was prepared in DMSO. CCL21 (250 ng/ml) together with the indicated concentration (absissa) of oleyl thiophosphate or vehicle (0 μM oleyl thiophosphate) was added to the bottom chamber. Incubation was carried out for 4 h at 37°C. Data are expressed as means ± S.D. for triplicate experiments. *p < 0.05 vs. vehicle control by Dunnett’s test.

**and B Cells** We examined whether LPA selectively inhibits CCL21-induced chemotaxis of T or B cells. Lymphocytes from mouse spleens were added to the top wells and CCL21 with or without LPA (1 μM) was added to the bottom chambers, and then the cells were allowed to migrate into the bottom chambers. Cells were stained with PE-conjugated anti-B220 and FITC-conjugated anti-CD3ε, and then analyzed by two-color flow cytometry (Table 1). CCL21 preferentially induced the chemotaxis of T cells, as revealed by the cell numbers recovered from the bottom chambers. When LPA was present together with CCL21 in the bottom chambers, the migration of both T and B cells was inhibited as compared with the absence of LPA. B cell chemotaxis was more strongly affected by LPA.

**LPA Receptor Agonist Oleyl Thiophosphate Inhibits CCL21-Induced Lymphocyte Chemotaxis** Oleyl thiophosphate is known to act as a partial agonist for LPA₁ and LPA₂ receptors, and as a full agonist for LPA₃ ones.⁵⁵ We observed that oleyl thiophosphate inhibited CCL21-induced lymphocyte chemotaxis at 0.1 μM (Fig. 3).

**DISCUSSION**

LPA is a phospholipid metabolite that has various effects on cellular responses through binding to specific receptors. LPA is generated from LPC by lysoPLD (ATX).⁹,¹⁹,²⁰ Alternatively, LPA can be generated through the reaction of phospholipase D (PLD), which converts phosphatidylcholine (PC) into PA, followed by the reaction of phospholipase A₂, which converts PA into LPA.³⁶—³⁸ Phospholipase A₂ is also known to give rise to a structurally distinct type of LPA.³⁹ Similar but not identical reactivity to LPA receptors has been demonstrated for this type of LPA.⁴⁰

LPA with a fatty acid at the C1-position of glycerol has been used in biological experiments so far. It exhibits various physiological functions depending on the type of receptor, LPA₁ to LPA₆. Many cellular responses to LPA are potentially mediated through these receptors. There have been several studies demonstrating LPA-induced cell migration, especially of cancer cells, such as human gastric cancer,⁴₀ human pancreatic cancer,⁴¹ human ovarian cancer,⁴² human hepatoma,⁴³ and mouse T lymphoma,⁴⁴ as well as mouse osteoblastic cells.⁴⁵ In addition, LPA has been reported to be secreted by human ovarian cancer,⁴⁶,⁴⁷ human multiple myeloma,⁴⁷ human prostate cancer,⁴⁸ and many other cancer cell types.⁴⁹ These reports may suggest that LPA plays roles in cancer cell accumulation and tumour formation in tissue environments.

On the other hand, there are several reports describing that cell migration was suppressed by LPA under certain conditions. Under the conditions where the function of G protein was inhibited, LPA suppressed the migration of Chinese hamster ovary (CHO) cells induced by insulin-like growth factor I.⁴⁰ When LPA was expressed in a human colon cancer cell line ectopically, LPA-induced cell migration was markedly inhibited.⁴⁰ In the presence of an antagonist to LPA₁ and LPA₄, epidermal growth factor (EGF)-induced migration of pancreatic cancer cells was inhibited by LPA.⁵¹ Inhibition of cell migration by LPA has also been reported for human breast cancer cells.⁵²

In contrast, the effect of LPA on lymphocyte migration has been paid little attention so far. LPA receptors have been reported to be expressed on human blood lymphocytes and leukocytes.²⁶,³² There has been a study showing the presence of LPA₁, LPA₂, and LPA₃ on human peripheral blood T cells.³⁴ In that study, LPA exhibited chemotactic activity toward CD4⁺-T cells that had been activated with anti-CD3, anti-CD28 and IL-2. The observed chemotactic activity of LPA appears to be similar in extent to that of S1P. In contrast, another study demonstrated that only LPA₁ was expressed on human T-lymphoma cells.⁵³ Changes in the expression of LPA receptors have been reported upon activation of T cells. Human peripheral blood CD4⁺ T cells were shown to predominantly express LPA₁ in the resting stage but to predominantly express LPA₂ after activation with phytohemagglutinin (PHA) for 24 h.⁵⁴ During the culture of CD4⁺ T cells, spontaneous IL-2 secretion in the absence of PHA was suppressed by LPA whereas PHA-induced IL-2 secretion was enhanced by LPA.⁵⁴

Our initial experiments demonstrated that LPA inhibited the migration of mouse splenic lymphocytes. Thus, LPA added to the bottom chambers of the transwell apparatus inhibited the spontaneous migration of lymphocytes. When LPA was added to both the top wells and bottom chambers, lymphocyte migration was also inhibited. This may indicate that LPA affects cell viability or cell movement itself. However, when LPA was only added to the top wells, the migration of lymphocytes was not affected at all. This indicated that LPA is less likely to inhibit chemokinesis. Furthermore, we found that LPA did not affect cell viability at a concentra-
tion at which lymphocyte migration was inhibited.

The dose–response curve of LPA indicated that there is an optimal range of effective concentrations of it. That is, a high concentration of LPA (10 μM) did not inhibit lymphocyte migration. The occurrence of such an optimal range of concentrations of a lipid mediator has been documented in the case of S1P, which exhibits chemotactic activity toward lymphocytes. A high dose of S1P (>0.1 μM) did not induce chemotaxis of lymph node CD4+ T cells.7) Such a common feature of the dose–response curve may suggest that LPA exerts its effect through specific receptors.

We demonstrated that LPA inhibited CCL21-induced chemotaxis of mouse splenic lymphocytes. Chemokines are known to be involved in leukocyte migration from blood to target tissue sites. This leukocyte migration is closely connected with immune defense and inflammatory diseases.55) S1P and FTY720 have been reported to enhance the migration of splenic T cells toward CCL19.13) Another study also revealed that FTY720 enhanced CCL21-induced chemotaxis of T cells.14) These results may indicate that ligation of S1P receptors coordinately enhances signal transduction through CCR7, which is the common receptor for CCL19 and CCL21. In contrast to these findings, signaling through LPA receptors seems to suppress CCL21-induced migration of splenic lymphocytes. Because CCL21 is a key chemokine for lymphocyte homing through the high endothelial venules of lymph nodes as well as DC migration through afferent lymphatics,56) our data may suggest that LPA is one of the key regulators of lymphocyte homing and DC trafficking.

Analysis of the phenotypes of the lymphocytes that migrated indicated that CCL21 exhibits preferential chemotactic activity toward CD3+ T lymphocytes rather than B220+ B lymphocytes, as expected.56) The inhibitory activity of LPA toward CCL21-induced chemotaxis was effective for both T and B cells. B cells seem to be more susceptible to the inhibitory effect of LPA.

Apoptotic cells have been shown to release chemotactic signals (find-me signals) for macrophages to facilitate the clearance of apoptotic cells.56) These signals include ATP, UTP, chemokine CCL1 and LPC.57) Unlike LPC, LPA was shown not to be a chemotactant for macrophages.58)

The involvement of LPA receptors was confirmed with oleyl thiophosphate, which is a partial agonist for LPA1 and LPA3, and a potent and full agonist for LPA2.35) Our finding that oleyl thiophosphate could suppress CCL21-induced chemotaxis of splenic lymphocytes is further evidence of the involvement of some LPA receptors in the regulation of chemokine signaling. To determine which receptors are responsible for this effect, further studies are required.

In conclusion, we found that LPA inhibits spontaneous and CCL21-induced lymphocyte migration. The inhibitory effect on CCL21-induced migration was seen for both T and B cells. The involvement of some LPA receptors was confirmed with a synthetic agonist, oleyl thiophosphate.

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