Inhibition of Lysophosphatidic Acid Receptor-2 Expression by RNA Interference Decreases Lysophosphatidic Acid-induced Urokinase Plasminogen Activator Activation, Cell Invasion, and Migration in Ovarian Cancer SKOV-3 Cells

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Aim To explore the role of lysophosphatidic acid receptor-2 (LPA₂) in regulating lysophosphatidic acid (LPA)-induced urokinase plasminogen activator (uPA) activation, cell invasion, and migration in human ovarian cancer cell line SKOV-3.

Methods SKOV-3 cells were stimulated with LPA. Cell supernatant uPA level and activity were measured using enzyme-linked immunosorbent assay. LPA₂ mRNA expression was inhibited with LPA₂-specific small interfering RNA (siRNA) and examined using semiquantitative reverse transcriptase-polymerase chain reaction. LPA-induced cell invasion and migration in transfected cells were evaluated by a Matrigel invasion chamber and a Transwell chemotaxis chamber, respectively.

Results LPA stimulation significantly enhanced in vitro uPA activity in time- and dose-dependent manner. The levels of LPA-induced uPA protein decreased by 55% in LPA₂ siRNA-transfected cells compared with negatively transfected cells at 24 hours after being treated with 80 μmol/L LPA (0.75 ± 0.03 vs 0.34 ± 0.04, P = 0.004). In the LPA₂ specific siRNA-transfected SKOV-3 cells, LPA treatment at 80 μmol/L induced considerably less invasion and migration compared with negative control siRNA-transfected SKOV-3 cells (invasion: 178 ± 17.2 vs 36.2 ± 3.3, P = 0.009; migration: 220.4 ± 25.5 vs 57 ± 7.6, P = 0.009).

Conclusion LPA₂ has an essential role in LPA-induced uPA activation and tumor cell invasion in ovarian cancer SKOV-3 cells.
Lysophosphatidic acid (LPA) is a naturally occurring lysophospholipid, which mediates diverse biological responses such as mitogenesis, differentiation, cell survival, angiogenesis, inflammation, and cell migration (1). Extracellular LPA has been shown to be involved in certain diseases, such as atherosclerosis (2) and cancer (3-5). In fact, LPA has been identified as a growth-promoting factor that supports the proliferation of ovarian cancer cells in malignant ascites from ovarian cancer patients (6,7). Indeed, LPA is present at high levels in the ascites of patients with advanced-stage ovarian cancer, in concentrations of 5-200 µmol/L (3,8).

Most of the biological responses to LPA are mediated through the lipid-specific endothelial differentiation gene (EDG) family G protein-coupled receptors, ie, LPA₁/EDG-2, LPA₂/EDG-4, and LPA₃/EDG-7 (9-11), although recent studies have suggested that LPA responses are potentially mediated through LPA₄/GPR23 (12) and peroxisome proliferator-activated receptor γ (13). These LPA receptors differ with respect to their distribution in various tissues. LPA₁ is the most widely expressed receptor subtype in normal and tumor tissues, whereas LPA₂ and LPA₃ are frequently overexpressed in human tumor tissues, such as ovarian cancer, gastric cancer, and ductal cancer, which may account for the various biological effects of LPA (9,10,14). Previous studies found that malignant transformation results in aberrant expression of LPA₁ in ovarian and thyroid cancers, suggesting that LPA may play a role in tumor biology and that shifts in LPA receptor expression are related to carcinogenesis (4,7).

Proteolysis of extracellular matrix (ECM) proteins is necessary for the invasion and metastasis of cancer cells. Urokinase plasminogen activator (uPA), a serine protease, can promote degradation of ECM and has been shown to correlate inversely with prognosis in ovarian cancer patients. In vitro studies have shown that uPA expression is induced by LPA in ovarian cancer cell lines but not in normal ovarian epithelial cell (15,16).

Previous studies showed that overexpression of LPA₁ induced the expressions of active uPA and vascular endothelial growth factor in the ovaries of transgenic mice (17). In breast carcinoma cells, LPA₃ was also shown by RNA interference approach to mediate LPA-stimulated cell migration (18). However, the effect of LPA₁ on these processes in ovarian cancer, especially when LPA₂ is inhibited, has not been investigated. In the present study, we inhibited the endogenous expression of LPA mRNA using specific small interfering RNA (siRNA), to investigate more directly the potential role of LPA₂ in LPA-induced uPA activity, cell invasion, and migration in ovarian cancer SKOV-3 cells.

Material and methods

Reagents

1-oleoyl-LPA was purchased from Avanti Polar Lipids Inc (Alabaster, AL, USA). The kit for quantification of uPA activity was obtained from American Diagnostica (Greenwich, CT, USA) and used according to the manufacturer’s guidelines.

Cell culture and LPA stimulation

The established ovarian cancer cell line SKOV-3 was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were maintained under standard conditions (37°C and 5% CO₂) in a plastic cell culture flasks and grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin, and 1% streptomycin (all from Sigma, St. Louis, MO, USA) until their confluence level reached about 80%. They were then washed twice with prewarmed phosphate-buffered saline and cultured in serum-free medium (Invitrogen, Carlsbad, CA,
USA) overnight. The appropriate amount of LPA was diluted in 1% delipidated bovine serum albumin (Sigma) on ice. After starvation, the serum-free medium was replaced, LPA was added to the culture at a concentration of 80 μmol/L, and incubation was carried out at 37°C for various time periods up to 24 hours. In a different experiment, various concentrations of LPA (0-80 μmol/L) were added to the culture and incubation was carried out at 37°C for 24 hours. Cell culture supernatant was collected and analyzed using uPA enzyme-linked immunosorbent assay (ELISA).

**uPA ELISA**

Extracts were diluted 1:5 in assay buffer and 100 μL aliquots of each extract were incubated overnight at 4°C in precoated microtest wells. Wells were washed thoroughly with wash buffer and a second, biotinylated antibody that recognizes a specific epitope on uPA molecule was added for each analysis. Wells were washed again after an incubation of 1 hour and 100 μL of enzyme conjugate was added (streptavidin-conjugated horseradish peroxidase), leading to the formation of the antibody-enzyme detection complex. After 1-hour incubation, wells were washed again. Then, 100 μL of perborate 3, 3′, 5, 5′-tetramethylbenzidine substrate was added to each well and reacted with horseradish peroxidase, producing a blue solution. We used 50 μL of 0.5 mol/L sulfuric acid as a stopping solution, which yielded a yellow color in the reaction.

**siRNA transfection**

The human LPA2 SMARTpool siRNA and control non-targeting siRNA were obtained from Dharmacon (Lafayette, CO, USA). SKOV-3 cells were seeded in 24-well plates at a density of 1.25 × 10^5 cells/well in a complete medium without antibiotics the day before transfection. The cells were transfected overnight with control and LPA2-specific siRNA (20 pmol) with LipofectAMINE™ 2000 (1μL) (Invitrogen) resuspended in OptiMEM (500 μL) (Invitrogen). After transfection, the cells were incubated with fresh complete medium for 36 hours for recovery, parts of the transfected cells were then used for the isolation of total cellular RNA followed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of LPA2 mRNA level. The remaining cells were starved in serum-free medium for 16 hours before stimulation with LPA. The uPA ELISA, Matrigel invasion assay, and cell migration assay were performed.

**Semiquantitative RT-PCR analysis**

The LPA2 and β-actin mRNA levels were evaluated by semiquantitative RT-PCR. Briefly, total cellular RNA was extracted by a single-step method using TRIzol (Invitrogen). For RT-PCR, 10 μg of total RNA was used for a 838-bp fragment of LPA2 complementary DNA (cDNA) synthesis according to the manufacturer’s protocol, using 5′-CAATCTGCTGGTCATAGC-3′ (forward) and 5′-AGAGGATGTATAGTGACGAC-3′ (backward) primer. The primers for β-actin (internal control) were also included as follows: 5′-CCTTCAACACCCAGCCAT-3′ (forward) and 5′-ATGCCAGGGTACATGGTGTT-3′ (backward) that amplified a 550-bp fragment of β-actin cDNA. The PCR cycling parameters were as follows: 5 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 45 seconds at 60°C, and 45 seconds at 72°C, and a final cycle at 72°C for 7 minutes. The PCR products were separated by agarose gel (1.5%) electrophoresis and stained with ethidium bromide. The fluorescence intensity (optical density) was automatically measured and integrated by gel data acquisition AlphaImager HP software (Alpha Innotech Corp., San Leandro, CA, USA). A ratio of LPA2 and β-actin represented the relative level of LPA2 expression.
In vitro migration and invasion assay

Cell migration was assayed using a Transwell chemotaxis chamber (8-μm pore filters). The transfected SKOV-3 cells (5 × 10⁴/well) were starved for 16 hours. Next, these cells were trypsinized and treated with vehicle or 80 μmol/L LPA and loaded into the top chamber. Fetal bovine serum was then placed into the bottom chamber as a chemoattractant. Cells were incubated at 37°C in a humidified atmosphere and allowed to migrate through the chemotaxis chamber for 24 hours. After incubation, the cells remaining at the upper surface were completely removed using a cotton carrier. The migrated cells on the bottom of chemotaxis chamber were stained with hematoxylin and eosin. The experiments were repeated in triplicate wells and the migrated cells were counted microscopically (200 × ) in five different fields per filter. Cell invasion capability was assessed by using a Matrigel invasion chamber (Becton-Dickenson, Bedford, MA, USA) according to the manufacturer’s instructions. The procedures were essentially the same as those for the migration assay using Transwell chemotaxis chambers.

Statistical analysis

Results are reported as mean ± standard deviation. Statistical analyses were performed using rank-sum test. P<0.05 was considered statistically significant. The Statistical Package for the Social Sciences for Windows, version 13.0 (SPSS, Inc., Chicago, IL, USA) was used to perform the analysis.

Results

LPA induces uPA activation in ovarian cancer SKOV-3 cells

The treatment of SKOV-3 cells with 80 μmol/L LPA for 0-24 hours showed that LPA significantly increased the levels of uPA activity in a time-dependent manner (Figure 1A). Also, the treatment with increasing concentrations (0-80 μmol/L) of LPA for 24 hours showed that LPA significantly increased the levels of uPA activity in a dose-dependent manner (Figure 1B). These results were consistent with those described previously (15,16). A maximum increase in uPA activity when incubated with LPA occurred at 24 hours and 80 μmol/L.

Silencing of LPA₂ reduces LPA-induced uPA activation

SKOV-3 cells were transfected by LPA₂-specific siRNA and control siRNA. The relative LPA₂ mRNA level was determined by semiquantitative RT-PCR analysis. It was shown

Figure 1. Stimulation of urokinase plasminogen activator (uPA) activity in SKOV-3 ovarian cancer cells by lysophosphatidic acid (LPA). The uPA activity was measured by enzyme-linked immunosorbent assay. (A) SKOV-3 cells were incubated with 80 μmol/L lysophosphatidic acid (LPA) for 0, 3, 8, 12, and 24 hours before the collection of conditioned media. (B) SKOV-3 cells in 24-well plates (5 × 10⁵ per well) were cultured, starved in serum-free medium overnight, and incubated with increasing concentrations of LPA (0, 2, 20, 40, and 80 μmol/L) for 24 hours before the collection of conditioned media. Measurements were made in three separate experiments, and data are shown as mean ± standard deviation. Asterisk indicates P = 0.004 vs control. OD = optical density.
that the transfection with LPA2-specific siRNA led to >80% decrease in LPA2 mRNA levels, when compared with non-target control siRNA (NC-siRNA) (Figure 2A). Meanwhile, we found that the silencing of LPA2 expression resulted in ~55% reduction in LPA-induced uPA activity. In the culture medium without LPA stimulation, however, this change was minor (Figure 2B).

**Down-regulation of LPA2 decreases LPA-induced cell invasion**

In the culture medium without LPA stimulation, inhibiting LPA2 expression showed no significant differences in the invasion activity of the cells in the NC-siRNA- and LPA2-specific siRNA-transfected SKOV-3 cells (25.0 ± 3.2 vs 25.0 ± 3.7). In the presence of 80 µmol/L LPA, however, the increase of invasion was down-regulated from 7.3-fold in NC-siRNA-transfected SKOV-3 cells (178.0 ± 17.2 vs 25.0 ± 3.2) to 1.7-fold in LPA2 siRNA-transfected SKOV-3 cells (36.2 ± 3.3 vs 25.0 ± 3.7), approximately a 4.3-fold decrease in comparison with a negative control (178.0 ± 17.2 vs 36.2 ± 3.3, P = 0.009) (Figure 3A).

**Figure 2.** Effect of lysophosphatidic acid receptor-2-specific small interfering RNA (LPA2-siRNA) transfection on lysophosphatidic acid (LPA)-induced urokinase plasminogen activator activity. (A) Semi-quantitative reverse transcriptase-polymerase chain reaction results showed the average knocking down of LPA2 mRNA by siRNA. The fragment of LPA2 and β-actin (internal control) cDNA were 838bp and 550bp, respectively. (B) Enzyme-linked immunosorbent assay showed the decrease of urokinase plasminogen activator (uPA) secreted in the presence of 80 µmol/L LPA in LPA2 siRNA-transfected SKOV-3 cells compared with non-target control siRNA (NC-siRNA)-transfected SKOV-3 cells. Measurements were made in three separate siRNA transfection experiments, and data were depicted as the mean ± standard deviation. Gray bars – NC-siRNA; open bars – LPA2 siRNA; OD – optical density; asterisk indicates P = 0.004, LPA2-siRNA vs NC-siRNA transfection.

**Figure 3.** Lysophosphatidic acid receptor-2-specific small interfering RNA (LPA2-siRNA) transfection reduces LPA-induced increase of SKOV-3 invasion and migration. (A) Matrigel invasion assay of nontarget control and LPA2 siRNA treated with vehicle (0) or LPA (80 µmol/L). The transfected SKOV-3 ovarian cancer cells were starved for 16 hours and then trypsinized and treated with vehicle or 80 µmol/L LPA and loaded into the top chamber. The cells were incubated at 37°C for 24 hours. The number of cells invaded through the Matrigel was counted as described in Material and Methods section. (B) The migration activity of the transfected cells was examined using Transwell chemotaxis assay. The procedures were essentially the same as those for the invasion assay, except that there was no Matrigel. Data were depicted as the mean ± standard deviation. Asterisk indicates P = 0.009, LPA2-siRNA vs non-target control siRNA (NC-siRNA) transfection. Gray bars – NC-siRNA; open bars – LPA2-siRNA.
Knockdown of LPA₂ decreases LPA-induced cell migration

The number of migrated cells in the NC-siRNA- and LPA₂-specific siRNA-transfected SKOV-3 cells showed no significant differences in the absence of LPA (30.0 ± 3.5 vs 30.0 ± 4.3). However, after being stimulated with 80 µmol/L LPA, the number of migrated cells was significantly decreased in LPA₂-specific siRNA-transfected SKOV-3 cells (57.0 ± 7.6) compared with the NC-siRNA-transfected SKOV-3 cells (220.4 ± 25.5; \( P = 0.009 \)) (Figure 3B).

Discussion

In this report, we investigated the effect of LPA₂ on LPA-induced uPA activation in human ovarian cancer cell line SKOV-3. The data revealed that LPA induced uPA up-regulation in a dose- and time-dependent manner. Furthermore, we inhibited the expression of LPA₂ by siRNA technique and found that LPA treatment at 80 µmol/L induced a ~55% reduction of uPA activity in LPA₂-specific siRNA-transfected SKOV-3 cells, when compared with control siRNA-transfected SKOV-3 cells. This represents an important signaling pathway from the LPA₂ receptor in the mediation of uPA activation by LPA. LPA mediates diverse responses by binding to its specific receptors. In ovarian cancer, up to 90% tumor specimens have been found by Western blot analysis to overexpress LPA₂ receptor (19).

Degradation of the basement membrane is a requisite to the metastasis of tumor cells. uPA, a key protease that degrades the basement membrane, has been shown to contribute to tumor cell invasion. Since the inhibition of LPA₂ expression could decrease the level of uPA, we analyzed the effect of LPA₂ on LPA-induced ovarian cancer invasion. Matrigel invasion assay showed that down-regulation of LPA₂ expression by siRNA approach in SKOV-3 cells was accompanied by 78% inhibition of invasive capacity in response to LPA. The result demonstrated that LPA₂ played a crucial role in LPA-induced ovarian cancer cell invasion. Another study also showed that LPA₂ receptor is overexpressed only in ovarian cancer cells and not in normal ovarian epithelial cells (20). The finding suggested that, by inhibiting the LPA₂ receptor expression, we could interfere in the ovarian cancer cells without an impact on normal ovarian surface epithelial cells.

The ability of tumor cells to migrate is a prerequisite for tumor metastasis. We observed that down-regulation of LPA₂ expression in SKOV-3 cells resulted in 74% decrease in the migration capacity of the cells in response to LPA, suggesting that the key receptor for this phenomenon was LPA₂. A previous study found that the migration activity of pancreatic cancer cells was increased by LPA, mainly through LPA₁ receptor (5). Shida et al (21) also proved that LPA enhanced the migration of human colon carcinoma DLD1 cells by LPA₁ receptor, and that LPA₂, rather than LPA₁, was highly expressed in these cell lines. In ovarian cancer, however, LPA₁ had a minor role in LPA-induced cell migration, because it was expressed at similar levels in normal ovarian surface epithelial cells and ovarian cancer cell lines (14) and the effect was markedly inhibited by the down-regulation of LPA₂. Our results strongly suggested that LPA₂ is also capable of inducing cell migration in ovarian cancer cell line SKOV-3, although the mechanism remains unknown.

In summary, our study proved that LPA₂ receptor involved in LPA-dependent uPA activation and LPA₂ down-regulation significantly decreased LPA-induced invasion and migration in SKOV-3 ovarian cancer cells. These results suggest the importance of LPA₂ in response to LPA-induced pathological ac-
tions in ovarian cancer cells. Although there is not LPA2-selective antagonists at present, LPA2 may be a viable target for biological therapy in ovarian cancer. Thus, further research is needed to develop a novel LPA2 receptor antagonist and to investigate the role of LPA-LPA2 axis in tumor growth and metastasis of ovarian cancer using mouse xenograft models.

References