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S1P stimulates chemotactic migration and invasion in OVCAR3 ovarian cancer cells

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Abstract

OVCAR3 ovarian cancer cells express three sphingosine 1-phosphate (S1P) receptors, $S1P_1$, $S1P_2$, and $S1P_3$, but not $S1P_4$. Stimulation of OVCAR3 cells with S1P induced intracellular calcium increases, which were partly inhibited by VPC 23019 (an $S1P_{1/3}$ antagonist). S1P-induced calcium increases were mediated by phospholipase C and pertussis toxin (PTX)-sensitive G-proteins in OVCAR3 cells. S1P stimulated extracellular signal-regulated kinase, p38 kinase, and Akt which were inhibited by PTX. S1P-stimulated chemotactic migration of OVCAR3 cells in a PTX-sensitive manner, indicating crucial role of G_i protein(s) in the process. S1P-induced chemotactic migration of OVCAR3 cells was completely inhibited by LY294002 and SB203580. Pretreatment of VPC 23019 (an $S1P_{1/3}$ antagonist) completely inhibited S1P-induced chemotaxis. S1P also induced invasion of OVCAR3 cells, which was also inhibited by VPC 23019. Taken together, this study suggests that S1P stimulate chemotactic migration and cellular invasion, and VPC 23019-sensitive S1P receptor(s) might be involved in the processes.

Keywords: Sphingosine 1-phosphate; Ovarian cancer cells; Chemotaxis; Invasion; Pertussis toxin-sensitive G-protein

Ovarian cancer is one of serious clinical setting in worldwide. Especially in the United States, ovarian cancer is the leading cause of death from gynecologic diseases. More than 24,000 women are newly diagnosed in each year in the United States [1,2]. Since most women are diagnosed after ovarian tumors have spread through the abdominal cavity, it has been regarded as an important issue to understand the mechanism involved in ovarian cancer migration and invasion. Furthermore, revealing the factors and cell surface receptors which play an important role in ovarian cancer cell migration and invasion is also important.

Bioactive lipid molecule, sphingosine 1-phosphate (S1P), has been reported to modulate various physiological activities in some cell types [3,4]. S1P induces chemotactic migration and angiogenesis in human umbilical vein endothelial cells [5]. S1P has been shown to stimulate cell proliferation in several cell types [6,7]. S1P has also been reported to be present in the ascites of ovarian cancer patients [8]. S1P exhibits inhibitory effect on cell attachment to extracellular matrix proteins including laminin, collagens I and IV and fibronectin [8]. On the cell surface receptors for S1P, a family of G-protein-coupled receptors ($S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$) has been reported [4,9–11]. Although S1P has been regarded as an important adhesion modulator for ovarian cancer cells [8], the role of certain S1P receptor on the chemotactic migration and invasion in ovarian cancer has not been previously studied.

In this study, we investigated the role of S1P receptor on ovarian cancer cell migration and invasion using S1P and an $S1P_{1/3}$-selective antagonist.

Materials and methods

Reagents and cell culture. S1P and VPC 23019 were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama). The reverse transcription-

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polymerase chain reaction kit and fetal bovine serum (FBS) were purchased from Invitrogen Corporation (Carlsbad, CA). Phospho-extracellular signal-regulated protein kinase (ERK)1/2, phospho-p38 kinase, and ERK2 antibodies from New England Biolabs (Beverly, MA). LY294002, U-73122, U-73343, PD98059, and SB203580 were obtained from Calbiochem (San Diego, CA). OVCAR3 human ovarian cancer cells were purchased from American Type Culture Collection, Manassas, VA) and cultured in RPMI 1640 medium with 10% FBS, 1% sodium bicarbonate buffer.

RT-PCR analysis. mRNA was isolated by using a QIAshredder and an RNeasy kit (Qiagen, Hilden, Germany). mRNA, M-MLV reverse transcriptase, and pd(N)6 primers (Gibco BRL, Gaithersburg, MD) were used to obtain cDNA. The sequences of the primer used were as follows; $S1P_1$ receptor (691 bp product): forward, 5′-CGGTCTCTGACTACGTCA ACTATGATATC-3′, reverse, 5′-GCTGGCCTTGGAAATGTTCTTGC GGAACG-3′. $S1P_2$ receptor (611 bp product): forward, 5′-GCAGCTTGT ACTCGGAGTACCTGAAC-3′, reverse, 5′-CGATGGCCAACAGGAT GATGGAGAAG-3′. $S1P_3$ receptor (723 bp product): forward, 5′-CGTC TGTGAATGCCAAGTGATGGCAACTG-3′, reverse, 5′-CGAGTTGT TGTGGTTGGCCACCTTACG-3′. $S1P_4$ receptor (569 bp product): forward, 5′-GCCGGCTCATTGTTCTGCACTACAACC-3′, reverse, 5′-GC AGAAGAGGATGTAGCGCTTGGAGTAG-3′. We ran 35 PCR cycles at 94 °C (denaturation, 1 min), 57 °C (annealing, 1 min), and 72 °C (extension, 1 min). PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Ca^{2+} measurement. Intracellular calcium concentration ($[Ca^{2+}]_i$) was determined by Grynkiewicz's method using fura-2/AM [12]. Briefly, prepared cells were incubated with 3 μM fura-2/AM at 37 °C for 50 min in fresh serum free RPMI 1640 medium with continuous stirring. Cells (2×10^6) were aliquoted for each assay into Locke's solution [12]. Fluorescence was measured at 500 nm at excitation wavelengths of 340 and 380 nm.

Western blot analysis. OVCAR3 cells (2×10^6) were stimulated with the indicated concentrations of S1P. Cell extracts were separated in 10% SDS–polyacrylamide gel and, the proteins were blotted onto a nitrocellulose membrane. Subsequently, membranes were incubated with specific antibodies. Antigen–antibody complexes were visualized after incubating the membrane with 1:5000 diluted goat anti-rabbit IgG antibody coupled to horseradish peroxidase and detected by enhanced chemiluminescence.

Chemotaxis assay. Chemotaxis assays were performed using multiwell chambers (Neuroprobe Inc. Gaithersburg, MD) [13]. Polycarbonate membrane of 96-well chemotaxis chamber was precoated with fibronectin (20 μg/ml). OVCAR3 cells were suspended in RPMI at 1×10^6 cells/ml, and 25 μl of this suspension was placed into the upper well of a chamber separated by an 8 μm precoated polyhydrocarbon filter from S1P containing lower well. After incubation for 4 h at 37 °C, migrated cells were then counted in three randomly chosen high power fields (400×) [13].

Invasion assay. Invasion assays were performed using Matrigel coated invasion chambers (Corning, Corning, NY), as described before [14]. Precoated filters (6.5 mm in diameter, 8 μm pore-size, Matrigel 100 μg/cm^2) were rehydrated with 250 μl medium, and 5×10^4 cells in 200 μl medium with or without S1P were seeded into the upper part of each chamber. After incubation for 24 h at 37 °C, nonmigratory cells on the upper surface of the filter were wiped with a cotton swab, and migrated cells on the lower surface of the filter were fixed and stained with 0.125% Coomassie blue in a methanol:acetic acid:water mixture (45:10:45 v/v/v). Random fields were counted under a light microscope.

Statistics. The results are expressed as means ± SE of the number of determinations indicated. Statistical significance of differences was determined by ANOVA. Significance was accepted when $P < 0.05$.

Results

Functional expression of S1P receptors in OVCAR3 human ovarian cancer cells

We investigated whether cell surface receptors for S1P are expressed on OVCAR3 human ovarian cancer cells. To determine which S1P receptor isoforms are expressed on OVCAR3 human ovarian cancer cells, we analyzed the mRNA expressions of different S1P receptors by semi-quantitative RT-PCR. As shown in Fig. 1A, OVCAR3 human ovarian cancer cells expressed three isoforms of S1P receptor, namely, $S1P_1$, $S1P_2$, and $S1P_3$. We were unable to detect $S1P_4$ expression (Fig. 1A).

Activation of some G-protein-coupled receptor for lysolipids such as $S1P_2$ or LPA_3 links to phospholipase C (PLC) activation and subsequent production of inositol-1,4,5-trisphosphate and $[Ca^{2+}]_i$ increase [15,16]. We, then, examined the effect of S1P on $[Ca^{2+}]_i$ in OVCAR3 cells. As shown in Fig. 1B, the stimulation of OVCAR3 cells with 2 μM of S1P caused a $[Ca^{2+}]_i$ increase. We also investigated the role of pertussis toxin (PTX)-sensitive G-proteins on S1P-induced $[Ca^{2+}]_i$ increase. Cultured OVCAR3

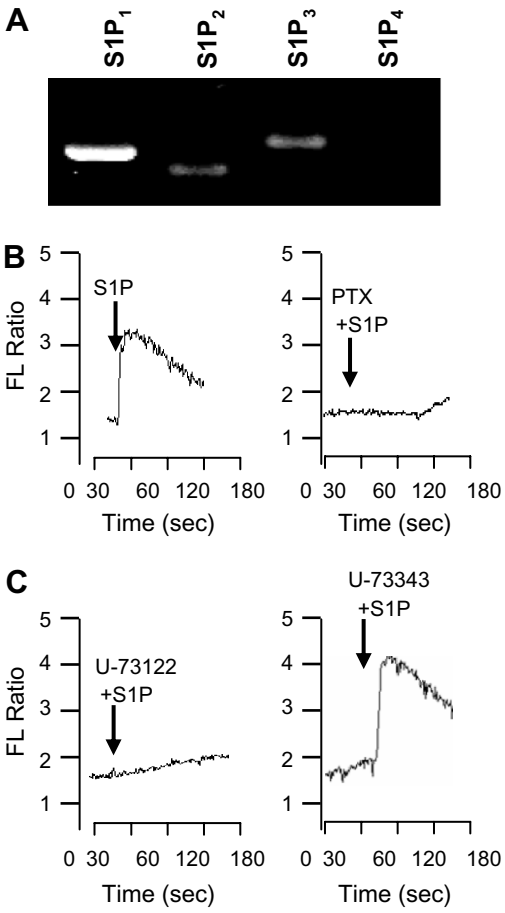

Fig. 1. Functional expression of S1P receptors in OVCAR3 human ovarian cancer cells. RT-PCR analysis was performed on mRNA isolated from cultured OVCAR3 cells. The data presented are representative of three-independent experiments (A). OVCAR3 cells were preincubated in the absence or presence of 100 ng/ml of PTX for 24 h. OVCAR3 cells were then loaded with fura-2/AM and $[Ca^{2+}]_i$ was determined fluorometrically after stimulation with 2 μM S1P (B). OVCAR3 cells were pretreated with 5 μM of U-73122 or 5 μM of U-73343 prior to 2 μM S1P (A), and $[Ca^{2+}]_i$ was determined. Relative intracellular Ca^{2+} concentrations are expressed as fluorescence ratios (340:380 nm). Data are representative of four-independent experiments (B,C).

cells were preincubated with 100 ng/ml of PTX prior to being stimulated with 2 μM of S1P. We found that pretreatment with PTX blocked $[Ca^{2+}]_i$ increase by S1P (Fig. 1B), showing that S1P induces $[Ca^{2+}]_i$ increase in a PTX-sensitive manner. One of the well-known mechanisms of $[Ca^{2+}]_i$ increase in the absence of extracellular calcium is the PLC-dependent response [17]. To determine the role of PLC on S1P-induced $[Ca^{2+}]_i$ increase, we pretreated the cells with a specific PLC inhibitor, U-73122 or with its inactive analogue U-73343. Fig. 1C shows that U-73122, but not U-73343, completely inhibited S1P-induced $[Ca^{2+}]_i$ increase. This result indicates that S1P stimulates $[Ca^{2+}]_i$ increase via PLC activation in OVCAR3 cells.

S1P stimulates ERK, p38 kinase, and Akt in OVCAR3 cells via PTX-sensitive manner

Mitogen-activated protein kinase (MAPK) has been reported to mediate extracellular signals to the nucleus in various cell types [18]. In this study, we examined whether S1P stimulate MAPKs by using Western blot analysis with anti-phospho-specific antibodies against each enzyme. When OVCAR3 cells were stimulated with 2 μM of S1P for different times, the phosphorylation level of ERK was transiently increased, showing dramatic activity within 2–5 min of stimulation, and returned to baseline 10 min after stimulation (Fig. 2A). Another important MAPK, p38 kinase was also transiently activated by S1P stimulation with kinetics that resembled those of ERK activation (Fig. 2A). We examined the effect of PTX, a specific inhibitor of G_i type G proteins, on S1P-induced MAPK phosphorylation. When OVCAR3 cells were preincubated with 100 ng/ml of PTX prior to being stimulated with 2 μM of S1P, ERK and p38 kinase phosphorylations by S1P were almost completely inhibited (Fig. 2B). The result indicates that S1P stimulate ERK and p38 kinase activation via PTX-sensitive G-proteins.

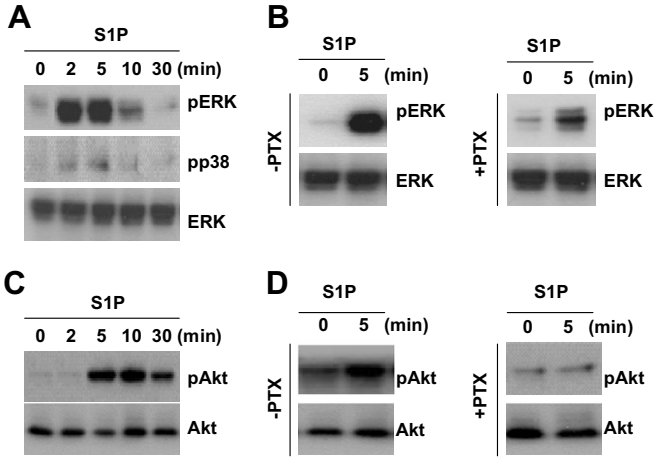

Fig. 2. S1P stimulates ERK, p38 kinase and Akt phosphorylation in a PTX-sensitive manner in OVCAR3 cells. OVCAR3 cells were stimulated with 2 μM of S1P for various times (A,C). OVCAR3 cells, which were preincubated in the absence or presence of 100 ng/ml of PTX for 24 h, were stimulated with 2 μM of S1P for 0 or 5 min (B,D). Each sample (30 μg of protein) was subjected to 10% SDS–PAGE, and phosphorylated ERK, p38 kinase (A,B) or Akt (C,D) was determined by immunoblotting using anti-phospho-specific antibodies. The results shown are representative of at least three-independent experiments (A–D).

Akt has been reported to play important roles in the regulation of several cellular responses, such as, cell migration and cell survival [19]. When OVCAR3 cells were stimulated with 2 μM of S1P for different times, Akt phosphorylation was transiently increased, showing maximal activity after 5–30 min of stimulation (Fig. 2C). S1P-induced Akt phosphorylation was found to be almost completely inhibited by PTX (Fig. 2D), indicating that S1P stimulates Akt activation via a PTX-sensitive pathway.

S1P induces OVCAR3 chemotaxis which is inhibited by VPC 23019

We examined the effect of S1P on the migration of OVCAR3 cells. Since intracellular signaling through several chemoattractant receptors results in the activation of some kinds of integrins which are involved in the leukocytes adhesion and migration [20], we investigated the effect of S1P on ovarian cancer cell migration on specific extracellular matrix. S1P induced a chemotactic migration of OVCAR3 cells on fibronectin (Fig. 3A). Fig. 3A shows the concentration–responsive curve of S1P-induced OVCAR3 cell migration, showing maximal activity at 10 nM. We also examined the effect of PTX on S1P-induced chemotaxis in human ovarian cancer cells. When OVCAR3 cells were preincubated with 100 ng/ml of PTX prior to the chemotaxis assay, the number of cells migrating toward S1P was completely reduced in comparison to the numbers of cell not treated with PTX (Fig. 3A), strongly suggesting the involvement of PTX-sensitive G protein(s).

In order to determine which receptor is involved in S1P-induced OVCAR3 chemotaxis, we utilized the $S1P_{1/3}$-selective antagonist, VPC 23019. As shown in Fig. 3B, S1P-induced OVCAR3 chemotaxis was completely inhibited by preincubating OVCAR3 cells with 10 μM of VPC 23019. These results strongly indicate that S1P act at VPC 23019-sensitive S1P receptors, resulting in OVCAR3 chemotaxis.

S1P-induced OVCAR3 chemotaxis is p38 kinase and phosphoinositide 3-kinase (PI3K)-dependent signaling

Several reports have shown that many chemoattractants stimulate PI3K-mediated Akt activity and that PI3K pathway is involved in the chemotaxis of leukocytes stimulated by these chemoattractants [21,22]. Since we observed stimulation of the cells with S1P led to a rapid increase in the phosphorylation of Akt (Fig. 2C), we examined whether PI3K pathway is required for S1P-induced OVCAR3 cell chemotaxis. Preincubation of the cells with 50 μM of LY294002, a well-known PI3K inhibitor, for 15 min at

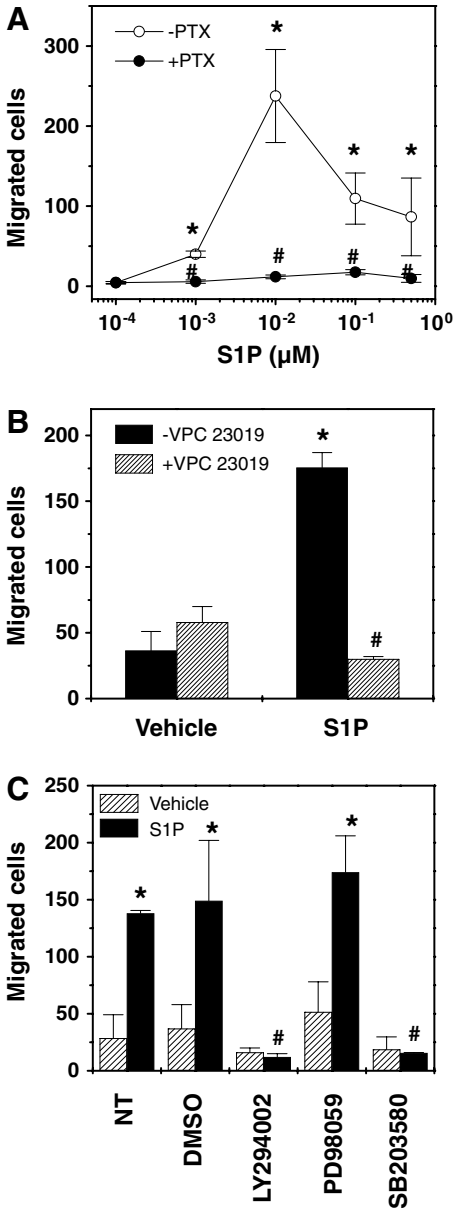

Fig. 3. Regulation of S1P-induced OVCAR3 chemotaxis. OVCAR3 cells, which were preincubated in the absence or presence of 100 ng/ml of PTX for 24 h, were used for chemotaxis assay with several concentrations of S1P (A). OVCAR3 cells were preincubated in the absence or presence of 10 μM of VPC 23019 for 30 min, and then chemotaxis assay was performed with several 10 nM of S1P (B). OVCAR3 cells were treated with effective concentrations of vehicle (DMSO), PD98059 (50 μM), SB203580 (20 μM), or LY294002 (50 μM) for 15 min (60 min for PD98059). And then the cells were used for chemotaxis assay with 10 nM of S1P (C) for 4 h. The numbers of migrated cells were determined by counting them in three high power fields (400×). The data are presented as means ± SE of three-independent experiments each performed in duplicates (A–C). $^{*}P < 0.05$ from vehicle treated. $^{\#}P < 0.05$ from S1P only treated.

37 °C prior to stimulation with S1P did indeed affect cellular chemotaxis (Fig. 3C). These results indicate that S1P activates PI3K pathway and that this signaling is required for S1P-induced chemotaxis of OVCAR3 human ovarian cancer cells.

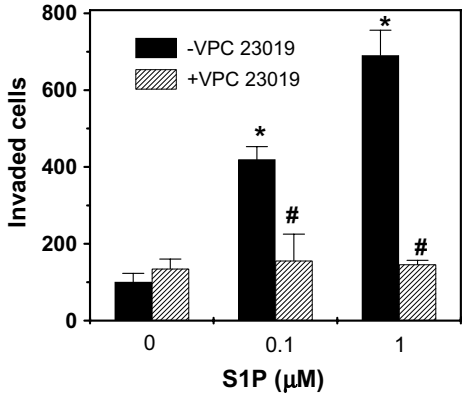

Fig. 4. S1P stimulates OVCAR3 cell invasion in a VPC 23019-sensitive manner. For invasion assays, the lower and upper parts of Transwells were coated with Matrigel. OVCAR3 cells, which were preincubated in the absence or presence of 10 μM of VPC 23019 for 30 min, were cultured in the absence or presence of S1P (0.1 or 1 μM). Cell invasiveness was determined by determining the ability of cells to pass through the Matrigel-coated filter. After 24 h, cells on the bottom of the filter were fixed, stained, and counted as described in Materials and methods. The data shown represent means ± SE of two separate experiments performed in duplicate. $^{*}P < 0.05$ from vehicle treated. $^{\#}P < 0.05$ from S1P only treated.

We also examined the role of ERK or p38 kinase on S1P-induced OVCAR3 chemotaxis. When OVCAR3 cells were preincubated with PD98059 or SB203580 prior to chemotaxis assay, S1P-induced OVCAR3 chemotaxis was significantly blunted by SB203580, however the S1P-induced chemotaxis of the cells was not inhibited by PD98059 (Fig. 3C). This implies p38 kinase-mediated signaling is involved in S1P-induced OVCAR3 chemotaxis.

S1P stimulates OVCAR3 in vitro invasion

The finding that S1P stimulates chemotactic migration in OVCAR3 cells led us to investigate the effect of S1P on the *in vitro* invasion of OVCAR3 cells. S1P significantly induced OVCAR3 *in vitro* invasion (Fig. 4), and preincubation with VPC 23019 prior to invasion assay completely inhibited S1P-elicited *in vitro* invasion (Fig. 4), thus demonstrating that the stimulating effect of S1P on *in vitro* invasion may be mediated by $S1P_1$ or $S1P_3$.

Discussion

Revealing extracellular factors and their target receptors which involve in the regulation of ovarian cancer cell migration, and invasion has been an important issue for the development of drugs for ovarian cancer therapy. Previously several groups have reported that various extracellular stimuli are involved in the regulation of ovarian cancer cell chemotactic migration and invasion [20,23]. However, the role of S1P and its receptors in ovarian cancer cell chemotaxis and invasion has not been studied. In the present study, we found for the first time that S1P stimulates the chemotactic migration and invasion of human

ovarian cancer cells. This result suggests that S1P and VPC 23019-sensitive S1P-specific receptor(s) have a potential role in various functional aspects related to ovarian cancer cell migration and invasion.

Previous reports have suggested that S1P might have its specific cell surface receptors, $S1P_{1-4}$ [4,9–11]. We also found that S1P induced chemotactic migration and invasion of OVCAR3 cells. Furthermore, as shown in Figs. 3 and 4, VPC 23019 completely inhibited S1P-induced OVCAR3 cell migration and invasion. VPC 23019 is an antagonist for $S1P_1$ and $S1P_3$ [24]. Since OVCAR3 express not only $S1P_1$ but also $S1P_3$, we suggest that S1P may stimulates OVCAR3 via $S1P_1$ or $S1P_3$ resulting in invasion. Previous reports have demonstrated controversial role of S1P receptor family on the chemotactic migration of some cells [25–27]. $S1P_1$ has been reported to be essential for the lymphocyte recruitment [25], however, $S1P_2$ has been demonstrated to have inhibitory regulation of cell migration [26]. In case of $S1P_4$, it does not induce migration of the cells, but does induce suppression of T cell proliferation and generation of several cytokines, including interleukin-2, and interferon-γ [27]. In this study, we also suggest that VPC-sensitive S1P receptor(s) ($S1P_1$ or $S1P_3$) may be involved for the S1P-induced OVCAR3 chemotactic migration and invasion.

In our study, we investigated the effect of PTX, which specifically inactivates $G_{i/o}$-mediated signaling pathways, on S1P-induced signaling. When OVCAR3 cells were treated with 100 ng/ml of PTX for 24 h prior to S1P stimulation, S1P-induced $[Ca^{2+}]_i$ increases were completely inhibited (Fig. 1B). Furthermore, activation of ERK, p38 kinase, and Akt, and chemotactic migration with S1P were completely inhibited by treatment of PTX, as shown in Figs. 2B and D. These results also imply that S1P utilizes PTX-sensitive G-protein-coupled receptor. Our investigation of the signaling triggering S1P-induced chemotaxis in OVCAR3 cells using specific inhibitors such as PD98059, SB203580, LY294002 and Western blot analysis, identified the critical roles of p38 kinase and PI3K. Taken together, it is considerable that S1P stimulate PTX-sensitive G-protein-coupled signaling, resulting in chemotactic migration and invasion via p38 kinase and PI3K.

In conclusion, we found that S1P induced chemotactic migration and invasion of OVCAR3 human ovarian cancer cells by modulating several activities of intracellular signaling molecules such as p38 kinase and PI3K. Since this study is the only report upon the role of S1P and VPC 23019-sensitive S1P receptor(s) ($S1P_1$ or $S1P_3$) on chemotaxis and invasion in human ovarian cancer cells, further studies on the pathological and physiological roles of S1P and on its specific cell surface receptor(s) in ovarian cancer cells are required.

Acknowledgments

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