

## Inhibitory Effects of Sphingosine 1-Phosphate on Proliferation of PC-3 Human Prostate Cancer Cells

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**Jia-Jun Liao, Yu-Ting Huang, and Hsinyu Lee (2005)** Inhibitory effects of sphingosine 1-phosphate on proliferation of PC-3 human prostate cancer cells. *Zoological Studies* 44(2): 219-227. Prostate cancer is the most-common cancer in adult men and the 2nd-leading cause of cancer deaths in Western countries. Although androgens and peptide growth factors have been implicated in this disease, determinants of the pathologic growth of prostate cancer are still unclear. Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are both potent lysophospholipid growth factors with diverse biological activities and have been suggested as being important in regulating the proliferation and metastasis of cancer cells. LPA activates the ERK pathway and induces proliferation of the human prostate cancer cell line, PC-3. However, the effect of S1P on prostate cancer is still poorly understood. In this study, we found that S1P inhibited cell proliferation through an apoptosis-independent and necrosis-dependent mechanism and caused cell cycle arrest in the G<sub>1</sub> phase of PC-3 cells. S1P also induced significant rounding of cells and actin reorganization. These effects are likely mediated through activation of the S1P<sub>5</sub> receptor. In conclusion, we propose that S1P might change cell-ECM interactions through cytoskeletal rearrangement, thereby influencing the proliferation of prostate cancer cells. <http://www.sinica.edu.tw/zool/zoolstud/44.2/219.pdf>

**Key words:** S1P, Prostate cancer, Cell death, Cell cycle arrest, Cytoskeleton rearrangement.

Prostate cancer is the most-commonly diagnosed malignant tumor in men and is the 2nd-leading cause of cancer deaths in Western countries. Cancer of the prostate is multifocal, and the cancerous tissue is commonly observed to contain multiple independent lesions, suggesting heterogeneity of the disease (Foster et al. 2000). Although steroid androgens and peptide growth factors have been implicated (Agus et al. 1999, Djakiew 2000), determinants responsible for the pathologic growth of prostate cancer remain poorly understood.

The bioactive lysophospholipids (LPLs), including lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), are membrane-derived lipid mediators, which are generated from phospholipid precursors in the membrane and secreted by platelets, macrophages, epithelial cells, and

some cancer cells (Goetzl and An 1998, Xie et al. 2002a). These lysophospholipids regulate migration, proliferation, and survival of multiple cell types (Goetzl et al. 2002, Spiegel and Milstien 2003b). They bind multiple G protein-coupled receptors of the endothelial differentiation gene (Edg) family. To date, 9 members of the family have been discovered in mammalian cells: LPA<sub>1</sub> (Edg-2), LPA<sub>2</sub> (Edg-4), LPA<sub>3</sub> (Edg-7), and LPA<sub>4</sub> are receptors for LPA, while S1P<sub>1</sub> (Edg-1), S1P<sub>2</sub> (Edg-5), S1P<sub>3</sub> (Edg-3), S1P<sub>4</sub> (Edg-6), and S1P<sub>5</sub> (Edg-8) are receptors for S1P (Moolenaar et al. 1997, An et al. 1998b). Activation of these receptors results in a wide spectrum of intracellular events, such as increases in inositol phosphates and intracellular calcium (An et al. 1998a), inhibition of adenylyl cyclase (Tigyi et al. 1996), and activation of kinases such as protein kinase C

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(PKC) and many other signaling pathways (Seewald et al. 1999). On the other hand, S1P might mediate VEGF-induced activation of Ras, thus consequently activating ERK/MAPK signaling and cell growth (Spiegel and Milstien 2003b). In addition, S1P also stimulates angiogenesis *in vivo* (Lee et al. 1998) and activates  $\alpha_2\beta_1$  integrin (Lee 2004).

LPA has been shown to act as an autocrine factor for prostate cancer cells (Xie et al. 2002b). It has been reported that LPA induces prostate cancer cell proliferation through activation of the ERK pathway (Qi et al. 1998, Kue et al. 2002). However, the effects of S1P on prostate cancer are largely unknown. In the present study, we show that S1P inhibits proliferation of the prostate cancer cell line, PC-3. In addition, S1P also causes cell rounding and actin cytoskeleton rearrangement. Our results suggest a novel role for S1P as a growth inhibitory mediator for prostate cancer cells.

## MATERIALS AND METHODS

### Reagents

Sphingosine 1-phosphate (S1P), oleoyl-lysophosphatidic acid (LPA), bovine serum albumin (BSA), 4',6-diamino-2-phenylindole (DAPI), propidium iodide (PI), and RNase A were purchased from Sigma (St. Louis, MO). Fetal bovine serum and RPMI-1640 were obtained from Hyclone Laboratories (Logan, UT). Trypan blue stain (0.4%), TRizol, penicillin-streptomycin, and trypsin-EDTA were purchased from Invitrogen (Grand I., NY). Rhodamine-labeled phalloidin was obtained from Molecular Probes (Eugene, OR). Triton X-100 was purchased from Amresco (Solon, OH).

### Cell culture

The prostate cancer cell line, PC-3, was kindly provided by Dr. Jih-Hua Guh's laboratory (Department of Pharmacology, National Taiwan University Hospital, Taipei, Taiwan). Cells were cultured in RPMI1640 with 10% FBS, penicillin (100 IU/ml), streptomycin (100 IU/ml), and glutamine (5 mM) in a humidified incubator with 5% CO<sub>2</sub> at 37°C. Trypsin-EDTA (0.05%) was used to detach cells for the experiments.

### RNA extraction and reverse-transcriptase polymerase chain reaction (RT-PCR)

Cells were cultured to around 80% confluence, and then were serum-starved overnight in serum-free medium. Total RNA was isolated using the TRizol reagent (Invitrogen) following the manufacturer's instructions. One microgram of total RNA was reverse-transcribed using a reverse-transcriptase enzyme (NEB, Beverly, MA). Polymerase chain reactions (PCRs) for Edg receptors were performed by 35 programmed cycles of 94 °C for 30 s (denaturation), different annealing temperatures of each respective primer set for 30 s, and 72 °C for 45 s (polymerization). Sequences of the oligonucleotide primers used and the expected sizes for the PCR products are shown in table 1. PCR products were resolved on 1% agarose gels, stained with ethidium bromide, and photographed. All data were quantified using TotalLab vers. 2.01 software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

### Lysophospholipid treatments

One-millimolar solutions of LPA and S1P were prepared in chloroform and methanol (1 : 9) and stored at -20°C. PC-3 cells were cultured to a subconfluent condition in complete media, washed with Dulbecco's phosphate-buffered saline (DPBS), and then serum-starved in serum-free medium for 16~24 h. LPA and S1P were then added to cultures of serum-free RPMI1640 supplemented with 0.005% fatty acid-free BSA as a carrier.

### Cell proliferation assay

The cell proliferation assay was carried out as described previously (Davaille et al. 2000). PC-3 cells at  $2 \times 10^4$  cells/well were seeded in 24-well plates in complete media for 24 h. Before the assays, media were changed to serum-free RPMI1640 for 16 h. Serum-free RPMI1640 medium containing serum and different concentrations of LPA or S1P was then added. Cell numbers were evaluated at various time points by directly counting the resuspended cells using a hemacytometer. Cell viability was evaluated by the trypan blue exclusion method. Cells were photographed using a Nikon (Tokyo, Japan) microscope with interference contrast optics (20x objective).

### Cell cycle analysis

Subconfluent cultures of PC-3 cells were serum-starved for 16 h, and incubated with 10% FBS or 10  $\mu$ M S1P for 16 h at 37°C. Cells were suspended in 1 ml of PBS and 300  $\mu$ l of staining solution containing 200  $\mu$ g/ml RNase A, 20  $\mu$ g/ml propidium iodide, and 0.1% Triton X-100. After incubation in a 37°C water bath in darkness for 20 min, the intensity of propidium iodide staining in each cell was analyzed by flow cytometry (CyFlow, Partec, Münster, Germany), and the cell cycle distribution was analyzed with a computer program (CyFlow® SL, Partec).

### Annexin-V staining

Subconfluent cultures of PC-3 cells were serum-starved for 16 h, and incubated with 10  $\mu$ M S1P for 24 h at 37°C. Arsenite (20  $\mu$ M) was used to induce apoptosis as the positive control (Maeda et al. 2001). Cells were suspended in 200  $\mu$ l PBS with FITC-conjugated Annexin-V (Strong Biotech Corporation, Taipei, Taiwan) according to the manufacturer's protocols. Treated cells were subsequently analyzed by flow cytometry (CyFlow, Partec).

### Propidium iodide uptake assay

The protocols of the PI uptake assay were

modified from Ogbourne et al. (2004). In brief, 1 x 10<sup>5</sup> cells/well of PC-3 cells was seeded in a 6-well plate for 1 d and subsequently serum-starved for 16 h. Cells were incubated with 10  $\mu$ M S1P or starvation medium for 24 h at 37°C and trypsinized using 0.05% trypsin-EDTA. After 500 xg centrifugation, cells were suspended in 1 ml PBS with 500 ng/ml propidium iodide at 37°C for 20 min in the dark. The intensity of propidium iodide uptake of PC-3 cells was analyzed by flow cytometry (CyFlow, Partec).

### Cell staining

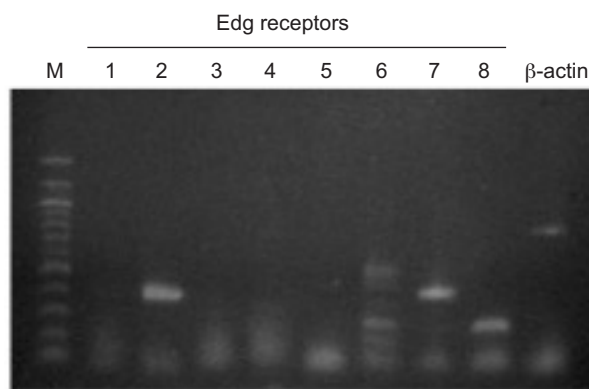
PC-3 cells were seeded on coverslips for 1 d, followed by 16 h of serum starvation. Serum-starved cells were then treated with 5  $\mu$ M LPA or S1P for 10 or 30 min. Treated cells were washed 3 times with DPBS and fixed with 3.7% formaldehyde in DPBS for 30 min at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100 in DPBS with 0.5% BSA for 30 min at room temperature. To investigate the cytoskeletal rearrangements, rhodamine-labeled phalloidin was added at 4°C overnight followed by 3 h at room temperature. Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI) before mounting. Cells were analyzed by fluorescence microscopy as described previously (Graler et al. 2003).

**Table 1.** Human Edg receptor primer sets

Primers		Sequences	Product size (bp)	Temp. (°C)
Edg1	S1P1	5'GACTCTGCTGGCAAATTCAAGCGAC 3'ACCCCTCCCAGTGCATTGTTACACAG	352	62
Edg5	S1P2	5'CTCTCTACGCCAAGCATTATGTGCT 3'TCAGACCACCGTGTGCCCCTC	512	62
Edg3	S1P3	5'CAAATGAGGCCTTACGACGCCA 3'TCCCATTCTGAAGTGTGCGTTC	701	61.2
Edg6	S1P4	5'AGCCTTCTGCCCCTCTACTC 3'GTAGATGATGGGGTTGACCG	339	62
Edg8	S1P5	5'GGAGTAGTTCGGAAGGACC 3'TCTAGAATCCACGGGTCTG	236	59.7
Edg2	LPA1	5'CGGAGACTGACTGTCAGCAC 3'GGTCCAGAACTATGCCGAGA	397	62
Edg4	LPA2	5'AGCTGCACAGCCGCTGCCCGT 3'TGCTGTGCCATGCCAGACCTTGTC	888	53
Edg7	LPA3	5'TTAGCTGCTGCCGATTCTT 3'ATGATGAGGAAGGCCATGAG	392	62
	$\beta$ -actin	5'TTCTACAATGAGCTGCGTGTGGC 3'CCTGCTTGCTGATCCACATCTGC	814	62

## Statistical analysis

Results are expressed as the mean  $\pm$  the standard error of the mean (SEM). After exclusion of outliers by the R-test or Tn-test, the significance of differences between the control and different treatment groups was tested using two-way ANOVA followed by Fisher's protected least-significant differences test (StatView; Abacus Concept, Berkeley, CA). A value of  $p < 0.05$  was considered statistically significant.



**Fig. 1.** Edg receptor expression profiles of PC-3 cells. RT-PCRs for human Edg receptors were performed with cDNA derived from PC-3 cells. PCR products were resolved on 1% agarose gels, stained with ethidium bromide, and photographed. Only Edg-2, Edg-7, and Edg-8 were found to be expressed in PC-3 cells.  $\beta$ -actin was used as the internal control.

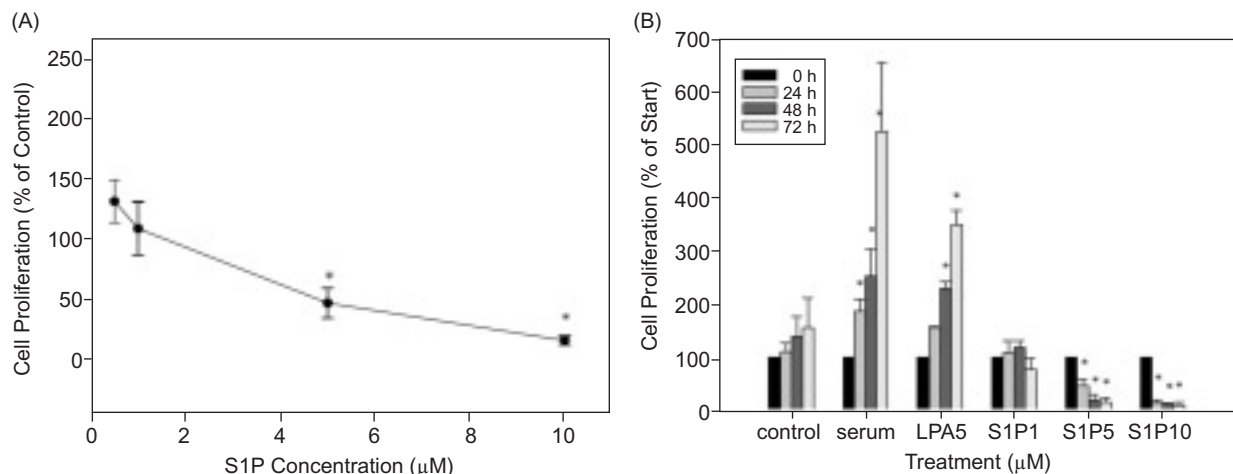
## RESULTS

### Expression of Edg-2, -7, and -8 by PC-3 cells

Lysophospholipids mediate their physiological functions through activation of multiple specific membrane receptors. Edg receptor expression profiles differed among different cell types and different developmental stages (Lee et al. 2002). To investigate the Edg receptor expression profile of PC-3 cells, we used RT-PCR to detect mRNA expressions of Edg-1 to Edg-8. As shown in fig. 1, only Edg-2 ( $LPA_1$ ), Edg-7 ( $LPA_2$ ), and Edg-8 ( $S1P_5$ ) were expressed in PC-3 cells. These results suggest that the effects of S1P on PC-3 cells likely occur through Edg-8.

### Inhibition of PC-3 cell proliferation by S1P

LPA is a well-known mitogen of PC-3 cells (Daaka 2002). However, even though it is considered to be a molecule with a similar structure and functions, the effects of S1P on PC-3 cells remain unknown. Therefore, we were interested in determining if S1P can also regulate the proliferation of PC-3 cells. We found that S1P inhibited the growth of PC-3 cells in a concentration-dependent manner (Fig. 2A). Using serum and 5  $\mu$ M LPA as a positive control, we did find significant induction of proliferation as described in previous work (Qi et al. 1998). The cell number of 10  $\mu$ M S1P-treated groups had decreased to 15.3% by 24 h. The cell number of 5  $\mu$ M S1P-treated groups had dropped



**Fig. 2.** S1P inhibition of PC-3 cell proliferation. PC-3 cells were treated with the indicated concentrations of S1P for 24 h (A) or 10% FBS, 5  $\mu$ M LPA, and either 1, 5, or 10  $\mu$ M S1P for 24, 48, and 72 h (B). Cells were washed by DPBS and trypsinized for counting using a hemacytometer. Similar experiments were repeated at least 3 times, and a representative result is shown in the figure. Data are presented as the mean  $\pm$  SEM. \*Statistically different as compared to the level in control cells ( $p < 0.05$ ).

to 48.6% by 24 h and to 15.5% by 72 h (Fig. 2B). However, with treatment at a low concentration of S1P (1  $\mu$ M), the difference was not significant.

### PC-3 cells arrested in the G<sub>1</sub> phase by S1P

S1P has been shown to be an antiapoptotic molecule in several reports (Spiegel and Milstien 2003a, Suomalainen et al. 2003). Therefore, we used flow cytometry to analyze the effect of S1P on the cell cycles of PC-3 cells (Fig. 3). After 16 h of 10  $\mu$ M S1P treatment, PC-3 cells were arrested in the G<sub>1</sub> phase (86.1% in the S1P-treated group and 69.7% in the control group). Compared with the 10% FBS-treated groups, only 3.36% of the cells entered the S phase after S1P treatment, while 13.47% of the 10% FBS-treated PC-3 cells were in the S phase. The percentage of S1P-treated groups which remained in the G<sub>2</sub>M phase (10.54%) was also only 1/2 of the control (21.6%) and 10% FBS-treated groups (21.53%).

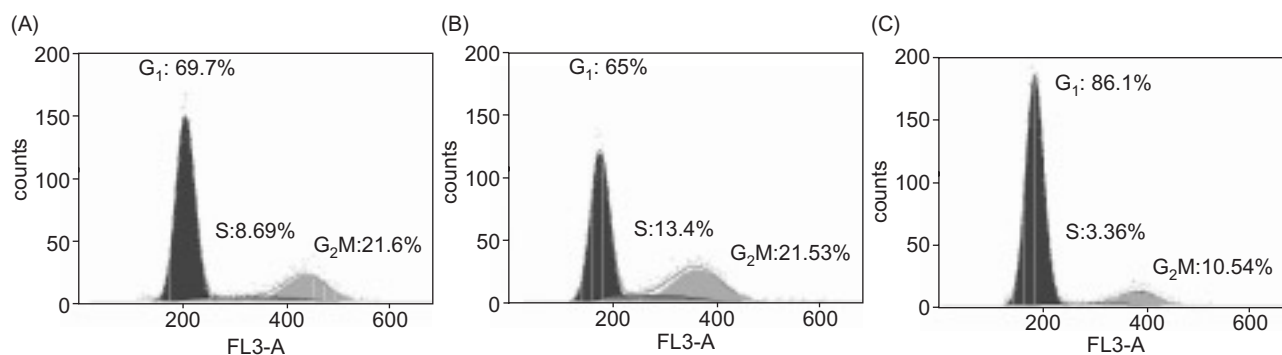
### PC-3 cell proliferation inhibited by S1P through an apoptosis-independent and necrosis-dependent pathway

Gennero et al. (2002) showed that in MEM cells under a low-cell-density culture condition, S1P activates stress-activated protein kinase (SAPK), which increases the degradation of S1P and the accumulation of sphingosine in the cytosol, and induces apoptosis. To investigate whether S1P inhibits PC-3 cell proliferation through inducing apoptosis, we used trypan blue to evaluate the viability of cells. No stained cells were observed in any of the experiments. Using flow cytometry to analyze the cell cycle of PC-3 cells after S1P treatment also showed no apoptotic populations (Fig. 3C). In addition, with DAPI stain-

ing of S1P-treated PC-3 cells, no DNA fragmentation was observed (Fig. 6I-L). We used FITC-conjugated annexin V to further confirm whether S1P induces apoptosis in PC-3 cells. Figure 4B shows that S1P treatment did not increase annexin-V staining. However, incubation of PC-3 cells with 20  $\mu$ M arsenite for 24 h induced apoptosis as reported in previous work (Maeda et al. 2001). Since cell membrane instability is an early indicator of necrosis (Walsh et al. 1998), we used a PI uptake assay to determine if S1P causes necrosis of PC-3 cells. As shown in Fig. 4D, PI uptake increased in the 10  $\mu$ M S1P-treated groups. These results suggested that S1P inhibits PC-3 cell proliferation through an apoptosis-independent and necrosis-dependent pathway.

### Cell rounding and cytoskeletal rearrangement of PC-3 cells induced by S1P

It is known that LPA and S1P treatments induce actin reorganization and cell morphological changes (Ridley and Hall 1992, Postma et al. 1996). However, the effects of S1P on the cytoskeleton of PC-3 cells remain unclear. Herein, we show that S1P also caused significant morphological changes in PC-3 cells. As shown in fig. 5B, most cells displayed a rounded morphology after 10  $\mu$ M S1P treatment for 24 h. Compared with the control (Fig. 5A), although some rounded cells were observed, the ratio of rounded cells in the S1P-treated group significantly increased. We propose that S1P might induce cytoskeletal rearrangement and cause cell rounding, which may lead to inhibition of proliferation. To investigate this assumption, rhodamine-labeled phalloidin was used to test whether S1P induces actin reorganization. Figure 6I shows that a large amount of stress fibers was expressed in PC-3 cells after 5



**Fig 3.** Cell cycle arrest of PC-3 cells caused by S1P. PC-3 cells were treated with the control (A), 10% FBS (B), or 10  $\mu$ M S1P (C) for 16 h. Cell cycles of PC-3 cells were analyzed by flow cytometry.



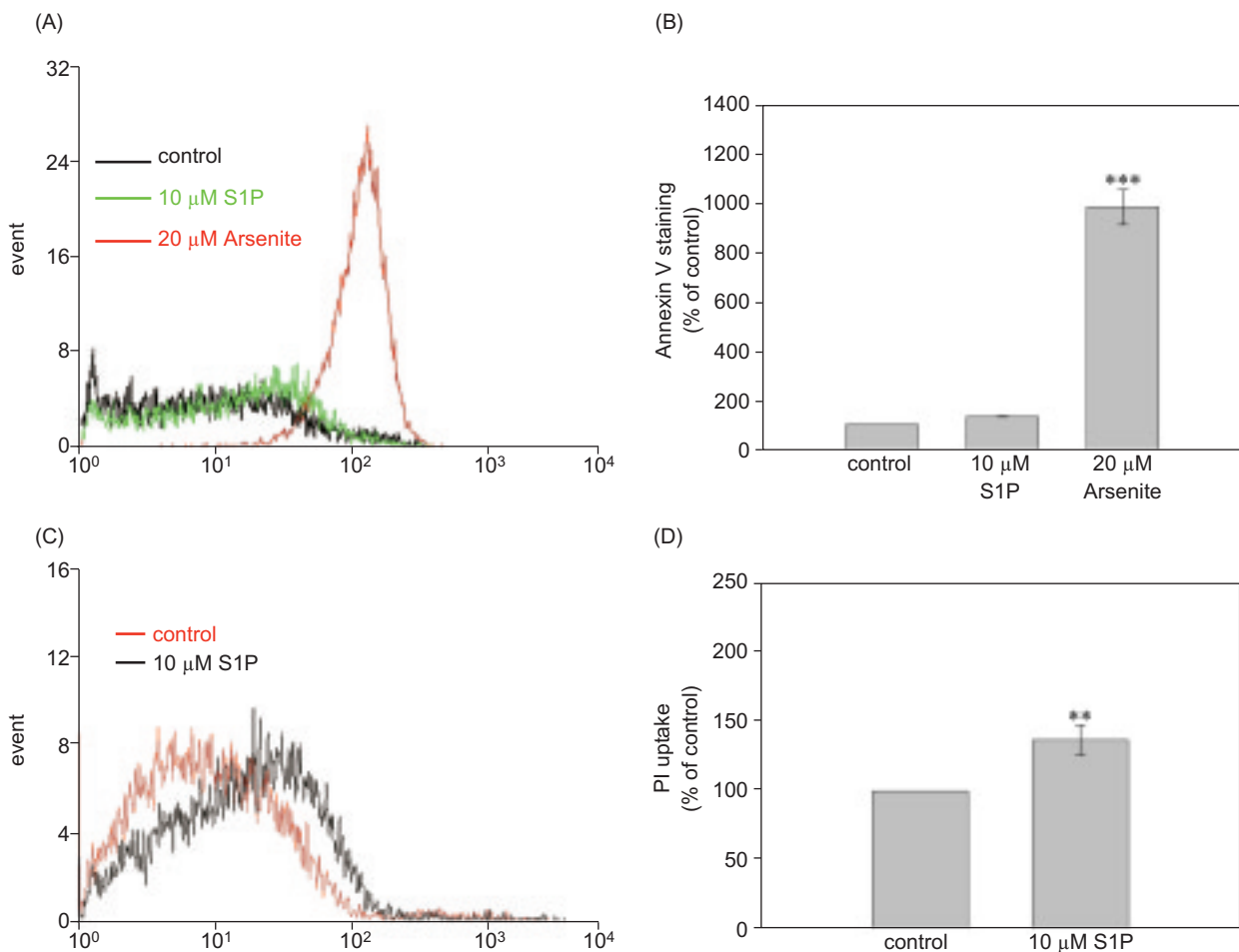
$\mu\text{M}$  S1P treatment for 10 min. After 30 min, actin fibers were concentrated in the region near the cell membrane, forming cortical rings (Fig. 6L), and some dark spots were also revealed near the nuclei (Fig. 6K). No similar pattern was observed in either the LPA-treated or control group. Additional pseudopodia formed after 5  $\mu\text{M}$  LPA treatment for 10 min. However, the stress fibers were obvious only after 30 min (Fig. 6H). Our results suggest that S1P induces actin reorganization and cell rounding in PC-3 cells.

## DISCUSSION

Lysophospholipids (S1P and LPA) are potent mitogenic mediators reported to influence cell behaviors in various pathophysiological situations

(Goetzl et al. 2002, Xu et al. 2003). LPA has also been shown to induce PC-3 cell proliferation through activation of the ERK pathway (Kue et al. 2002). In the present study, we demonstrate that exposure to S1P inhibits proliferation by PC-3 cells and causes cell cycle arrest in the  $G_1$  phase. At a physiological concentration, S1P also induced cell rounding and cytoskeletal rearrangement. We propose that the cytoskeletal rearrangement might be highly related to the inhibition of proliferation caused by S1P. Although the actual concentration of S1P in prostate cancer tissue is unknown, the S1P concentration can rise to micromolar levels in the plasma (Igarashi et al. 1998). Therefore, we attempted to use S1P concentrations in our experiments which are likely within the physiological range.

S1P has been reported to act as a mitogen in



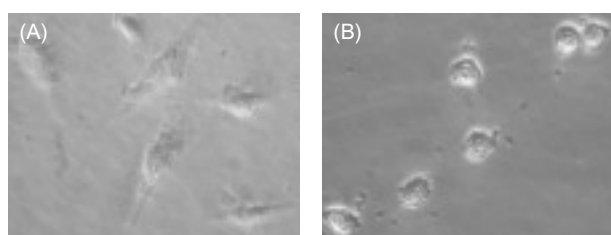
**Fig. 4.** S1P induction of necrosis but not apoptosis in PC-3 cells. PC-3 cells were treated with control media, 10  $\mu\text{M}$  S1P, or 20  $\mu\text{M}$   $\text{As}_2\text{O}_3$  for 24 h to induce apoptosis as the positive control. FITC-conjugated annexin V (A) or a non-detergent PI staining solution (C) was added to the cell suspensions after the indicated treatments, and cells were analyzed by flow cytometry. All experiments were repeated at least 3 times, and similar results were obtained. Data are presented as the mean  $\pm$  SEM (B, D). Asterisks are used to indicate a statistically significant difference as compared to the level in control cells (\*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ).

several cell systems (Goetzl et al. 2002). However, it has been noted that S1P also inhibits cell growth of certain cell types (Van Brocklyn et al. 1998, Kim et al. 2004). It is currently unclear whether the growth inhibitory actions of S1P are membrane receptor-mediated. For instance, S1P-induced apoptotic signals have been shown to be mediated through a receptor-independent mechanism in human hepatic myofibroblasts (Davaille et al. 2002) and rat mesangial cells (Gennero et al. 2002). However, other reports suggested that S1P-induced apoptosis is associated with activation of Edg-3/Edg-5 Gq-coupled receptors (Moore et al. 1999, Van Brocklyn et al. 1999). Our results suggest that S1P's inhibition of the proliferation of

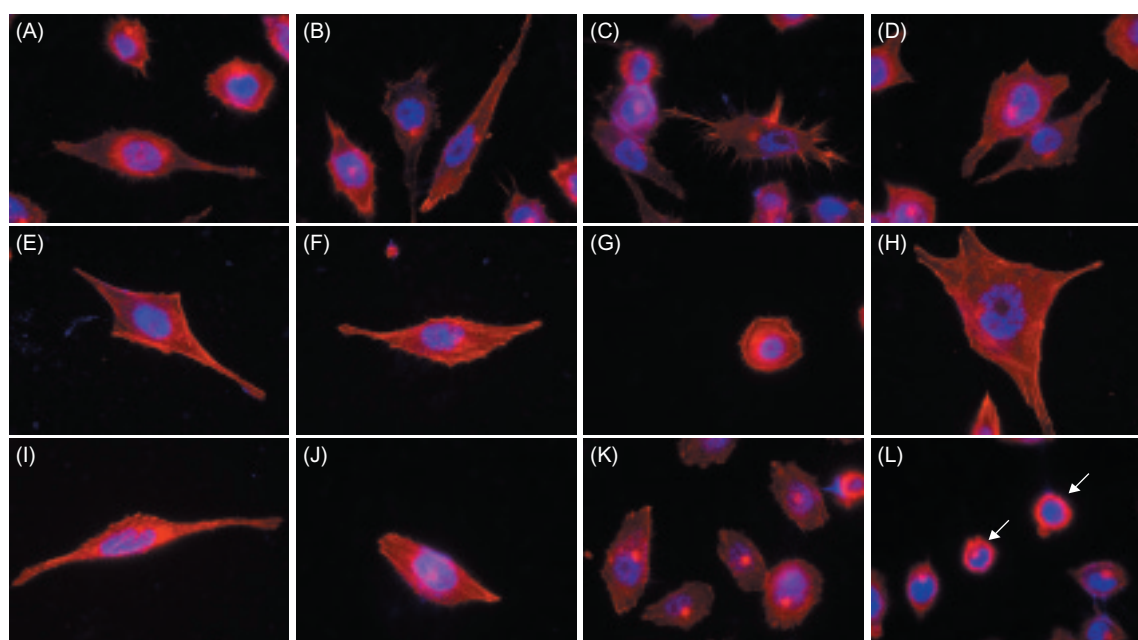
PC-3 cells is mediated through an apoptosis-independent and necrosis-dependent pathway. Currently, we cannot clearly distinguish if the inhibitory effect of S1P is due to a receptor-mediated mechanism. The specific small interference RNA technique for Edg-8 should be helpful for clarifying this issue in the near future.

Niedernberg et al. (2003) recently showed that overexpression of Edg-8 (S1P<sub>5</sub>) in CHO-K1 cells induces S1P-dependent cell rounding. In our experimental system, the only endogenously expressed S1P receptor in PC-3 cells was Edg-8, and we also found that S1P treatment induced cytoskeletal rearrangement and cell rounding. These results suggest that the cell rounding and actin reorganization induced by S1P might occur through Edg-8 and a downstream signaling pathway.

In the present study, we found that S1P inhibits PC-3 cell proliferation. To determine whether cell rounding is correlated with apoptotic cell death, nuclei were stained with DAPI. As shown in fig. 6, none of the rounded cells contained fragmented DNA in their nuclei, suggesting that S1P does not induce apoptosis in PC-3 cells. In addition, trypan blue exclusion, annexin V staining (Fig. 4A), and flow cytometric analysis (Fig. 3) also showed that S1P did not induce apoptosis.



**Fig. 5.** S1P induction of morphological changes in PC-3 cells. PC-3 cells were treated with control (A) or 10  $\mu$ M S1P (B) for 24 h and observed under interference contrast optics (with a 20x objective).



**Fig. 6.** Actin reorganization of PC-3 cells caused by lysophospholipids. PC-3 cells were starved in serum-free media for 16 h and then treated with 5  $\mu$ M LPA or S1P for the indicated times. Rhodamine-labeled phalloidin was used to detect the location of actin fibers in the cells. Cell nuclei were stained with DAPI. (A, B) control, 10 min; (C, D) control, 30 min; (E, F) LPA, 10 min; (G, H) LPA, 30 min; (I, J) S1P, 10 min; and (K, L) S1P, 30 min. The arrow indicates the formation of cortical rings.

Therefore, we exclude apoptosis as the cause of the inhibition of proliferation by S1P. In the PI uptake experiment, we found that S1P causes cell membrane instability without increasing annexin V staining (Fig. 4). Our results suggest that the growth inhibitory effect of S1P on PC-3 cells might be due to necrosis but not apoptosis.

The 2nd explanation for the inhibitory effect of S1P on PC-3 cells we propose is cell rounding. Our results showed that S1P induces actin fibers to concentrate in the region near the cell membrane where they form cortical rings, which was also correlated with cell rounding. This phenomenon might have 2 effects on the inhibition of PC-3 cell proliferation. First, cell rounding might cause PC-3 cells to more-easily detach from the culture dish. Loss of cell attachment may be a possible cause for the observed inhibition of proliferation. Second, cell rounding might weaken the interaction between integrins and the extracellular matrix (ECM). Interactions between integrins and the ECM have been shown to be important sources of cell growth and survival signals (Slack-Davis and Parsons 2004). Previous work demonstrated that cell morphological changes influence cell growth and survival through the integrin-ECM pathway (Tzima et al. 2002). In addition, changes of integrin-ECM interactions also regulate cell cycle arrest (Cordes and van Beuningen 2003). Therefore, we propose that the cell rounding caused by S1P might be correlated to the inhibition of PC-3 cell proliferation through integrin-ECM interactions.

In summary, our data demonstrate that S1P has antiproliferative properties in PC-3 cells, and this effect might be correlated with cell rounding and actin cytoskeletal rearrangement. This is the 1st study on the effects of S1P in PC-3 cells, and we also demonstrate the endogenous function of Edg-8 (S1P<sub>5</sub>). As only 1 S1P receptor (Edg-8) is expressed in PC-3 cells, we propose that PC-3 may be a good model system to investigate the physiological function and downstream signaling pathway of Edg-8.

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