

Original Article

Role of sphingosine 1-phosphate in human pancreatic cancer cells proliferation and migration

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Abstract: Deregulation of production or degradation of sphingosine 1-phosphate (S1P), a bioactive lipid, involves in tumor progression, metastasis and chemoresistance. Since the tumor progression effects of S1P and its mechanism in human pancreatic cancer is not fully understood, we investigated the role of S1P in Capan-1 and Panc-1 cells proliferation and migration. The effects of S1P on proliferation, invasion and migration were studied using MTT and transwell assay, respectively. The concentrations of MMP2 and MMP9 were detected by ELISA assay. Role of S1P on the expressions of tyrosine kinase and cell proliferation related proteins were assessed by western blot. Our results showed that cell proliferation and migration were mediated by low concentration of S1P treatment in both cell lines. In addition, we also investigated another survival mechanism of S1P in cell survival and tumor progression, Src signaling pathway. These results indicated that roles of S1P in tumor progression were S1P receptor-dependent through interaction with Src signaling pathway. In conclude, our data demonstrated the importance of this molecule as a target to design novel anticancer drugs in future through S1P receptors and Src signaling pathway.

Keywords: S1P, human pancreatic cancer, proliferation, invasion, Src, MMP

Introduction

In recent years, role of sphingolipids on the cancerous process has been the new field of cancer research, from their constitutive roles in the cell membrane to the signaling aspects of cellular bio-behaviours. The metabolic products of sphingolipids, such as ceramide, sphingosine, and their phosphorylated forms ceramide 1-phosphate (C1P) and sphingosine 1-phosphate (S1P), are biologically active and vital for variety of different cellular functions related to cell growth and migration [1, 2]. More and more experimental data over the past decade have indicated that S1P as one of the most important sphingolipid metabolites [3] and a source of some bioactive signaling molecules. The functions of S1P in physiological processes, for example, cellular proliferation and survival rely on the manner of an intracellular receptor-independent mechanism [4, 5]. Researches have shown that S1P not only as an intracellular signaling molecule, but also acts extracellularly through G-protein coupled

receptors (GPCRs) [6]. Researches indicated that S1P and S1P receptors (S1PRs) are involved in regulation of cell proliferation, migration and angiogenesis [7]. The balance between synthesis and degradation of S1P is firmly regulated by S1P kinase (SphK) and S1P phosphatase (SPP1, SPP2) [8, 9].

Modulation of normal tyrosine kinase level in blood or tissues can contribute to pathophysiological events in cardiovascular diseases, chronic inflammation, cancer, drug resistance and metastasis [10, 11]. Additionally various studies have shown the role of Src involved in migration and invasion of tumor cells and cancer recurrence after chemotherapy in breast, lung and pancreatic cancer [12, 13].

In this study we assessed the effect of S1P on malignant behavior of Capan-1 and Panc-1 cells such as proliferation, migration and invasion of these cells through Src pathway. Our results confirm the key role of S1P in tumor development. Blocking S1P receptors or its down-

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stream signaling can be considered as the novel strategies to control tumor growth and metastasis.

Materials and methods

Capan-1 and Panc-1 cell lines were purchased from American Type Culture Collection (Maryland, American). S1P, S1PR1 inhibitor (W123), Src inhibitors (PP2), BSA (bovine serum albumin), fibronectin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) and agar obtained from Sigma-Aldrich (St. Louis, MO, USA), Transwells® (polycarbonate, 6.5 mm, 8 µm pore size) were obtained from Corning (Corning, NY, USA). Antibodies to Src, phosphate Src (Tyr416), p38 MAPK, AKT (Thr308) were purchased from cell signaling technology (Danverse, USA). RPMI-1640 and FBS (Fetal Bovine Serum) were purchased from Invitrogen (Auckland, New Zealand).

Cell culture and treatment

Capan-1 and Panc-1 cells were maintained in RPMI-1640 supplied with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin on culture plates at 37°C in a humidified incubator supplemented with 5% CO₂. Prior to the experiment, cultured cells at 70-80% confluence were digested by 0.5% EDTA-Typsin, followed with centrifugation at 500 g for 5 min and the cell pellet was re-suspended in fresh culture media to be used in the experiments.

S1P was reconstituted in methanol at 10 mmol/L and stored at -20°C. Cells were trypsinized and then plated in 48-well plates (5×10³ cells/well). Before cell stimulation, growth medium was replaced with factors-reduced medium (RPMI-1640 contained with 5% FBS). Cells were stimulated with varying concentrations of S1P or pretreated with 10 µM Src inhibitor in serum-starved medium (RPMI-1640 contained with 0.5% FBS).

MTT assay

The logarithmic growth phase cells were cultured and treated as previous description for indicated time. The media in each well was replaced with 200 µL fresh media containing 20 µL of 5% MTT and incubated for 4 h at 37°C. After incubation period, media was removed and 200 µL DMSO was added to each well. The

absorbance of each well was measured at 570 nm after shaking for 10 min, employing a microplate reader (MultiPlex 600, USA). Wells with MTT solution and DMSO (without cells) were used as blank control.

Transwell assay

Two million cells were seeded in a 25 cm² flask with growth medium for at least 48 h, followed with serum starvation for 24 h before the migration experiment. Cells were centrifuged and re-suspended in starvation media and counted. Cells (about 5×10³) were seeded into each of the fibronectin coated Transwell® filters and incubated for 45 min. Filters were then transferred into bottom chambers which contained 500 µL serum starvation medium containing S1P. Chambers were incubated at 37°C and cells were allowed to migrate through the pores in the filter for 12 h. The cells of the upper side of the filter was then scraped free. The cells attached on the filter were fixed with methanol and stained with a Diff-Quick staining kit (Life Science Products, IHC World). The number of cells on the lower side of membranes was determined by measuring optical densities at 595 nm using a 96-well microplate reader model 355 (Bio-Rad).

Enzyme-linked immunosorbent assay

Capan-1 and Panc-1 cells were treated as described previously. MMP-9 and MMP-2 Elisa kits (R&D Systems, USA) were used for detection the concentrations of MMP-9 and MMP-2 in the cell culture supernatants. Each sample was analysed in triplicate and manipulated according kit protocol.

Western blot

Cells were lysed in RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% TritonX-100, 0.1% SDS, 1% sodium deoxycholate, 5 mM EDTA, 100 mM NaF, and 1 mM Na₃VO₄] containing a protease inhibitor cocktail for 30 min on ice, followed by centrifugation for 30 min at 16000 g. Protein concentrations were determined by the BCA method (Pierce, USA). Equal total proteins were electrophoresis by 12% SDS-PAGE gel, followed by transferred to PVDF membranes using a wet transblot system (Bio-Rad, Hercules, CA). The membranes were blocked for 1 h at room temperature with 5% nonfat milk and incubated

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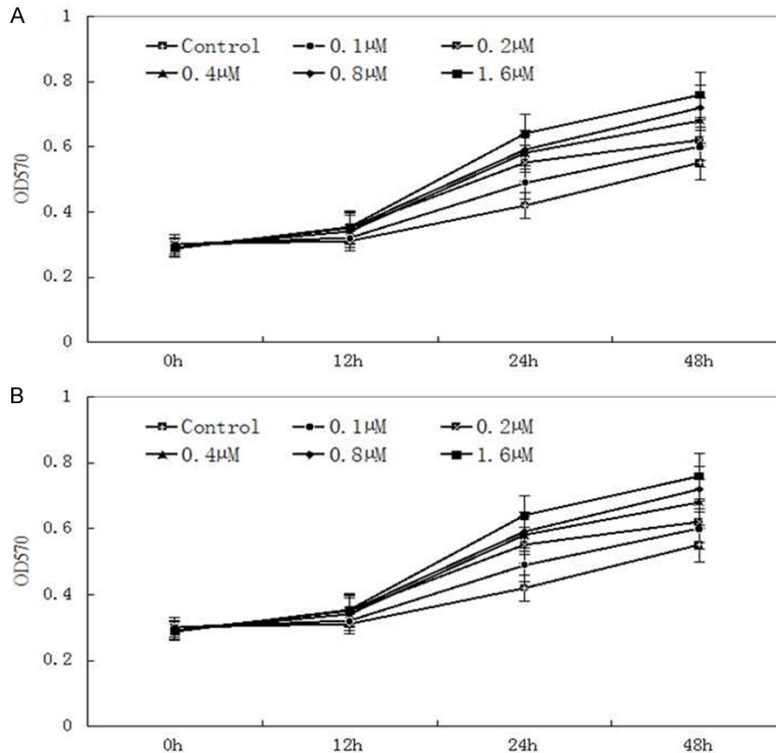


Figure 1. Effect of S1P on proliferation of Capan-1 and Panc-1 cells. MTT assay was used for detection the proliferation of cells that treated with 0.1-1.6 μ M S1P for 24 h (A, B).

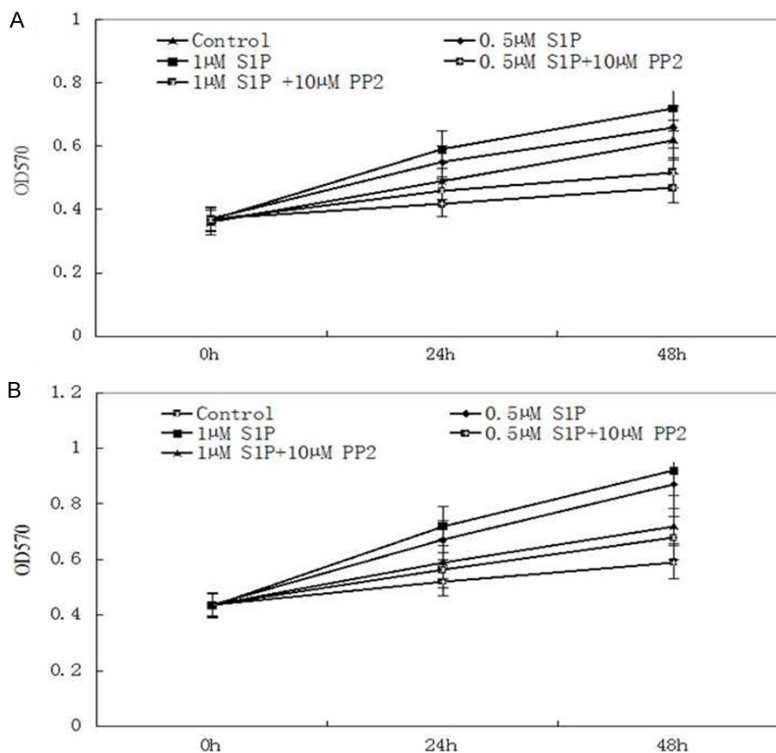


Figure 2. Effect of S1P and Src inhibitor on proliferation of Capan-1 and Panc-1 cells. Pre-incubation of Capan-1 and Panc-1 cells with 10 μ M PP2 for 30 min, the proliferation effect of S1P was monitored by MTT assay.

overnight at 4°C with antibodies against phosphate-Src (Tyr416), p38 MAPK, AKT (Thr308), β -actin (1:1000). After washing, the membrane was incubated for 1 h with HRP-conjugated goat anti-rabbit secondary antibody diluted 1:5,000 in PBST. After further washing and processing with Super Signal West Pico chemiluminescent substrate (Pierce, USA), the membrane was exposed to Fujifilm LAS 3000 Imager (Fuji, Japan). The band densities of the western blots were normalized relative to the relevant β -actin with Image J analyst software (NIH).

Statistical analysis

Results were presented as means \pm SD from three independent experiments. Statistical analysis was performed using SPSS software through ANOVA or student t-tests. $P < 0.05$ was considered as significant statistically.

Results

Proliferation effects of S1P on Capan-1 and Panc-1 cells

The effects of S1P in the proliferation of Capan-1 and Panc-1 cells were evaluated via MTT assay. We first treated Capan-1 and Panc-1 cells with increasing concentrations of S1P for 24 h and 48 h in starvation media to determine the optimal concentration of S1P that shows maximum proliferation effect. MTT results in both cell lines showed cell proliferation increased remarkably upon treatment with a range

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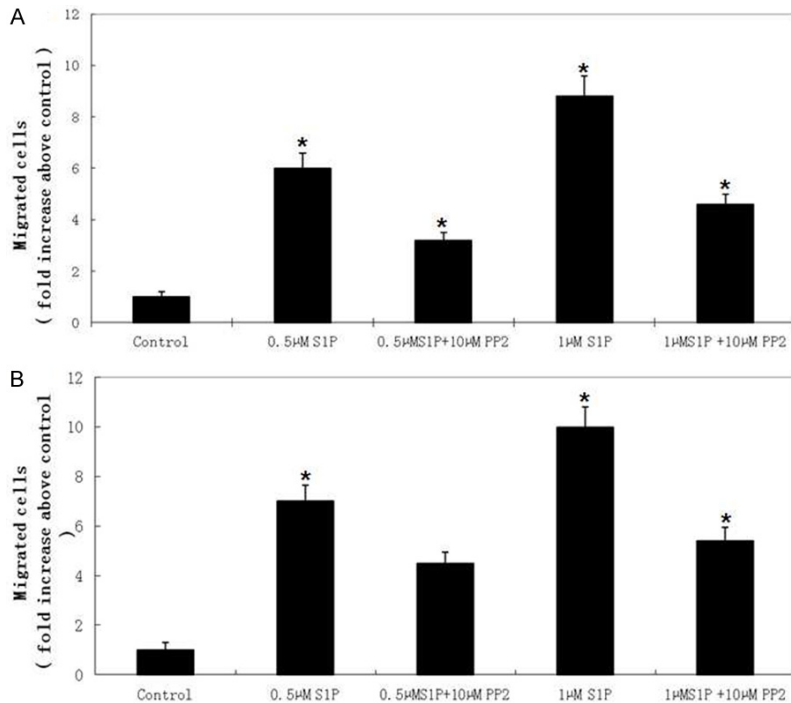


Figure 3. Effect of S1P in Capan-1 and Panc-1 cell migration. Transwell assay showed that S1P increased cell migration and invasion (A for Capan-1 cells and B for Panc-1 cells). Pre-incubation of Capan-1 and Panc-1 cells with 10 µM PP2 for 30 min almost abolished the effects of S1P in induction of tumor cell migration. Columns, average number of migratory cells (triplicated wells) in five random fields in 100× magnification per insert; error bars, SD. *, $P < 0.05$ by Student's t test compared with the control group.

of concentrations S1P between 0.1 and 1 µM S1P (**Figure 1A** and **1B**).

S1P induced Capan-1 and Panc-1 cell proliferation through c-Src signaling pathway

To investigate the signaling mechanisms by which S1P induced proliferation in Capan-1 and Panc-1 cells, we applied PP2 to inhibit Src signaling pathway on cell proliferation. We found that, pre-treatment cells with PP2 inhibited S1P-induced cell proliferation significantly (**Figure 2A** and **2B**), indicating that the role of S1P in induction of cell proliferation is Src dependent.

Effect of S1P on the migration and invasion of Capan-1 and Panc-1 cells

To evaluate migration effects of S1P on Capan-1 and Panc-1 cells, at first, about 50,000 cells were seeded in starvation media in each of the fibronectin coated Transwell filters. Total 500 µL starvation media contain-

ing indicated concentrations of S1P was added to the lower chamber and incubated for 12 h. Our results showed that S1P exert a significant effect on migration of both Capan-1 and Panc-1 cells (**Figure 3A** and **3B**). However, the effect was reduced significantly when cells pretreated with Src inhibitors. Our results showed that the migration of PP2-treated cells from both Capan-1 and Panc-1 cell lines were decreased remarkably in comparison to control cells. These data strongly suggested the involvement of Src-sensitive in induction of migration and invasion of Capan-1 and Panc-1 cells by S1P.

Effect of S1P on the expressions of MMP2 and MMP9 in Capan-1 and Panc-1 cells

The concentrations of MMP2 and MMP9 in cell culture medium were detected by ELISA methods. We found that the concentrations of MMP2 and MMP9 increased significantly compared with the control group. However, the effect reduced when the cells pretreated with 10 µM PP2 for 30 min (**Figure 4A** and **4B**).

S1P induce Src signaling pathway activated in Capan-1 and Panc-1 cells

Western blot experiment was done for the Capan-1 and Panc-1 cells that were treated with variable concentrations of S1P (0, 0.5 and 1 µM) for 30 min. We found that the activated Src levels (pY416) increased remarkably in contrast to the cells without of S1P (**Figure 5**). We also found that cell proliferation related proteins were increased significantly compare to the control group. However, cells that pre-incubated with PP2 (10 µM for 30 min) significantly decreased the expressions of proliferation related proteins.

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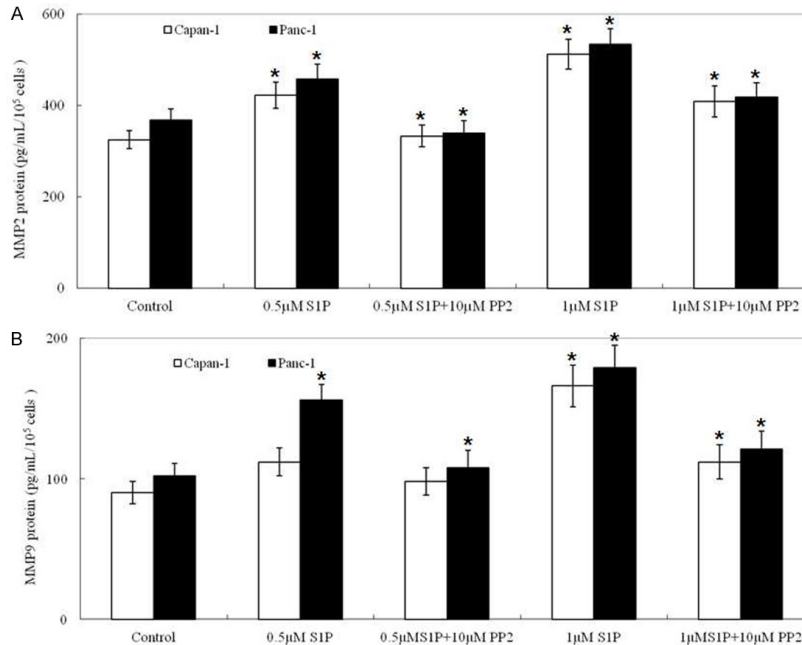


Figure 4. Effect of S1P on the expression of MMP2 and MMP9 in Capan-1 and Panc-1 cells. ELISA assay was used for detection the concentrations of MMP2 and MMP9 in cell culture supernatants. Columns, average protein concentration from three independent experiments; error bars, SD. *, $P < 0.05$ by Student's t test compared with the control group.

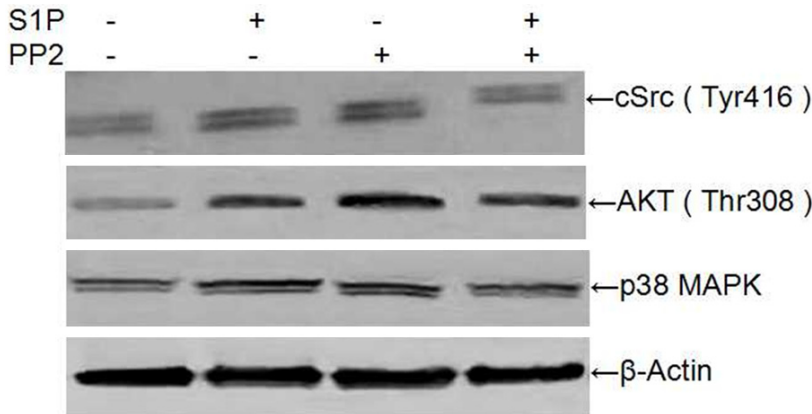


Figure 5. Proteins expression analysis of Capan-1 and Panc-1 cells treated with S1P. Western blot assay was used for detection the expressions of activated Src and cell proliferation related proteins.

Discussion

Different intracellular signaling pathways can be activated by S1P through binding to its receptors, which depending on intracellular Gα protein they couple to. Signaling through Gα can promote the activation of small GTPase Rho, Rho-associated kinase or other signaling pathway [15].

Our data showed that S1P increased the proliferation effect in both cell lines in a dose-dependent manner. However, cell proliferation effect was reduced upon PP2 pretreatment in contrast to the control cells. These results indicated that combination of S1P via Src inhibitor attributes to neutralize S1P effect on cell proliferation.

Previous research has shown that involvement of S1P in cell survival through S1PRs by activating PI3K/AKT pathway as well activation of sur-

Previous research showed that S1P regulation of cellular migration occurs through the Rho family of GTPases (Rac1, Rho, and CDC42) signaling [16]. In our research, we demonstrated cell migration increased evidently in both of cell lines upon S1P signaling pathway activated. Furthermore, decreasing of cell migration was seen after treatment of cells with c-Src inhibitors, which suggested that c-Src pathway involved in the S1P-S1PR1 signaling in regulation Capan-1 and Panc-1 cell migration.

To detect whether c-Src pathway was activated by S1P, we detected the expression of phosphory c-Src upon S1P treatment. Our results showed that the expression of activated c-Src increased significantly in the presence of S1P mediated through S1PR receptor. And we also found that the effect reduced evidently when cells pretreated with S1PR inhibitor. It can be concluded that S1P-induced c-Src activated is S1P receptor-dependent.

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Survival through PI3K/AKT pathway [17]. In our current research, we also demonstrated one of the mechanisms of S1P in cell survival, tumor proliferation and even invasion through c-Src related pathway. We found the levels of MAPK, ERK and AKT increased evidently upon S1P treatment, and the effect also was Src signaling pathway dependent. Although more investigations are required to find some signals downstream of c-Src when cells were treated with S1P.

Conclusion

In this study, we studied the involvement of S1P and its receptors in tumor progression of Capan-1 and Panc-1, we also investigated another survival mechanism of S1P in cell survival through interaction with Src signaling pathway. In conclusion, our data demonstrated the importance of this molecule as a target to design novel anticancer drugs in future.

Disclosure of conflict of interest

None.

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