

〈Regular Article〉

Sphingosine-1-phosphate receptor 1 expression in angiosarcoma: Possible role in metastasis and a potential therapeutic target

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ABSTRACT Sphingosine-1-phosphate (S1P) is a potent lipid mediator that has been implicated in the migration of lymphocytes and endothelial cells through S1P receptors. S1PR1 is strongly expressed in angiosarcoma, a malignant tumor of endothelial cell origin that has a high propensity for metastasis and poor prognosis; however, the pathological significance of S1PR1 expression is not clear. In this study, we investigated the effect of S1PR1 modulation on cell migration, and examined its potential role as a therapeutic target against metastatic dissemination of angiosarcoma. S1PR1 expression in the human angiosarcoma cell line MO-LAS was assessed by immunocytochemical examination and Western blotting. Effects of S1PR1-specific small interfering RNA (siRNA) and that of FTY720-P (a functional S1PR1-antagonist) on MO-LAS cell chemotactic migration towards sphingosine-1-phosphate (S1P) or 10% fetal bovine serum (FBS) were assessed by Transwell migration assay; wound healing assays for random cell migration were performed using a live cell analyzer. Immunostaining revealed high expression of S1PR1 on the MO-LAS cell membrane. Transwell and wound-healing assays showed that S1P enhanced chemotactic and random migration of MO-LAS cells, respectively. Inhibition of S1PR1 expression with siRNA significantly attenuated chemotaxis of cells towards S1P and 10% FBS. Further, FTY720-P strongly induced the internalization and degradation of S1PR1 even in the presence of serum containing S1P. It attenuated chemotactic cell migration of MO-LAS towards both S1P and serum, as well as the random motility of cells at nanomolar concentrations. These results suggest that the S1P/S1PR1 axis may be a potential therapeutic target for inhibition of angiosarcoma metastasis by controlling its cell motility.

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INTRODUCTION

Angiosarcomas are aggressive malignant tumors that originate from vascular or lymphatic endothelial cells¹⁾. Despite radical surgery and adjuvant radiotherapy for localized tumors, angiosarcomas show a high rate of metastasis, most commonly to the lungs. Accordingly, development of novel treatment modalities for angiosarcoma, such as those that aim to prevent metastasis, is a key research imperative²⁾. However, molecular research to identify potential therapeutic targets against invasive and metastatic properties of angiosarcoma has been largely lacking^{2, 3)}.

Sphingosine-1-phosphate (S1P), a potent lipid mediator, is a by-product of sphingolipid metabolism, which is generated in cells by the action of sphingosine kinase (SK)^{4, 5)}. S1P receptors are ubiquitously expressed in different tissues and are coupled to a variety of G proteins. S1P is known to be an important transducer of intracellular signals that are involved in biological processes such as proliferation, survival, migration, and morphogenesis of normal and malignant cells, via activation of S1P receptors 1-5 (S1PRs 1-5)^{6, 7)}. These receptors preferentially couple to different downstream signaling pathways, and the receptor-dependent biological response elicited by S1P in a specific cell type appears to critically depend on the cell type and the expression levels of receptor subtypes. S1P/S1PR1 or S1P/S1PR3 signaling promotes cell migration via activation of Rac through Gi⁸⁾, while S1P/S1PR2 signaling was shown to inhibit cell migration by inducing activation of Rho/Roh kinase⁹⁾. S1P is secreted by platelets, erythrocytes, and endothelial cells^{10, 11)}; thus, blood plasma contains a higher concentration of S1P (191 ± 79 nM) as compared to that in

tissues¹⁰⁾.

Cell migration is fundamental to metastatic dissemination of malignant tumors¹²⁾. Tumor cells with S1PR1 expression may have high migration activity by transducing S1P/S1PR1 signaling to regulate actin reorganization and pseudopod formation¹³⁾. This hypothesis is supported by the known significance of S1PR1 in regulating trafficking of normal lymphocytes by means of S1P concentration gradients formed between circulatory blood and tissues *in vivo*¹⁴⁾. Based on the current body of evidence, a S1P gradient may control metastasis of tumors with high expression of S1PR1, which presents a novel opportunity for therapeutic intervention¹⁵⁾.

In our previous studies, *in-situ* localization of S1PR1 in human tissues and strong expression of S1PR1 in angiosarcomas and normal endothelial cells were observed after immunostaining of formalin-fixed paraffin-embedded (FFPE) sections with a well-defined commercially available anti-S1PR1 antibody^{16, 17)}. Moreover, Krump-Konvalinkova *et al.*¹⁸⁾ demonstrated that the siRNA targeting S1PR1 inhibited S1P-induced actin reorganization and expression of adhesion molecules VE-cadherin and PECAM-1 in the angiosarcoma cell line AS-M.5. These findings suggest that the invasive properties and the metastatic potential of angiosarcoma cells may be mediated via the S1P/S1PR1 axis, and that this axis may be a potential therapeutic target.

In this study, we investigated expression of S1PR1 in the human angiosarcoma cell line MO-LAS, sourced from a patient with cutaneous angiosarcoma¹⁹⁾, and examined its role in cell migration. To this end, we studied the effect of a functional antagonist of S1PR1, the phosphorylated

form of FTY720 (FTY720-P), on MO-LAS cell migration stimulated by S1P and also by 10% fetal bovine serum (FBS), the latter being more akin to *in vivo* conditions. Our results underline S1P/S1PR1 signaling as a potential therapeutic target against angiosarcoma metastasis, by virtue of its critical role in cell motility.

MATERIALS AND METHODS

The study design (452-3, 1590-1) were approved by the research ethics committee at the Kawasaki Medical School and Hospital.

Patients and tissue samples

A total of five biopsy specimens of cutaneous angiosarcoma and one autopsy specimen of a pericardial angiosarcoma metastatic lesion in the lung, which were diagnosed in Kawasaki Medical School Hospital during 2002-2014, were used for immunohistochemical (IHC) study of S1PR1 expression. All cases were confirmed on histopathological and IHC examination using antibodies against von Willebrand factor (DAKO, Tokyo, Japan), CD31 (platelet endothelial cell adhesion molecule, PECAM-1; DAKO), and CD34 (QbEnd10, DAKO).

Reagents

D-erythro-S1P, FTY720, FTY720-phosphate (FTY720-P), and JTE013 (antagonist of S1PR2), were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Fatty-acid free bovine serum albumin (FAF-BSA) was purchased from Sigma (St. Louis, MI, USA). S1P was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mM and stored at -20°C. The 2 mM solution was further diluted to 0.5% FAF-BSA/PBS and added to cell migration and wound healing assays. FTY720 was dissolved in water at a concentration of 10 mM and stored at -20°C. FTY720-P was prepared as a 1 mM solution in DMSO and stored at -20°C. FTY720-P stock solution was warmed at 37°C for 10 min prior

to its use in experiments. JTE013 was dissolved in phosphate buffered saline containing methanol at a concentration of 1 mM.

Cell lines and cell culture

A human angiosarcoma cell line, MO-LAS¹⁹⁾, derived from specimens collected from pleural effusion of the patient with cutaneous angiosarcoma of the scalp, was obtained from the Cell Resource Center for the Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. This cell line was expanded and placed in stock within a month of receipt. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) containing 10% fetal bovine serum (FBS) (Sigma) that was heat inactivated at 56°C for 30 min, 200 µg/mL kanamycin, and were incubated at 37°C in a Personal Multi Gas Incubator APM-30D (Astec, Fukuoka, Japan).

Immunocytochemistry (ICC) /Immunohistochemistry (IHC)

MO-LAS cells were solidified using iPGell (Genostaff, Tokyo, Japan) according to the manufacturer's instructions. The cell blocks were fixed with 10% formalin neutral buffer solution and embedded in paraffin using a standard method. Paraffin-embedded MO-LAS cells and skin and lung tissues were cut into 3µm sections and deparaffinized. Subsequently, sections were incubated in antigen retrieval CC1 solution for 60 min at 98°C followed by staining with the Ventana Discovery XT system using an avidin-biotin detection system. Primary antibodies included the well-defined rabbit polyclonal anti-S1PR1/EDG-1 (Santa Cruz Biotechnology, CA, USA; 1:20 dilution as described elsewhere)^{16, 17)}. Sections were incubated with primary antibodies against S1PR1 for 60 min. A diaminobenzidine hydrochloride solution with hydrogen peroxide was used as the chromogen, and slides were counterstained with

hematoxylin.

Western blot (WB) analysis

MO-LAS cell lysates were directly resuspended and boiled in lysis buffer containing 1% sodium dodecyl sulfate, 1.0 mM sodium orthovanadate and 10 mM Tris (pH 7.4). Lysates were then homogenized, boiled for 5 min, passed through a 26 G needle 5-10 times, and centrifuged. Protein concentrations of cell extracts were determined using NanoDrop 1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Proteins (20 μ g) were loaded onto NuPAGE 4%-12% gels (Thermo Fisher Scientific) along with molecular weight marker (Novex Sharp Pre-Stained Protein Standard) and MagicMark XP Western Protein Standard (Life Technologies). After electrophoretic separation, proteins were transferred to polyvinylidene difluoride membranes using iBlot (Life Technologies). Membranes were treated with a blocking reagent (Roche Diagnostics, Basel, Switzerland) at room temperature for 1 h and were then incubated with rabbit polyclonal anti-S1PR1 (1:500 dilution) at 4°C, with rabbit monoclonal antibodies against STAT3 (1:1000 dilution, Abcam, Tokyo, Japan), phospho-STAT3 (Tyrosine705) (1:1000 dilution, Abcam), p44/42MAPK (Erk1/2) (1:1000 dilution; Abcam), p-p44/42MAPK (Thr202/Tyr204) (1:1000 dilution; Abcam), Akt (1:5000 dilution; Abcam), p-Akt (Ser473) (1:1000 dilution; Abcam) at room temperature for 1 h, or with mouse anti- β -actin monoclonal antibody (1:5000 dilution; Sigma-Aldrich, St Louis, MO, USA) at room temperature for 1 h. Membranes were then washed with Tris-buffered saline (TBS) for 30 min, incubated at room temperature (RT) for 1 h with a horseradish peroxidase-conjugated anti-mouse/rabbit IgG antibody (Roche Diagnostics), washed with TBS for 30 min, and finally treated with BM chemiluminescence Western blotting kit (Roche Diagnostics). Specific bands were visualized

using LAS-1000 UVmini (GE Healthcare, Tokyo, Japan) and analyzed for density using Quantity One 1-D analysis software, ver. 4.5 (BIORAD, Tokyo, Japan).

Quantitative reverse transcription PCR (qRT-PCR)

Total mRNA was extracted using a RiboPure kit (Life Technologies) and quantified using NanoDrop 1000; cDNA was synthesized from extracts containing 1 μ g of mRNA using a QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). Real-time PCR primers were purchased from Qiagen (QuantiTect Primer Assay) for human S1PR1 (QT00208733), S1PR2 (QT00230846), S1PR3 (QT00244251), S1PR4 (QT01192744), S1PR5 (QT00234178), SK1 (QT01011927), SK2 (QT00085386), and RPS18 (QT00248682). Gene expression levels were analyzed in triplicate using an Applied Biosystems StepOne Plus PCR System (Life Technologies) with a QuantiFast SYBR Green PCR kit (Qiagen). PCR amplification was performed by exposure to 95°C for 5 min to activate the HotStarTaq DNA polymerase, followed by 40 cycles at 95°C for 10 s each and a combined annealing/extension step at 60°C for 30 s. Owing to comparable PCR efficiency of reaction between target and endogenous reference (RPS18) genes, normalized S1PR and SK expression were calculated using StepOne software, version 2.2.2 (Life Technologies) and $2^{-\Delta\Delta Ct}$ analysis. Some data are expressed as mean $\Delta Ct \pm SEM$ ($\Delta Ct = Ct$ value of target mRNA minus Ct value of 18S rRNA). Greater expression corresponds to smaller ΔCt values of mRNA.

S1PR1 knockdown by small interfering RNA (siRNA) transfection

MO-LAS cells were seeded onto a 6-well plate (2.5×10^5 cells/well) and incubated for 24h. The cells were transfected with 5 nM Silencer Select Validated siRNA targeting S1PR1 s4448 (Ambion,

Life Technologies, Paisley, UK, #ASO0Z0KI), and 5 nM siRNA targeting S1PR1 s4449 (Ambion, #ASO0Z0KF), 5 nM glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA (Ambion, #ASO0YFP1), 5 nM non-specific siRNA (Ambion, #ASO0YGGG) and negative controls using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA, #1687504) according to the manufacturer's instruction. The knockdown was confirmed 24 h and 72 h after transfection by qRT-PCR and WB, respectively. After transfection for 72 h, cells were harvested and used for further assays.

Cell migration assay

Chemotactic cell migration assay was carried out using Transwell chambers (Corning, NY, USA) with an 8 μm pore polycarbonate filter insert. MO-LAS (2.0×10^5 cells/well) cells were seeded in the upper inserts with DMEM. The lower compartment was supplemented with reagents tested. 20 h after incubation, cells remaining in the upper chamber were wiped off with a cotton swab, and cells that had migrated to the lower chamber side of the filter were fixed in methanol for 30 sec and stained with 0.05% toluidine blue. The filter was removed, the stain was solubilized in 0.1 mL of 10% acetic acid, and the color intensity quantitated by an ELISA reader at 630 nm. The cells on the lower surface of the filters were fixed with methanol and stained with toluidine blue for 30 min as described elsewhere²⁰.

Wound healing assay

A wound healing assay was performed for measuring random cell motility. MO-LAS cells were cultured in a 60 mm tissue culture dish (FALCON) at 2.5×10^6 cells/dish as confluent monolayers. The monolayers were incubated in serum for 6 h and wounded by drawing a line across the well with a 1 mL standard pipette tip, separated by a 300 μm thick wall. The wounded monolayers were then washed twice with serum-free media to

remove cell debris and incubated in reagents tested. The cell-free wound area was recorded at indicated time points using the JuLi Br, Live cell analyzer (NanoEnTek Inc, Seoul, Korea) for 24 h. The wound healing effect was calculated as the percentage of the remaining cell-free area compared with the area of the initial wound.

Effects of FTY720-P on cell survival of MO-LAS cells

A 100 μL aliquot of MO-LAS cells (1×10^6) from each sample was centrifuged and re-suspended in 100 μL apoptosis buffer added to 5 μL Annexin V (conjugated with Alexa Fluor[®] 488) and incubated at RT in the dark for 20 min. Samples were then centrifuged and re-suspended in 100 μL of the same buffer with 1 μL PI (Propidium Iodide) added at RT in the dark for 5 min and analyzed by Tali[®] Image-Based Cytometer (Invitrogen). For each analysis, a dot plot and real percentages have been elaborated with the Attune Cytometric software (Invitrogen).

Statistical analysis

Data are expressed as mean \pm standard error of mean (SEM) of three determinations. Student's *t*-test was used for statistical analysis. A value of $p < 0.05$ was considered to be significantly different from controls.

RESULTS

Strong expression of S1PR1 in angiosarcoma cases

We investigated the expression of S1PR1 in five cutaneous angiosarcomas by immunostaining formalin-fixed, paraffin-embedded (FFPE) sections. Tumor cells of all five cases showed membranous and cytoplasmic S1PR1 staining as observed previously¹⁷. Interestingly, immunostaining for lung metastatic lesion of autopsy sample showed strong expression of S1PR1 as well as CD31 (Fig. 1).

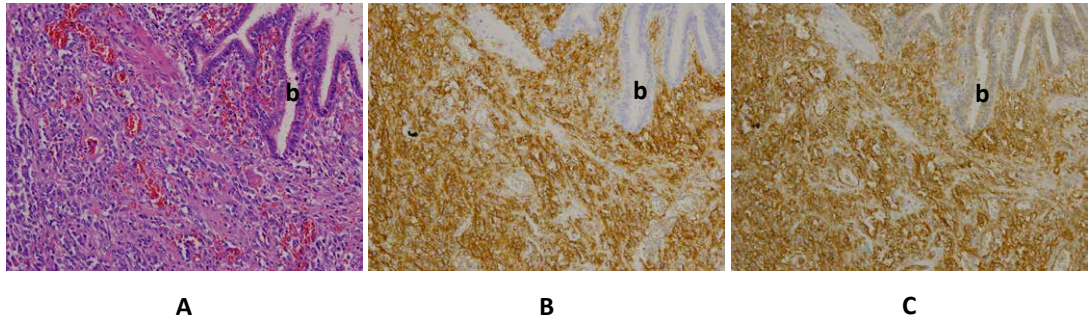


Fig. 1. Immunohistochemical expression of S1PR1 in an autopsy specimen of metastatic angiosarcoma of lung of a 61-year-old man. Formalin-fixed and paraffin-embedded lung tissue serial sections were immunostained. The metastatic lesion is nodular and composed of spindle cells having a large nuclear-to-cytoplasmic ratio (A: hematoxylin and eosin stain). The spindle cells exhibit strong signals for CD31 (B) and S1PR1 (C). (b): Bronchiole

Expression profile of S1P receptors in MO-LAS cells

A comparative study of S1PRs and SK expression were performed by qRT-PCR. S1PR1 mRNA was more abundantly expressed than other S1PRs (Fig. 2).

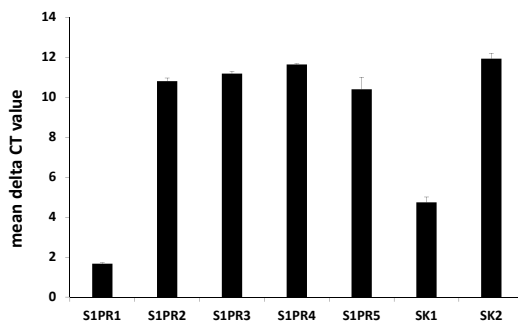


Fig. 2. Results of qRT-PCR for mRNA expression of S1PR, SK1 and SK2 in MO-LAS cells cultured for 6 days. Δ Ct values represent mean \pm SEM of triplicate assay.

Effect of selective knockdown of S1PR1 on migration of MO-LAS cells

The effect of transfection of siRNA targeting S1PR1 on the expression of S1PR1 in MO-LAS was assessed by qRT-PCR at 24 h and WB at 72 h after transfection. Transfection with the siRNA targeting S1PR1 significantly reduced both mRNA and protein levels of S1PR1 as compared to that observed after transfection with non-specific siRNA at 24 h and 72 h post-transfection, respectively (Table 1, Fig. 3A, B).

The functional consequences of MO-LAS transfected with siRNA were assessed on a Transwell migration assay, which measures chemotaxis across 8 μ m polycarbonate filters that separate the upper and lower chambers (Fig. 4). The addition of 0.5% FAF-BSA containing 1 μ M or 5 μ M S1P in lower chamber promoted greater cell migration ($141 \pm 4.8\%$, $p < 0.001$ and $156 \pm$

Table 1. Results of qRT-PCR showing effects of S1PR1 siRNA transfection on mRNA expression of MO-LAS cells

	S1PR1	S1PR2	S1PR3	S1PR4	S1PR5	GAPDH
non-specific siRNA	0.95 \pm 0.02	0.92 \pm 0.03	0.88 \pm 0.02	1.12 \pm 0.05	1.18 \pm 0.06	0.87 \pm 0.07
S1PR1 siRNA s4448	0.39 \pm 0.07**	0.86 \pm 0.09	0.93 \pm 0.05	1.35 \pm 0.04	1.27 \pm 0.03	0.81 \pm 0.09
GAPDH siRNA	1.06 \pm 0.05	1.27 \pm 0.09	1.12 \pm 0.01	1.56 \pm 0.01	1.34 \pm 0.10	0.44 \pm 0.03**

** indicates $p < 0.001$ vs. the other two groups

24h after transfection, S1PR1 expression was decreased by S1PR1-specific siRNAs as compared to GAPDH siRNA (positive control) and non-specific siRNA (negative control). The relative gene expression level was normalized to RPS18 and is presented as the relative amount of the group without transfection. Data expressed as mean \pm SEM.

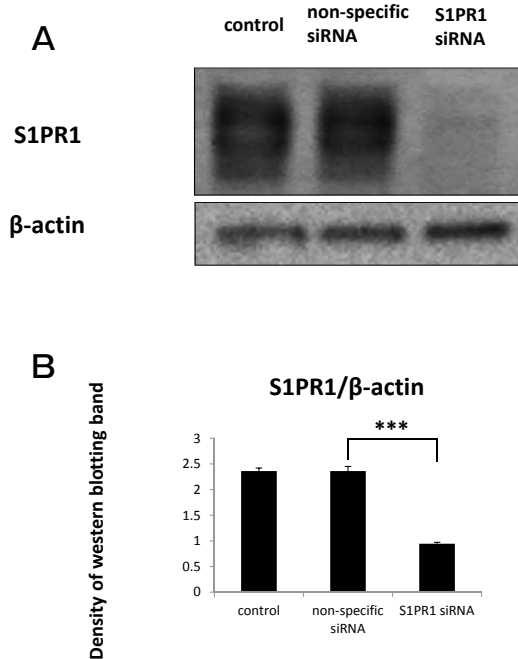


Fig. 3.

(A) Western blot for S1PR1 protein expression in MO-LAS cells, three days after transfection with S1PR1-specific siRNA. S1PR1 expression was significantly decreased after transfection with siRNA specific for S1PR1, as compared to that with non-specific siRNA.

(B) Western blot analysis of effects of S1PR1-specific siRNA transfection on expression level of S1PR1 (S1PR1/β-actin) in MO-LAS cells. Specific bands were analyzed for density using Quantity One 1-D analysis software, ver. 4.5. Data represents mean \pm SEM of three independent experiments. *** $p < 0.0001$ compared to the control.

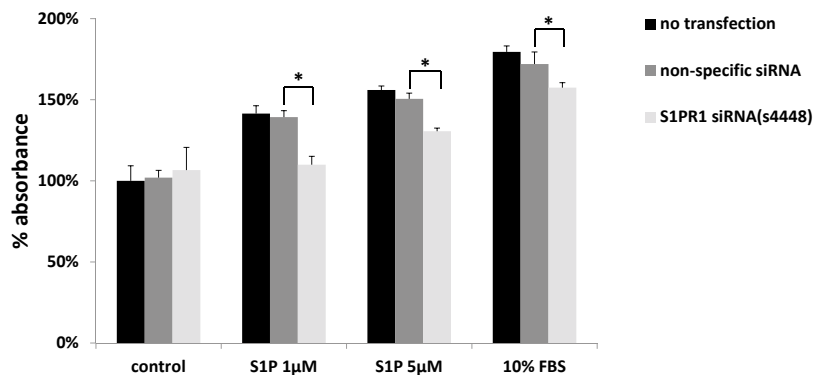


Fig. 4. Results of Transwell migration assay showing effects of S1PR1 knockdown on MO-LAS chemotactic cell migration. Transwell migration assay was performed 72h after transfection with siRNA targeting S1PR1. Cell migration was promoted by addition of 1 μ M or 5 μ M S1P in 0.5% FAF-BSA, or 10% FBS, when compared to that seen with use of 0.5% FAF-BSA only (control). After selective knockdown of S1PR1 with siRNA targeting S1PR1, the promoted migration toward 1 μ M or 5 μ M S1P or 10% FBS was significantly inhibited as compared with non-specific siRNA. Values (% absorbance) are mean \pm SEM of triplicate assay. * $p < 0.05$ compared to the non-specific siRNA group.

2.48%, $p < 0.001$) as compared to that observed with 0.5% FAF-BSA, respectively. The addition of 10% FBS in the lower chamber promoted migration more ($179 \pm 3.66\%$, $p < 0.001$ vs. 0.5% FAF-BSA). By selective knockdown of S1PR1 with targeted siRNA, the increase in migration induced by 1 μ M S1P ($139 \pm 4.00\%$ vs. $109 \pm 5.16\%$, $p < 0.003$), 5 μ M S1P ($150 \pm 3.54\%$ vs. $130 \pm 1.90\%$, $p < 0.002$), and 10% FBS ($172 \pm 7.46\%$ vs. $157 \pm 3.05\%$, $p < 0.002$) was significantly reversed as compared to that observed after treatment with non-specific siRNA. The siRNA transfection did not affect viability of MO-LAS cells (Table 2A).

Effects FTY720 and FTY720-P on MO-LAS cell migration

In the next step, we examined the effect of FTY720, a S1PR1 modulator, and its active form, FTY720-P, on MO-LAS migration towards 10% FBS using a Transwell well migration assay. Addition of FTY720 or FTY720-P (Fig. 5A, B) into the lower chamber appeared to inhibit migration of MO-LAS cells towards 10% FBS.

Moreover, we studied changes in S1PR1 expression of MO-LAS pre-incubated with

Table 2. Effects of S1PR1 siRNA (A) and FTY720-P and JTE013 (B) on survival of MO-LAS cells

(A)			
	Live cells (%)	Dead cells (%)	Apoptosis (%)
non-specific siRNA	98 ± 0.79	1 ± 0.30	1 ± 0.61
S1PR1 siRNA s4448	97 ± 0.57	2 ± 0.30	1 ± 0.47
(B)			
	Live cells (%)	Dead cells (%)	Apoptosis (%)
Control	97 ± 0.55	1 ± 0.33	2 ± 0.60
FTY720-P (100 nM)	94 ± 0.88	4 ± 0.70	2 ± 0.49
JTE013 (100 nM)	98 ± 0.88	1 ± 0.49	1 ± 0.51
FTY720-P (100 nM) + JTE013 (100 nM)	98 ± 0.87	1 ± 0.30	1 ± 0.66

(A) MO-LAS cells were transfected with non-specific siRNA or siRNA specific for S1PR1. Eight hours post-transfection, apoptosis was analyzed by the Tali[®] apoptosis assay using Annexin V and PI.

(B) MO-LAS cells were treated with FTY720-P (100 nM) for 24 h and/or JTE013 (100 nM) for 30 min, and assessed for apoptosis. Data expressed as mean ± SEM.

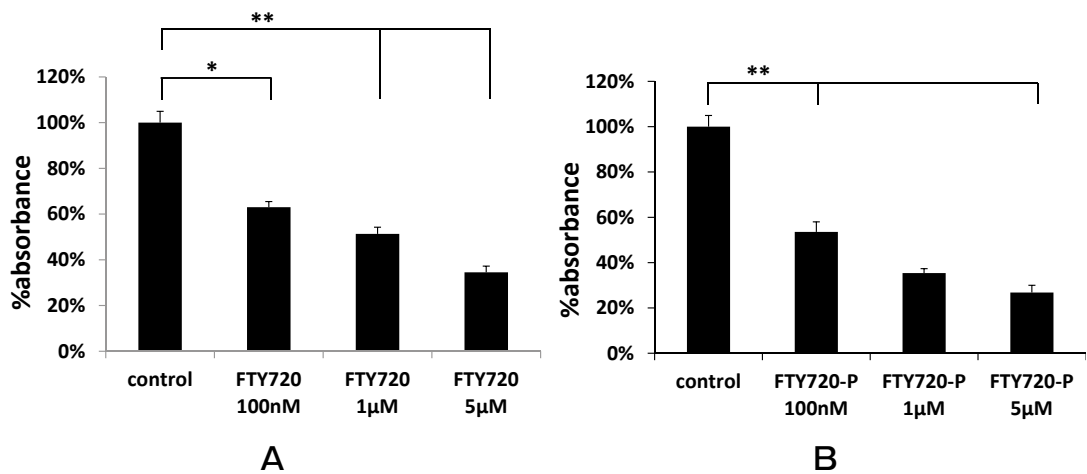


Fig. 5. (A) (B) Inhibitory effect of FTY720-P or FTY720 on chemotactic migration of MO-LAS cells in response to 10% FBS (control) or 10% FBS plus FTY720-P or FTY720. A dose-dependent inhibitory effect of FTY720-P and FTY720 are observable. Values (% absorbance) represent mean ± SEM of three assays. * $p < 0.05$, ** $p < 0.001$ compared to the 10% FBS group.

FTY720-P at the respective time points, because FTY720-P has been shown to induce internalization and degradation of S1PR1 and to work as a functional antagonist of S1PR1²¹. ICC analysis of a MO-LAS cell block with an anti-S1PR1 antibody revealed expression of S1PR1 on the cell membrane even when the cells were cultured in the presence of 10% FBS (Fig. 6A). Exogenous FTY720-P (100 nM) treatment at 37°C for 30 min decreased

cell membrane signals and increased vesicular localization signals of S1PR1. At 4 h after treatment, the signals were still observed on the cell membrane but most were observed as vesicular signals in the cytoplasm of perinuclear region. At 8 h, the signals were very faintly observed in cytoplasm.

To elucidate the mechanism underlying the inhibitory effect of FTY720-P on cell migration, we pre-incubated MO-LAS with culture medium

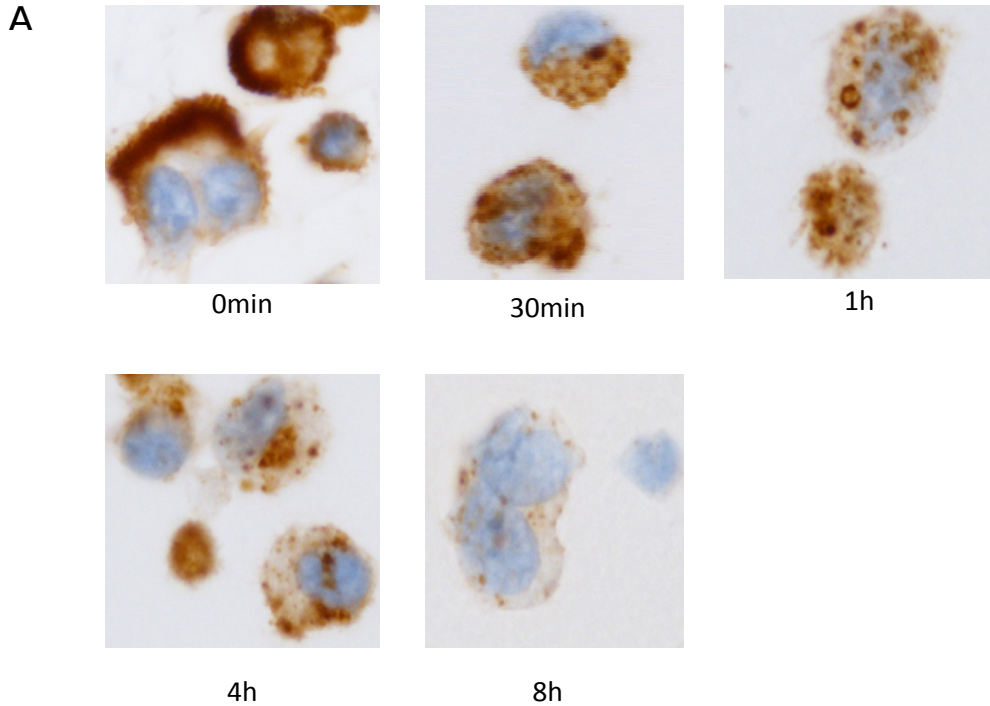
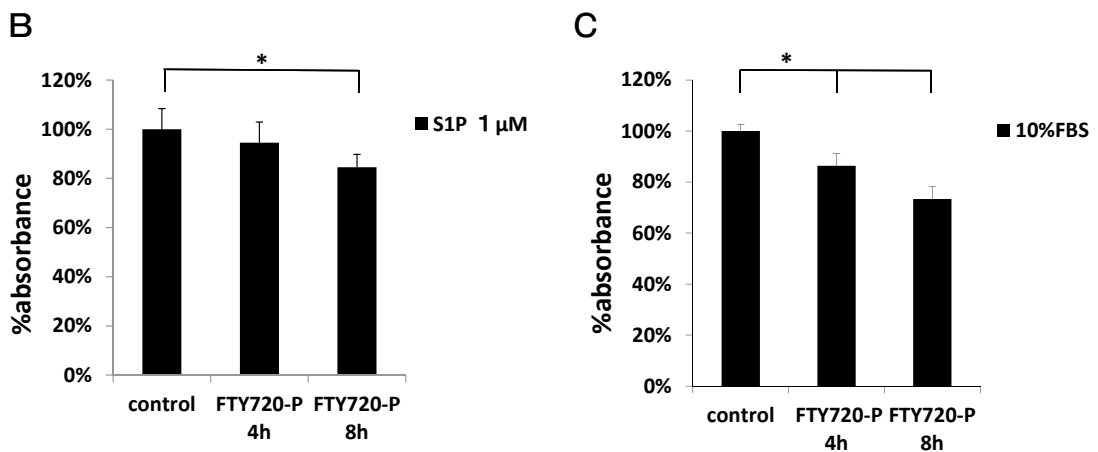


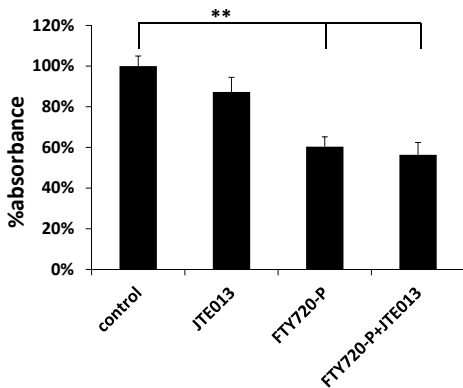
Fig. 6.

(A) Internalization of S1PR1 in MO-LAS cells after addition of FTY720-P (100 nM) in culture medium containing 10% FBS. S1PR1 signals were observed on cell membrane and cytoplasm on immunocytochemistry with anti-S1PR1 antibody 30 min after additional of FTY720-P. After 1h, S1PR1 was observed circular in the cytoplasm. After 4h, dot-like S1PR1 signals were observed, and by 8h S1PR1 signals had all but disappeared.



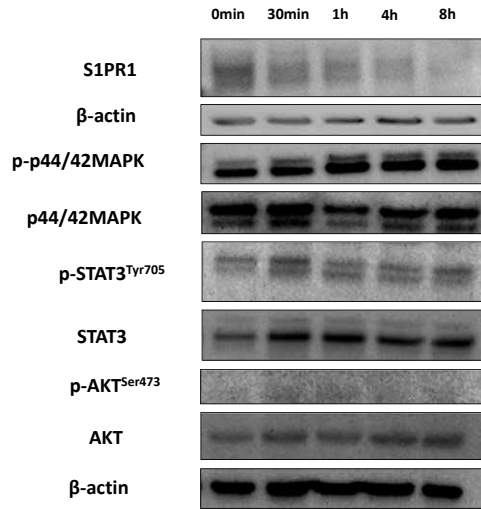
(B) (C) Inhibition of MO-LAS cell migration in Transwell toward S1P plus 0.5% FAF-BSA (control) or 10% FBS after FTY720-P pretreatment. MO-LAS cells were pre-incubated with FTY720-P for 4 h to 8 h before Transwell migration assay. Cell migration was evaluated at 24 h. Values (% absorbance) represent mean \pm SEM of three assays. * p < 0.05, compared to the no treatment group (control).

D



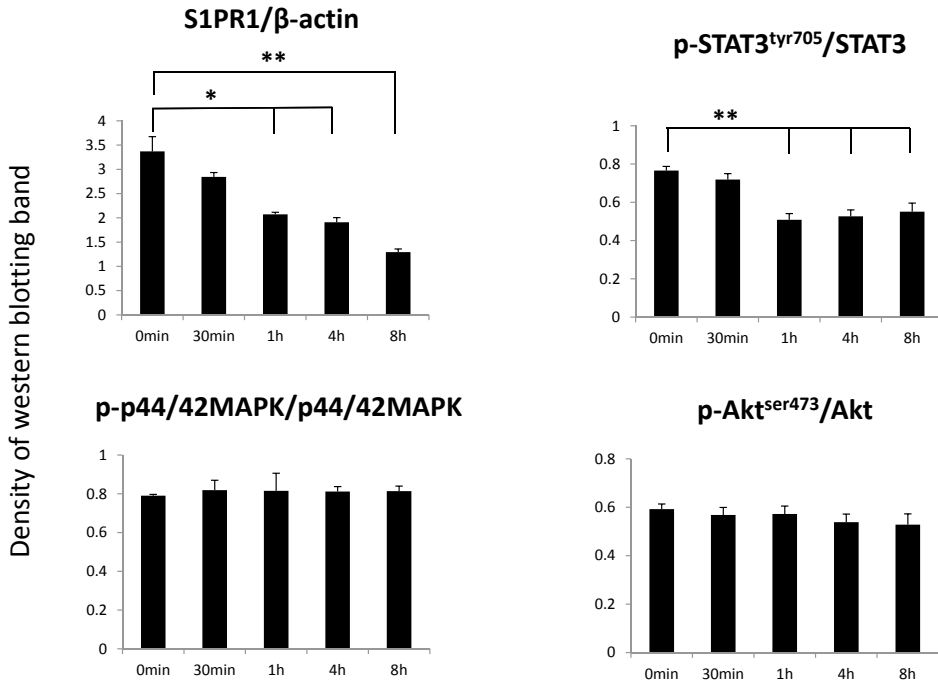
(D) Effects of pretreatment with S1PR2-antagonist JTE013 on MO-LAS cell migration induced by 10% FBS. No promotion or recovery from inhibition of cell migration with FTY720-P was observed in MO-LAS cells pre-incubated with JTE013. Values (% absorbance) represent mean \pm SEM from three assays. $**p < 0.001$ compared to the no treatment group (control).

E



(E) Western blot results showing the effect of FTY720-P (100 nM) on S1PR1 protein expression and on phosphorylation of p44/42MAPK (Erk1/2) (Thr202/Tyr204), STAT3 (Tyr 705), and Akt (Ser473) in MO-LAS in the presence of 10% FBS. Cells were exposed to FTY720-P for various times (30 min to 8 h).

F



(F) Western blot analysis of time-dependent effects of FTY720-P (100 nM) on expression level of S1PR1 (S1PR1/ β -actin) and phosphorylation level of p44/42MAPK (p-p44/42MAPK/p44/42MAPK), STAT3 (p-STAT3/STAT3), and Akt (p-Akt/Akt) in MO-LAS cells. Specific bands were analyzed for density using Quantity One 1-D Analysis Software, ver. 4.5. Data represents mean \pm SEM of three independent experiments. $*p < 0.05$, $**p < 0.001$ compared to the 0min group.

containing 10% FBS and FTY720-P and then examined its effect on cell migration. Pre-incubation with FTY720-P inhibited MO-LAS cell migration towards S1P and 10% FBS in pre-incubation time-dependent manner (Fig. 6B, C).

S1PR1 has been shown to promote cell motility of endothelial cells while S1PR2 has the opposite effect²². We also examined a possible role of S1PR2 in the cell migration of MO-LAS cells by pre-incubation with a S1PR2 antagonist, JTE013, for 30 min and/or FTY720-P for 4 h. As shown in Fig. 6D, no promotion or recovery from inhibition of cell migration with FTY720-P was observed in MO-LAS cells pre-incubated with JTE013. Further, pre-incubation of cells with FTY720-P and/or JTE013 did not appear to affect cell viability (Table 2B).

We further studied the effects of FTY720-P on S1PR1 protein expression and activation status of p44/42MAPK (Erk1/2), STAT3, and Akt on WB analysis. MO-LAS cells were exposed to 10% FBS containing FTY720-P (100 nM) for different

durations of time (1 h, 4 h, and 8 h) (Fig. 6E, F). S1PR1 protein expression decreased in a time-dependent manner, which correlated with IHC results. Interestingly, FTY720-P slightly inhibited phosphorylation of STAT3 but not of p44/42MAPK (Erk1/2) and AKT during this time.

Effects of FTY720-P on wound healing of MO-LAS

The effect of S1P and FTY720-P on random motility of MO-LAS was assessed by wound healing assay and monitored by time-lapse video (Fig. 7). 10% FBS promoted cell migration, which was reflected in the higher rate of wound closure and 100% coverage of the scratched area. One micromolar S1P also covered $95 \pm 2.3\%$ of the scratched area at 24 h. 0.5% FAF-BSA-induced cell migration was slower as the associated coverage of the scratched area at 24 h was $75 \pm 2.3\%$ ($p < 0.001$). In contrast, FTY720-P inhibited cell migration, which was reflected in the lowest rate of wound closure ($55 \pm 1.9\%$ ($p < 0.0001$)) at 24 h.

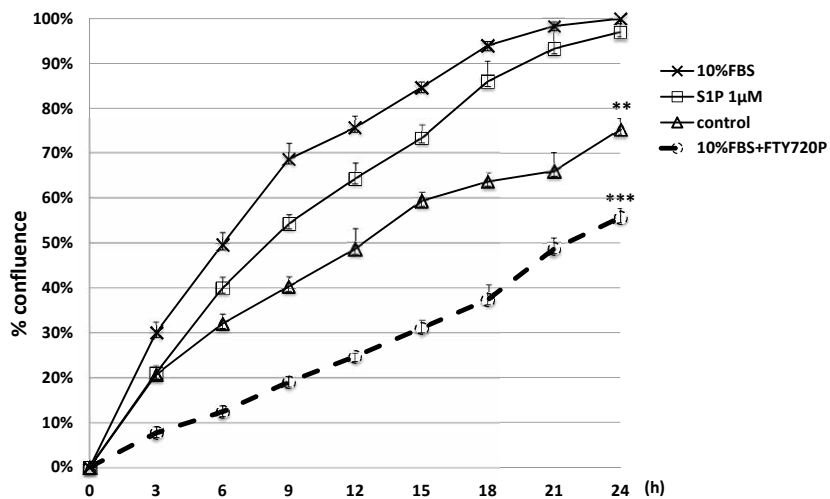


Fig. 7. Effects of FTY720-P on wound healing of MO-LAS. Cells were grown to a monolayer. A scratch wound was made. Images were taken using the JuLi Br, Live cell analyzer. FTY720-P treatment impairs MO-LAS scrape wound healing. Time *vs.* % confluence effect of serum, FAF-BSA (control), FTY720-P (100 nM) and S1P (1 μ M) are shown. Almost complete closure occurred after 21 h for S1P (1 μ M) plus 0.5% FAF-BSA or 10% FBS. FTY720-P (100 nM) significantly inhibited migration of MO-LAS cells and only 55% of wound closure was observed 24 h the treatment. Data represent mean \pm SEM of three independent experiments. ** $p < 0.001$, *** $p < 0.0001$ compared to the 10% FBS group at 24 h.

DISCUSSION

Although direct evidence of a relationship between S1PR1 expression and tumor dissemination has not been obtained *in vivo* so far, the relevance of S1PR1 expression to metastatic activity of tumors was suggested by recent clinicopathological studies of malignant lymphomas using IHC with S1PR1-specific antibodies²³⁻²⁵. In the present study, we examined the impact of S1PR1 knockdown on migration of angiosarcoma (MO-LAS) cells by Transwell assay. Expression level of S1PR1 in the angiosarcoma cells showed a positive correlation with cell migration induced by S1P or 10% FBS. These results underline the important role S1P/S1PR1 axis on migratory potential of angiosarcoma cells.

FTY720 (Fingolimod) has been used as an immune-modulator for treatment of multiple sclerosis²⁶. FTY720 has been shown to inhibit lymphocyte egress from lymphoid organs and induce lymphopenia in both mice and humans^{14, 26}. FTY720 is phosphorylated *in vivo* by SK2 and converted to FTY720-phosphate (FTY720-P), which acts as a functional antagonist by inducing irreversible internalization and degradation of S1PR1^{22, 26}. In other words, FTY720-P appears to desensitize the lymphocytes to the S1P gradient between blood and tissue, and thereby, lead to sequestration of S1PR1-positive lymphocytes in the secondary lymphoid organs¹⁴.

In the present study, FTY720-P induced S1PR1 internalization and degradation in an angiosarcoma cell line, MO-LAS, even in the presence of 10% FBS. Surprisingly, FTY720-P inhibited chemotactic cell migration induced by S1P as well as that induced by 10% FBS containing S1P and multiple cytokines^{10, 12}, at nanomolar concentrations that lie within the therapeutic range for multiple sclerosis. The results imply that FTY720-P-induced loss of S1PR1 expression on the angiosarcoma cell surface caused desensitization of S1P/S1PR1 signaling in

the presence of the serum, and thereby, reduced the cell migration activity²⁷.

In the wound healing assay, > 50% of the scratched area was covered with MO-LAS cells after 24 h, even in the absence of extrinsically added stimulants other than FAF-BSA. This result implies that S1P and other factors, including vascular endothelial factor (VEGF), released from MO-LAS induce random cell motility in an autocrine manner^{11, 28}. MO-LAS showed a substantial level of SK1 expression and still constitutively activated p44/42MAPK (Erk1/2), Akt, and STAT3 post 16 h serum-starvation. However, the confluence rate of MO-LAS cells treated with FTY720-P plus 10% FBS was lower as compared to that observed on treatment with FAF-BSA, which confirmed the potent inhibitory effect of FTY720-P against angiosarcoma cell migration. A possible explanation is that homeostatic activation of S1PR2 by serum S1P increased after down-regulation of S1PR1, and that led to a shift in S1P signaling balance from S1PR1 to S1PR2, which suppressed the cell migration activity^{21, 29}. However, such a possibility appears unlikely because the inhibitory effects of 100 nM FTY720-P were not reversed on pretreatment with a S1PR2-antagonist, JTE013, which has been shown to be effective *in vitro* as well as *in vivo* studies³⁰. Therefore, the functional consequence of FTY720-P against angiosarcoma cell motility appears to be based on modification of S1PR1 expression plus other unknown mechanisms.

S1PR1 has a crucial role in the persistent activation of STAT3³¹. Targeting of S1PR1 by administration of FTY720 reduces S1PR1 expression and downregulates STAT3 activity in diffuse large B cell lymphoma (DLBCL), both *in vitro* and *in vivo*³². Our clinicopathological findings also showed S1PR1 overexpression in 16% of DLBCL cases; S1PR1 expression correlated with STAT3 phosphorylation in fresh samples of the cases²⁵. The present study showed that FTY720-P at nanomolar concentrations

did not affect phosphorylation of p44/42MAPK (Erk1/2) or Akt, but induced a significant decrease in the phosphorylation level of STAT3, an effect that correlated well with internalization and degradation of S1PR1, as detected by ICC and WB. Because S1P/S1PR1 signaling activates STAT3 phosphorylation³¹⁾, reduction in STAT3 activation level after FTY720-P treatment may be due to decreased expression level of S1PR1.

FTY720 has been shown to be successful in preclinical antitumor studies in several cancers³³⁾. Administration of FTY720 remarkably suppressed Bcr/Abl-driven leukemogenesis³⁴⁾, prolonged the survival of mice with lymphoblastic leukemia/lymphoma³⁵⁾, and inhibited growth of mantle cell leukemia cell lines xenografted in SCID mice³⁶⁾. More recently, FTY720 was shown to decrease S1PR1 expression and induce apoptosis in a canine hemangiosarcoma cell line³⁷⁾. However, at least 10 to 100-fold higher concentrations were required than those used for treatment of multiple sclerosis to obtain these effects. This implies that the inhibitory effects of FTY720 on tumor progression are not mediated through FTY720 to FTY720-P conversion but through direct suppressive effects of FTY720 on signals related to cell proliferation and survival, i.e., on the activation of PP2A that leads to suppression of p44/42MAPK (Erk1/2), Akt activation³⁴⁻³⁶⁾, and on inhibition of SK1 that produces S1P³³⁾.

In conclusion, our study demonstrates that S1PR1 is abundantly expressed and is functionally activated in MO-LAS cell migration through S1P/S1PR1 signaling. FTY720/FTY720-P inhibited not only S1P-induced but also serum-induced cell migration of angiosarcoma cells at clinically used concentrations. The present results suggest the potential of the S1P/S1PR1 axis as a therapeutic target for angiosarcoma.

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