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Negative regulation of endothelial morphogenesis and angiogenesis by $S1P_2$ receptor

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Abstract

We speculated that the sphingosine-1-phosphate (S1P) receptor $S1P_2$, which uniquely inhibits cell migration, might mediate inhibitory effects on endothelial cell migration and angiogenesis, different from $S1P_1$ and $S1P_3$. Mouse vascular endothelial cells, which endogenously express $S1P_2$ and $S1P_3$, but not $S1P_1$, responded to S1P and epidermal growth factor (EGF) with stimulation of Rac, migration and the formation of tube-like structures on the Matrigel. The $S1P_3$-antagonist VPC-23019 abolished S1P-induced, G_i-dependent Rac stimulation, cell migration and tube formation, whereas the $S1P_2$-antagonist JTE-013 enhanced these S1P-induced responses, suggesting that $S1P_2$ exerts inhibitory effects on endothelial Rac, migration and angiogenesis. $S1P_2$ overexpression markedly augmented S1P-induced, G_i-independent inhibition of EGF-induced migration and tube formation. Finally, the blockade of $S1P_2$ by JTE-013 potentiated S1P-induced stimulation of angiogenesis in vivo in the Matrigel implant assay. These observations indicate that in contrast to $S1P_1$ and $S1P_3$, $S1P_2$ negatively regulates endothelial morphogenesis and angiogenesis most likely through down-regulating Rac.
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Keywords: Sphingosine-1-phosphate; $S1P_2$; Anti-angiogenesis; Tube formation; Morphogenesis; Migration; Rac; Rho

Angiogenesis plays a pivotal role in various physiological and pathological conditions such as embryonic development, tumor growth, inflammation, and diabetic retinopathy. Since suppression of angiogenesis is likely beneficial for treating these diseases, substantial efforts have been made to identify endogenous anti-angiogenic molecules [1,2]. A number of molecules that exhibit anti-angiogenic activities under certain assay conditions have been identified, however, there are few whose therapeutic efficacy has been verified in the clinical settings [3–5].

Sphingosine-1-phosphate (S1P) is an endogenous lipid mediator that exerts pleiotropic effects in diverse cell types through the specific G protein-coupled receptors, $S1P_{1–5}$ [5–11]. Among these, $S1P_1$, $S1P_2$ and $S1P_3$ are widely expressed subtypes [6,9,7,8,11]. In vascular endothelial cells S1P stimulates cell proliferation and migration in vitro, and angiogenesis in vivo in the Matrigel implant assay [12,13]. These S1P actions were mediated via $S1P_1$ and $S1P_3$ receptors. A recent investigation [14] showed that RNA interference-mediated $S1P_1$ silencing inhibited in vivo tumor angiogenesis and tumor growth. Investigations of $S1P_1$-knockout mice demonstrated that $S1P_1$ is also essential for the process of recruitment of medial smooth muscle cells and pericytes to the newly formed blood vessel, i.e. vascular maturation [15]. S1P is released from activated

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platelets and other cell types on their activation, and present at a substantial concentration in the circulating plasma [10,16]. Thus, S1P may be one of the key regulators that promote angiogenesis and vascular maturation under physiological and pathological conditions.

We previously demonstrated that S1P receptors exert receptor subtype-specific, distinct effects on cell migration; $S1P_1$ and $S1P_3$ mediate S1P-directed cell migration, whereas $S1P_2$ inhibits migration directed toward chemoattractants such as platelet-derived growth factor (PDGF) and insulin-like growth factor-I [17,18]. The Rho family small GTPases, Rac, Cdc42 and Rho, are essential regulators of the reorganization of actin cytoskeletons and thus cell motility [19] in a variety of cell types including vascular endothelial cells. $S1P_1$ and $S1P_3$ mediate Rac activation like many other chemoattractant receptors whereas the inhibitory receptor $S1P_2$ mediates Rac inhibition [20,21], suggesting that Rac inhibition is at least in part responsible for $S1P_2$-mediated inhibition of cell migration. Rac and also Rho are shown to be essential for the capillary morphogenesis of vascular endothelial cells [22].

Although $S1P_1$ and $S1P_3$ appear to be major S1P receptor subtypes expressed in vascular endothelial cells, certain endothelial cells were also shown to express $S1P_2$ [23,24]. However, the biological effect of $S1P_2$ on angiogenesis remains unknown. In the present study, we examined the roles of $S1P_2$ in endothelial cell migration, Rho GTPases regulation and morphogenesis in vitro and angiogenesis in vivo, and found that $S1P_2$ exerts inhibitory effects on endothelial migration, morphogenesis and angiogenesis in vivo likely through Rac inhibition.

Methods

Materials. S1P was purchased from Biomol (Plymouth meeting, PA, USA). The $S1P_2$-selective antagonist JTE-013 was kindly donated by Japan Tobacco (Tokyo, Japan). The $S1P_1$- and $S1P_3$-selective antagonist VPC-23019 was bought from Avanti (Alabaster, AL, USA). Recombinant human epidermal growth factor (EGF) was purchased from R&D systems. Pertussis toxin (PTX) was from List biological. Anti-von Willebrand factor antibody was purchased from DAKO. Matrigel and dispase were purchased from BD Biosciences.

Cell culture. Mouse vascular endothelial cells, SVEC4-10 (SVEC), were purchased from American Type Tissue Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 ng/ml streptmycin (Wako, Osaka, Japan) and 100 U/ml of penicillin G (Wako) under the humidified atmosphere of 5% CO_2 and 95% air. Human umbilical vein endothelial cells (HUVECs) and human skin microvascular endothelial cells (MVECs) were bought from Sanko Junyaku (Osaka, Japan).

Plasmids, adenoviruses and Northern blot analysis. To establish SVEC-$S1P_2$ cells which stably overexpress $S1P_2$, PCAGGS-$S1P_2$ [20,25] and the blastcidin resistance gene-expression vector pMAM2-BSD (Funakoshi, Tokyo, Japan) were co-transduced into SVEC cells using Lipofectamine (Life Technologies) and selected in the presence of 2 μg/ml of blastcidine (Funakoshi). The cells were infected with adenoviruses at a multiplicity of infection of 100 as described previously [20,26] and cultured in the FCS-containing medium for 24 h before tube formation assays. Under this condition, more than 95% of the cells were transfected as estimated by using LacZ-expressing adenovirus and X-gal staining method. For Northern analysis of mRNA expression, total RNA was separated in formaldehyde 1% agarose gel and transferred onto a nylon membrane (Perkin-Elmer Life Sciences, Boston, MA, USA), followed by hybridization with ^{32}P-labeled $S1P_1$, $S1P_2$, $S1P_3$, and GAPDH cDNA probes and visualized as previously described [18].

Cell migration assay, and Rac and Rho pull-down assay. Transwell migration of SVEC cells was determined with a modified Boyden chamber method using fibronectin-coated polycarbonate filters (8 μm-pores, Neuroprobe) as previously described [18,20,26]. S1P and/or epidermal growth factor (EGF) was added to the lower chamber in FCS-free Ham F-12 media. The S1P receptor antagonists were added in both the upper and lower chambers. After incubation at 37 °C in 5% CO_2 for 5 h, the number of the migrated cells onto the lower side of the filter were determined. For determinations of amounts of GTP-bound forms of Rac and Rho, the cell lysates were prepared and incubated with glutathione *S*-transferase (GST)-rhotekin (for determination of Rho activity) or GST-p21-activated kinase (rat αPAK) (for determination of Rac activity) bound to glutathione–Sepharose 4B beads (Amersham Bioscience) as described previously [20,21]. After washing three times, bound Rho and Rac were quantitatively analyzed by Western blotting using monoclonal antibodies against RhoA (Santa Cruz Biotechnology) and Rac1 (Upstate Biotechnology), respectively.

Tube formation assay. The tube formation assay was carried out on the growth factor-free Matrigel (BD Biosciences, Tokyo, Japan) as extracellular matrix, as described previously [3]. Five hundred microliters of the Matrigel was dispensed into a 24-well plate and allowed for gelation at 37 °C for an hour. The cells that were serum-deprived for 24 h and suspended in serum-free Dulbecco's modified Eagle's minimal essential medium containing S1P, EGF, and/or other compounds were plated onto the Matrigel at the density of 5×10^4 cells/well. After the cells were incubated at 37 °C in 5% CO_2 for 4 h, each well was photographed and tubular length of the cells was quantitated in five different areas in 0.25 mm^2 using the NIH Image.

Matrigel implant model of angiogenesis in mice and RT-PCR analysis of S1P receptor mRNAs. The animal experiments were performed according to the guidelines approved by institutional Animal Use Committee in Kanazawa University. In vivo angiogenesis assay using Matrigel injection model in mice was performed as previously described [4,27]. Briefly, 500 μl of Matrigel containing either PBS, S1P, or S1P plus JTE-013 was injected subcutaneously near the abdominal midline of male C57BL/6 mice aged 6–8 weeks ($n = 5$ in each procedures). Five days later, the mice were sacrificed and implanted Matrigel plug was excised after the injection, followed by fixation with 4% paraformaldehyde in PBS. The sections were stained with hematoxylin–eosin. The degree of angiogenesis was determined by counting the number of blood vessels with apparent luminal areas in five different areas in 0.25 mm^2. The sections were also immunostained with rabbit anti-von Willebrand factor antibody (DAKO) by using the EnVision Kit (DAKO) [3]. To evaluate the expression pattern of S1P receptor subtype mRNAs in the cells that invaded into Matrigel plug, reverse transcription-mediated polymerase chain reaction (RT-PCR) was performed as follows. The Matrigel plug containing S1P was excised and digested with 10 ml of dispase solution (BD Biosciences) at 37 °C for 2 h. The isolated cells were plated and incubated in DMEM supplemented with 10% FCS for 3 h. Then RNA was extracted from the cells and reverse-transcribed by using the reverse transcriptase ReverTra Ace (TOYOBO, Tokyo Japan). PCR-based subtype specific gene amplification for $S1P_1$, $S1P_2$, and $S1P_3$ receptors was performed with LA *Taq* (TAKARA, Tokyo, Japan) using the sets of primers as follows: 5′-aactttgcgagtgagctggt-3′ and 5′-tagctgctaattgtgtcccc-3′ for $S1P_1$, 5′-cagtctccaaaaccaaccac-3′ and 5′-agtaaggtgttggccacgaa-3′ for $S1P_2$, 5′-ttagctgagacacggcagcat-3′ and 5′-ggtaccaagaagaggatggt-3′ for $S1P_3$, and 5′-aatcatgtttgagaccttcaacac-3′ and 5′-ggatcttcatgaggtagtcagt-3′ for β-actin.

Statistical analysis. Measured values were expressed as mean ± SE of 3–5 determinations. Statistical analysis was performed with Kruskal–Wallis test and p values less than 0.05 were considered statistically significant. The experiments were repeated with similar results at least twice.

Results

Stimulatory and inhibitory regulation of migration by $S1P_3$ and $S1P_2$

Mouse vascular endothelial (SVEC) cells expressed readily detectable levels of $S1P_2$ and $S1P_3$ mRNAs and a barely detectable level of $S1P_1$ mRNA, while human umbilical vein endothelial cells (HUVECs) and microvascular endothelial cells (MVECs) expressed readily detectable levels of $S1P_1$ and $S1P_3$ mRNAs, but not $S1P_2$ mRNA (Fig. 1A). The SVEC cells that were stably transfected with the $S1P_2$ expression vector (SVEC-$S1P_2$) overexpressed $S1P_2$ mRNA.

S1P stimulated trans-well migration of SVEC cells in a dose-dependent manner with a peak response observed at 10^{-7} M (Fig. 1B). In the presence of the $S1P_1$- and $S1P_3$ -selective antagonist VPC-23019 [28], however, the stimulatory effect of S1P was abolished. On the other hand, the $S1P_2$-selective antagonist JTE-013 [29] enhanced S1P -induced cell migration. Thus, S1P appears to exert both stimulatory and inhibitory effects on cell migration via

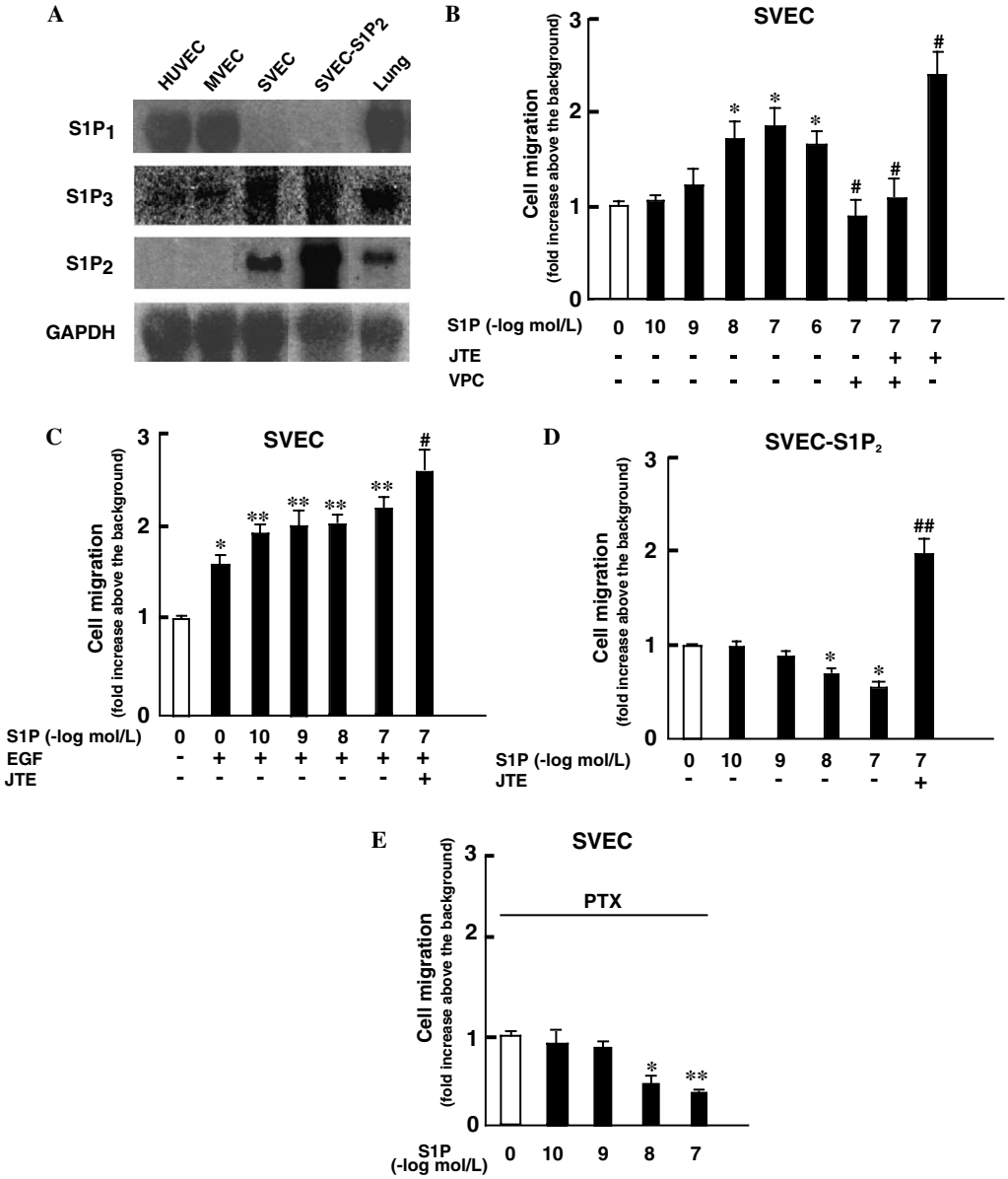

Fig. 1. S1P stimulates and inhibits endothelial cell migration via $S1P_3$ and $S1P_2$, respectively, in SVEC cells. (A) Expression of S1P receptor mRNAs in vascular endothelial cells. Twenty micrograms of total RNA isolated from the cultured cells and mouse lung was analyzed for the expression of $S1P_1$, $S1P_2$ and $S1P_3$ mRNAs by Northern blotting. (B–E) Effects of S1P and EGF on trans-well cell migration with or without the $S1P_2$ antagonist JTE-013 (JTE) (10^{-6} M), the $S1P_3$ antagonist VPC-23019 (VPC) (10^{-6} M) and PTX pretreatment (100 ng/ml for 24 h) in SVEC cells (B,C,E) and in SVEC-$S1P_2$ cells (D). S1P and EGF (20 ng/ml) were present only in the lower chamber. JTE-013 and VPC-23019 were present both in the lower and upper chambers. * and **, $p < 0.05$ and $p < 0.01$, respectively, compared with non-treated control. # and ##, $p < 0.05$ and $p < 0.01$, respectively, compared with S1P at 10^{-7} M.

$S1P_3$ and $S1P_2$, respectively. EGF modestly stimulated cell migration (Fig. 1C). The simultaneous addition of S1P with EGF induced further stimulation of migration above the level obtained with EGF alone. The addition of JTE-013 again augmented migration stimulated by the combination of EGF and S1P.

To gain further insight into the role of $S1P_2$ in the regulation of migration, we examined a cell migratory response to S1P in $S1P_2$-overexpressing SVEC-$S1P_2$ cells. Different from SVEC cells, S1P alone dose-dependently inhibited cell migration in SVEC-$S1P_2$ cells (Fig. 1D). When $S1P_2$ was blocked by JTE-013, S1P stimulated migration to the similar extent as naive SVEC cells. In SVEC-$S1P_2$ cells, S1P inhibited EGF-induced cell migration, which was also reversed by JTE-013 (data not shown). In SVEC cells pretreated with pertussis toxin (PTX), S1P dose-dependently inhibited migration (Fig. 1E), suggesting that S1P-induced stimulation of migration was mediated by G_i whereas S1P inhibition of migration is mediated by a PTX-insensitive G protein.

Stimulatory and inhibitory regulation of tube formation by $S1P_3$ and $S1P_2$

We performed tube formation assays to evaluate the effect of S1P on capillary morphogenesis in vitro. SVEC cells formed capillary tube-like structures on the Matrigel without any stimulation (Fig. 2A). The addition of either S1P or EGF modestly stimulated tube formation. The combination of S1P and EGF induced larger stimulation than either alone. JTE-013 substantially augmented S1P-induced tube formation, whereas VPC-23019 abolished S1P-induced tube formation. JTE-013 and VPC-23019 also enhanced and inhibited tube formation induced by EGF plus S1P, respectively. In SVEC-$S1P_2$ cells, S1P substantially inhibited the basal and EGF-induced tube formation (Fig. 3). In the presence of JTE-013, S1P by itself stimulated tube

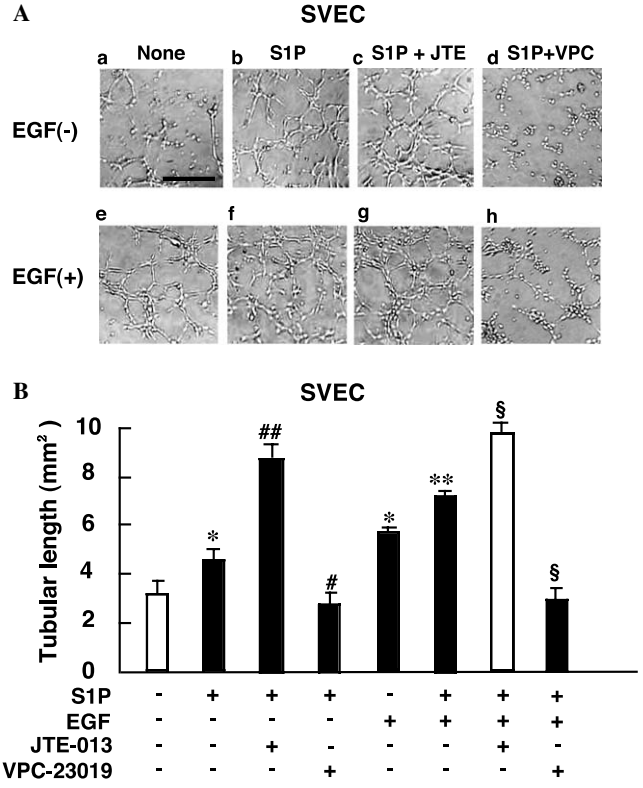

Fig. 2. S1P stimulates and inhibits endothelial morphogenesis via $S1P_3$ and $S1P_2$, respectively, in SVEC cells. SVEC cells were plated onto the Matrigel and cultured in the presence and absence of S1P, EGF and the S1P receptor antagonists. (A) Representative photographs. None (a); S1P (10^{-7} M) (b); S1P (10^{-7} M) and JTE-013 (10^{-6} M) (c); S1P (10^{-7} M) and VPC-23019 (10^{-6} M) (d); EGF (20 ng/ml) (e); EGF (20 ng/ml) and S1P (10^{-7} M) (f); EGF (20 ng/ml), S1P (10^{-7} M) and JTE-013 (10^{-6} M) (g); EGF (20 ng/ml), S1P (10^{-7} M) and VPC-23019 (10^{-6} M) (h). Bar in (a), 250 μm. (B) Quantitative summaries of tube formation. The cells were photographed and the tubular length was measured in five different areas in 0.25 mm^2 using NIH image. * and **, $p < 0.05$ and $p < 0.01$, respectively, compared with non-treated control; # and ##, $p < 0.05$ and $p < 0.01$, respectively, compared with S1P alone; §, $p < 0.05$ compared with S1P and EGF.

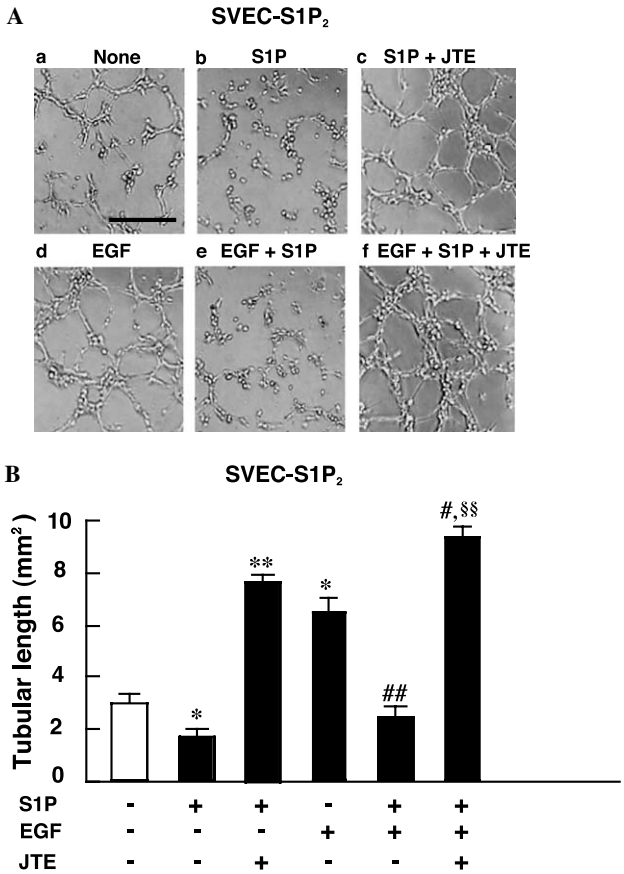

Fig. 3. S1P inhibits endothelial morphogenesis in SVEC-$S1P_2$ cells. (A,B) The SVEC-$S1P_2$ cells were plated and cultured as in Fig. 2. (A) None (a); S1P (10^{-7} M) (b); S1P (10^{-7} M) and JTE-013 (10^{-6} M) (c); EGF (20 ng/ml) (d), EGF (20 ng/ml) and S1P (10^{-7} M) (e), EGF (20 ng/ml), S1P (10^{-7} M) and JTE-013 (10^{-6} M) (f). Bar in (a), 250 μm. (B) Quantitative summaries. Tubular formations were determined as in Fig. 2. * and **, $p < 0.05$ and $p < 0.01$, respectively, compared with non-treated control; # and ##, $p < 0.05$ and $p < 0.01$, respectively, compared with EGF. §§, $p < 0.01$ compared with S1P and EGF.

formation and when combined with EGF, S1P further stimulated tube formation above the level induced by EGF alone. In SVEC cells pretreated with PTX, S1P inhibited the basal and EGF-induced tube formation (data not shown). These observations were similar to those of cell migration (Fig. 1B–E), suggesting that $S1P_2$ and $S1P_3$ mediated inhibition and stimulation of endothelial morphogenesis via a PTX-insensitive G protein and G_i, respectively.

Roles of Rho GTPases in morphogenesis and negative regulation of Rac by $S1P_2$

We analyzed the involvement of the Rho family GTPases Rac and Rho in S1P- and EGF-induced capillary morphogenesis. We infected SVEC cells with adenoviruses carrying the genes of either a dominant negative form of Rac1 (N^{17}-Rac1), a dominant negative form of RhoA

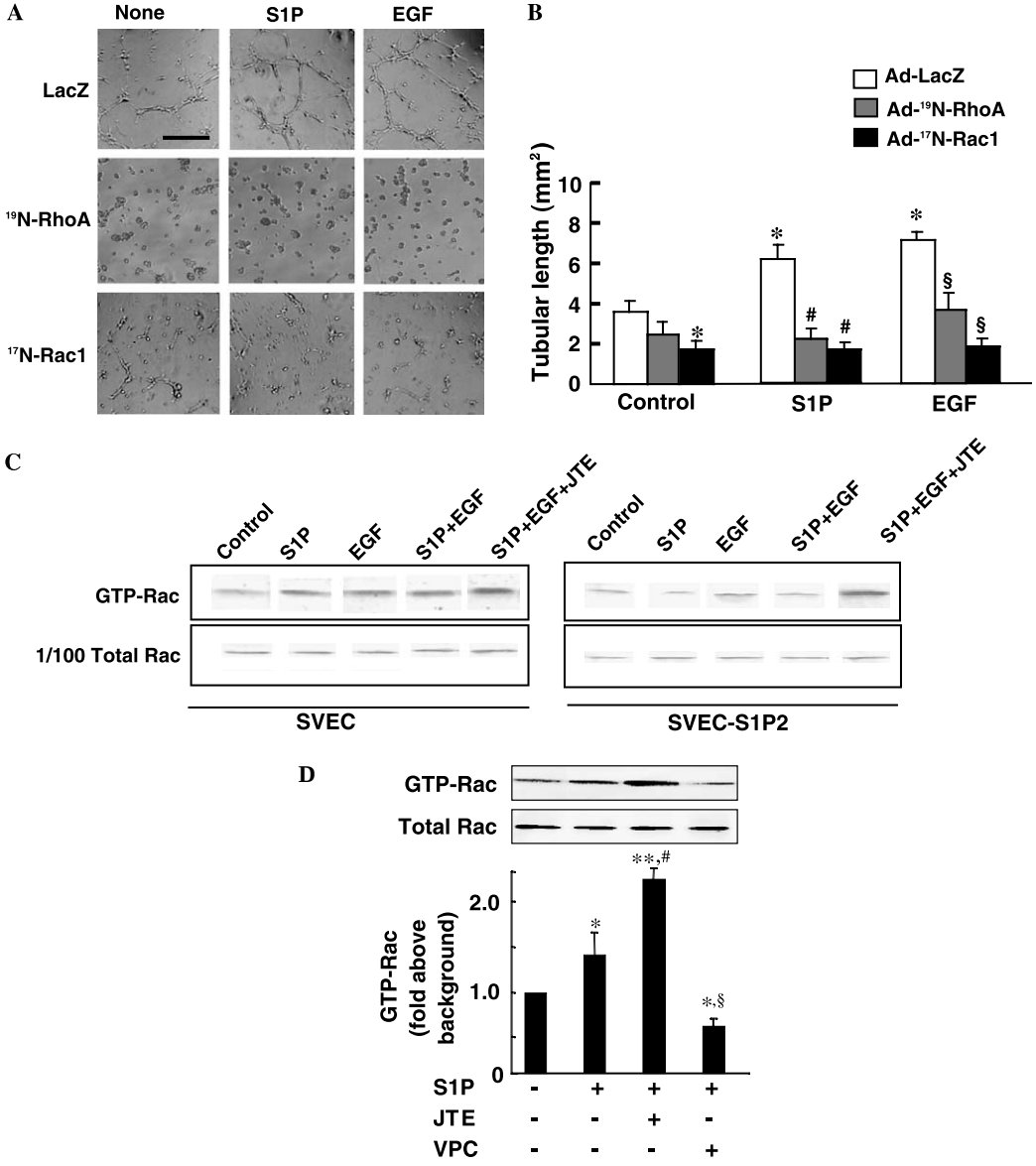

Fig. 4. Dependence of endothelial morphogenesis on Rac and Rho, and regulation of Rac by S1P. (A) Effects of ^{17}N-Rac and ^{19}N-Rho expression on S1P- and EGF-induced morphogenesis. The SVEC cells were infected with adenoviruses carrying the cDNAs encoding either of LacZ (Ad-LacZ), ^{17}N-Rac1 (Ad-^{17}N-Rac) and ^{19}N-RhoA (Ad-^{19}N-RhoA), and incubated for 24 h. The cells were then plated onto the Matrigel in the presence and absence of S1P (10^{-7} M) or EGF (20 ng/ml). Bar in (a), 250 μm. (B) Quantitative summaries of tube formation. The tube formations were determined as in Fig. 2. *, $p < 0.05$ compared with LacZ-infected non-treated control. # and §, $p < 0.05$ compared with LacZ-infected cells stimulated with S1P (10^{-7} M) alone and EGF alone (20 ng/ml), respectively. (C) Effects of S1P, EGF, JTE-013, and their combinations on the cellular amounts of GTP-bound Rac. The SVEC and SVEC-$S1P_2$ cells were non-treated or treated with either JTE-013 (10^{-6} M for 5 min), S1P (10^{-7} M for 2 min), the combination of JTE-013 and S1P stimulation, or the combination of JTE-013, S1P and EGF (20 ng/ml for 2 min). JTE-013 was added 3 min before the addition of S1P and EGF. (D) Effects of S1P on GTP-Rac in the presence and absence of JTE-013 or VPC-23019. The SVEC cells were pretreated or non-pretreated with either JTE-013 (10^{-6} M) or VPC-23019 (10^{-6} M) for 3 min, and stimulated with S1P (10^{-7} M for 2 min). (C,D) The amounts of GTP-loaded Rac and Rho were determined by the pull-down assay. A portion (1/100) of cell lysates was subjected to Western analysis for evaluating the amount of total Rac and Rho in each sample.

(N^{19}-RhoA), or LacZ as control. In LacZ-transfected cells, S1P and EGF stimulated tube formation to the similar extents as in the naïve cells. The expression of either N^{19}-RhoA or N^{17}-Rac1 tended to inhibit the basal tube formation and abolished S1P- and EGF-induced stimulation of tube formation (Fig. 4A and B).

We determined the effects of S1P and EGF on cellular amounts of GTP-bound, active forms of Rac and Rho (GTP-Rac and GTP-Rho, respectively). S1P induced a modest increase in the amount of GTP-Rac with a maximal 70% elevation at 2 min in SVEC cells (Fig. 4C, left) and GTP-Rho (data not shown). EGF also stimulated Rac approximately by twofold. S1P-induced Rac activation was enhanced by the $S1P_2$-selective antagonist JTE-013, but was inhibited below the basal level by the $S1P_3$-antagonist VPC-23019 (Fig. 4D). JTE-013 augmented Rac stimulation by the combination of S1P and EGF (Fig. 4C, left). In SVEC-$S1P_2$ cells, S1P reduced the basal level and EGF-induced increase in the amount of GTP-Rac (Fig. 4C, right). JTE-013 abolished S1P inhibition of EGF-induced Rac activation.

Negative regulation of angiogenesis in vivo by $S1P_2$

We evaluated the in vivo role of $S1P_2$ by utilizing the Matrigel implant model of subcutaneous angiogenesis in mice. The implanted Matrigel without S1P showed infiltration of a relatively small number of spindle-shaped cells and blood vessels (Fig. 5A-a). The inclusion of S1P in the Matrigel increased the numbers of infiltrating cells and blood vessels in the plug (Fig. 5A-b). The addition of JTE-013 together with S1P augmented increases in the numbers of infiltrating cells and blood vessels (Fig. 5A-c and B). Moreover, it was noted that blood vessels in the Matrigel implant containing S1P and JTE-013 were dilated and contained more red blood cells compared with S1P alone. Many of infiltrating cells and blood vessel-forming cells in the Matrigel were positive for staining for the endothelial marker von Willebrand factor (Fig. 5A-d). RT-PCR analysis showed that all of $S1P_1$, $S1P_2$ and $S1P_3$ were expressed in the cells migrated into the plug, as in Swiss 3T3 cells used as a positive control. These observations together suggest that $S1P_2$ exerts an inhibitory effect on angiogenesis in vivo.

Discussion

S1P has a variety of physiological and pathological effects on the blood vessels, which include endothelium-dependent vasorelaxation, smooth muscle contraction, stimulation of tumor angiogenesis and blood vessel maturation during development [6,8–11]. We have shown in the present study that S1P exerts inhibitory effects on vascular endothelial cell migration and morphogenesis in vitro and angiogenesis in vivo, and that the novel, angiostatic effects of S1P are mediated by $S1P_2$ most likely through Rac inhibition. This is the first study to directly examine the angiostatic activities of S1P and the involvement of $S1P_2$ in the inhibitory actions of S1P.

S1P was previously shown to stimulate vascular endothelial cell migration and tube-like formation in vitro and angiogenesis in the implanted Matrigel and agarose in vivo via $S1P_1$ and $S1P_3$ [12]. In native SVEC cells employed here, S1P by itself stimulated migration and morphogenesis (Fig. 1B and 2). When the major angiogenic receptor $S1P_3$ in this cell type was blocked by the receptor antagonist, the stimulatory effects of S1P on migration and morphogenesis were abolished. On the other hand, the selective blockade of $S1P_2$ augmented S1P stimulation of migration and morphogenesis in the absence of the $S1P_3$ antagonist. Thus,

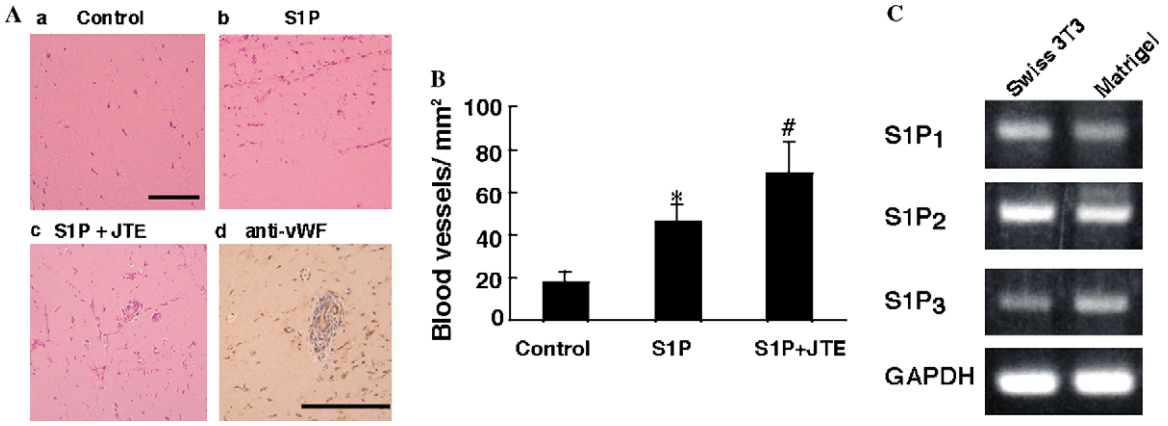

Fig. 5. $S1P_2$ negatively regulates angiogenesis in vivo in the Matrigel implant assay. (A) Matrigel plugs with or without S1P (10^{-7} M) and JTE-013 (10^{-6} M) were injected into the subcutaneous tissues in C57BL/6 mice, and 5 days later excised, followed by staining with hematoxylin–eosin (a–c) and immunohistochemistry using anti-von Willebrand factor antibody (d). Note that almost all of the cells are immuno-positive for von Willebrand factor. Bars, 500 μm. (B) Quantitative analysis of angiogenesis in the Matrigel. The number of vessels with lumen was counted in five different areas in 1 mm^2. *, $p < 0.05$ compared with non-treated control. #, $p < 0.05$ compared with S1P (10^{-7} M). (C) Expression of $S1P_1$, $S1P_2$ and $S1P_3$ mRNAs in the cells that invaded into the plug. The plug containing S1P was excised from surrounding tissues and digested with dispase for the isolation of cells. Total RNA was isolated from the cells and subjected to RT-PCR. The S1P receptor mRNA expression in Swiss 3T3 cells is shown as a positive control.

S1P exhibits both stimulatory and inhibitory effects on endothelial cell migration and morphogenesis in receptor subtype-specific, distinct manners, i.e. via $S1P_3$ and $S1P_2$, respectively. Our results suggest that $S1P_2$ counteracts $S1P_3$-mediated stimulation of migration and morphogenesis by exerting inhibitory effects on these. The net effects of S1P on migration and morphogenesis appear to depend on relative expression levels of the angiogenic $S1P_3$ and angiostatic $S1P_2$, because we observed that the overexpression of $S1P_2$ converted the S1P-induced stimulatory responses into the inhibitory ones (Fig. 1 and 3). Consistent with these in vitro observations, the blockade of $S1P_2$ potentiated S1P-induced angiogenesis in the Matrigel in mice (Fig. 5). It has not been well understood how the expression of $S1P_2$ is regulated during angiogenic processes, however, our results in in vivo experiments suggest that $S1P_2$ is expressed in the cells in the vascularlized plug and plays a role in the negative regulation of angiogenesis.

We [18] and others [12] previously showed that S1P-stimulated migration of vascular endothelial cells was dependent on the small GTPase Rac, but not Rho. However, the morphogenic response in SVEC cells was dependent on both Rac and Rho (Fig. 4). Because $S1P_2$ mediated inhibition of Rac but not Rho in SVEC cells, it is most likely that Rac inhibition at least in part contributes to the angiostatic effect of $S1P_2$. PTX pretreatment abolished S1P-induced stimulation of migration, morphogenesis and Rac activation. This is likely because the actions of the major angiogenic receptor $S1P_3$ depend on G_i [12,17,20].

A number of endogenous angiostatic molecules have been reported. Among these, certain chemokines [30] and parathyroid hormone-related peptide (PTHrP) [31] exert angiostatic effects by binding to the G protein-coupled receptors, like S1P. However, the PTHrP receptor is coupled to adenylate cyclase via G_s and exerts an angiostatic effect via protein kinase A [31]. The major angiostatic chemokine receptor is CXCR3, which is coupled to the G_i signaling pathway [30]. The exact signaling pathway that mediates an angiostatic effect of CXCR3 has not been identified yet. These observations together suggest that G protein-coupled receptors could mediate angiostatic effects by more than a single mechanism, including the G_s-cyclic AMP-protein kinase A and the Rac inhibition pathways.

In conclusion, the present study demonstrates that $S1P_2$ mediates the negative regulation by S1P of endothelial cell migration and capillary morphogenesis, and in vivo angiogenesis. Since S1P is released locally from activated platelets, endothelial cells and inflammatory cells upon their activation by thrombin, growth factors and oxidized lipoproteins etc., S1P could likely exert stimulatory and inhibitory effects on angiogenesis via the angiogenic receptors $S1P_1$ and $S1P_3$ and the angiostatic receptor $S1P_2$. The development and application of $S1P_2$-selective agonists and antagonists in conjunction with $S1P_1$- and $S1P_3$-antagonists and agonists might provide novel opportunities for angiostatic and angiogenic therapies [32].

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