

Sphingosine 1-phosphate stimulates proliferation and migration of human endothelial cells possibly through the lipid receptors, Edg-1 and Edg-3

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Sphingosine 1-phosphate (S1P) stimulates thymidine incorporation (DNA synthesis), cell growth and cell migration in human aortic endothelial cells (HAECs). The extent of the S1P-induced responses are comparable to those stimulated by vascular endothelial growth factor, one of the most potent stimulators of angiogenesis. These responses to S1P were mimicked by dihydro-sphingosine 1-phosphate, an S1P receptor agonist, and inhibited by pertussis toxin (PTX), an inactivator of G_i/G_o-proteins. S1P also induced activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAP kinase). The activation of these enzymes was inhibited again by PTX and also by suramin, a non-selective receptor antagonist. S1P-induced DNA synthesis and ERK activation were inhibited

by PD98059, an ERK kinase inhibitor, but not by SB203580, a p38 MAP kinase inhibitor. In contrast, cell migration and p38 MAP kinase activation, in response to S1P, were inhibited by SB203580 but not by PD98059. In HAECs, high-affinity S1P binding activity and expression of Edg-1 and Edg-3 mRNA were detected. These results suggest that S1P might be a novel angiogenesis factor and that the lipid-induced proliferation and migration of endothelial cells are possibly mediated through cell-surface S1P receptors, Edg-1 and Edg-3, which are linked to signalling pathways.

Key words: angiogenesis, ERK, extracellular signal-regulated kinase, p38 MAP kinase, p38 mitogen-activated protein kinase.

INTRODUCTION

Blood vessels are formed via two processes; (1) vasculogenesis, in which endothelial cells differentiate from progenitor cells and a primitive vascular network is established, and (2) angiogenesis, in which new capillaries sprout from the existing vessels to form new vessels [1,2]. In both processes, endothelial cells play an important role. They migrate, proliferate, and finally assemble to form a new capillary tube. Recent studies revealed essential roles of receptor tyrosine kinases, such as the vascular endothelial growth factor (VEGF)/VEGF receptor and angiopoietin/Tie systems, in these processes [3,4]. In addition, based on the analysis of gene-deficient mice, the G₁₃-protein also participates in cell assembly and angiogenesis [5]. Hence, G-protein-coupled receptors and their ligands are also implicated in vessel formation.

Recently, it has been suggested that sphingosine 1-phosphate (S1P), a sphingolipid metabolite, affects a variety of cellular processes, including proliferation, differentiation and cell motility [6,7]. The cellular responses elicited by S1P have been ascribed to intracellular actions. On the other hand, S1P-induced responses are also accompanied by the stimulation of several early signalling events, which are usually regulated by cell-surface receptors [8–16]. These signalling events include activation of phospholipase C [8–11], an increase in cytoplasmic free Ca²⁺ concentration [8–13,16], regulation of adenylyl cyclase [8,11,12], activation of K⁺ channels [12] and activation of Rho [14,15].

Edg-1 was first cloned from endothelial cells as a gene inducible by phorbol ester and was classified as an endothelial differentiation gene [17]. Based on sequence similarity, several cDNAs, possibly encoding G-protein-coupled receptors, have been cloned from various types of cells and tissues as orphan receptors

[18–20]. Among these cDNAs, three kinds of orphan receptor cDNAs, Edg-1, Edg-3 and AGR16/H218 (Edg-5) have recently been identified as S1P receptors [21–23]. It has been suggested that S1P is released into the bloodstream from activated platelets [24]. Therefore, it is reasonable to speculate that the cellular function of vascular endothelial cells may be influenced by platelets through S1P. In endothelial cells, however, only a few studies have been reported on the S1P actions and, furthermore, the site of action of S1P has not yet been characterized [16,25,26]. In the present study, we have examined the actions of S1P in human aortic endothelial cells (HAECs), especially focusing on the effect of the lipid on the proliferation and migration of the cells, which are essential events in angiogenesis, and their signalling mechanisms. We found that S1P is a potent stimulator, comparable to VEGF, of proliferation and migration of endothelial cells. Furthermore, the lipid actions may be mediated through the putative S1P receptors, Edg-1 and Edg-3.

EXPERIMENTAL

Materials

S1P was purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.), VEGF was obtained from Petro Tech EC Ltd. (London, U.K.), SB203580 was purchased from Calbiochem–Novabiochem (La Jolla, CA, U.S.A.) and the p38 MAP kinase assay kit was obtained from New England Biolabs (Beverly, MA, U.S.A.). [³H]S1P was enzymically synthesized from [³H]-sphingosine by sphingosine kinase-catalysed phosphorylation as described previously [27]. The sources of all other reagents were the same as described previously [27–30].

Abbreviations used: ATF, activating transcription factor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HAECs, human aortic endothelial cells; HUVECs, human umbilical vein endothelial cells; p38 MAP kinase, p38 mitogen-activated protein kinase; VEGF, vascular endothelial growth factor; PTX, pertussis toxin; S1P, sphingosine 1-phosphate.

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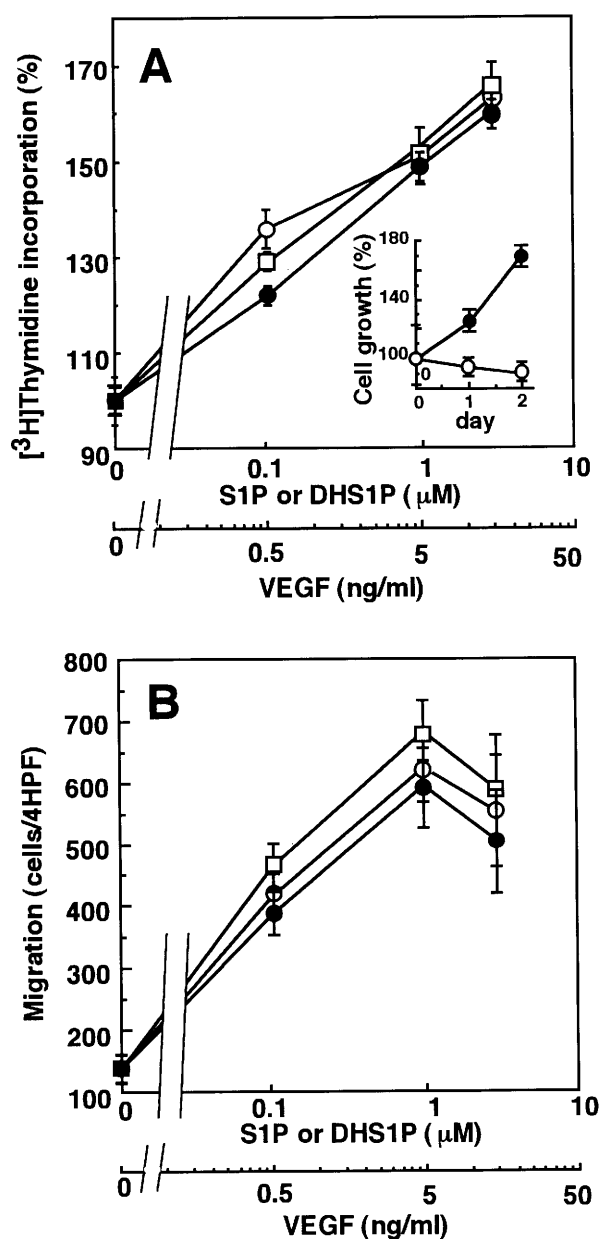


Figure 1 Effects of S1P, dihydrosphingosine 1-phosphate and VEGF on proliferation and migration of HAECs

The cells were incubated with the indicated concentrations of S1P (\circ), dihydrosphingosine 1-phosphate (\bullet) and VEGF (\square) to measure [3 H]thymidine incorporation (**A**) and cell migration (**B**). In (**A**) results are expressed as percentages of the basal value without test agents (1094 ± 73 dpm/well). The inset in (**A**) shows cell growth, the cells were incubated with (\bullet) or without (\circ) 1μ M S1P for the indicated length of time and the cell number was counted. Results are expressed as percentages of initial cell number (85000 ± 5000 cells/well). Data are means \pm S.E.M. of four separate experiments. DHS1P, dihydrosphingosine 1-phosphate.

Cell culture

HAECs and human umbilical vein endothelial cells (HUVECs) (passage number 3) were purchased from Whittaker Bioproducts (Walkersville, MD, U.S.A.). The cells (passage number 5–12) were cultured in RPMI 1640 medium supplemented with 15% (v/v) fetal bovine serum (FBS) (Sigma, St. Louis, MO, U.S.A.), 15μ g/ml endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY, U.S.A.), 10 units/ml heparin,

10 ng/ml epidermal growth factor and 1μ g/ml hydrocortisone in a humidified air/ CO_2 (19:1) atmosphere. The culture dishes and plates were coated with 100μ g/ml rat tail collagen. Where indicated, pertussis toxin (PTX, 100 ng/ml) or its vehicle (2 mM urea, final concn.) were added to the culture medium 24 h before experiments, unless otherwise stated.

[3 H]Thymidine incorporation and cell growth

The cells were cultured for 24 h with S1P or the test agent in fresh RPMI 1640 medium containing 5% FBS and for another 6 h with [3 H]thymidine (2μ Ci in 1 ml). The radioactivity in the trichloroacetic acid-insoluble fraction was measured as described previously [30]. For cell growth, when the cells became approx. 30% confluent, they were further cultured with or without S1P (1μ M) for 1–2 days in fresh RPMI 1640 medium containing 5% FBS. FBS was included in the assay medium to avoid cells from detaching from the dishes during culture. The cells were harvested following treatment with trypsin, and cell numbers were counted under a microscope.

Migration assay

The migration of endothelial cells was quantified using a blind Boyden chamber apparatus using procedures essentially the same as those described in [27], except that endothelial cells and RPMI 1640 medium were used instead of CHO cells and Dulbecco's modified Eagle's medium. The number of cells that had migrated during 4 h to the lower surface was determined by counting the cells in four places under microscopy at $\times 400$ magnification (4HPF). Since the incubation time for migration experiments was short compared with experiments measuring activity of DNA synthesis, FBS was not included in the assay medium.

Measurements of extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (p38 MAP kinase) activity

The HAECs were washed once, preincubated for 20 min with or without PD 98059 (10μ M) or SB203580 (1μ M) at 37°C in a HEPES-buffered medium consisting of 20 mM HEPES, 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 2 mM CaCl_2 , 2.5 mM NaHCO_3 , 5 mM glucose and 0.1% (w/v) BSA (fraction V), pH 7.5, and then incubated for 5 min with test agents. For measurement of ERK1/2 activity, the incubation was terminated by washing twice with ice-cold PBS and adding 0.5 ml of lysis buffer, as described previously [29]. The same lysate was also analysed by Western-blotting with an ERK-specific antibody, as described previously [29]. For measurement of p38 MAP kinase activity, active (phospho) p38 MAP kinase was immunoprecipitated and its activity was evaluated by its ability to phosphorylate the activating transcription factor (ATF)-2 fusion protein, according to the manufacturer's instructions (p38 MAP kinase assay kit from New England Biolabs). Briefly, the phosphorylated ATF-2 was separated by electrophoresis and detected by a phospho-ATF-2 antibody. Phosphorylation of ATF-2 was quantified by densitometry.

S1P receptor binding

S1P receptor binding was performed essentially as described in [27]. The cells were incubated with [3 H]S1P, from 3.1–50 nM, for dose-dependent binding. The dissociation constant (K_d) and maximal binding were estimated from Scatchard analysis. To estimate the affinity of the S1P-related lipids for the S1P binding site, the cells were incubated with 1 nM [3 H]S1P (approx.

15000 dpm in 0.4 ml) in the presence of appropriate doses of test agents. Other experimental procedures have been described previously [27]. Specific S1P binding to its receptor was estimated by subtracting the radioactivity in the presence of 10 μ M unlabelled S1P for Scatchard analysis and 1 μ M unlabelled S1P for the competition experiments.

RNA extraction and Northern-blot analysis

Total RNA was prepared from HAECs according to the manufacturer's instructions for TRIZOL reagent (Life Technologies, Frederick, MD, U.S.A.). Northern-blot analysis was performed as described previously [29].

Data presentation

All experiments were performed in duplicate or triplicate. The results of multiple observations were presented as means \pm S.E.M. of at least three separate experiments unless otherwise stated.

RESULTS

S1P induces DNA synthesis and migration of endothelial cells

In Figure 1, we show the effects of S1P on DNA synthesis and

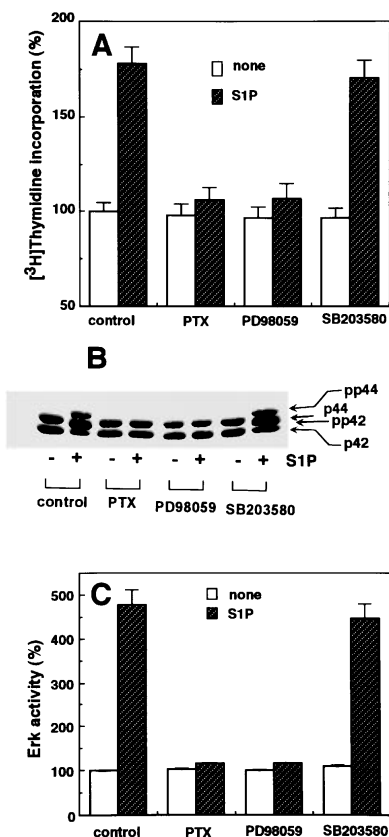


Figure 2 Effects of PTX, PD98059 and SB203580 on S1P-induced DNA synthesis and ERK activation

HAECs, which had been treated or not treated with PTX, PD98059 (10 μ M for 20 min) or SB203580 (1 μ M for 20 min), were incubated with or without 1 μ M S1P to measure [3 H]thymidine incorporation (A), the change in gel mobility of ERK (B) and ERK activity (C). In (A) and (C), results are expressed as percentages of the basal values in control cells; 1129 \pm 71 dpm/well for [3 H]thymidine incorporation and 0.66 \pm 0.09 pmol/min for ERK activity. Data are means \pm S.E.M. for (A) and (C), and representative for (B), of four separate experiments.

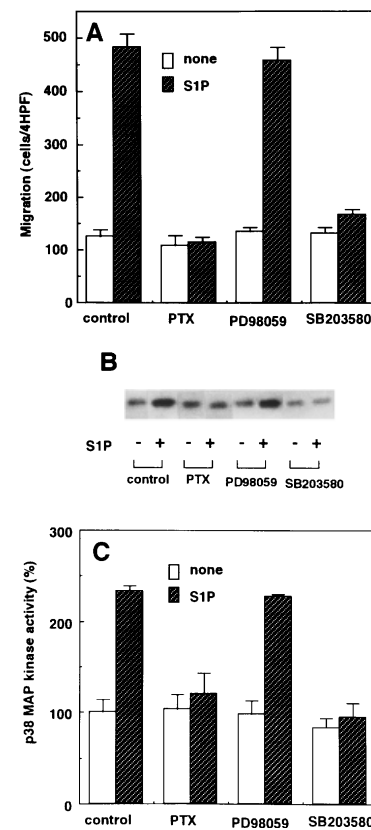


Figure 3 Effects of PTX, PD98059 and SB203580 on S1P-induced migration and p38 MAP kinase activation

Experimental conditions are essentially the same as those for Figure 2 except for measurements of cell migration (A) and p38 MAP kinase activity (B and C). In (B) the representative electrophoresis pattern of the ATF-2 fusion protein, phosphorylated by activated p38 MAP kinase, is shown. In (C) the activity was evaluated by densitometry and results are expressed as percentages of the basal value (arbitrary units) in control cells. Data are means \pm S.E.M. for (A) and (C), and representative for (B), of four separate experiments.

cell migration as compared with the effects of VEGF, a potent angiogenesis factor, in HAECs. As expected, VEGF clearly stimulated [3 H]thymidine incorporation and migration of HAECs, with a similar affinity for both responses. S1P also stimulated both responses. The maximal activity was attained at 3 μ M for [3 H]thymidine incorporation (Figure 1A) and at 1 μ M for cell migration (Figure 1B). Increasing the concentration of the lipid to 10 μ M resulted in the reduction of these activities to almost basal levels (results not shown). Such bell-shaped dose-response curves might be explained in part by the toxic effect related to its lipophilic nature. Thus, the magnitude of the response to S1P was almost identical to that of the corresponding response to VEGF. The increase in [3 H]thymidine incorporation by S1P was actually associated with an increase in cell growth (Figure 1A, inset). In the experiments measuring DNA synthesis, 5% FBS was included in the assay medium to avoid detachment of the cells from the dishes. However, we observed a stimulatory action of S1P on DNA synthesis even without FBS with a similar potency (EC_{50} approx. 100 nM), although the magnitude of the activity without FBS, regardless of the presence of S1P, was about one-third of the activity with FBS (results not shown). We also examined the effects of dihydrosphingosine 1-phosphate, which is thought to be an agonist for S1P receptors [31]. We found that the S1P derivative mimicked the S1P-induced actions

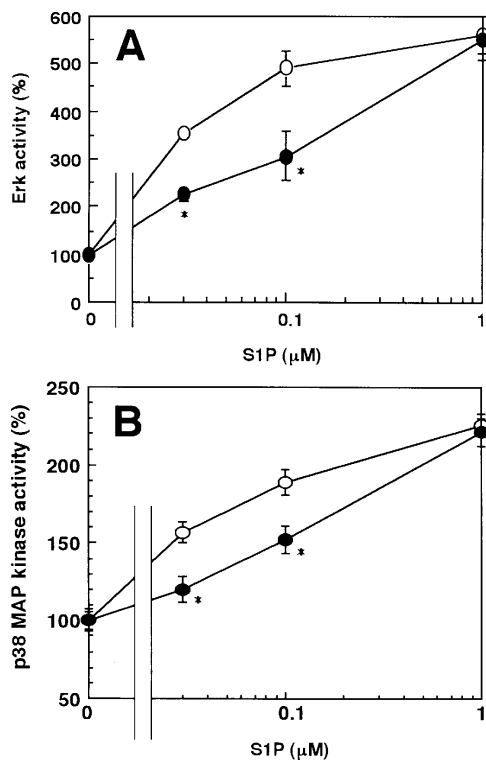


Figure 4 Effects of suramin on S1P-induced activation of ERK and p38 MAP kinase

The cells were incubated with the indicated concentrations of S1P in the presence (●) or absence (○) of 100 μM suramin to measure ERK activity (A) or p38 MAP kinase activity (B). In (A) results are expressed as percentages of the basal ERK activity without S1P. The basal activity was 0.63 ± 0.06 pmol/min in control cells and 0.66 ± 0.09 pmol/min in the suramin-treated cells. In (B) the activity was evaluated by densitometry and results are expressed as percentages of the basal activities (arbitrary units) in the cells treated with or without suramin. Suramin hardly affected the basal activity. Data are means \pm S.E.M. of three separate experiments. *Significant effect of suramin ($P < 0.05$).

with a potency similar to that of S1P for both responses (Figure 1). Similar effects of S1P were observed in HUVECs as well. S1P stimulated [3 H]thymidine incorporation and migration of HUVECs, with a similar potency for both responses (results not shown).

S1P-induced DNA synthesis and migration of cells seem to be mediated by PTX-sensitive G-proteins but by different signalling pathways

The S1P-induced [3 H]thymidine incorporation was markedly suppressed by treatment of the cells with either PTX or PD98059, an ERK kinase inhibitor (Figure 2A). These results suggest that G_i/G_o -proteins and an ERK pathway may be involved in S1P-induced DNA synthesis. Actually S1P stimulated the phosphorylation of the ERK-specific substrate peptide (Figure 2C). The S1P-induced activation of ERK was associated with a change in the gel mobility of ERK1/2 on electrophoresis, suggesting phosphorylation of the enzyme by S1P (Figure 2B). The enzyme activation and the gel mobility-shift were completely inhibited by PD98059, an ERK kinase inhibitor, but not by SB203580, a p38 MAP kinase inhibitor (Figures 2B and 2C).

Migration of HAECs was also inhibited by PTX, but the sensitivity to the MAP kinase inhibitors was totally different: the p38 MAP kinase inhibitor (SB203580) markedly inhibited the

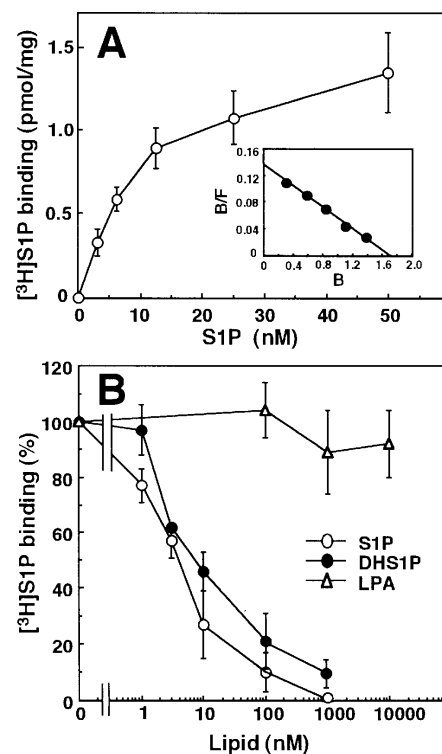


Figure 5 Expression of a high-affinity S1P binding activity in HAECs

(A) Specific [3 H]S1P binding is plotted against S1P concentrations. Data are means \pm S.E.M. of three separate experiments. The mean value was used for calculation of the binding parameters based on the Scatchard plot (inset in A), where B is S1P binding activity (pmol/mg protein) and F is free S1P concentration (nM). (B) Cells were incubated with 1 nM [3 H]S1P in the presence of the indicated concentrations of lipids. Results are expressed as percentages of the basal activity without these test agents. The basal value was 134 ± 7 fmol/mg protein. Data are means \pm S.E.M. of three separate experiments. DHS1P, dihydrosphingosine 1-phosphate; LPA, lysophosphatidic acid.

S1P-induced cell migration, whereas the ERK kinase inhibitor (PD98059) was ineffective in suppressing it (Figure 3A). S1P actually stimulated p38 MAP kinase, and the change in its enzyme activity (Figures 3B and 3C) was parallel to the activity of the lipid-induced cell migration (Figure 3A).

Suramin inhibits the S1P-induced activation of ERK and p38 MAP kinase

Suramin has been shown to suppress angiogenesis activity [32]. Furthermore, this agent has been shown to be a non-selective antagonist of G-protein-coupled receptors [14,15]. As shown in Figure 4 S1P-induced activation of ERK and p38 MAP kinase was competitively inhibited by suramin. We also examined the effect of suramin on DNA synthesis and cell migration. Unexpectedly, however, suramin did not exert significant effects on these S1P-induced functions (results not shown).

Detection of a high-affinity S1P binding activity and expression of Edg-1 and Edg-3 mRNA in HAECs

In Figure 5 we show the S1P binding activity in HAECs. The K_d and maximal binding activity were estimated as 12.1 nM and 1.70 pmol/mg protein, respectively, based on Scatchard analysis (Figure 5A, inset). The K_d value was very similar to that for S1P-receptors expressed in CHO cells [27]. The binding affinity of

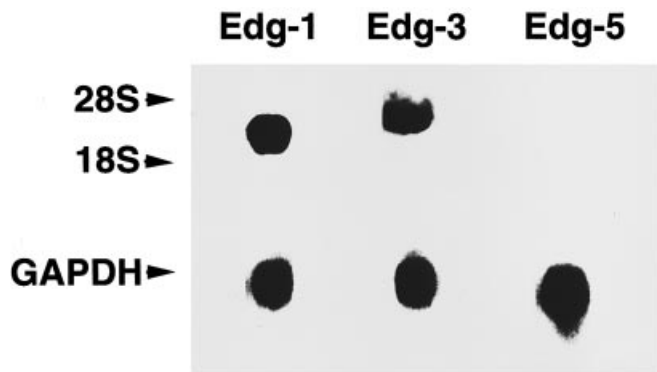


Figure 6 Northern-blot analysis of putative S1P receptors

Total RNA (10 μ g) prepared from HAECs was electrophoresed to analyse the expression of Edg-1, Edg-3 and AGR16 (Edg-5) mRNA. To compare the amount of RNA in each lane, the membrane filters were rehybridized with a probe of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results are representative of four separate experiments.

dihydrosphingosine 1-phosphate to the S1P receptor was evaluated based on competition with [3 H]S1P binding (Figure 5B). As expected from the experiments shown in Figure 1, the affinity of dihydrosphingosine 1-phosphate was almost the same as S1P. The specificity of [3 H]S1P binding was confirmed by the lack of competition by lysophosphatidic acid (Figure 5B).

Of three types, Edg-1, Edg-3 and AGR16 (Edg-5), which have been identified as S1P receptors so far, mRNA of both Edg-1 and Edg-3 were clearly detected, but we could not detect AGR16 mRNA expression in HAECs (Figure 6).

DISCUSSION

In endothelial cells, Edg-1 was first cloned as an endothelial differentiation gene, which was upregulated by phorbol ester-induced differentiation [17]. Later, several related genes to Edg-1 were cloned, and a group of Edg family proteins were identified as S1P receptors. However, the role of S1P and its receptor systems in endothelial cells has not been well defined. In the present study, we have shown that S1P, probably through the Edg family of G-protein-coupled receptors, induced the proliferation and migration of endothelial cells. The stimulation of proliferation and migration of endothelial cells by S1P is supported by several recent reports [26,33–35], performed independently of the present study.

DNA synthesis and cell migration in response to S1P were inhibited by PTX, suggesting that both S1P-induced actions were mediated through PTX-sensitive G_i/G_o -proteins. However, the downstream signalling pathways leading to DNA synthesis and cell migration in response to S1P are largely different. S1P-induced DNA synthesis was inhibited by an ERK kinase inhibitor, but not by a p38 MAP kinase inhibitor. In contrast, the migration response to S1P was inhibited by the p38 MAP kinase inhibitor, but not by the ERK kinase inhibitor. The EC_{50} value for enzyme activation (approx. 30 nM for both ERK and p38 MAP kinase, see Figure 4) was slightly lower than that for migration or proliferation (approx. 100 nM for both responses, see Figure 1). However, this may simply reflect the difference in the incubation time, i.e. 5 min for enzyme activity measurements and 4–30 h for migration and proliferation activities. Actually, the decline in the apparent affinity of S1P to stimulate receptor-mediated actions has been observed by increasing the incubation time at the expense of an increase in the efficacy (increase in the

maximal response) [27,28]. This may be explained by the rapid metabolism of S1P through degradation or uptake into the cells. Thus, we concluded that ERK is essential for DNA synthesis and that p38 MAP kinase is essential for cell migration. The involvement of p38 MAP kinase in the migration of endothelial cells has recently been suggested for VEGF as well [36]. The activation of ERK and p38 MAP kinase in response to S1P was also inhibited by PTX, suggesting that both ERK and p38 MAP kinase may be located downstream of the G_i/G_o -proteins in S1P signalling. Recent studies suggested that G_{12}/G_{13} -proteins and Rho, one of the small G-proteins, are also upstream regulators of cell migration [5,15,37]. Thus, G_i/G_o -proteins may regulate, in collaboration with G_{12}/G_{13} -proteins and Rho, the migration of endothelial cells.

There are two ways for exogenous S1P to induce cellular responses; intracellular mechanisms through intracellular unidentified targets and extracellular mechanisms through cell-surface receptors. In previous studies, in fibroblasts [6,31], the intracellular mechanisms have been proposed for S1P action on cell proliferation. Our results, however, favour the extracellular mechanism, through S1P receptors, for the S1P-induced actions on the proliferation and migration of endothelial cells. Firstly, the S1P-induced actions were suppressed by PTX treatment, suggesting the involvement of G_i/G_o -protein-coupled receptors in the lipid actions. Secondly, dihydrosphingosine 1-phosphate, which has been suggested to be a cell-surface receptor agonist but not a ligand for the intracellular targets [31], was as equipotent as S1P in activation of DNA synthesis, ERK activation and cell migration. Thirdly, the S1P-induced activation of ERK and p38 MAP kinase was suppressed by suramin, a non-selective receptor antagonist, although this drug failed to inhibit the lipid-induced DNA synthesis and cell migration. The failure of suramin to inhibit these parameters might be explained in part by the difference in incubation time for the assays. ERK and p38 MAP kinase activities were measured after 5 min, while DNA synthesis and cell-migration activities were measured at more than 4 h after addition of test agents. Suramin has been suggested to be incorporated into the cells during incubation [38]. Finally, we could detect a high-affinity S1P binding activity, and expression of Edg-1 and Edg-3 mRNA in HAECs.

In HAECs, both Edg-1 and Edg-3 may be responsible for the S1P-induced actions. Suramin has recently been reported to selectively antagonize Edg-3, but not Edg-1- and AGR16-induced actions, suggesting that it is an Edg-3-selective antagonist [39]. The finding that suramin competitively inhibited S1P-induced activation of ERK and p38 MAP kinase suggests that at least Edg-3 may be involved in these S1P-induced actions. These results, however, do not rule out the possible involvement of Edg-1 in these actions; the suramin-insensitive part of S1P actions might be mediated through Edg-1. Recent studies have shown that S1P actually stimulated ERK activity and cell migration activity when the S1P receptors were overexpressed in mammalian cells [21–23,27,40], although an involvement of p38 MAP kinase in cell migration was not demonstrated in these previous studies. For ERK activity, all S1P receptors seem to be effective, whereas, for cell migration activity, Edg-1 and Edg-3, but not AGR16, was effective [27,40]. Furthermore, recent studies on endothelial cells performed by several groups independently of ours suggested an important role for Edg-1 in the S1P-induced ERK activation, proliferation and migration [33–35]. These results further support the possible coupling of both Edg-1 and Edg-3 to the signalling pathways leading to ERK activation/cell proliferation and p38 MAP kinase activation/cell migration in endothelial cells.

Angiogenesis is thought to be an important event in the

process of the repair of female reproductive tissues during the menstrual cycle and in the abnormal growth and metastasis of tumour cells [1–4]. Furthermore, the proliferation and migration of endothelial cells may be important for the remodelling of the vascular system in the case of wounds and vascular inflammation, such as atherosclerosis. SIP is released from activated platelets and a few transformed cells [7]. Although the role of platelets in these physiological and pathological conditions has not been well defined, the released SIP might be involved in these processes. Based on experiments using gene-deficient mice, the G_{13} -protein has been suggested to participate in angiogenesis [5]. Among a variety of G-protein-coupled receptors, it has been suggested that a group of receptors couples with the G_{12}/G_{13} family of G-proteins. These include thrombin, lysophosphatidic acid and probably SIP receptors [15,41]. Thus, certain types of SIP receptors expressed in endothelial cells might be one of the G_{13} -protein-coupled receptors implicated in angiogenesis, although their coupling has not yet been demonstrated in this cell type.

In conclusion, SIP stimulates the proliferation and migration of endothelial cells to an extent comparable with that stimulated by VEGF, one of the most potent angiogenesis factors. The SIP-induced actions seem to be mediated by MAP kinase pathways, which are linked to cell-surface G-protein-coupled SIP receptors. The proliferation activity may be regulated by the ERK pathway, and the migration activity by p38 MAP kinase. Thus, SIP receptors might be the members of G-protein-coupled receptors involved in angiogenesis.

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