Inhibition of chemotactic motility and trans-endothelial migration of human neutrophils by sphingosine 1-phosphate

Shigeyuki Kawa, Satoshi Kimura, Sen-itiro Hakomori, Yasuyuki Igarashi*

The Biomembrane Institute and Department of Pathobiology, University of Washington, Seattle, WA 90104, USA

Abstract In previous studies, we reported that sphingosine 1-phosphate (Sph-1-P) inhibits the chemotactic motility of some cancer cell lines such as mouse melanoma cells, as well as human smooth muscle cells, at a very low concentration, as demonstrated by a transwell migration assay method (Proc. Natl. Acad. Sci. USA 89, 9698, 1992; J. Cell Biol. 130, 193, 1995). In this study, we investigated the effect of Sph-1-P on the chemotactic motility and invasiveness of human neutrophils, utilizing three different assay systems: (a) a transwell migration assay where IL-8 or fLMP was added as a chemotactic factor, (b) a phagokinetic assay with gold colloids, and (c) a trans-endothelial migration assay with human umbilical vein endothelial cells (HUVECs) plated on collagen layers. We found that among various sphingosine derivatives, Sph-1-P specifically inhibited the IL-8- or fLMP-induced chemotactic migration of neutrophils at concentrations below 1 μM. Phagokinetic activity of neutrophils was also suppressed by Sph-1-P, but more moderately than by the PKC inhibitory sphingosine analog, trimethylsphingosine. Finally, Sph-1-P inhibited trans-endothelial migration and invasiveness of neutrophils into HUVEC-covered collagen layers, whereas no effect on their adhesion to HUVECs was observed. These observations strongly suggest that Sph-1-P can act as a specific and effective motility regulator of human neutrophils, raising the possibility of future applications of Sph-1-P, or its analogs, as anti-inflammatory agents regulating invasive migration of neutrophils through endothelial layers at injured vascular sites.
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Key words: Sphingosine 1-phosphate; Neutrophil motility; Neutrophil migration

1. Introduction

Sphingosine 1-phosphate (Sph-1-P), the initial product of the catabolism of sphingosine by sphingosine kinase, had once been regarded simply as an intermediary metabolite in sphingolipid metabolism in cells [1]. However, recent studies have shown that Sph-1-P has several important physiologic functions other than its role as a metabolite of Sph. Although originally proposed as a mitogenic messenger [2,3], Sph-1-P has since been reported to be involved in a variety of cellular functions, including activation of human platelets acting in an autocrine manner [4,5], activation of muscarinic K^+ current in atrial myocytes [6], mediation of FcεRI antigen receptor signalling [7], and neurite retraction [8,9].

In our previous studies, we also found that exogenously added Sph-1-P inhibits the chemotactic motility of various cancer cells as well as human smooth muscle cells [10,11] by interfering with actin filament reorganization in the leading edges of pseudopodia stimulated by chemotactic factors [12]. Furthermore, very recently, we found that Sph-1-P inhibits cell motility of melanoma cells by acting through its cell surface receptors [13]. However, in these studies, the sensitivity for Sph-1-P was very different among various cell types and motility inhibition by Sph-1-P seemed a cell-type specific phenomenon [10]. Therefore, in the present study, we utilized different in vitro assay systems to investigate whether Sph-1-P can affect the chemotactic motility and trans-endothelial invasiveness of human neutrophils, fast migrating cells, the trans-endothelial migration of which seems to be relevant to the development of inflammatory reactions in blood vessels.

2. Materials and methods

2.1. Chemicals

Sphingosine, trimethylsphingosine (TMS), C8-ceramide (*N*-octanoyl sphingosine), sphingosine 1-phosphate (Sph-1-P), dihydro-Sph-1-P, and [1-³H]Sph were prepared in this lab as previously described [14–17]. Stock solutions of Sph-1-P and other sphingosine derivatives (2 mM) were prepared in ethanol:water (1:1) solution, stored at −20°C and sonicated for 30 s at room temperature just before use, with appropriate dilution. Control experiments were performed with ethanol (final concentration, < 0.5%) as a vehicle, which did not affect neutrophil functions. IL-8 and IL-1β were purchased from UBI (Lake Plasid, NY), phorbol 12-myristate 13-acetate (PMA) was from Calbiochem (Indianapolis, IN). Other chemicals were reagent grade and purchased from Sigma (St. Louis, MO).

2.2. Human neutrophils and other cells

Human neutrophils were isolated with Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) centrifugation method, as previously described [18]. The final preparation consisted of > 98% neutrophils, as determined by Wright-Giemsa staining. Cell suspensions were kept on ice and used within 3 h. Human umbilical vein endothelial cells (HUVEC) and CS-C culture medium (acidic fibroblast growth factor and heparin supplemented) were obtained from Cell System (Kirkland, WA), and cultured to monolayer on a collagen bed in 24-well culture plates as previously described [19,20].

2.3. Neutrophil myeloperoxidase assay

For quantification of neutrophil cell number, we measured the myeloperoxidase activity of neutrophils, as previously described [21]. Briefly, neutrophils sedimented with light centrifugation were solubilized in 10 μl of 0.1% Triton-X 100 with sonication for 10 min. To this lysate, 300 μl o-dianisidine solution (10 mg o-dianisidine in 20 ml 0.05 M phosphate-citrate buffer, pH 5, containing 0.003% sodium perborate) was added, the color reaction was developed for 30 min, terminated by the addition of 50 μl 0.5 N HCl, and the OD_{405} was measured with reference at 630 nm. The linear relationship between the

*Corresponding author. Present address: Fred Hutchinson Cancer Research Center, 1124 Columbia Street, M621, Seattle, WA 98104, USA. Fax: +1 (206) 667-6519. E-mail: yigarash@fhcrc.org

absorbance and cell counts was observed at the 1×10^4–1×10^5 cells. This correlation was unaffected under various medium conditions used in this study, trypsin-EDTA, RPMI 1640, 10^{-8} M fMLP, or 5 ng/ml IL-8.

2.4. Transmigration assay with HUVEC monolayer

Transmigration assay of neutrophils (1.6×10^6 cells) was performed on IL-1β-stimulated HUVEC monolayer, as previously described [19]. After formaldehyde fixation, the collagen bed with surface HUVECs and migrated neutrophils, was embedded in paraffin, stained with hematoxylin-eosin, and sliced for slide preparation. Photomicrographs were taken for each slide ($\times 100$). For quantification of migrated cells, cells in the collagen bed were counted per 100 μm length of HUVEC in pictures of 23 to 63 different fields for each experiments.

2.5. Adhesion assay with HUVEC monolayer

Human neutrophil suspension (300 μl, 1×10^6 cells/ml) in M199 with 1% heat-inactivated FCS was added on HUVEC monolayer, prestimulated with IL-1β (10 U/ml) for 4 h, and incubated for 15 min at 37°C. Non-adherent neutrophils were removed by gentle shaking and aspiration. After the plates were washed with PBS, neutrophils attached on HUVEC were treated with 300 μl trypsin-EDTA solution, and detached cells were transferred to a microcentrifuge tube and mixed with 100 μl RPMI 1640 with 10% heat-inactivated FCS. Adherent neutrophils were counted by measuring the myeloperoxidase activity as described above. As a control, the enzyme activity of neutrophil attached to unstimulated HUVEC and that of HUVEC alone was measured.

2.6. Chemotaxis assay with modified Boyden chamber

Neutrophil chemotaxis was measured using a transwell chamber (Costar, Cambridge, MA) with 6.5 mm diameter polycarbonate filters (3 μm pore size) as previously described [10]. Briefly, human neutrophils (1×10^6 cells/ml) in RPMI 1640 with heat-inactivated FCS were preincubated with test compounds for 10 min at 37°C, and a 100 μl cell suspension was loaded into the upper chamber. 600 μl of medium containing a chemoattractant (5 ng/ml IL-8, or 10^{-8} M fMLP) and a test compound was loaded in the lower chamber. Then, the upper chamber was combined to the lower chamber, and incubated at 37°C for 30 min in a CO_2 incubator. Migrated cells (those in the lower chamber and attached to the lower surface of the filter) were trypsinized and counted by measuring myeloperoxidase activity.

2.7. Phagokinetic activity with gold colloid glass plates

Phagokinetic assays with gold colloid-coated plates were performed as described [19,22]. Briefly, fresh human neutrophils (4×10^3 cells/plate) were seeded on gold colloid-coated coverslips, in RPMI 1640 with or without Sph derivatives, and incubated at 37°C for 30 min in a CO_2 incubator. After formaldehyde fixation, the coverslips were mounted onto glass microscope slides. The tracks of phagocytosis were traced onto transparent sheets from a television monitor connected to a microscope, and the areas of phagocytosis were calculated by measuring the weight of photocopied tracks [10,19].

2.8. O_2 consumption and O_2^- production

O_2 consumption of neutrophils stimulated with PMA in the presence of Sph derivatives was performed using a Clark-type electrode with a Model 5300 biological O_2 monitor and micro O_2 chamber (Y.S.I. Inc. Yellow Springs, OH), as recently described [19]. O_2^- production was measured by monitoring O_2^--mediated cytochrome C reduction as described [19].

3. Results

3.1. Inhibition of chemotactic migration of neutrophils with Boyden chamber

First, effects of exogenously added Sph-1-P and other sphingosine derivatives on human neutrophil migration were examined utilizing a modified Boyden chamber with Costar Transwell tissue culture plates. In this experiment, neutrophils migrated into the lower chamber under the influences of neutrophil chemotactic factors, fLMP and IL-8 [23], and in the presence or absence of tested compounds, were quantified by
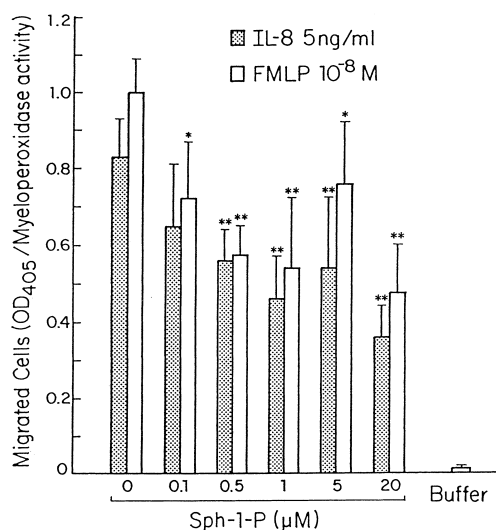

Fig. 1. Sph-1-P effects on IL-8- and fMLP-induced neutrophil chemotaxis with Boyden chamber. 100 μl of human neutrophils (1×10^6/ml) in RPMI 1640 supplemented with 1% heat-inactivated FCS was preincubated with tested compounds for 10 min at 37°C and put into the upper chamber of a transwell assay system with a 3 mm pore size polycarbonate membrane. IL-8 (5 ng/ml) or fMLP (10^{-8} M), together with the lipid compounds in 600 μl of the same buffer was put into the lower chamber. After 30 min incubation, migrated cells (in lower chamber media and detached cells from lower surface of membrane with trypsin-EDTA) were collected, and cell numbers were counted by myeloperoxidase measurement. In this assay system, spontaneous migration with buffer alone was negligible. The data were mean ± S.D. of four independent experiments. $*P < 0.05$, $**P < 0.01$.

measuring their myeloperoxidase activity. Under these experimental conditions, spontaneous migration of neutrophils was negligible. Addition of both fMLP (10^{-8} M) or IL-8 (5 ng/ml) stimulated chemotactic migration of neutrophils about 100-fold. The fLMP- or IL-8-induced chemotaxis was significantly inhibited at 0.5 μM or higher concentrations of added Sph-1-P with about 50% of inhibitions being observed at the optimal concentration, 1 μM (Fig. 1). A transient decrease of inhibition was observed at 5 μM, as we reported previously in melanoma experiments [10], but the underlying mechanism for the U-shaped inhibition curves is not known. It should be noted that viability of neutrophils remained similar from controls up to 75 μM of Sph-1-P, by trypan-blue exclusion assay. Therefore, these effects were not due to cytotoxicity of this compound even at the highest concentration. Inhibition of neutrophil migration by Sph-1-P was very specific among various related sphingolipids. No other derivatives tested (sphingosine, N-formylsphingosine, C8-ceramide, TMS) showed any significant inhibitory effect even at 1 μM concentration (Table 1). Interestingly, a closely related analog (without 4,5-double bond) of Sph-1-P, dihydro-Sph-1-P, was also totally ineffective.

3.2. Effects on phagokinetic tracks of neutrophils and O_2^- formation

As shown in Table 2, Sph-1-P showed significant effects on phagokinetic activity of neutrophils, as measured by gold colloid assay, in a similar dose-dependent manner, although TMS, a lipid PKC inhibitor, showed much stronger effects even at a lower concentration, as we already reported previously [19]. On the other hand, C8-ceramide did not show

Table 1
Effects of Sph-1-P and other sphingosine derivatives on IL-8- and fLMP-induced chemotactic migration in a Boyden chamber

Tested compound 1 μM	% of control	
	IL-8 (10^{-8} M)	fMLP (5 ng/ml)
None (control)	100 ± 12	100 ± 23
Sph-1-P	46 ± 13**	54 ± 18**
dihydro-Sph-1-P	81 ± 18	93 ± 12
sphingosine	87 ± 8	90 ± 17
N-formylsphingosine	81 ± 14	88 ± 17
TMS	79 ± 29	90 ± 22
C8-ceramide	95 ± 12	92 ± 6

The data were mean ± S.D. of three independent experiments.
**$P < 0.01$.

inhibition even at a higher concentration, 5 μM. These results probably reflect the facts that the gold colloid phagokinetic assay method may measure mixed abilities of random cell motility and phagocytosis [22], and that phagocytotoxic ability of neutrophils requires PKC activation [19]. Consistent with these results, as we reported previously, PMA-induced O_2^- formation in neutrophils was strongly inhibited by TMS [19], whereas Sph-1-P as well as ceramide, which have no PKC inhibitory activity, had no effects on the formation of O_2^- in PMA-activated neutrophils (data not shown).

3.3. Inhibition of in vitro trans-endothelial invasion of neutrophils

To examine the effects of Sph-1-P on trans-endothelial invasion of human neutrophils, we utilized the experimental system of a HUVEC monolayer plated on a collagen matrix, roughly resembling the in vivo situation of transmigration of neutrophils, through attachment to inactivated endothelial cells, into the vascular matrix. In this experiment, human neutrophils, plated on the IL-1β-activated (4 h preincubation) HUVEC monolayer, attached to HUVECs and migrated into the collagen matrix during 90 min incubation. As shown in Fig. 2A and B, trans-endothelial migration (invasion) of neutrophils was significantly inhibited by pretreatment with 0.1–1.0 μM Sph-1-P, and even more strongly suppressed by pretreatment with higher concentrations around 5–15 μM. Here, the viability of both HUVEC and human neutrophils was not affected up to 75 μM. On the other hand, sphingosine or C8-ceramide did not show any effects under these conditions (data not shown).

To exclude the possibility that Sph-1-P inhibited the migration through inhibiting neutrophil adhesion, we next examined the effects of Sph-1-P on neutrophil adhesion to the HUVEC monolayer. As shown in Fig. 3, IL-1β stimulated neutrophil adhesion about 10-fold compared to unstimulated control experiments, but no effect of Sph-1-P pretreatment was observed for this stimulation in concentrations of 0.1 to 5 μM. Therefore, the inhibitory effect of Sph-1-P on neutrophil trans-endothelial migration is apparently not due to inhibiting the initial adhesion, indicating that Sph-1-P mainly affects the motility of neutrophils in the trans-endothelial invasive migration processes.
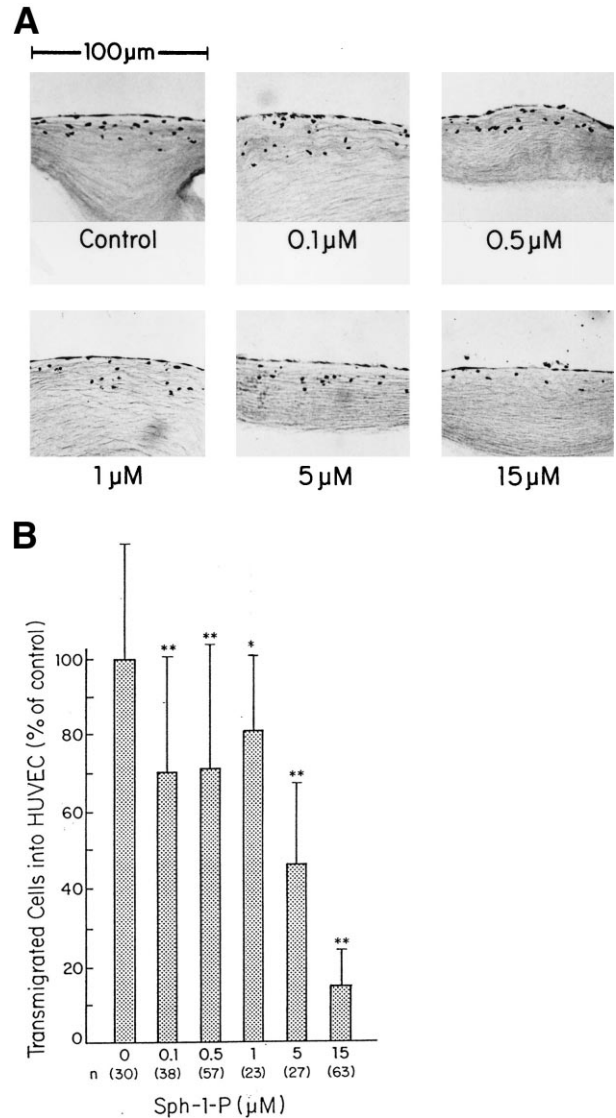

Fig. 2. Sph-1-P suppressed trans-endothelial migration of neutrophils. Human neutrophils in M199 medium supplemented with heat-inactivated FCS were preincubated with test compounds for 10 min at 37°C. Neutrophil suspension was then added to HUVEC monolayer on collagen bed, which was prestimulated with 10 U/ml IL-1β for 4 h. Culture plates were incubated further for 90 min at 37°C, then fixed with 10% formaldehyde, paraffin-embedded, stained hematoxylin-eosin. A: Representative pictures of paraffin-embedded samples at various Sph-1-P concentrations. B: Quantification of migrated neutrophils, counted as invaded neutrophil numbers per 100 μm length of HUVEC monolayer, and expressed as the percentage of control. The data are mean ± S.D. (sample numbers). *$P < 0.05$, **$P < 0.01$.

Table 2
Effects of Sph-1-P, TMS, and C8-ceramide on neutrophil phagokinetic activity

Tested compound		Area swept ($\times 10^3$ μm^2)	Number of measurements
None (control)		6.3 ± 2.3	141
Sph-1-P	0.5 μM	5.5 ± 2.7*	80
	1.0 μM	5.4 ± 2.4*	74
	5.0 μM	3.5 ± 1.4**	123
TMS	0.5 μM	5.4 ± 2.7*	77
	1.0 μM	3.6 ± 1.4**	100
C8-ceramide	5.0 μM	5.7 ± 3.0	75

The data were mean ± S.D. (number of measurements).
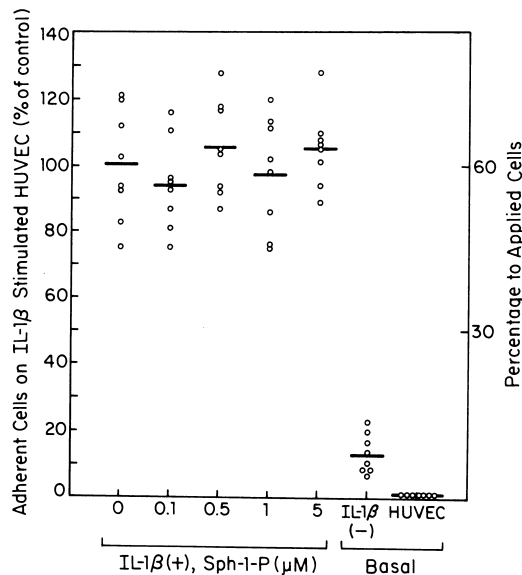*$P < 0.05$, **$P < 0.01$.

strong specific binding of [³H]Sph-1-P was observed on the surface of human neutrophils. Sph-1-P may inhibit actin filament reorganization in leading edges of neutrophil pseudopodia by acting through the cell surface receptor, thus resulting in the observed inhibition of motility and trans-endothelial migration.

We recently reported that Sph-1-P acts as an autocrine stimulator of human platelets, being stored in platelets and released extracellularly upon thrombin stimulation [4,5], and also identified Sph-1-P as a normal constituent in human plasma and serum [24]. These observation strongly suggest that Sph-1-P may act not only as an exogenously added tool for motility regulation but also acts as a physiological cell motility regulator in blood vessels. This possibility seems to be important especially when considering the roles of platelet-neutrophil-endothelial cell interaction in inflammation development [25].

The recruitment of circulating neutrophils to specific sites plays a crucial role in a number of physiological and pathophysiological events including protection from microorganisms and, on the contrary, development of inflammatory processes. In case of inflammation, circulating neutrophils first attach to activated endothelial cells in blood vessels through various induced adhesion molecules, then migrate through the endothelial cell layer to injured sites, and, finally, produce toxic substances such as active oxygen species which may destroy the surrounding tissues [26,27]. To prevent overreaction of neutrophils in the injured sites, the processes consisting of neutrophil adhesion, trans-endothelial migration, or even the toxic substance production itself must be controlled properly [26,27]. Our present study raised the future applicability of Sph-1-P, or an analog, as an anti-inflammatory agent regulating invasion of neutrophils through endothelial layers at vascular injured sites, working as an anti-motility factor. On the other hand, in our previous studies we reported that administration of one of sphingosine derivatives, TMS, effectively inhibited neutrophil adhesion to endothelial cells by blocking P/E-selection expression on the endothelial cells [28,29], and that TMS also inhibited PMA-induced O_2^- production in neutrophils [19]. In both models, TMS seemed to act by inhibiting cellular PKC activities in neutrophils or endothelial cells as a cell permeable PKC inhibitor. The combination of TMS and Sph-1-P, which show totally distinct effects on neutrophils or endothelial cells, might be very effective in anti-inflammation treatment, as indicated in cancer metastasis prevention experiments in animals [30].

Fig. 3. No effect of Sph-1-P on adhesion of neutrophils to HUVEC monolayer. Human neutrophils (1×10^6/ml) in M199 medium were preincubated with or without Sph-1-P for 10 min at 37°C. After washing, 300 μl of neutrophil suspension was added to HUVEC monolayer which was cultured in 48-well plates and stimulated with IL-1β for 4 h prior to the experiments. After 15 min incubation, non-adherent neutrophils were removed by gentle shaking and aspiration. HUVECs and adherent neutrophils were detached from wells by trypsin-EDTA treatment, and neutrophils were quantified by measuring myeloperoxidase activity (HUVECs have no myeloperoxidase activity), and expressed as a percentage of control (without Sph-1-P). Bar indicates the mean value of eight experiments.

4. Discussion

Our previous studies showed that exogenously added Sph-1-P inhibits the chemotactic motility of cancer cell lines, such as mouse melanoma cells, as well as human smooth muscle cells in in vitro experiments [10,11] by interfering the actin filament reorganization in activated cells [12]. However, in these previous studies we realized that the sensitivities for Sph-1-P in motility regulation depends on cell type. For example, the motilities of mouse melanoma cells and human smooth muscle cells were very sensitive for this lipid, whereas endothelial cells such as HUVEC and CPAE were much less sensitive [10]. In this study, we investigated the effect of Sph-1-P on regulating chemotactic motility and invasiveness of human neutrophils, utilizing three different in vitro assay systems (chemotactic, phagokinetic, and trans-endothelial models). We found that (1) Sph-1-P, alone of the sphingolipids tested, specifically inhibited the IL-8- of fLMP-induced chemotactic migration of neutrophils at less than 1 μM concentration; (2) phagokinetic activity of neutrophils was suppressed by Sph-1-P, but more moderately than by the PKC inhibitory sphingosine analog, trimethylsphingosine; (3) Sph-1-P inhibited trans-endothelial migration and invasiveness of neutrophils into HUVEC-covered collagen layers, whereas no effects on their adhesion to HUVEC was observed. These observations strongly suggest that Sph-1-P can act as a specific and effective motility regulator of neutrophils.

The underlying mechanism for different sensitivities for Sph-1-P among various cells is uncertain at present, but it may relate to the expression of its receptor molecules on the cell surface [13]. Indeed, in our preliminary experiments, the

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