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Original article

Sphingosine 1-Phosphate Stimulates Cell Migration and Active Seprase Expression in Human Endothelial Cells

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Abstract: *Objective*: Sphingosine 1-phosphate (Sph-1-P), a bioactive lysophospholipid present in the plasma, is released from activated platelets. Our previous study demonstrated that Sph-1-P promoted the spreading on and migration of human umbilical vein endothelial cells (HUVEC) through the extracellular matrix (ECM), suggesting a possible induction of cell surface proteases in the Sph-1-P activated endothelial cells. In the present study, we examined whether seprase, a type II transmembrane serine protease (TTSP) usually absent in tissue cells, can be induced in endothelial cells activated by Sph-1-P. *Methods*: HUVEC migration through ECM was examined by modified Boyden chamber assay. Western blotting and immunoprecipitation, using anti-seprase monoclonal antibodies (mAbs), were used to confirm the seprase expression of HUVEC. *Results*: We show that Sph-1-P enhanced expression of active form seprase in a time- and dose-dependent manner in HUVEC. The Sph-1-P inducible active form seprase could be blocked by pertussis toxin and by C3 transferase, which inactivate Gi-type heterotrimetric G proteins and Rho, respectively. *Conclusion*: These results show that Sph-1-P can regulate migration of endothelial cells by inducing active form seprase expression, which, in turn, is mediated through a Gi-coupled cell surface receptor and the Rho protein.

Key Words: Sphingosine 1-phosphate, Seprase, Angiogenesis, Invasion, Extracellular matrix

Introduction

Sphingosine 1-phosphate (Sph-1-P) is a bioactive lysophospholipid that is capable of inducing a wide spectrum of biological responses including cell growth, differentiation, survival, and motility[1,2)]. Originally, it was reported that Sph-1-P can serve as an intracellular second messenger regulating intracellular Ca^{2+} mobilization and cell growth and survival[3,4)]. Furthermore, a dynamic balance between the intracellular levels of ceramide (Cer) and Sph-1-P, with the consequent regulation of opposing signaling pathways, was proposed to be an important factor that determines the cell's fate[5)]. However, recent evidence has indicated that Sph-1-P also acts as an inter-

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cellular mediator, interacting with the S1P family of G protein-coupled receptors/Endothelial Differentiation Gene (EDG)[1,2,6]. These receptors, $S1P_1$ (EDG-1), $S1P_2$ (EDG-5), $S1P_3$ (EDG-3), $S1P_4$ (EDG-6), and $S1P_5$ (EDG-8), exhibit overlapping as well as distinct patterns of expression in various tissues, and they function as Sph-1-P receptors; the cellular responses to extracellular Sph-1-P depend on the types of EDG receptors that are expressed on the cell surface[6,7]. Sph-1-P is considered to be a unique lipid mediator that is involved in a variety of physiological and pathological processes. Recently, Sph-1-P was reported to induce endothelial cell migration and morphogenesis into capillary-like networks, suggesting it may be an angiogenic molecule[8–11].

Angiogenesis, the development of new blood vessels from pre-existing endothelium, is a critical process in many physiological and pathological conditions including embryonic development, organ regeneration, chronic inflammation, and solid tumor growth[12,13]. The process of angiogenesis is complex and involves several discrete steps, including extracellular matrix (ECM) degradation, proliferation and migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes[14]. It has also been suggested that ECM-degrading proteases are fundamental to several steps of angiogenesis[15–17].

Recently, a panel of membrane-bound serine proteases implicated in angiogenesis, called type II transmembrane serine proteases (TTSPs) that contain C-terminal extracellular serine protease domains, are thought to play central roles in cell surface-activating events[16–18]. Seprase is a TTSP with non-classical catalytic site; it is absent in normal differentiated cells; it is selectively expressed in malignant melanoma and breast carcinoma, as well as in stromal cells of healing wounds[16]. Seprase interacts with other major proteolytic enzymes on the cell surface and the presence of seprase complexes correlates with activation of the invasiveness of both normal and tumor cells[19–28]. However, it is still not known how seprase expression is induced in tissue cells.

In the present study, we investigated the effect of Sph-1-P on proteolytic stimulation of endothelial cells. We found that Sph-1-P induced seprase expression in endothelial cells and enhanced the migration and matrix invasion of activated endothelial cells, suggesting that Sph-1-P may be an inducer for seprase-dependent cell migration.

MATERIALS AND METHODS

Chemicals and reagents

Sph-1-P was obtained from Biomol (Plymouth Meeting, PA). *Clostridium botulinum* C3 exoenzyme, a specific inhibitor of Rho, was purchased from Upstate Biotechnology (Lake Placid, NY). Pertussis toxin (PT) was from Kaken Pharmaceutical Co. (Tokyo, Japan). The following materials were obtained from the indicated suppliers: the reconstituted basement membrane matrix Matrigel (Becton Dickinson Labware, Bedford, MA); Protein G-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden); fetal calf serum (FCS) (ICN Biomedicals, Aurora, OH); recombinant human basic fibroblast growth factor (Becton Dickinson Labware, Bedford, MA); Biotinylated rabbit anti-rat IgG and Peroxidase-conjugated streptavidin (DAKO A/S, Glostrup, Denmark); Anti-rat IgG (Cappel, Aurora, OH). Anti-seprase monoclonal antibody (mAb) was prepared according to a previously described method[21,22]. Rat anti-seprase mAb, D8, D43 and E97 (all subclass IgG 2a) are directed against human placental seprase.

Culture of human endothelial cells

HUVECs were isolated from human umbili-

cal cord by trypsin treatment and plated onto 0.2% gelatin-coated dishes, as described previously[30]. They were then grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 ng/m*l* of recombinant human basic fibroblast growth factor, 20% fetal calf serum, penicillin G (100 units/m*l*), and streptomycin sulfate (100 mg/m*l*) at 37°C under an atmosphere of 5% CO_2 and 95% room air. HUVECs were not used after the sixth passage. The cells were transferred to 100 mm dishes coated with 0.2% gelatin (Sigma) and were used when they reached subconfluency. In all experiments, HUVECs were serum-starved for 1 hr before stimulation.

Endothelial cell migration assay using Matrigel

HUVEC migration assays were carried out using a Transwell cell culture chamber (Costar, Cambridge, MA), which is a modified Boyden chamber, as previously described[31]. Polycarbonate filters with 8-μm pores were used to separate the upper and lower chamber and these were coated with 500 μg/m*l* of Matrigel. The coated filters were washed with serum-free medium and dried immediately. Next, HUVECs were added to the upper compartment of the chamber at a density of 1×10^5/100 μl of medium containing 0.1% BSA and incubated at 37°C. HUVECs were allowed to migrate toward Sph-1-P in the lower chamber. After the removal of non-migrated cells by wiping with cotton swabs, the filters were removed, fixed, stained with trypan blue, and mounted on glass slides. Cells that had migrated through the filter to the lower surface were counted manually under a microscope, using five predetermined fields.

Preparation of cell lysates

HUVECs were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0 containing 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) with the aid of a cell scraper. The lysates were incubated on ice for 1 hr with occasional mixing, followed by centrifugation at 15,000 × g for 10 min. The protein concentrations of the supernatant were determined by Bradford assay[32]. Approximately 500 μg of protein was then used for immunoprecipitation and isolation of cell surface glycoproteins.

Immunoprecipitation

The samples were pre-cleaned with protein G-Sepharose and the resulting supernatants were incubated overnight with anti-seprase mAb D8, which recognizes 170-kDa seprase dimer and 97-kDa monomer. Protein G-Sepharose was then added and the samples were incubated for another 2 hr. All immunoprecipitation steps were carried out at 4°C. The Sepharose beads were then washed with the lysis buffer three times. Next, they were either treated with SDS sample buffer at room temperature overnight or they were boiled in SDS sample buffer for 10 min.

Immunoblotting

The proteins were resolved on an SDS-PAGE (8.0% polyacrylamide gel) under non-reducing or reduced conditions, and were then transferred electrophoretically to a PVDF membrane. The membranes were blocked with 1% bovine serum albumin in phosphate buffered saline (PBS). After extensive washing with PBS containing 0.1% Tween 80, blots were incubated with anti-seprase mAb D43, which recognizes the 170-kDa seprase dimer, or with anti-seprase mAb E97, which labels the 97-kDa seprase monomer. After adding biotinylated anti-rat IgG (diluted 1:10,000) and Peroxidase-conjugated streptavidin, antibody binding was visualized with ECL chemiluminescence reaction reagents (Amersham Pharmacia

Biotech).

Immunoblotting for assaying seprase dimer and monomer

The 170-kDa seprase homodimer was assayed by immunoblotting using anti-seprase mAb D43 on seprase proteins enriched from mAb D8 immunoprecipitates that were solublilized under mild conditions (not reduced and not boiled), as previously described [19,22,33]. The 97-kDa seprase monomer was assayed by immunoblotting using anti-seprase mAb E97 on seprase proteins enriched from mAb D8 immunoprecipitates that were solublilized under reduced and boiled conditions [22].

Statistics

All experiments were conducted in triplicate and were repeated three times. When indicated, statistical analysis was performed using the Student's *t*-test, and $p<0.05$ was considered significant.

RESULTS

Extracellular matrix degradation is an important step in vascular endothelial cell migration, and therefore it is of interest to determine the specific roles played by proteases that act on the ECM. We first examined the morphological change on endothelial cell migration using a Transwell cell culture chamber, as described in Materials and Methods. HUVECs had migrated through the filter toward Sph-1-P in the lower chamber as shown in Fig. 1A. Next, we investigated the seprase activity by immunoblotting, to characterize the involvement of seprase on endothelial cell migration. Fig. 1B shows the effects of 100 nM Sph-1-P on endothelial cell migration over several time periods. The maximum migratory activity was attained at the 4 hr time point. Similar quantities of HUVEC lysate proteins were resolved by SDS-PAGE under the non-reducing conditions and without boiling. The seprase activation was confirmed using immunoprecipitation with the mAb D8 and probing with the mAb D43, which recognizes the active dimeric form of seprase. As shown in Fig. 1C, Sph-1-P induced self-association of the 170-kDa seprase to form the active dimer. This dimer formation reached a maximum level 15-30 min after stimulation. Same samples were reproved with the mAb E97, which recognized the inactive 97 kDa monomer. Inactive monomer was comparatively decreased.

Sph-1-P induces seprase activation, as well as HUVEC migration, in a dose-dependent manner. As shown in Fig. 2A, Sph-1-P induced HUVEC migration in a dose-dependent manner up to a concentration of 100 nM. Expression of the active dimeric form in seprase HUVECs was evaluated by immunoblotting. Unstimulated HUVECs expressed low amounts of active seprase. Sph-1-P treatment significantly up-regulated the expression of active seprase. This up-regulation peaked at 100-250 nM concentration (Fig. 2B). Same samples were reproved with the mAb E97, inactivated seprase was gradually decreased.

To explore the signal transduction pathways responsible for the observed seprase activation by Sph-1-P in HUVECs, we examined the Gi- and Rho- pathways after Sph-1-P stimulation. Sph-1-P induced HUVEC migration were inhibited by pretreatment with pertussis toxin and C3 exoenzyme (Fig. 3A), although the basal response (without stimulation) was not affected by either reagent. In addition, PMSF, a serine protease inhibitor, also inhibited HUVEC migration. Inhibition of seprase activity by PT, C3, and PMSF (Fig. 3B) was also examined by immunoblot analysis. This inhibition suggested

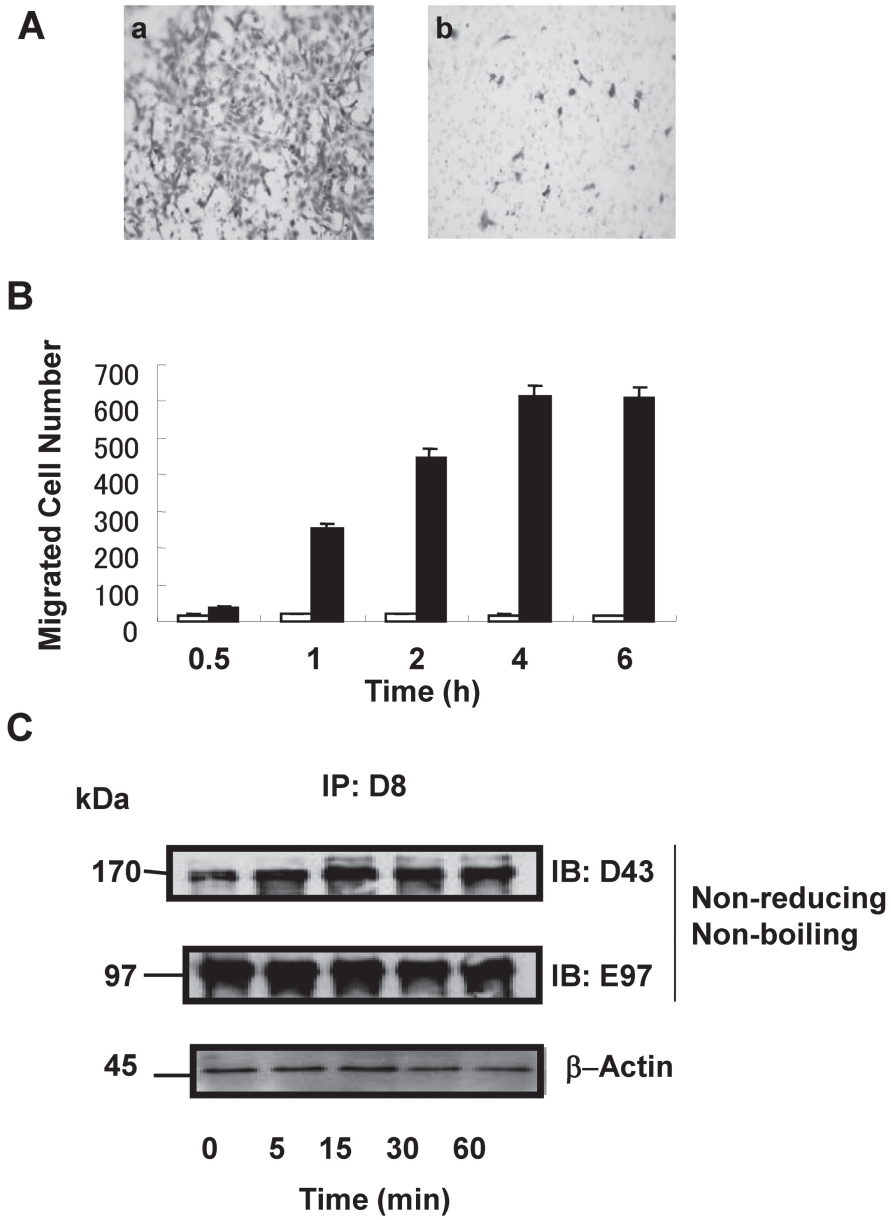

Fig. 1. Coincidental stimulation of cellular migration and expression of 170-kDa seprase dimer in endothelial cells. **A**, Morphorogy of HUVEC migration. HUVECs were added to the upper compartment of a 24-Transwell chamber and incubated in the presence (**a**) or absence (**b**) of 100 nM Sph-1-P in the lower compartment for 4 hr. **B**, A time-dependent study of stimulation of HUVEC migration with 100 nM Sph-1-P. HUVECs were incubated in the presence (solid square) or absence (open square) of 100 nM Sph-1-P in the lower compartment for various time durations. Data are expressed as the mean ± SD of three separate experiments. **C**, The cell lysates were immunoprecipitated (IP) with the D8 anti-seprase antibody and immunoblotted with the D43 or E97 anti-seprase antibody under non-reducing conditions without boiling. Same sample were immunoblotted with β-actin antibody as a control. The data shown are representative of three separate experiments.

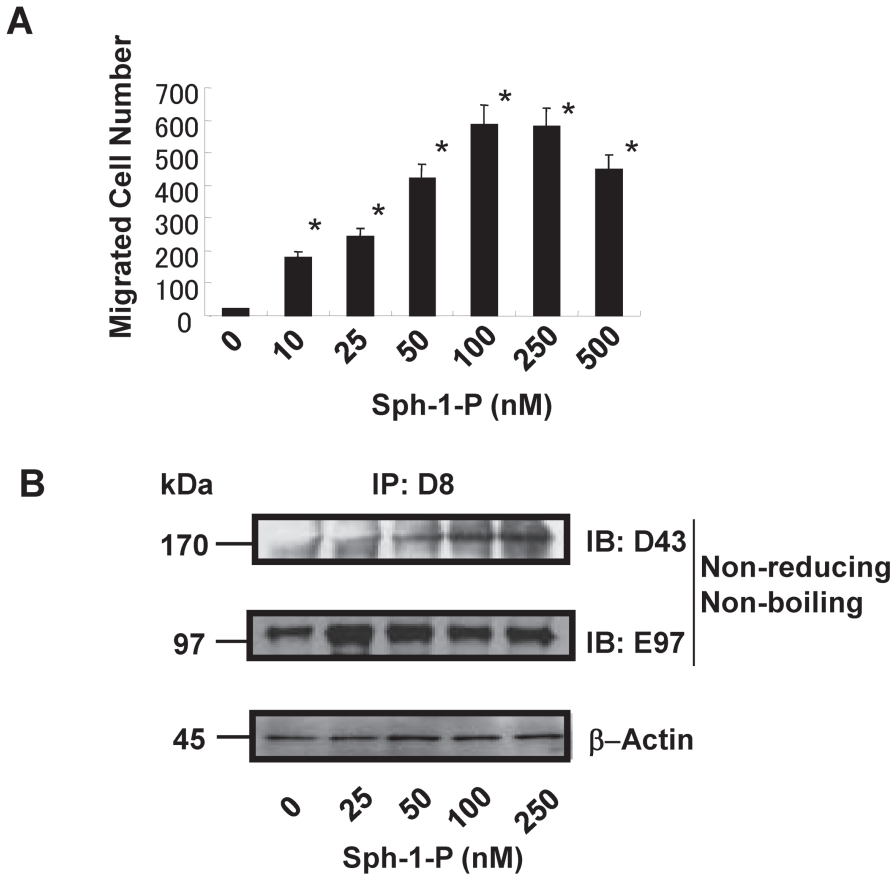

Fig. 2. Coincidental stimulation of cellular migration and expression of 170-kDa seprase dimer in endothelial cells. **A**, Stimulation of HUVEC migration with various concentrations of Sph-1-P. HUVECs were added to the upper compartment of a 24-Transwell chamber and incubated with various concentrations of Sph-1-P in the lower compartment. Solid squares and error bars represent the mean ± SD of three separate experiments. *Statistically significant compared with control (without Sph-1-P stimulation). **B**, HUVECs were challenged with various concentrations of Sph-1-P. The cell lysates were immunoprecipitated (IP) with the D8 anti-seprase antibody and immunoblotted with the D43 or E97 anti-seprase antibody under non-reducing conditions without boiling. Same sample were immunoblotted with β-actin antibody as a control. The data shown are representative of three separate experiments.

that that Gi- and Rho- dependent activation of seprase is critical in promoting migration of endothelial cells on Matrigel.

DISCUSSION

This study identifies seprase, which occurs on the surface of endothelial cells, as a novel downstream effector for Sph-1-P. Our results suggest a new mechanism by which Sph-1-P may modulate physiological and pathological states. Specifically, we have shown here that Sph-1-P activates seprase on the surfaces of endothelial cells. The gelatinase activity of seprase was

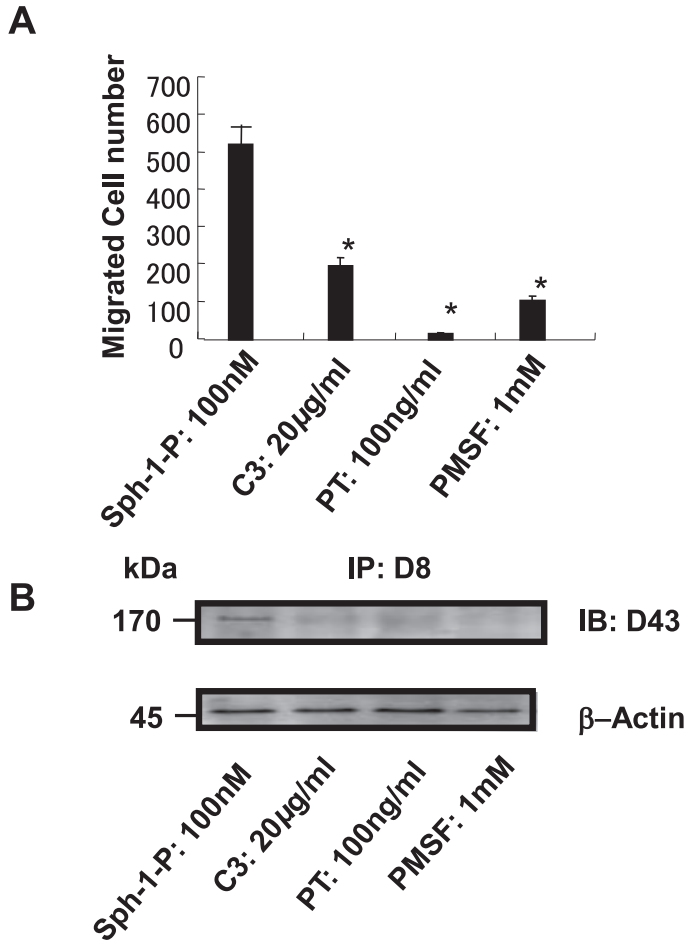

Fig. 3. Inhibition of Sph-1-P-induced HUVEC migration by C3 exoenzyme, pertussis toxin, and PMSF. **A**, HUVECs were pretreated without (control) or with 20 μg/m*l* of C3 exoenzyme (C3), or with 100 *n*g/m*l* of pertussis toxin (PT) for 24 hr, or with 1 mM PMSF for 1 hr. HUVECs were added to the upper compartment of a 24-Transwell chamber and incubated with 100 nM of Sph-1-P in the lower compartment. Open columns and error bars represent the mean ± SD of three separate experiments. *Statistically significant compared with control (without Sph-1-P stimulation or without pretreatment). **B**, HUVECs were challenged with 100 nM of Sph-1-P, after being pretreated with 20 μg/m*l* of C3 exoenzyme (C3), or with 100 *n*g/m*l* of pertussis toxin (PT) for 24 hr, or with 1 mM PMSF for 1 hr. The cell lysates were immunoprecipitated (IP) with the D8 anti-seprase antibody, and then immunoblotted with the D43 anti-seprase under non-reducing conditions without boiling. Same sample were immunoblotted with β-actin antibody as a control. The data shown are representative of three separate experiments.

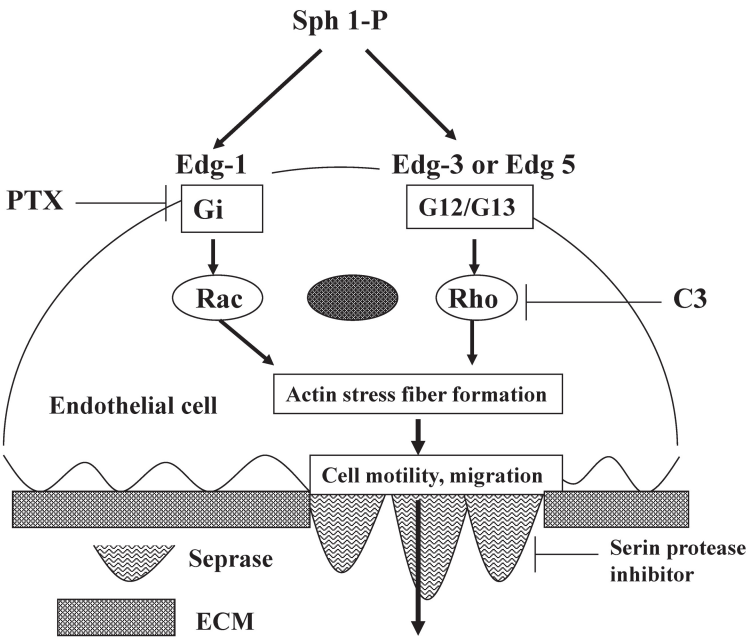

Fig. 4. Schematic illustration of a potential seprase activation process and endothelial cell migration. Sph 1-P is bound to cell membrane by Edg-1 or Edg-3 or -5 Sph 1-P receptor. Edg-1 signal transduces via Gi-protein, which is blocked by PT (Pertussis toxin), to Rac. Edg-3 or-5 signal transduces via G12/G13 to Rho, which is also blocked by C3. Activated Rho and /or Rac induce actin stress fiber formation, leading to cell motility and migration with degradation of ECM (Extra-cellular matrix) by seprase activation.

shown to be sensitive to heat, acid pH, phenyl methyl sulfonyl fluoride (PMSF) and N-methyl maleimide (NEM), but it was insensitive to β-mercaptoethanol, dithiothreitol, EDTA, 1,10 phenanthroline, pepstatin, and leupeptin[19,33]. Consistent with these findings, we observed that seprase was inactivated by heat and PMSF treatments of samples.

$S1P_1$ (Edg-1) is abundantly expressed in HUVECs, while the $S1P_3$ (Edg-3) transcript is expressed at a much lower level[8,9]. We can also confirm $S1P_1$ and $S1P_3$ expression in HUVECs (data not shown). $S1P_1$ couples to a number of signaling pathways, primarily via the pertussis toxin-sensitive Gi protein[34–36]. In the present study, we observed that pertussis toxin suppressed both Sph-1-P-mediated activation of seprase and HUVEC migration, suggesting a role for $S1P_1$ in seprase activation and in HUVEC migration. Recently, it was documented that Sph-1-P activates matriptase, which is a type II integral membrane serine protease, in epithelial cells[37]. In the current study, pertussis toxin failed to suppress Sph-1-P-mediated activation of matriptase, suggesting it has another target besides the $S1P_1$ receptor that may be involved in initiation of the proteolytic activation cascade. Differences between seprase and matriptase in signal transduction may be due to different signaling mechanisms used by the two proteases, or

they may be caused by differences in cell types. For example, HUVECs and mammary epithelial cells may utilize different pathways and/or mechanisms.

It has been documented that the Sph-1-P receptor $S1P_3$, which is expressed in HUVECs, is coupled predominantly with G_{13}/G_q[8,38,39]. In the present study, Sph-1-P-induced seprase activation of HUVECs, as well as migration, were abolished by the C3 exoenzyme or by Y-27634 (a specific Rho kinase inhibitor)(data not shown). This suggests involvement of the Rho/Rho kinase pathway involving G_{13}/G_q. Seprase is localized and concentrated on invadopodia, specialized protrusions of the plasma membrane that carry out proteolysis of the extracellular matrix[21,33]. Activation of the β1 integrin induced the recruitment of seprase to invadopodia and stimulated tyrosine phosphorylation of p190*RhoGAP*. Seprase is also associated with F-actin organization at invadopodia[20,40], suggesting that seprase may be linked to cytoskeletal signaling involving Rho. Our results showing inhibition of HUVEC migration and inhibition of seprase activation by C3 transferase support this suggestion. It was also reported that Sph-1-P induced endothelial cell adherent junction assembly, migration, and capillary tube formation. These effects were mediated via both Edg-1 and Edg-3, which coupled with G_i and G_{13}/G_q, respectively[8]. Furthermore, in HUVECs stimulated with Sph-1-P, cytoskeletal signaling pathways may be divided into Gi-mediated pathways (via Edg-1) and Rho-mediated pathways (via Edg-3). Coordinated signals from both pathways are required for Sph-1-P-stimulated enhancement of HUVEC motility[41]. In taking these findings into consideration, we conclude it is likely that seprase activation stimulated by Sph-1-P is mediated by and/or is carried out cooperatively with both the Gi protein and Rho pathways in HUVECs.

Recent pathological study showed that seprase immunoreactivity was recognized in endothelial cells especially adjacent to tumor nests, indicating the involvement of seprase on tumor angiogenesis[27,28]. Further investigation indicated that the protease complex including seprase and dipeptidylpeptidase IV facilitated the local degradation of the extracellular matrix and the invasion of the human endotheial cells[29]. This study supports our present investigation.

An abundant amount of Sph-1-P is stored in platelets (possibly due to the presence of a highly active Sph kinase and a lack of Sph-1-P lyase), and Sph-1-P is released extracellularly upon platelet activation[42,43]. This is consistent with the fact that Sph-1-P is a normal constituent of plasma and serum; the Sph-1-P levels of serum are quite high[44]. Sph-1-P has diverse physiological effects, and the Sph-1-P released from activated platelets may be involved in a variety of pathological processes, especially those involving angiogenesis. Further elucidation of details of Sph-1-P biology may lead to new therapeutic approaches to control vascular diseases.

Acknowledgments

No conflicts of interest

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