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Sphingosine-1-phosphate inhibits extracellular matrix protein-induced haptotactic motility but not adhesion of B16 mouse melanoma cells

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Abstract

Sphingosine-1-phosphate (Sph-1-P), the initial product of Sph catabolism, inhibited chemotactic motility of a few lines of tumor cells [(1992) Proc. Natl. Acad. Sci. USA 89, 9686]. We now report that Sph-1-P even at very low concentration (10–100 nM) inhibits integrin-dependent motility of melanoma cells induced by extracellular matrix (ECM), although it did not affect integrin-dependent adhesion to ECM. Other Sph-related compounds tested (including sphinganine-1-P) were much less effective than Sph-1-P at inhibiting motility, and also had no effect on integrin-dependent adhesion of tumor cells to ECM. Our findings suggest that Sph-1-P inhibits actin filament reorganization by affecting cytoplasmic connection to integrin in ECM-stimulated motility of melanoma cells.

Key words: Sphingosine-1-phosphate; Cell motility; Cell adhesion; Matrix protein; Actin filament; Haptotaxis

1. Introduction

Migration of tumor cells through basement membrane and ECM is considered an essential process for tumor invasion and metastasis [1–3]. ECM proteins such as FN and LM promote integrin-dependent cell adhesion as well as cell motility in vitro [4,5]. Studies of signal transduction mechanisms and their control through ECM–integrin interactions have led to considerable current interest in the molecular basis of tumor cell invasiveness, which is partially dependent on enhanced haptotactic motility of tumor cells. Recent studies have indicated that sphingolipid breakdown products such as Cer, Sph, and their metabolites (e.g. *N,N*-dimethyl-Sph, Sph-1-P, Cer-1-P) regulate cell growth and differentiation through PKC or receptor-associated kinases [6–8]. Sph-1-P has also been reported to be an endogenous modulator of intracellular Ca^{2+} release [9,10] and phosphatidic acid synthesis [11]. Very recently, Sph kinase, which converts Sph to Sph-1-P, was shown to be regulated by serum factors and/or PDGF in Swiss 3T3 cells [12], and by phorbol ester in Balb/c 3T3 A31 cells [13]. In our previous studies [14,15], using Boyden chamber Transwell assay and phagokinetic assay with gold colloid-coated plates, we found that Sph-1-P strongly and specifically inhibits conditioned medium- or TPA-induced chemotactic motility (migration and invasion) and random motility of B16/F1 mouse melanoma cells at 10–100 nM concentration. In the present study, we examined the effect of Sph-1-P on ECM/integrin-dependent haptotactic motility of F1 cells, and found that Sph-1-P is also a strong and specific inhibitor of ECM–integrin-induced haptotaxis.

2. Materials and methods

2.1. Chemical synthesis of Sph-1-P

In our previous study, we utilized both enzymatically and chemically synthesized Sph-1-P [14,15]. In the present study, we modified our chemical synthesis method. Instead of utilizing an acid-stable pivaloyl group as before, we utilized benzylbromide as an allylic-OH protection agent (Fig. 1). Benzylation of D-erythro-olefinic alcohol and its selective deprotection yielded 2-*N*-Boc-3-*O*-benzyl-D-erythro-Sph. The primary hydroxyl group was then phosphorylated with dibenzyl *N,N*-diisopropylphosphoramidite [16], with tetrazole as an activating agent, and subsequently oxidized in situ with *m*-chloroperbenzoic acid. Finally, sequential deprotection successfully yielded D-erythro-Sph-1-P. Structural elucidation (NMR and FAB-MS) and biological activities of the newly synthesized compound were identical to those reported previously [15,17].

2.2. Other Sph derivatives, and chemicals

Sph, TMS, and DMS were synthesized as previously described

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Abbreviations: Cer, ceramide; DMS, dimethyl-sphingosine; ECM, extracellular matrix; FN, fibronectin; FNR, fibronectin receptor(s); LM, laminin; Sph, sphingosine; Sph-1-P, sphingosine-1-phosphate; TMS, trimethyl-sphingosine.

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[18,19]. Dihydro Sph-1-P (sphinganine-1-P) was synthesized as described by Weiss [20]. C2-Cer (*N*-acetyl Sph) and C8-Cer (*N*-octanoyl Sph) were synthesized as described by Vunnam and Radin [21]. These preparations were free of endotoxin as determined by *Limulus* amebocyte lysate assay kit (sensitivity limit, 0.125 ng/ml; Associates of Cape Cod, Woods Hole, MA). All lipid samples except C8-Cer were dissolved in ethanol/water (1:1) as a 1 or 2 mM stock solution. C8-Cer was solubilized in ethanol. These samples were incubated in a water bath warmed to 56°C before use, then appropriately diluted in culture medium containing 2% FCS. Control experiments were performed with ethanol (0.1% or below) as vehicle, which did not affect haptotactic motility.

LM and RGDS (arginylglycylasparatylserine) peptide were purchased from Sigma Chemical Co., St. Louis, MO. Matrigel was from Collaborative Research, Bedford, MA. FN and FNR were purified in our laboratory [22,23].

2.3. Cell culture

B16 melanoma cell variant F1 was obtained from Dr. I.J. Fidler (M.D. Anderson Cancer Center, University of Texas, Houston, TX) and cultured in DMEM supplemented with 10% FBS (Hyclone, Logan, UT), 2 mM L-glutamine, 2 mM pyruvic acid, 4.5 mg/ml D-glucose, 100 U/ml penicillin G, and 100 μg/ml streptomycin.

2.4. Haptotactic cell motility assay

Haptotaxis was assayed using Transwell chambers (Costar, Cambridge, MA) with 6.5 mm diameter polycarbonate filters (8 μm pore size) [4]. Briefly, the lower surface of the filter was coated with 25 μl Tris-HCl buffer containing human plasma FN (5 μg/filter), LM (1 μg/filter), or Matrigel (1 μg/filter). The filter was dried at 37°C overnight. Cells were harvested after brief exposure to 0.05% trypsin and 0.02% EDTA, and resuspended to a concentration of 4×10^5 cells/ml in DMEM supplemented with 0.1% BSA. 100 μl of the suspension was placed in the upper compartment andincubated for 30 min in a CO_2 incubator. 0.6 ml DMEM with 0.1% BSA and the test compound were placed in the lower chamber, which was then connected to the upper chamber. After CO_2 incubation for 18 h at 37°C, cells on the lower surface of the filter were stained and counted as previously described [14].

2.5. Cell adhesion and cell spreading assay

Adhesion assay was performed as previously described [23]. Briefly, 96-well plates were coated with adhesion protein in PBS (100 μl/ml) at 37°C for 3 h and then blocked with 1% denatured BSA in PBS (200 μl/ml) at 37°C for 2 h. ^{3}H-labeled (1 μCi/ml, 24 h) F1 cells (5×10^4 cells/well) were incubated for 15 min at room temperature with Sph derivatives or medium alone, and suspended cells were transferred into ECM protein-coated wells and incubated for 30 min at 37°C. Nonadherent cells were removed by washing with PBS three times. Adherent cells were dissolved with 1% SDS in 0.1 NaOH and quantified by scintillation counter.

For spreading assay, 24-well plates were coated with Matrigel (20 μg/ml) or human plasma FN (20 μg/ml) in distilled water for 2 h at room temp. F1 cells (5×10^4 cells/ml) were suspended in DMEM with or without test compounds. 0.5 ml of the suspension was seeded in the coated wells and allowed to adhere at 37°C. After each period of incubation, cells were fixed with 4% formaldehyde. Spreading (%) was observed under phase contrast microscopy and quantified as [(shadow cells)/ (shadow cells+bright cells)] × 100. Spread cells and adherent but non-spread cells were identified as shadow and bright (spherical) cells, respectively [24]. At least 100 cells were scored.

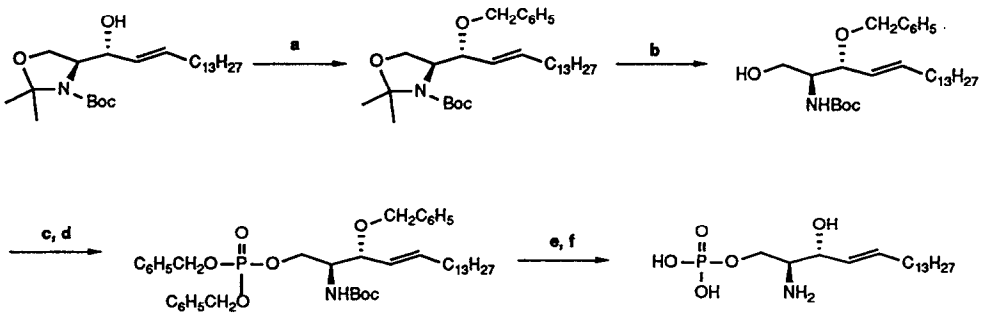
2.6. Liposome binding assay

Binding of FNR-containing liposomes to FN was measured using phosphatidylcholine/[^{14}C]cholesterol/FNR (α5β1)-containing liposomes prepared as previously described [22,23]. Liposome binding assay was performed in Pro-bind Assay Plates (Falcon) precoated with FN (50 μg/100 μl). Liposomes were suspended in TBS containing 1% BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. Aliquots of this suspension were mixed with varying concentrations of Sph derivatives or 1% BSA alone, added to wells (100 μl/well containing 1.25 μg [^{14}C]cholesterol), and allowed to bind with gentle shaking at 4°C for 24 h. Non-specifically bound liposomes were removed by washing with TBS. Bound liposomes were dissolved in 1% SDS and quantified by scintillation counter.

3. Results

3.1. Sph-1-P inhibits haptotaxis of B16/F1 cells

McCarthy and Furcht reported that LM and FN promote haptotactic migration of B16 melanoma cells [4,5]. We confirmed that this effect is dependent on the amount of ECM protein loaded on the lower surface of the Transwell filters. The optimal concentration of FN was 5 μg/filter. No haptotaxis of F1 cells was observed in the absence of FN. We first examined the effect of Sph-1-P on F1 cell haptotaxis through FN-loaded Transwell filters (Fig. 2). This FN-induced haptotaxis was inhibited up to 55% by 300 μM RGDS synthetic peptide, indicating that the phenomenon is dependent on FN–integrin interaction. Sph-1-P added to the lower chamber strongly inhibited haptotaxis at 10–100 nM concentration. Similar to our previous findings for conditioned medium-induced F1 cell motility, higher concentrations



(a) $C_6H_5CH_2Br$, NaOH, DMF; (b) p-TsOH, CH_3OH; (c) $(iPr)_2NP(OCH_2C_6H_5)_2$, tetrazole, CH_2Cl_2; (d) MCPBA, CH_2Cl_2; (e) Na, Liq. NH_3, THF; (f) TFA, CH_2Cl_2.

Fig. 1. New chemical synthetic scheme for Sph-1-P. See text for explanation.

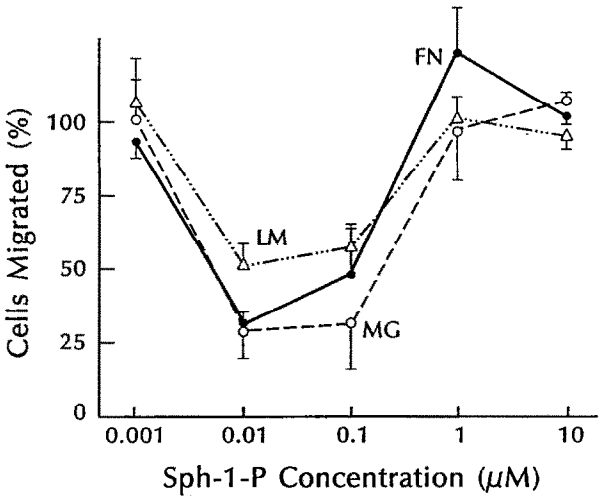


Fig. 2. Effect of Sph-1-P on ECM protein-induced B16/F1 cell haptotaxis. Haptotaxis was measured by a Transwell filter assay as described in section 2. The lower filter surface was coated with FN (●, 5 μg/filter), LM (△, 1 μg/filter), or Matrigel (○, 1 μg/filter). F1 cells (4×10^5) were placed on a filter in the upper chamber and cultured for 16 h in the presence of various concentrations of Sph-1-P in the lower chamber. Values represent the mean ± S.E.M. ($n = 3$) of% cells migrated relative to control experiments (defined as 100%). The actual OD values of the control experiments were 0.156 (LM), 0.144 (FN), and 0.130 (Matrigel), respectively.

(1–10 μM) of Sph-1-P did not have an inhibitory effect. Haptotaxis induced by LM (1 μg/filter) or Matrigel (1 μg/filter) was similarly inhibited by Sph-1-P in a concentration-dependent manner.

3.2. Specificity

Next, we compared the haptotaxis-inhibitory effect of Sph-1-P with those of various structurally related Sph derivatives, some of which have been claimed to be second messengers or modulators of PKC or other signal transduction systems [6–8]. C2-Cer and C8-Cer are cell membrane-permeable synthetic analogs of natural Cer [25–27]. As shown in Table 1, TMS had no inhibitory effect over a wide range of concentrations (1 nM–1 μM). Sph showed moderate inhibition at 1 μM, possibly because of its conversion to Sph-1-P in cells [14]. Only dihydro Sph-1-P showed significant (but still 10-fold weaker than Sph-1-P) inhibition at the 10–100 nM concentration. These findings indicate that inhibition of FN-induced haptotaxis is a specific property of Sph-1-P.

Table 1
Effect of Sph-1-P and other Sph-related compounds on F1 cell haptotaxis induced by FN

Compound	Concentration (μM)			
	0.001	0.01	0.1	1.0
Sph-1-P	73	12	38	163
C8-Cer	119	100	146	138
C2-Cer	112	113	144	119
Dihydro Sph-1-P	102	58	15	34
Sph	83	111	120	44
TMS	133	132	91	131

FN-induced F1 cell haptotaxis was measured as described in section 2. Values represent the mean of two determinations of migrated cell number expressed as a percentage of the control experiment (defined as 100%) with vehicle (0.1% ethanol) alone. The actual OD value of the control experiment was 0.088 ± 0.010 (mean ± S.E.M.).

3.3. Effect of Sph-1-P on F1 cell adhesion and spreading on ECM proteins

When F1 cells were seeded on plastic dishes precoated with ECM proteins (FN and Matrigel), the cells became attached, spread isotropically, and assumed a discoid shape. In the cell adhesion assay, Sph-1-P and Sph very weakly affected F1 cell adhesion to Matrigel-coated plastic surface (about 20% inhibition at 0.1–1 μM for Sph-1-P and at 1–5 μM for Sph). Other Sph derivatives had no significant effect (Fig. 3). Similar results were obtained in FN-coating experiments (data not shown). Attachment of liposomes containing human FNR to FN-coated plates was not inhibited in this range of Sph-1-P concentration (Table 2). However, even 10 nM Sph-1-P produced significant inhibition of cell spreading (Table 3). This inhibition is due to prolonging of spreading time (data not shown). No cytotoxic effect of Sph-1-P was seen up to 20 μM. Other Sph derivatives (TMS, DMS, C2-Cer, C8-Cer) did not inhibit cell spreading up to 5 μM (data not shown).

4. Discussion

Three types of cell motility can be distinguished: (i) random motility; (ii) directed motility toward a soluble

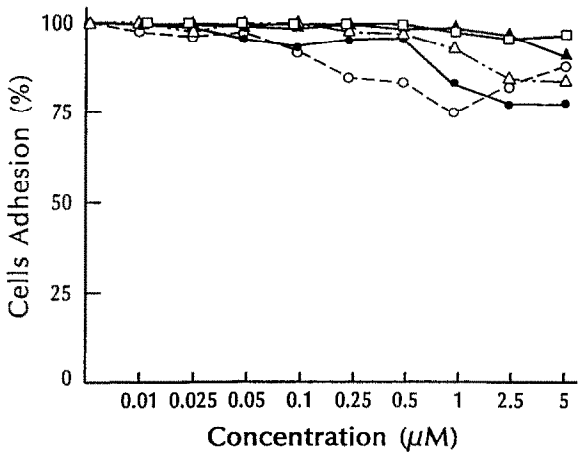


Fig. 3. Effect of Sph-1-P and other Sph derivatives on adhesion of [^{3}H]thymidine-labeled F1 cells to Matrigel-coated plates. Cell adhesion was assayed as described in section 2. Values represent the mean ($n = 2$) of% cells adhered relative to control experiments (defined as 100%). The actual value from control experiment was 2.5×10^4 cpm/well (3.8×10^4 cells/well). ○, Sph-1-P. ●, Sph. △, DMS. ▲, C2-Cer. □, TMS.

Table 2
Effect of Sph-1-P and other Sph-related compounds on binding of FNR-liposome to FN

Compound	Concentration (μM)			
	0.03	0.3	1.0	3.0
Sph-1-P	NI	NI	4	6
C2-Cer	NI	NI	4	7
Cer	NI	NI	3	8
Sph	NI	NI	6	12
TMS	NI	NI	3	5
DNS	NI	NI	4	7

Liposome binding assay was performed as described in section 2. Values represent the mean of three determinations of binding of FNR-containing liposomes to FN, expressed as a percentage of inhibition, based upon [^{3}H]cholesterol scintillation counting. The actual value of the control experiment was 40.5 ng/well. NI, no inhibition (< 1%).

attractant (chemotaxis); (iii) directed motility toward substratum-bound, insolubilized ECM proteins (haptotaxis). Although the distinction between chemotaxis and haptotaxis is not always clear, they are known to be induced and regulated by distinct signal transduction mechanisms [27]. Haptotaxis may depend on integrin receptors [4]. Remarkable progress has been made recently in integrin-related signal transduction systems, especially in studies of ECM protein–integrin interaction-induced activation of focal adhesion tyrosine kinase ($P125^{FAK}$) [28–31].

In our previous study [14], using both Transwell and phagokinetic assays, we found that random motility and chemotaxis with conditioned medium of mouse melanoma cells were specifically inhibited by a low concentration (10–100 nM) of Sph-1-P. The present study shows that ECM protein-induced haptotaxis is also inhibited by a low concentration of Sph-1-P. This inhibitory effect is specific to Sph-1-P as opposed to other Sph-related compounds. However, Sph-1-P had little effect on attachment of F1 cells to ECM proteins. Sph-1-P differs in this respect from unknown integrin-modulating lipids (similar M_W to Sph-1-P) recently described by Wright et al. [32], which were claimed to directly affect the affinity of integrin molecules for ECM proteins [27].

Table 3
Inhibition of F1 cell spreading on Matrigel- and FN-coated plates by Sph-1-P

Concentration (μM)	Percent of spreading cells	
	Matrigel	FN
0	84 ± 3	89 ± 3
0.001	79 ± 3	87 ± 5
0.01	74 ± 3	82 ± 2
0.1	64 ± 8	67 ± 5
1.0	81 ± 3	64 ± 5

Spreading of F1 cells on ECM protein-coated plates was measured as described in section 2. Values represent the mean ± S.D. ($n = 3$) of percent spreading of cells expressed as [(shadow cells)/(shadow cells ± bright cells)] × 100.

The fact that Sph-1-P inhibits both chemotaxis and haptotaxis of F1 cells suggests that this naturally occuring Sph derivative affects cell motility (and spreading) through modulation of a common signaling pathway. Our preliminary experiments demonstrated no effect of Sph-1-P on ECM-dependent tyrosine kinase $P125^{FAK}$ activity (data not shown). It is possible that the active site of Sph-1-P is located downstream from cell surface receptors, and affects actin filament reorganization in the stimulated cells (Sadahira, Y., Ruan, F., Zheng, M., Hakomori, S. and Igarashi, Y., unpublished data)

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