Sphingosine-1-phosphate Inhibits Motility of Human Breast Cancer Cells Independently of Cell Surface Receptors


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ABSTRACT

Exogenous sphingosine-1-phosphate (SPP) inhibits chemotactic motility of several transformed cell lines. We have found that SPP at high molar concentrations decreased chemotaxis of estrogen-independent (MDA-MB-231 and BT 549) and estrogen-dependent (MCF-7 and ZR-75-1) human breast cancer cells. Because SPP has been implicated as a lipid-signaling molecule with novel dual intracellular and intercellular actions, it was of interest to determine whether the effect of SPP on chemotactic motility of human breast cancer cells is mediated intracellularly or through the recently identified endothelial differentiation gene (EDG) family of G protein-coupled SPP receptors. There was no detectable specific binding of [32P]SPP to MDA-MB-231 or MCF-7 cells; however, reverse transcription-PCR analysis revealed that both MDA-MB-231 and MCF-7 cells expressed moderate levels of EDG-3, neither expressed EDG-1, and EDG-5 mRNA was expressed in MCF-7 but not in MDA-MB-231 cells. In contrast to SPP, sphingosine-1-phosphate, which binds to and signals through SPP receptors EDG-1, EDG-3, and EDG-5, had no effect on chemotactic motility of MDA-MB-231 or MCF-7 cells. To further discriminate between intracellular and receptor-mediated actions of SPP, we used caged SPP, a photolyzable derivative of SPP that elevates intracellular levels of SPP after illumination. Caged SPP inhibited chemotactic motility of MDA-MB-231 cells only upon UV irradiation. In addition, in MCF-7 cells, overexpression of sphingosine kinase, the enzyme that produces SPP, inhibited chemotactic motility compared with vector-transfected cells and markedly increased cellular SPP levels in the absence of detectable secretion. Our results suggest that the inhibitory effect of SPP on chemotactic motility of human breast cancer cells is likely mediated through intracellular actions of SPP rather than through cell surface receptors.

INTRODUCTION

The sphingolipid metabolite, SPP, has been shown to act on various signaling pathways and to affect numerous biological functions (reviewed in Ref. 1). SPP has been implicated as a lipid second messenger in mitogenesis and calcium mobilization and also antagonizes ceramide-mediated apoptosis (1). The resting level of SPP is very low in most cells. The enzyme that catalyzes its formation, sphingosine kinase, is increased by a wide variety of stimuli, including nerve growth factor, basic fibroblast growth factor (6), sphingosine kinase, is increased by a wide variety of stimuli, including nerve growth factor, basic fibroblast growth factor (6), and vitamin D3 (8). Although many studies indicate an intracellular site of action for SPP, pertussis toxin-sensitive G proteins have been shown to be involved in some of the signaling pathways regulated by SPP, suggesting that it might activate a receptor coupled to a Gαi protein (21). In agreement, low concentrations of SPP activated Gi protein-gated inwardly rectifying potassium channels only when SPP was applied to the extracellular face of guinea pig atrial myocytes (22). Also, nanomolar concentrations of SPP (EC50, 2 nM) rapidly induced Rho-dependent neurite retraction and cell rounding of mouse NIE-115 neurons (23) and platelet activation (24).

Recently, the GPCRs EDG-1, EDG-3, and EDG-5 were identified as high-affinity receptors for SPP (25–29). EDG-1 causes morphogenetic differentiation in response to SPP when expressed in HEK293 cells (23), whereas EDG-5, and to a lesser extent EDG-3, cause cell rounding in HEK293 cells and neurite retraction in PC12 cells (28). Binding of SPP to EDG-1, EDG-3, and EDG-5 stimulates different Gαs and βγ dimers to signal through cyclic AMP, phospholipase C, Ras, mitogen-activated protein kinase, Rho, and several protein tyrosine kinases (25, 26, 29–32). Collectively, these studies suggest that SPP is capable of acting as a second messenger to regulate cell proliferation and survival and as a first messenger through the EDG family of GPCRs to regulate diverse biological responses.

Migration of cells is important in a variety of normal physiological processes, including embryogenesis, reproduction, inflammation, and wound healing. Moreover, cell motility plays a notable role in pathological processes important for malignant progression such as metastasis. Cell migration is regulated by both expression of adhesion molecules and deposition of basement membrane or matrix proteins and soluble extracellular molecules interacting with specific cell surface receptors (33). Previously, many studies have shown that exogenous SPP inhibits the chemotactic motility of various cancer cells at very low nanomolar concentrations (10–100 nM; Refs. 34–36). SPP also inhibits integrin-dependent motility (haptotactic motility) of mouse melanoma B16 cells by inhibiting actin nucleation and pseudopodia formation, without reducing integrin-dependent adhesion to the extracellular matrix (35). Furthermore, SPP immobilized on controlled pore glass beads inhibits motility of mouse melanoma cells (37), indicating that this effect may be mediated through cell surface receptors. In contrast, we found previously that inhibition of chemotactic motility of human breast cancer MCF-7 and MDA-MB-231 cells requires micromolar concentrations of SPP (38). Thus, it is of interest to determine whether SPP acts extracellularly as a ligand for cell surface receptors or hydroxymvitamin D3, ligation of Fc receptors FcεRI (9) and FcγRI (10), muscarinic acetylcholine receptors (11), and the B subunit of cholera toxin (12). The elevation in SPP as a result of sphingosine kinase activation is relatively short-lasting because of the action of two specific enzymes involved in its catabolism, SPP lyase and SPP phosphatase (13–17). Prevention of the increase in SPP by competitive inhibitors of sphingosine kinase selectively blocks cellular proliferation induced by PDGF and serum (2, 18), as well as FcεRI- and FcγRI-mediated calcium release from internal sources (9, 10), calcium influx induced by carbachol (11), and the cytoprotective effects of 12-O-tetradecanoylphorbol-13-acetate, cyclic AMP activators (19, 20), nerve growth factor (6), and vitamin D3 (8).

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3 The abbreviations used are: SPP, sphingosine-1-phosphate; C8-Cer-1-P, N-octanoyl ceramide-1-phosphate; dihydro-SPP, sphinganine-1-phosphate; EDG, endothelial differentiation gene; PPMI, Rickett’s Improved Minimal Essential Medium; LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; RT-PCR, reverse transcription-PCR; FAK, focal adhesion kinase; SAP-1, SAP-2, SAP-3, SAP-4, SAP-5; RIC, G protein-coupled receptors; IMEM, Richter’s Improved Minimal Essential Medium; LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; RT-PCR, reverse transcription-PCR; FAK, focal adhesion kinase; GRAF, GTPase regulator associated with FAK; PMSF, phenylmethylsulfonyl fluoride.
Intracellularly as a second messenger to inhibit chemotactic motility of human breast cancer cells.

The use of exogenously administered SPP is a major limitation of most previous studies because this approach cannot distinguish between receptor-mediated and intracellular effects because SPP is readily taken up by cells in culture (2, 26, 39). In this study, we demonstrated that motility of human breast cancer cells was inhibited when intracellular levels of SPP were increased either after photolysis of caged SPP or by overexpression of sphingosine kinase; both approaches bypass cell surface receptors. Our results suggest that SPP can inhibit motility through intracellular actions.

**MATERIALS AND METHODS**

**Materials.** SPP, dihydro-SPP, sphingosine, and N,N-dimethylsphingosine were purchased from Biomol Research Laboratory, Inc. (Plymouth Meeting, PA). SPP was >99% pure by TLC analysis. C8-SPP, C8-ceramide-1-phospate, octyl β-D-glucopyranoside, and bovine brain ceramides (type IV) were from Calbiochem (La Jolla, CA). Cyclic SPP was purchased from Alexis Biochemicals (San Diego, CA). Other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). Medium, serum, and supplements were from Biofluids, Inc. (Rockville, MD); insulin and transferrin were from Collaborative Research (Lexington, MA); BSA, alkaline phosphatase (type VII-T), and Quik-fix kit were from Sigma Chemical Co. (St. Louis, MO); and G418 was from Mediatech (Herndon, VA). Collagen type IV was purchased from Collagen Corp. (Palo Alto, CA). Polycarbonate filters were from Poretics (Livermore, CA). γ-32P]ATP (3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL).

**Cell Culture.** The human breast cancer cell lines MDA-MB-231, MCF-7, BT 549, and ZR-75-1 were obtained from the Cell Culture Core Resource, Lombardi Cancer Center (Washington, DC). Cells were maintained in Rich- ter’s IMEM supplemented with 10% fetal bovine serum. Unless indicated, cells were seeded and incubated in a humidified incubator at 37°C in 5% CO2/95% air for 4 or 24 h.

**Boyden chamber as chemoattractant.** Cells were harvested by trypsinization and incubated in a humidified incubator at 37°C in 5% CO2/95% air for 4 or 24 h.

**RT-PCR.** Total RNA was isolated from cells using TRIZOL reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s instructions and treated with RNase-free DNase I (RQ-1; Promega Corp., Madison, WI) to eliminate contaminating DNA. Reverse transcription was performed using MULV-RT (Perkin Elmer, Branchburg, NJ) for 15 min at 42°C. The primers (Life Technologies) used for PCR amplification were: 5′-GATATCATGTTGCGGATTAC and 5′-ACCCCTCCAGTGCATTGTC for EDG-1 (40); 5′-CAGTACAGATGTACCTGTTCC and 5′-AACACT-CAGATCAGTTGTCGAC for EDG-5 (41, 42); and 5′-GACTGCTTCAC-CATCTGCCC and 5′-GTAAGATGACAGGCTCATGGC for EDG-3 (43). PCR reactions were performed for 30 cycles with denaturation at 95°C for 45 s, annealing at 55°C for 45 s, and elongation at 72°C for 50 s. The PCR products were analyzed by agarose gel electrophoresis after staining with ethidium bromide.

**Expression of Sphingosine Kinase.** SPHK1a was subcloned into a modified pcDNA3 vector (Invitrogen, Carlsbad, CA) to express it with an NH2-terminal c-myc epitope tag by PCR using a 5′ primer with a BamHI restriction site c-TACGATCAGAATGCTCTCGAGGA (44) and as the 3′ primer, the last 21 nucleotides of the SPHK1a sequence with an authentic ATG start codon (41, 42); and 5′-GACTGCTTCAC-CATCTGCCC and 5′-GTAAGATGACAGGCTCATGGC for EDG-3 (43). The specific activity of [32 P]SPP was 6 × 104 cpm/nmol. Cells (5 × 105 cells) were harvested by trypsinization and incubated in a humidified incubator at 37°C in 5% CO2/95% air for 4 or 24 h.

**Chemotaxis Assay.** Boyden chamber chemotactic motility assays were carried out essentially as described previously (38). Polycarbonate filters (13-mm diameter, 12-μm pore size) were coated with collagen IV (5 μg/filter) and placed into the lower chamber. Collagen IV coating promotes uniform attachment to and migration across the filter, without formation of a barrier. FM, obtained by incubating confluent NIH 3T3 cells for 48 h with IMEM supplemented with 10% fetal bovine serum. Unless indicated, cells were seeded at 1.8 × 105 cells/filter. Twenty-four h prior to experiments, the medium was changed to serum-free IMEM without phenol red supplemented with 2 mM HEPES, 1% (v/v) nonessential amino acids, 1% (v/v) trace elements, 0.4% (v/v) insulin, transferrin, and selenium, 0.2% (w/v) fibronectin, and 1% (v/v) vitamins (38).

**Measurement of Sphingosine Levels.** Cells were harvested by a brief freeze-thawing in buffer A [20 mM Tris (pH 7.4), 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 μM glicophosphatase, 15 mM NaF, 10 μg/ml leupeptin, aprotinin, and soybean trypsin inhibitor, 1 mM PMSF, and 0.5% 4-deoxyriboxidine]. Sphingosine kinase activity was determined in the presence of 10 μM sphingosine, 0.25% Triton X-100, and [32 P]ATP (10 μCi, 20 mM) containing MgCl2 (200 mM) in buffer A as described previously (45). The labeled SPP was separated by TLC on silica gel G60 with chloroform:methanol:acetone:acetic acid:water (10:3:4:3:1, w/v) and visualized by autoradiography. Radioactive spots corresponding to authentic SPP were identified as described (39) and quantified with a Molecular Dynamics Storm PhosphorImager (Sunnyvale, CA). Sphingosine kinase-specific activity was expressed as pmol of SPP formed per min per mg of protein.

**Extraction of Lipids.** Transfected cells were washed with PBS and scraped in 1 ml of 25 mM HCl/methanol. Lipids were extracted with 2 ml of chloroform:1 M NaCl (1:1, v/v) plus 100 μl of 3 M NaOH, and phases were separated (46). Phospholipids, ceramide, and sphingosine levels were determined in aliquots of the organic layer, whereas SPP levels were determined from aqueous phase extracts. Extraction of SPP from the medium of cells labeled with 40 μCi of [32 P]SPP for 48 h was performed similarly. To 1 ml of medium, 2 ml of chloroform:methanol:water (11:1:v/v) plus 100 μl of 3 M NaOH were added, and phases were separated. The aqueous phase containing SPP was then acidified with 50 μl of concentrated HCl and reextracted twice with 1 ml of chloroform, and organic fractions were pooled (46).

**SPP Binding Assay.** [32 P]SPP was synthesized enzymatically using recombiant sphingosine kinase as described previously (26). The specific activity of [32 P]SPP was 6 × 104 cpm/nmol. Cells (5 × 105) were incubated with 1 nm [32 P]SPP in 200 μl of binding buffer [20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 15 mM NaF, 2 mM deoxyriboxidine, 0.2 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin] for 30 min at 4°C. Cells were washed twice with 200 μl of ice-cold binding buffer containing 0.4 mg/ml fatty acid-free BSA and resuspended in PBS, and specific binding of [32 P]SPP was determined in the presence of 1 μM unlabeled SPP (26).

**Western Blotting.** Transfected cells were lysed in a buffer containing 0.1% Triton X-100, and 0.6% SDS-PAGE PAGE Pharmacia (Piscataway, NJ) suspended in 2 M glycine (pH 9.0) plus 50 units of alkaline phosphatase were added to the cells. Bands were visualized with Super Signal chemiluminescent reagent (Pierce, Rockford, IL) for horseradish peroxidase-conjugated antibody lgG (1:5000).

**Sphingosine Kinase Assay.** Transfected cells were harvested and lysed by repeated freeze-thawing in buffer A [20 mM Tris (pH 7.4), 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 μM β-glucophosphatase, 15 mM NaF, 10 μg/ml leupeptin, aprotinin, and soybean trypsin inhibitor, 1 mM PMSF, and 0.5% 4-deoxyriboxidine]. Sphingosine kinase activity was determined in the presence of 50 μM sphingosine, 0.25% Triton X-100, and [32 P]ATP (10 μCi, 20 mM) containing MgCl2 (200 mM) in buffer A as described previously (45). The labeled SPP was separated by TLC on silica gel G60 with chloroform:methanol:acetone:acetic acid:water (10:3:4:3:1, w/v) and visualized by autoradiography. Radioactive spots corresponding to authentic SPP were identified as described (39) and quantified with a Molecular Dynamics Storm PhosphorImager (Sunnyvale, CA). Sphingosine kinase-specific activity was expressed as pmol of SPP formed per min per mg of protein.

**Measurement of SPP Levels.** SPP levels were measured essentially as described (46). Briefly, buffer B [200 mM Tris-HCl, (pH 7.4), 75 mM MgCl2 in 2 M glycine (pH 9.0)] and 50 units of alkaline phosphatase were added to the aqueous phase containing extracted SPP. After incubating 1 h at 37°C, 50 μl of concentrated HCl were added, and sphingosine was extracted and quantified with sphingosine kinase as described (46). For each experiment, known amounts of SPP were used to generate a standard curve.

**Measurement of Sphingosine Levels.** Sphingosine was measured by minor modifications of a method described previously (47). Briefly, aliquots of lipid extracts containing ≥50 nmol of total phospholipid were dried under nitrogen and resuspended in buffer A containing 0.25% Triton X-100, and
Fig. 1. SPP decreases motility of human breast cancer cells. A, effect of SPP on chemotaxis. MDA-MB-231, MCF-7, ZR-75-1, and BT-549 cells were allowed to migrate through polycarbonate filters coated with collagen IV for 4 h in the absence (C) or presence (D) of SPP (10 μM). FCM was present in the lower chamber as a chemoattractant, and chemotaxis was measured as described in “Materials and Methods.” B, effect of SPP on random motility. MDA-MB-231 cells were incubated in the absence or presence of SPP (10 μM) or sphingosine (Sph, 15 μM), and random motility was measured in the absence of FCM. The results are from a representative experiment repeated three times. All treatments were significantly different from controls as determined by Student’s t test (P ≤ 0.05). Bars, SD.

Fig. 2. Detection of EDG receptor mRNA. RT-PCR analysis of glyceraldehyde-3-phosphate dehydrogenase (G), EDG-1 (1), EDG-3 (3), and EDG-5 (5) mRNA expression was performed on RNA isolated from MCF-7 MDA-MB-231 and MCF-7 cells stably expressing myc epitope-tagged EDG-1 (MCF-7-EDG-1), without (+) or with (+) MULV-RT (RT) as described in “Materials and Methods.” Similar results were obtained in two independent experiments.

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sphingosine converted to SPP with sphingosine kinase was measured as described (47). For each experiment, known amounts of sphingosine were used to generate a standard curve.

Measurements of Ceramide Levels. Mass amounts of ceramide in cellular extracts were measured by the diacylglycerol kinase enzymatic method exactly as described (6).

Measurement of Total Cellular Phospholipids. Total phospholipids in cellular lipid extracts were quantified as described previously (48).

RESULTS

SPP Inhibits Chemotactic Motility of Human Breast Cancer Cells. In agreement with our previous report (38), we found that SPP inhibits chemotaxis of estrogen-independent MDA-MB-231 and BT 549 human breast cancer cells by 45 and 28%, respectively. Treatment of estrogen-dependent human breast cancer cells with SPP and BT-549 human breast cancer cells by 45 and 28%, respectively. The results are from a representative experiment repeated three times. All treatments were significantly different from controls as determined by Student’s t test (P ≤ 0.05). Bars, SD.

EDG-1, EDG-3, and EDG-5, bind SPP with high specificity. D-
The results are from a representative experiment repeated three times.

Chemotactic motility was determined. The results are from a representative experiment repeated at least two times. *, significant differences from untreated controls as determined by Student’s t test (P ≤ 0.05). Bars, SD.

hydro-SPP, which is similar to SPP but lacks the trans double bond, binds to and signals through all three SPP receptors with similar potency to SPP (25, 26, 28). Thus, if inhibition of chemotactic motility is mediated through one or more of these SPP receptors, dihydro-SPP should mimic the effect of SPP. However, 10 μM dihydro-SPP had no significant effect on chemotactic motility of MDA-MB-231 or MCF-7 cells (Fig. 3B). Collectively, these data suggest that the ability of SPP to inhibit chemotaxis may not be related to binding to EDG cell surface receptors.

Inhibition of Chemotaxis by Caged SPP. To determine whether SPP formed intracellularly regulates cell motility, it is useful to be able to experimentally increase intracellular SPP bypassing cell surface receptors. Recently, we synthesized a photolyzable derivative of SPP, termed caged SPP, which is taken up by cells and upon UV irradiation, is photolyzed to form SPP and o-nitrosoacetophenone (52). The introduction of caged SPP into cultured cells permits rapid and controlled elevation of intracellular SPP levels upon illumination (data not shown). The effect of caged SPP photolysis on chemotactic motility of MDA-MB-231 cells was compared with that of exogenous added SPP (Fig. 4). Exogenous SPP (10 μM) inhibited chemotactic motility of MDA-MB-231 cells by 50%, whereas caged SPP (5 or 10 μM) had no significant effect on chemotactic motility in nonilluminated cells or in cells illuminated prior to its addition. However, UV irradiation of caged SPP-loaded cells caused strong inhibition of chemotaxis, whereas UV irradiation alone had no effect (Fig. 4), nor did it alter the inhibitory effect of exogenous SPP. To exclude the possibility that the by product of caged SPP photolysis, o-nitrosoacetophenone, may affect chemotaxis, the motility of cells treated with caged cyclic SPP was measured. Unlike caged SPP, caged cyclic SPP had no effect on chemotaxis of MDA-MB-231 cells with or without UV irradiation (Fig. 4). Moreover, incubation of cells with o-nitrosoacetophenone had no effect on chemotaxis, even when cells were incubated for up to 4 h at very high concentrations (100 μM; data not shown).

Overexpression of Sphingosine Kinase Decreases Chemotactic Motility of MCF-7 Cells. As an alternative method to increase the level of intracellular SPP, MCF-7 cells were transfected with a murine sphingosine kinase expression vector (44). Transient expression of sphingosine kinase in MCF-7 cells decreased chemotaxis toward FCM (Fig. 5A). Moreover, although the number of migrating cells was much lower in the absence of chemoattractant, transient expression of sphingosine kinase also significantly reduced this random motility (Fig. 5A).

To further examine the effect of overexpression of sphingosine kinase on chemotaxis and to correlate this with protein expression, MCF-7 cells were transfected with c-myc-tagged sphingosine kinase and selected on the basis of G418 resistance. Pools of stably transfected cells were used to avoid potential phenotypic changes attributable to selection and propagation of clones derived from single individual cells. Stable expression of sphingosine kinase decreased chemotactic motility of MCF-7 cells by 30 and 46% after 4 and 24 h, respectively (Fig. 5B). Sphingosine kinase activity in the cytosolic fraction of these transfected cells was elevated by 36-fold over control vector-transfected cells (Table 1), whereas SPP levels were elevated by only 3.3-fold (Table 1). This is in agreement with previous results (44), where the large fold increase in transfected sphingosine kinase activity measured in vitro did not correspond with the fold increase in SPP levels. To further examine the correlation between expression of sphingosine kinase and increased SPP levels with inhibition of chemotaxis, we generated a separate independent pool of transfected cells that express even higher levels of sphingosine kinase (Table 1). Western blot analysis of the cytosolic fractions of these cells using anti-c-myc antibody revealed a band migrating at the expected molecular weight of c-myc-tagged sphingosine kinase, which was absent in vector-transfected cells (Fig. 6A, inset). Interestingly, although
expression of sphingosine kinase in this second pool of transfected MCF-7 cells resulted in even more marked increase in mass levels of SPP (Fig. 6B), there were no concomitant changes in the other sphingolipid metabolites, sphingosine (Fig. 6C), or ceramide (Fig. 6D). Nevertheless, chemotaxis of the MCF-7 cells with higher sphingosine kinase expression and cellular SPP levels was more strongly inhibited (Fig. 6E).

Because it has been suggested that SPP inhibits melanoma cell motility through an extracellular action by specific binding to cell surface receptors (37), it was of interest to determine whether MCF-7 cells overexpressing sphingosine kinase, which have increased cellular levels of SPP, can secrete SPP. We have recently developed a sensitive assay for the measurement of SPP (46) that is able to detect as little as 1 pmol of SPP. Using this assay, we were unable to detect secretion of SPP into the medium by either of the stable pools of sphingosine kinase-transfected MCF-7 cells (Table 1), suggesting that SPP is not released by these cells in appreciable amounts. To increase the sensitivity of detection of secreted SPP, we also labeled cells to isotopic equilibrium with $[^{32}P]$P$^i$ and analyzed the labeled SPP in cells as well as in the medium. Despite the large increases in $[^{32}P]$SPP detected in MCF-7 cells overexpressing sphingosine kinase, there was no detectable labeled SPP released into the medium. On the basis of the sensitivity of these methods, we estimate that the concentration of SPP in the extracellular medium is $\approx 0.4$ nm, a concentration well below the $K_d$ for binding of SPP to its EDG receptors (26, 28).

**DISCUSSION**

SPP has been shown to function both intracellularly as a second messenger and at the cell surface through specific SPP receptors to regulate numerous biological processes (29, 53). Previously, on the basis of addition of SPP to cells, many studies have suggested that SPP inhibits cell motility and chemotaxis by binding to a putative cell surface receptor (reviewed in Ref. 37). Recently, EDG-1 was identified as a high-affinity ($K_d$ 8 m),$ specific receptor for SPP (25). Two related receptors, EDG-3 and EDG-5 (also known as AGR16/H218), also bind SPP with low nanomolar affinities (28). However, it has not yet been determined whether EDG-1, EDG-3, or EDG-5 plays a role in mediating the effects of exogenous SPP on inhibition of motility.

SPP has been shown to inhibit cell motility, chemoinvasion, and haptotactic motility (35) of human B16 melanoma cells in a low concentration range (10–100 nm; Ref. 34). These effects appear to be mediated through a cell surface receptor, because SPP immobilized on glass beads, which cannot traverse the cell membrane, mimicked the effects of SPP (37). SPP also inhibited the chemotactic motility and transendothelial migration of human neutrophils (36) and PDGF-induced chemotaxis of aortic smooth muscle cells (3) at nanomolar concentrations. In contrast, micromolar concentrations of SPP were necessary to inhibit chemotactic motility of human breast cancer cell lines (MCF-7 and MDA-MB-231; Ref. 38) and human HT1080 fi-brosarcoma cells (34). It was thus important to determine whether inhibition of breast cancer cell motility by SPP is mediated intracellularly or through a cell surface receptor.

Surprisingly, in this study, several lines of evidence indicated that SPP inhibits chemotactic motility of MDA-MB-231 and MCF-7 cells through an intracellular action rather than by signaling through cell surface receptors: (a) SPP had no effect on motility of MDA-MB-231 cells at concentrations below 1 $\mu$m, approximately two orders of magnitude higher than the $K_d$ for binding of SPP to EDG-1, EDG-3, or EDG-5 (25, 28); (b) although MDA-MB-231 cells express SPP receptor EDG-3 mRNA and MCF-7 cells express EDG-3 and EDG-5, no specific SPP binding could be detected to either MCF-7 or MDA-MB-231 cells, indicating that either the receptor proteins are not present on the cell surface or that they are expressed at very low levels; and (c), dihydro-SPP, which binds to and signals through all three SPP receptors (26, 28)$^4$ had no effect on the chemotactic motility of MDA-MB-231 or MCF-7 cells. In agreement, it was reported previously that dihydro-SPP did not affect chemotactic motility of human neutrophils (36). Moreover, sphingosine, which is rapidly taken up by cells and converted intracellularly to SPP by sphingosine kinase, also inhibits chemotaxis of MCF-7 and MDA-MB-231 cells (38).

Additionally, two independent approaches were used to elevate intracellular SPP, bypassing cell surface SPP receptors: treatment of cells with caged SPP, which is taken up by cells and forms SPP intracellularly upon UV irradiation (52); and overexpression of sphingosine kinase, the enzyme that forms SPP within cells. After UV photolysis of intracellular caged SPP, chemotactic motility was inhibited to the same extent as after treatment with exogenous SPP. Overexpression of sphingosine kinase by transfection in MCF-7 cells led to increased intracellular SPP and drastically inhibited chemotactic motility as well as random motility. Although the intracellular levels of SPP were elevated 3–4.6-fold in stably transfected cells, no

Table 1  Sphingosine kinase activity and SPP levels in pooled clones of MCF-7 cells stably expressing sphingosine kinase

<table>
<thead>
<tr>
<th>Pool</th>
<th>Sphingosine kinase activity (fold increase)</th>
<th>Secreted SPP (pmol/ml)</th>
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<tbody>
<tr>
<td>Pool 1</td>
<td>36 ± 3</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>Pool 2</td>
<td>115 ± 7</td>
<td>4.6 ± 0.2</td>
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$^4$ ND, not detected (<0.4 pmol/ml).

Fig. 5. Overexpression of sphingosine kinase decreases chemotactic motility of MCF-7 cells. A, MCF-7 cells transiently cotransfected with pCEFLGFP and either vector c-myc-pcDNA3 (☐) or c-myc-pcDNA3-SPHK1a (●) were trypsinized after 48 h, washed with serum-free medium, and allowed to migrate in a Boyden chamber for 4 h toward either medium alone (IMEM) as a measure of random motility or toward FCM as chemoattractant to measure chemotaxis. Bars, SD. B, pooled clones of MCF-7 cells stably expressing c-myc-pcDNA3 (☐) or c-myc-pcDNA3-SPHK1a (●) were allowed to migrate toward FCM for 4 or 24 h, and chemotaxis was measured. *, significant differences from untreated controls as determined by Student’s t test ($P \leq 0.05$). Bars, SD.
detectable amounts of SPP were released into the medium. Thus, it seems highly unlikely that SPP inhibits motility of breast cancer cells by binding to cell surface receptors.

Although we have now shown that elevation of the intracellular levels of SPP inhibits motility of human breast cancer cells, our studies cannot exclude the possibility that in other cell types, the mode of action of SPP may vary, depending on the expression of different SPP receptors and the signaling pathways that the receptors couple to in different cell types. It is possible that SPP could effect the same biological response through separate mechanisms, even in the same cells. For example, microinjected SPP is mitogenic for Swiss 3T3 cells, however, to a lesser extent than exogenously added SPP (26). Although the mitogenic effect of microinjected SPP is insensitive to pertussis toxin treatment, the effect of exogenous SPP is partially inhibited by pertussis toxin, decreasing the response to approximately the same level as that induced by microinjection (26). These results suggested that in Swiss 3T3 cells, which express all three known SPP receptors, the mitogenic effect of SPP is attributable to the additive contributions of receptor-mediated responses and intracellular actions. Thus, it is possible that cell motility could also be regulated by both intracellular actions of SPP, as in breast cancer cells, and possibly by receptor-mediated actions of SPP, as in melanoma cells.

The mechanism of the inhibitory effect of SPP on motility has not yet been elucidated. It is possible that the inhibitory effect of SPP on chemotactic motility may be mediated by FAK and Rho. Activation of Rho results in the formation of actin stress fibers and focal adhesions (54–56), and it is well established that cytoskeletal changes regulated by Rho GTPases form the basis for the coordination of cell motility (57, 58). SPP induces tyrosine phosphorylation of FAK and actin stress fiber formation in a Rho-dependent manner (59). Although the exact connection between the expression and tyrosine phosphorylation of FAK and cell motility is not completely understood, it has been suggested that maximal migration requires optimal levels of FAK tyrosine phosphorylation (60), and inhibition of FAK signaling in focal adhesions decreases cell motility (61). Intriguingly, it was found recently that GRAF, which preferentially stimulates the GTPase activity of RhoA and Cdc42, can regulate cytoskeletal changes induced by SPP (62). In Swiss 3T3 cells, where GRAF is not expressed at detectable levels, GRAF overexpression inhibited SPP-induced, Rho-mediated stress fiber formation. Conversely, in PC12 cells that express high levels of GRAF, overexpression of GRAF stimulated Rho-mediated neurite retraction induced by SPP (62). These results may suggest that an optimal level of Rho activity is required for maximal cell motility. Another possibility is that the effect of SPP on motility is related to regulation of cytosolic free calcium. It has been shown that large (micromolar) global increases in the cytosolic free calcium concentration in neutrophils are usually associated with termination of chemotaxis (63). Recently, it was found that cells move forward during the phase of transient calcium elevation and remain stationary during the troughs (64). Consequently, changes in the frequency of Ca2+ fluctuations directly affect cerebellar granule cell movement, i.e., reducing the frequency of calcium fluctuations slows down the speed of cell movement (64). Interestingly, SPP has been found to elicit a nonsessory increase of intracellular free calcium in CG4, NIH 3T3, and PDGF receptor-transfected PC12 cells (65). Moreover, inhibition of sphingosine kinase with di-threo-dihydrosphingosine significantly reduced the percentage of cells responding to PDGF exposure with calcium oscillations in transformed oligodendrocytes (66). Thus, it is possible that SPP inhibits chemotactic motility of MDA-MB-231 and MCF-7 cells by inducing a nonsessory increase in intracellular free calcium. Additional studies are needed to clarify the importance of different signaling pathways for controlling motility that are regulated by SPP. Because cell movement is considered an important step in invasion and metastasis of cancer cells, the finding that endogenous SPP regulates cell migration and chemotactic signaling may have substantial biological ramifications.

REFERENCES


