Bioactive Lipids S1P and C1P Are Prometastatic Factors in Human Rhabdomyosarcoma, and Their Tissue Levels Increase in Response to Radio/Chemotherapy

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Introduction

Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma of adolescence and childhood and accounts for 5% of all malignant tumors in patients under 15 years of age (1, 2). There are 2 major histologic subtypes of RMS, alveolar RMS (ARMS) and embryonal RMS (ERMS). ARMS is more aggressive and has a significantly worse outcome than ERMS. Moreover, ARMS is characterized by the t(2;13)(q35;q14) translocation in 70% of cases or the variant t(1;13)(p36;q14) in a smaller percentage of cases (3). These translocations disrupt the PAX3 and PAX7 genes on chromosome 2 or 1, respectively, and the FKHR gene on chromosome 13, generating PAX3-FKHR and PAX7-FKHR fusion genes. These fusion genes encode the fusion proteins PAX3-FKHR and PAX7-FKHR, which have enhanced transcriptional activity compared with wild-type PAX3 and PAX7, and are postulated to play a role in cell survival and dysregulation of the cell cycle in ARMS (1).

It is well known that RMS cells, particularly in ARMS, are highly metastatic, belonging to the family of so-called “small round blue tumor cells,” which often infiltrate bone marrow and, because they can resemble hematologic blasts, may sometimes be misdiagnosed as acute leukemia cells (2). A significant effort has been made to find chemoattractants that lead to metastasis of RMS cells to bone marrow, and we and others have shown that the α-chemokine stromal-derived factor-1 (SDF-1) and hepatocyte growth factor/scatter factor (HGF/SF) are secreted by bone marrow stroma and play an important role in RMS cell metastatic behavior (4, 5). The robust chemotactic response to these factors is also observed in in vitro migration assays in which both factors are used as chemoattractants at supraphysiologic concentrations (4, 5).

Because the concentrations of SDF-1 and HGF/SF in biologic fluids and tissues are usually very low (6, 7), we began a search for other chemoattractants that could induce metastasis of RMS cells and turned our attention to bioactive lipids, such as sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P), as potential candidates. Both bioactive lipids, such as sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P), strongly enhanced the in vitro motility and adhesion of human rhabdomyosarcoma (RMS) cells. Importantly, this effect was observed at physiologic concentrations for both bioactive lipids, which are present in biologic fluids, and were much stronger than the effects observed in response to known RMS prometastatic factors such as stromal derived factors-1 (SDF-1/CXCL12) or hepatocyte growth factor/scatter factor (HGF/SF). We also present novel evidence that the levels of S1P and C1P were increased in several organs after γ-irradiation or chemotherapy, which indicates an unwanted prometastatic environment related to treatment. Critically, we found that the metastasis of RMS cells in response to S1P can be effectively inhibited in vivo with the S1P-specific binder NOX-S93 that is based on a high-affinity Spiegelmer. These data indicate that bioactive lipids play a vital role in dissemination of RMS and contribute to the unwanted side effects of radio/chemotherapy by creating a prometastatic microenvironment.

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Abstract

Evidence suggests that bioactive lipids may regulate pathophysiologic functions such as cancer cell metastasis. Therefore, we determined that the bioactive lipid chemoattractants sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P) strongly enhanced the in vitro motility and adhesion of human rhabdomyosarcoma (RMS) cells. Importantly, this effect was observed at physiologic concentrations for both bioactive lipids, which are present in biologic fluids, and were much stronger than the effects observed in response to known RMS prometastatic factors such as stromal derived factors-1 (SDF-1/CXCL12) or hepatocyte growth factor/scatter factor (HGF/SF). We also present novel evidence that the levels of S1P and C1P were increased in several organs after γ-irradiation or chemotherapy, which indicates an unwanted prometastatic environment related to treatment. Critically, we found that the metastasis of RMS cells in response to S1P can be effectively inhibited in vivo with the S1P-specific binder NOX-S93 that is based on a high-affinity Spiegelmer. These data indicate that bioactive lipids play a vital role in dissemination of RMS and contribute to the unwanted side effects of radio/chemotherapy by creating a prometastatic microenvironment.

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lipids have been reported to play an important role in development of skeletal muscle (8, 9), and, in addition, S1P has been shown to direct migration of various types of tumor cells (10–12). It is known that S1P is secreted from several types of cells and binds to serum albumin and lipoproteins, all of which explains its relatively high (μmol/L) concentration in peripheral blood and lymph (13). Similarly, the concentration of C1P is also comparably high in peripheral blood, and this bioactive lipid, which is retained intracellularly, may also be released from “leaky” cells following damage (13, 14). Although 5 signaling receptors for S1P (S1PR1–5) have been cloned and characterized, not a single receptor for C1P has been identified yet (15, 16). As reported already, exposure of cells to S1P and C1P results in activation of signal transduction pathways involving MAPKp42/44 and AKT (16, 17).

One of the challenging problems of current radio/chemotherapy is recurrence and metastasis of cancer cells that survive initial treatment. We have proposed that one of the unwanted effects of radio/chemotherapy is induction of a prometastatic microenvironment in normal tissues damaged by treatment (18, 19), which suggests that standard antitumor treatment should be augmented by antimetastatic therapy. Therefore, we were looking for a possibility to interfere pharmacologically with prometastatic factors such as S1P. To this end, a promising candidate is NOX-S93, a mirror-image oligonucleotide that binds with high affinity and selectivity to S1P, thereby inhibiting its activity (Unpublished Data). NOX-S93 is an inhibitor belonging to the class of so-called Spiegelmers (Spiegel = German word for mirror). These are biostable oligonucleotides (20). Currently, 3 Spiegelmer-based compounds are in clinical development (21).

In this article, we present novel evidence that S1P and C1P enhance motility and adhesive properties of RMS cells, and, as the levels of both bioactive lipids increase in several organs after γ-irradiation or chemotherapy, induce an unwanted prometastatic environment after treatment. Most importantly, we found that the metastasis of RMS cells in response to S1P can be effectively inhibited in vivo by administration of this novel Spiegelmer NOX-S93 that binds and neutralizes S1P present in biologic fluids. On the basis of this finding, we propose that antimetastatic treatment with this anti-S1P compound could follow standard radio/chemotherapy.

Materials and Methods

Cell lines

We used several human RMS cell lines (gifts from Dr. Peter Houghton, World Children’s Cancer Center, Columbus, OH and Prof. Fred Barr, University of Pennsylvania, Philadelphia, PA), including both ARMS (RH4, RH5, RH18, RH28, RH30, RH41, and CW9019) and ERMS (JR, SMS-CTR, RD, and RH36) cell lines. SMS-CTR and RH36 cells were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% FBS, 100 U/mL penicillin, and 10 μg/mL streptomycin. All other cell lines were maintained in RPMI-1640, containing 10% FBS, 100 U/mL penicillin, and 10 μg/mL streptomycin. Stromal cells were maintained in DMEM containing 20% FBS, 100 U/mL penicillin, and 10 μg/mL streptomycin. All cells were cultured in a humidified atmosphere of 5% CO2 at 37°C, and the media were changed every 48 hours.

Murine bone marrow stromal cells

Bone marrow–derived stromal cells (MSC) were expanded ex vivo from murine bone marrow mononuclear cells (BMMNC) as described previously (17). Briefly, BMMNC were expanded in DMEM supplemented with 20% FBS and 50 U/mL penicillin/streptomycin for 7 to 10 days at 37°C in a 5% CO2 incubator.

Real-time quantitative reverse-transcription PCR

Total RNA was isolated from RMS cells with the RNeasy Kit (Qiagen). Human muscle RNA was obtained from Ambion. The RNA was reverse transcribed with MultiScribe reverse transcriptase and oligo(dT) primers (Applied Biosystems). Quantitative assessment of mRNA levels was done by quantitative reverse transcription real-time PCR (qRT-PCR) on an ABI 7500 instrument with Power SYBR Green PCR Master Mix reagent. Real-time conditions were as follows: 95°C (15 seconds), 40 cycles at 95°C (15 seconds), and 60°C (1 minute). According to melting point analysis, only one PCR product was amplified under these conditions. The relative quantity of a target, normalized to the endogenous β2-microglobulin gene as control and relative to a calibrator, is expressed as 2−ΔΔCt (fold difference), where Ct is the threshold cycle, ΔCt = Ct of target genes − Ct of endogenous control gene, β2-microglobulin, and ΔΔCt = ΔCt of samples for target gene − (ΔCt of calibrator for the target gene). The following primer pairs were used: S1PR1-F, 5′-GCA TTA AAC GTA CCT CGG TGG TGT-3′; S1PR1-R, 5′-GGT CGG TGG AAT TTC TTG GTT-3′; S1PR2-F, 5′-CCT GAA ACA AGA GTT ACG TTT GAT-3′; S1PR2-R, 5′-CAG AAG GAA TCA TTT AGC TAC-3′; S1PR3-F, 5′-GGG CTT TTC CTT-3′; S1PR3-R, 5′-TTT GTG GCC CTT TGT-3′; S1PR4-F, 5′-CAG CCG AGC TAC ACT CCG GA-3′; S1PR4-R, 5′-GGG GTT CCC GCA TCC GAA AG-3′; S1PR5-F, 5′-TCA TGT GCC ATT CAC AGA CGA-3′; S1PR5-R, 5′-TCG TCG AGG CTC ACT CCG GA-3′; S1PR6-F, 5′-GGG CTT TGC TCA AAG-3′; S1PR6-R, 5′-ATG TGG TGC TTT TGT-3′; S1PR7-F, 5′-GGG GTT CCC GCA TCC GAA AG-3′; and S1PR7-R, 5′-TCA TGT GCC ATT CAC AGA CGA-3′.

Cell proliferation

Cells were plated in culture flasks at an initial density of 1.25 × 10⁴ cells/cm² in the presence or absence of S1P (1 μmol/L) or C1P (0.5 μmol/L). The cell number was calculated at 24, 48, and 72 hours after culture initiation. At the indicated time points, cells were harvested from the culture plates by trypsinization and scored using fluorescence-activated cell sorting (FACS) analysis.
Chemotaxis assay
Chemotaxis assays were conducted in a modified Boyden’s chamber with 8-μm pore polycarbonate membrane inserts (Costar Transwell; Corning Costar) as described previously (22). In brief, cells detached with 0.05% trypsin were seeded into the upper chamber of an insert at the density of \(3.5 \times 10^4\) in 110 μL. The lower chamber was filled with prewarmed culture medium containing test reagents. Medium supplemented with 0.5% bovine serum albumin (BSA) was used as a negative control. In some experiments, cells were pretreated with 1 μg/mL pertussis toxin (Sigma-Aldrich) and the inhibitors U0126 (1 μmol/L; Promega), LY294002 (10 μmol/L; Sigma-Aldrich), MK2206 (1 μmol/L; Selleckchem), W146 (1 μmol/L; Cayman Chemicals), W140 (1 μmol/L; Cayman Chemicals), JTE206 (1 μmol/L; Cayman Chemicals), or BML241 (10 μmol/L, Cayman Chemicals) for 15 minutes at 37°C. Inhibitors were also added to the lower chambers and were present throughout the duration of the experiment. After 24 hours, the inserts were removed from the Transwell supports. The cells that had not migrated were scraped off with cotton wool from the upper membrane and the cells that had transmigrated to the lower side of the membrane were fixed and stained with HEMA 3 (Protocol; Fisher Scientific) and counted on the lower side of the membrane using an inverted microscope.

Phosphorylation of intracellular pathway proteins
RMS cell lines were kept overnight in RPMI medium containing low levels of BSA (0.5%) to render the cells quiescent. After the cells were stimulated with S1P (0.1, 0.5, or 1 μmol/L) or C1P (0.5 or 1 μmol/L) for 5 minutes at 37°C, they were lysed for 10 minutes on ice in radio-immunoprecipitation assay (RIPA) lysis buffer containing protease and phosphatase inhibitors (Santa Cruz Biotechnology). Extracted proteins were separated on a 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The phosphorylation of serine/threonine kinase AKT (p-AKT473) and 44/42 mitogen-activated protein kinase (MAPK) was detected by phosphospecific mouse p44/42 and rabbit phosphospecific polyclonal antibodies (Cell Signaling Technology) with horseradish peroxidase (HRP)–conjugated goat antimouse and anti-rabbit secondary antibodies (Santa Cruz Biotechnology). Equal loading in the lanes was evaluated by stripping the blots and reprobing with monoclonal p42/44 anti-MAPK antibody (clone no. 9102; Cell Signaling Technology) and polyclonal anti-AKT antibody (Cell Signaling Technology). The membranes were developed with an enhanced chemiluminescence (ECL) reagent (Amersham Life Sciences), dried, and subsequently exposed to film (Hyperfilm; Amersham Life Sciences). The effect of different inhibitors on phosphorylation of AKT and MAPK kinases has been checked in experimental setting in which U0126, LY294002, MK2206, and pertussis toxin were added to the medium 2 hours before cell stimulation at concentrations used in our chemotaxis experiments.

Adhesion assay to fibronectin
Cells were made quiescent for 3 hours with 0.5% BSA in RPMI-1640 before incubation with S1P (1 μmol/L) or C1P (0.5 μmol/L) for 1 hour. Subsequently, cell suspensions (5 \(\times\) 10^7/100 μL) were added directly to 96-well plates covered with fibronectin and incubated for 10 minutes at 37°C. The wells were coated with fibronectin (10 μg/mL) overnight at 4°C and blocked with 0.5% BSA for 2 hours before the experiment. Following incubation, the plates were vigorously washed 3 times to remove nonadherent cells, and the number of adherent cells was counted using an inverted microscope.

Adhesion assay to bone marrow–derived stroma cells
RMS cells were labeled before assay with the fluorescent dye acetoxyethyl ester (calcein AM) and were made quiescent by incubation for 3 hours at 37°C in RPMI-1640 medium supplemented with 0.5% BSA. The cells were then stimulated with S1P (1 μmol/L) or C1P (0.5 μmol/L) for 1 hour at 37°C, then added to plates covered by mouse stromal cells, and incubated for 15 minutes at 37°C. After the nonadherent cells had been discarded, cells that adhered to the stromal cells were counted under a fluorescent microscope as described previously (4).

Fluorescent staining of RMS cells
RMS cells were fixed in 3.5% paraformaldehyde for 20 minutes, permeabilized by 0.1% Triton X-100, washed in PBS, preblocked with 2% BSA, and subsequently stained with paxillin [1:200, mouse monoclonal immunoglobulin G (IgG); eBioscience] and phalloidin-Alexa Fluor 488 (1:400; Molecular Probes). Appropriate secondary Alexa Fluor 594 goat anti-mouse IgG antibodies were used (1:400; Molecular Probes). The nuclei were identified by staining with 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes). The fluorescence images were collected with a confocal microscope (Olympus).

Knockdown of S1PR1 with short hairpin RNA
In RNA interference (RNAi) experiments, the short hairpin RNA (shRNA)-generating plasmid pSUPER.retro.puro (Oligoengine) was used. The targeting base sequence for human S1PR1 was: 5′-AAG CAC TAT ATC CTC TTC TGC-3′. As a control, shRNA against Renilla was used with the targeting base sequence 5′-AAC AAA GGA AAC GGA TGA TAA-3′ (23). RMS cells were plated at 80% confluency and transfected with shRNA vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Selection of pSUPER.retro.puro–expressing cells was conducted by exposure in in vitro cultures to puromycin at a final concentration of 5 μg/mL for 3 weeks.

Preparation of conditioned media
Pathogen-free C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD), allowed to adapt for at least 2 weeks, and used for experiments at age 7 to 8 weeks. Animal studies were approved by the Animal Care and Use Committee of the University of Louisville.
(Louisville, KY). Mice (4 per group) were irradiated with 250, 500, 1,000, or 1,500 cGy. Twenty-four hours later, bone marrow, liver, lungs, and plasma were isolated. Conditioned media were obtained by 1-hour incubation of bone marrow, liver, or lung cells (mechanically homogenized). Conditioned media were obtained by 1-hour incubation of bone marrow, liver, lungs, and plasma were isolated. Cultures of human cells with a constant number of murine cells.

**Transplants of RMS cells into immunodeficient mice**

To evaluate the in vivo metastatic behavior of 2 populations of RH30 cell lines (RH30 transfected with shRenilla and RH30 with knockdown of S1PR1), cells were injected intravenously (3 × 10⁶ per mouse) into severe combined immunodeficient (SCID)/Beige inbred mice (5 mice per group) that were either (i) untreated (control), (ii) irradiated with 750 cGy 24 hours earlier, or (iii) exposed to 4-deoxy-yridoxine (DOP) in drinking water as described previously. Murine-human chimerism was evaluated as the difference in the level of human α-satellite DNA expression. DNA was amplified in the extracts isolated from bone marrow-, liver-, and lung-derived cells using RT-PCR. Briefly, DNA was isolated using the QiAamp DNA Mini kit (Qiagen). Detection of human satellite and murine β-actin DNA levels was conducted using RT-PCR and an ABI Prism 7500 Sequence Detection System. A 25-μL reaction mixture containing 12.5 μL SYBR Green PCR Master Mix, 300 ng DNA template, 5′-ACC ACT CTG TGT CCT TCG TTC G-3′ forward and 5′-ACT GGG CTC TCA AAA GGA GTG T-3′ reverse primers for the α-satellite, and 5′-TTT AAT TCC AAC GTC CTG TCT TTTG-3′ forward and 5′-CTG TGG AGT GAC TAA ATG GAA ACC-3′ reverse primers for the β-actin were used. The Ct value was determined as before. The number of human cells present in the murine organs (the degree of chimerism) was calculated from the standard curve obtained by mixing different numbers of human cells with a constant number of murine cells. In long-term experiments, cells (7 × 10⁶ per mouse) were inoculated into the hind limb muscles of SCID/Beige inbred mice (5 mice per group). Five weeks later, the mice were sacrificed for evaluation of the RMS cells present in blood, bone marrow, liver, and lungs. Detection of human cells was conducted as described earlier.

To study the effects of a pharmacologic inhibition of S1P signaling on the metastasis of RMS in vivo, the lyophilized S1P-binding Spiegelmer NOX-S93 (synthesized at NOXXON Pharma AG), a 44mer oligonucleotide conjugated to 40-kDa polyethylene glycol to increase circulation half-life was dissolved in 5% glucose and administered in summary 7 times intraperitoneally, with the last dose of 20 mg/kg body weight (in 50 μL) every 12 hours starting 30 minutes after irradiation (750 cGy) to SCID/Beige inbred mice. Control animals received 5% glucose only. Twenty-four hours after irradiation, RMS cells were injected intravenously (3 × 10⁶ per mouse). Bone marrow, liver, and lungs were analyzed for the presence of RMS cells 48 hours after injection (72 hours after irradiation) by PCR as described earlier.

**Quantitation of C1P and S1P by tandem mass spectrometry**

Analysis of bioactive lipid content in tissue samples was conducted on the frozen tissue organ samples. Frozen tissue was weighed and homogenized in methanol followed by lipid extraction using acidified organic solvents, as previously described (24). Analysis of S1P and C1P was conducted using a Shimadzu UFLC coupled with an AB Sciex 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. Detailed liquid chromatography/tandem mass spectrometry (LC/MS-MS) conditions for analysis of S1P were previously described in Selim and colleagues (25). Various C1P species were separated using an Agilent Zorbax Eclipse XDB C8 column, 5 μm, 4.6 mm × 150 mm column. The mobile phase consisted of 75/25 of methanol/water with formic acid (0.5%) and 5 mmol/L ammonium formate (0.1%) as solvent A and 99/1 of methanol/water with formic acid (0.5%) and 5 mmol/L ammonium formate (0.1%) as solvent B. For the analysis of various C1P species, the separation was achieved by maintaining 75% of solvent B for 3 minutes, then increasing to 100% B over the next 3 minutes, and maintaining at 100% B for the last 18 minutes. Column was equilibrated back to the initial conditions in 3 minutes. The flow rate was 0.5 mL/min with a column temperature of 60°C. The sample injection volume was 10 μL. The mass spectrometer was operated in the positive electrospray ionization mode with optimal ion source settings determined by synthetic standards with a declustering potential of 46 V, entrance potential of 10 V, collision energy of 19 V, collision cell exit potential of 14 V, curtain gas of 30 psi, ion spray voltage of 5,500 V, ion source gas 1/gas 2 of 40 psi, and temperature of 550°C. MRM transitions monitored were as follows: 644.5/264.4 and 646.5/264.4 for C18-C1P.

**Flow-cytometric analysis of S1PR1 expression**

RH30-shS1PR1 and RH30-shRenilla cells were harvested at 50% to 60% confluence using CellStripper (Cellgro) then washed twice. Cells were stained against S1P receptor-1 [phycoerythrin (PE)-conjugated antibodies, clone 218713; R&D Systems] and PE IgG isotype controls (R&D Systems). Staining was carried out in PBS with 2% FBS (Invitrogen), at 4°C for 30 minutes. Cells were subsequently washed, resuspended, and analyzed using an LSR II (BD Biosciences). At least 2 × 10⁶ events were acquired from each sample. FlowJo software was used for analysis (Tree Star).

**Statistical analysis**

All results were presented as mean ± SD. Statistical analysis of the data was done using the nonparametric
Mann–Whitney test (animal studies) and Student t test (cell line experiments) for unpaired samples, with $P < 0.05$ considered significant.

Results

While S1P and C1P do not affect proliferation and survival of RMS cells, they strongly induce chemokinetic migration of these cells

Both S1P and C1P have been reported to stimulate proliferation of normal skeletal muscle cells (8, 26). To our surprise, however, neither S1P nor C1P affected proliferation of the RMS cell lines investigated in this study. Furthermore, they also did not enhance survival of RMS cells cultured in serum-free conditions (data not shown). In contrast, both bioactive lipids strongly enhanced robust migration of RMS cells (Fig. 1).

The motility of cancer cells plays a crucial role in the process of tumor metastasis, and we have investigated the role of various chemokines and growth factors in the migration of RMS cells (4, 5, 27, 28). Figure 1A shows the migration of ARMS cells (RH30) in response to physiologic

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Bioactive lipids S1P and C1P are chemoattractants for RMS cells at concentrations corresponding to physiologic concentrations in plasma. A, chemotaxis of RH30 cells in response to S1P [1 µmol/L, considered as physiologic dose (13)], C1P [0.5 µmol/L, considered as physiologic dose (13)], SDF1 (5 or 300 ng/mL), and HGF (0.3 or 10 ng/mL). Data are pooled from 3 independent experiments. B, dose-dependent effect of S1P and C1P on migration of RH30. C, chemotaxis of different RMS cell lines across Transwell membranes in response to S1P (1 µmol/L) or C1P (0.5 µmol/L). D, chemotaxis and chemokinesis of RH30 and RD cells in response to S1P (1 µmol/L) and C1P (0.5 µmol/L). The chemotaxis assay was done at least twice in duplicate, with similar results. Results are presented as mean ± SD, with a statistical significance relative to the control of *, $P < 0.05$ and **, $P < 0.01$. 

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doses of S1P and C1P present in biologic fluids in comparison with gradients of α-chemokine SDF-1 and HGF/SF, which, as we have shown, are potent chemoattractants for human RMS cells (4, 5). We found that at physiologic doses, S1P and C1P increase migration of these cells, and the chemotactic response of RMS cells to a gradient of biologic lipids was much higher than observed for physiologic doses of SDF-1 and HGF/SF and comparable with optimal supraphysiologic SDF-1 and HGF/SF doses. Interestingly, a dose–response migration assay (Fig. 1B) revealed that RH30 cells respond robustly to both bioactive lipids used at physiologic doses, and increases in S1P and C1P concentrations in lower Transwell chambers inhibit migration. Figure 1C shows that S1P and C1P are chemoattractants for 10 and 8 out of 10 different human RMS cell lines, respectively.

To address whether the observed increase in motility is a result of a chemotactic or a chemokinetic response (29), we conducted a checkerboard assay in which S1P or C1P were added at the same time into the upper and the lower Transwell chambers. Figure 1D shows that the migration of RH30 and RD cells was not significantly changed in these conditions, which indicates random chemokinetic rather than gradient-enforced chemotactic motility of RMS cells.

### Human RMS cells express functional G protein–coupled S1P and C1P receptors

To address whether RMS cells express functional receptors for S1P and C1P, all 10 RMS cells lines were first made quiescent and then subsequently stimulated by S1P or C1P to see a potential effect on the phosphorylation of MAPK p42/44 and AKT (Fig. 2A). These signaling pathways have been selected because of their known role in migration and adhesion of normal (30, 31) and malignant cells (32, 33). We observed that 5 of 7 ARMS and 2 of 3 ERMS cell lines

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**Figure 2.** S1P and C1P activate MAPK and AKT intracellular pathway proteins and induce migration of human RMS cell lines through G protein–coupled receptors. A, phosphorylation of MAPK p42/44 and AKT in human RMS cell lines stimulated for 5 minutes by S1P (0.1, 0.5, or 1 μmol/L) or C1P (0.5 or 1 μmol/L). The experiment was repeated 3 times with similar results, and a representative study is shown.
responded robustly to S1P and C1P stimulation and, more importantly, the activation of MAPKp42/44 and/or AKT correlated with their migratory responsiveness (Fig. 1C). Furthermore, while the chemokinetic responsiveness of RH30 cells (ARMS cell line) and RD cells (ERMS cell line) to S1P gradient was inhibited by mitogen-activated protein/extracellular signal–regulated kinase (MEK), AKT and G_{αi} protein inhibitors—UO126, MK2206, and pertussis toxin, respectively, the responsiveness of these cells to C1P was inhibited in the presence of the phosphoinositide 3-kinase (PI3K), AKT, the G_{αi} protein inhibitory molecules—LY294002, MK2206, and pertussis toxin, respectively (Fig. 2B and Supplementary Fig. S1). This confirms involvement of MAPKp42/44 and/or AKT in S1P and C1P signaling.

In contrast to C1P receptor(s), 5 G protein–coupled receptors (S1PR1–5) have been described and cloned. However, while RT-PCR revealed that most of the RMS cell lines express all 5 receptors (data not shown), we focused on the 3 most important: S1PR1 and S1PR3, which promote migration of several cell lines (34, 35), and S1PR2, which has the opposite effect (35, 36). To unravel this complexity, we used quantitative RT-PCR (qRT-PCR) to compare the relative expression among RMS cell lines (Fig. 2C). We observed that while promigratory S1PR1 and S1PR3 were predominantly expressed in ARMS cell lines, migration-inhibiting S1PR2 was highly expressed in ERMS cells.

Figure 2. (Continued) B, the effect of LY294002, UO126, MK2206, and pertussis toxin (PTx) or vehicle alone on the migration of RH30 and RD cells in response to S1P (1 μmol/L) or C1P (0.5 μmol/L). The experiment was done twice with similar results. **P < 0.05 or ***P < 0.01 indicates statistical significance relative to the control (cells migrating in response to S1P or C1P alone). C, qRT-PCR for S1PR1–3 revealed differences in expression of these receptors between ARMS and ERMS cell lines. The experiment was repeated twice on 2 different batches of cells, with similar results. D, chemotaxis of RMS cell lines to S1P (1 μmol/L) in the absence or presence of the S1PR1 inhibitor W146, the S1PR2 inhibitor JTE-013, or the S1PR3 inhibitor BML241. Chemotaxis of cells in the presence of vehicle or inactive analog of W146—W140 are also presented. The results from 2 independent experiments are shown as mean ± SD. **P < 0.05 or ***P < 0.01 compared with the control (cells stimulated with S1P alone).
To better understand the role of S1PR\textsubscript{1-3} in S1P-induced motility of RMS cells, we used commercially available specific S1PR\textsubscript{1-3} inhibitors (Fig. 2D) and found that inhibition of S1PR\textsubscript{1}, S1PR\textsubscript{3} receptors by W146 and BML-241, respectively, decreased motility of RH30 cells in the presence of S1P, whereas exposure of RH30 cells to the specific S1PR\textsubscript{3} antagonist JTE-012 lead even to a small increase in the number of migrating cells. In contrast, the migration of RD cells was only affected by inhibiting the S1PR\textsubscript{3} receptor (Fig. 2D), which suggests that S1P-induced cell motility depends on S1PR\textsubscript{1} and S1PR\textsubscript{3} signaling and varies with cell line.

S1P and C1P increase adhesion of RMS cells and induce changes in the β-actin cytoskeleton

Another important feature of metastasizing cancer cells is their adheriveness at the site of metastasis. Therefore, we next evaluated the adhesion of RMS cell lines to fibronectin-covered dishes and to a bone marrow stromal cell monolayer. We found that both bioactive lipids strongly induce adhesion of RMS cells to fibronectin in a dose-responsive manner (Fig. 3A) and that S1P, but not C1P, also strongly enhances adhesion of RH30 and RD cells to bone marrow–derived stroma (Fig. 3B). However, while we noticed that an increase in C1P dose resulted in more robust adhesion to fibronectin (Fig. 3A), an increase in a dose of this bioactive lipid had in contrast slightly opposite effect on cell migration (Fig. 1B).

It is well known that the dynamic reorganization of the cytoskeleton is a necessary step for cell adhesion and migration. Thus, we examined the β-actin cytoskeleton organization and the localization of the focal adhesion-associated protein paxillin by using confocal microscope analysis in S1P- or C1P-stimulated and nonstimulated RH30 and RD cells (Fig. 3C). We observed that RMS cells placed in control medium display well-developed bundles of F-actin arranged parallel to the long axis of cells, with paxillin mainly present in the cytoplasm. In contrast, in response to S1P or C1P, the β-actin cytoskeleton became reorganized toward the leading edge of the cell. Furthermore, in stimulated cells, paxillin was translocated close to the cell membrane at focal adhesion sites. These data confirm that both bioactive lipids increase adhesion of RMS cells.

The effect of S1PR\textsubscript{1} downregulation on in vivo tumor growth of RH30 cells

To assess the role of S1P in the metastasis of RMS cells, we downregulated the S1PR\textsubscript{1} receptor in RH30 cells by using an shRNA strategy that reduced S1PR\textsubscript{1} expression by approximately 75% (Fig. 4A). RH30 cells were selected for this study based on their robust S1P-mediated motility (Fig. 1) and because this responsiveness was strongly affected by the presence of the S1PR\textsubscript{1} small-molecule inhibitor W146 (Fig. 2D). Additional FACS analysis conducted on shS1PR\textsubscript{1} -transfected RH30 cells revealed decrease in S1PR\textsubscript{1} expression by approximately 85% (Fig. 4B). Of note, we did not observed significant differences in proliferation or apoptosis ratio between shS1PR\textsubscript{1} and shRenilla RH30 cells (data not shown).

Figure 4C shows that downregulation of S1PR\textsubscript{1} in RH30-shS1PR\textsubscript{1} cells severely reduced the responsiveness of these cells to S1P; however, their response to C1P remain unchanged. In the next step, to test the effect of S1PR\textsubscript{1} downregulation on metastatic behavior of RH30 cells, we inoculated RH30-shRenilla (control) and RH30-shS1PR\textsubscript{1} cells into the femoral muscles of SCID/Beige mice and observed that RH30-shS1PR\textsubscript{1} cells formed significantly smaller tumors compared with control RH30-shRenilla cells (Fig. 4D). Furthermore, 5 weeks after inoculation of RMS cells, we observed a lower number of RH30-shS1PR\textsubscript{1} cells in bone marrow, lungs, as well as in liver and circulating peripheral blood (Fig. 4E), which clearly shows involvement of the S1P–S1PR\textsubscript{1} axis in RMS metastasis in vivo.

Irradiation and chemotherapy increase S1P and C1P levels in bone marrow

We have proposed that one of the unwanted side effects of radio/chemotherapy is induction of a prometastatic environment in different tissues (18). To see whether radio/chemotherapy may in fact increase S1P and C1P levels in different organs, S1P and C1P levels were measured in supernatants harvested from murine bone marrow, liver, brain, and lungs, which are frequent sites of RMS metastasis, before and after treatment, by using a sensitive mass spectrometry–based approach. We observed that the concentrations of S1P and C1P increased predominantly in irradiated bone marrow, but also in irradiated liver, and lungs (Supplementary Fig. S2) as well as in supernatants harvested from cell suspensions prepared from these organs (data not shown).

To test whether supernatants from these organs induce motility of RMS cells, we tested the responsiveness of RH30 cells in the Transwell migration assay and found that RH30 cells respond robustly to these supernatants (Fig. 5A). We were aware that such conditioned media contain several potential chemotactic factors, and thus to assess the importance of the contribution by S1P, we repeated these migratory assays with control RH30-shRenilla and RH30-shS1PR\textsubscript{1} cell lines. Figure 5B shows that the response to conditioned media harvested from irradiated bone marrow, liver, and lungs was affected by downregulation of S1PR\textsubscript{1} on RH30 cells, which suggests a significant contribution by the S1P–S1PR\textsubscript{1} axis. The prometastatic involvement of S1P in RMS metastasis has been evaluated by using 2 different approaches to increase in vivo S1P level in the tissues (irradiation or exposure to DOP). As it is shown in Fig. 5C, in both experimental models we observed increased number of RH30 cells in murine organs after intravenous injection of RH30 cells, and this effect was S1PR\textsubscript{1} mediated.

In a similar type of experiment, we evaluated the effect of chemotherapy on induction of a prometastatic microenvironment. Supplementary Fig. S3A shows an increase in S1P and C1P levels in the bone marrow of mice treated with vincristine, which is the most common cytostatic agent used in chemotherapy protocols for RMS (37). Conditioned media harvested from bone marrow of mice
treated with vincristine induced increased motility activity of RH30 cells (Supplementary Fig. S3B), which again was reduced if RH30-shS1PR1 cells were used in the Transwell migration assay (Supplementary Fig. S3C).

**Spiegelmer NOX-S93 inhibits S1P-dependent migration of RMS cells**

Finally, we modulated the unwanted in vivo S1P-induced motility of RMS cells by using the anti-S1P Spiegelmer NOX-S93 that prevents S1P binding to its receptors (Unpublished Data, manuscript in preparation). As mentioned earlier, Spiegelmers are biostable and immunologically passive mirror-image (L-stereoisomer) oligonucleotides that can be identified to bind to pharmacologically relevant targets with high affinity and specificity.

Figure 6A shows that NOX-S93 inhibits dose-dependently the promigratory activity of RH30-shRenilla, but not RH30-shS1PR1 cells, to bone marrow extracts that were irradiated with 1,500 cGy. The selective inhibitory effect of NOX-S93–pretreated bone marrow supernatants against S1PR1-expressing RH30-shRenilla cells shows a selective effect of this anti-S1P compound on the S1P–S1PR1 axis.

Finally, to check whether NOX-S93 inhibits the S1P-dependent spread of RMS cells to tissues damaged by
irradiation in vivo, we compared the seeding efficiency to different organs (bone marrow, lungs, and liver) of RH30 cells injected into control nonirradiated and 750-cGy-irradiated SCID/Beige mice (Fig. 6B). We found that the irradiation-increased seeding efficiency of RH30 cells, especially into bone marrow, was significantly reduced after administration of NOX-S93. This corroborates the observation that the S1P level is highly elevated in irradiated bone marrow (Supplementary Fig. S2).

Discussion

Two major problems in cancer therapy are the recurrence of tumor growth after successful initial treatment and the fatal tendency of cancerous cells to spread and metastasize to different vital organs (38, 39). The ability to metastasize is one characteristic of highly malignant and primitive tumors including RMS (2), and different tumors often have preferred organs to which they metastasize (39). The tropism of cancer cells to metastasize to selected organs pinpoints the involvement of organ-specific factors that direct metastasis.

Figure 4. Downregulation of S1PR1 inhibits RH30 cell growth in vivo and in metastasis. A, qRT-PCR for S1PR1 in RH30 cells transfected with plasmids encoding shRNA against Renilla (control) and against S1PR1. The experiment was repeated twice on 3 different batches of cells with similar results. **, P < 0.01. B, FACS analysis of S1PR1 expression in RH30-shRenilla and RH30-shS1PR1 cells. The experiment was repeated 3 times with similar results. **, P < 0.01. C, chemotaxis of RH30 cells transfected with plasmid encoding shRNA against Renilla (control) and against S1PR1 across Transwell membranes in response to S1P (1 µmol/L). The results from 3 independent experiments are shown as mean ± SD. **, P < 0.01 compared with the control. D, RH30 tumor formation after downregulating S1PR1 expression. Tumor formation by RH30-shRenilla (control) and RH30-shS1PR1 cells inoculated into the hind limb muscles of SCID/Beige inbred mice. Five weeks later, mice were sacrificed and femora harvested to evaluate the size of the growing tumor. **, P < 0.01 compared with the control. E, detection of human RMS cells in bone marrow (BM), liver, lung, and peripheral blood (PB) by qRT-PCR. The results are shown as mean ± SD. **, P < 0.01.
These factors may promote the formation of a pre-metastatic niche that provides metastasizing tumor cells a favorable growth and survival environment. In support of this notion, we have shown an important role of the α-chemokine SDF-1–CXCR4 axis in metastasis of RMS cells to bone marrow, which is one of the common metastatic sites for RMS cells (2).

As with other cancer types, the metastasis of RMS cells is directed by several growth factors, including HGF/SF (5), insulin-like growth factor-I (IGF-I; ref. 41), and several chemokines such as SDF-1 (4), interferon-inducible T-cell alpha chemoattractant (I-TAC; ref. 22), and macrophage migration inhibitory factor (MIF; ref. 28). However, in addition to these prometastatic, peptide-based factors, evidence has accumulated that a family of bioactive lipids plays an important and underappreciated role (42). In particular, S1P has been shown to be involved in metastasis of lung (10), breast (11), prostate (43), and liver (12) cancer cells. In contrast to S1P, there are no reports of the involvement of another bioactive lipid, C1P, in cancer metastasis. However, C1P, as reported by others and us, is a potent modulator of the trafficking of monocytes (15), hematopoietic stem progenitor cells (44), mesenchymal stromal cells (17), endothelial progenitors (17), and murine myoblasts (8). Similarly, S1P has also been proposed to play an important role in trafficking of nonmalignant cells such as hematopoietic stem

Figure 5. S1P and C1P levels create a prometastatic microenvironment in irradiated organs. A, conditioned media (CM) from irradiated bone marrow (BM), liver, and lung cells enhance migration of RH30 cell lines across Transwell membranes. The results from 3 independent experiments are shown as mean ± SD. *, P < 0.05 or **, P < 0.01 compared with the control (conditioned media from cells from nontreated animals). B, differences in migration in response to conditioned media between control (shRenilla) and cells with downregulation of S1PR1 (shS1PR1). C, detection of human RMS cells in organs after irradiation (IR) or DOP administration. In the experiment, 5 mice were used per group. The results are shown as mean ± SD. *, P < 0.05 or **, P < 0.01.
progenitor cells (6, 44), mesenchymal stromal cells (45), and endothelial progenitors (17). It thus plays a role in skeletal muscle development (46), angiogenesis (47), and tissue regeneration from injury (9) and acts directly on skeletal muscle satellite stem cells (26).

The effects of S1P and C1P in myogenesis (8, 46) could be relevant to the effect on RMS proliferation. However, we did not observe any effect of either bioactive lipid on proliferation or survival of RMS cells. Nevertheless, our data showing an effect of S1P and C1P on trafficking of RMS cells corroborates their effect in modulating the trafficking of normal skeletal muscle-derived cells (26).

The metastasis of a tumor is a multistep process, and in the first step, cells endowed with a higher motility potential detach from the primary tumor mass and migrate into the peripheral blood, lymph vessels, or internal body cavities. In this process, we distinguish 2 major types of cell motility: chemotaxis (directed migration to the gradient) and chemokinesis (random migration of cells in response to a chemoattractant). Although the first type of motility may explain tropism of cancer cells to a particular organ that is a source of specific chemoattractant, the other type of motility reflects the ability of tumor cells to detach from the primary tumor in a search for a new environment where they can grow and...
expand. However, these processes are tightly connected and together result in tumor metastatic growth.

In this article, by using a checkerboard migration assay (Fig. 1D), we show for the first time that the roles of bioactive phospholipids S1P and C1P are mainly in inducing random chemokinetic migration of RMS cells. It would be interesting to see whether this type of motility is also involved in metastasis of other types of cancer cells (e.g., lung, breast, or prostate). However, it is worth mentioning that, in contrast to RMS cells, we have already excluded chemokinesis in S1P- and C1P-induced migration of normal hematopoietic cells (44), mesenchymal stromal cells (17), and endothelial progenitors (17). This suggests that differences in migratory responsiveness to bioactive lipid gradients may exist between normal and malignant cells.

One of the most important observations in this current work is that RMS cells respond to S1P and C1P at doses normally encountered in peripheral blood and lymph. Thus, we can envision a scenario that, while S1P and C1P are involved in increasing overall motility of RMS cells and promote their egress from the primary tumor, other factors such as SDF-1 or HGF/SF tune and direct their migration to distant places that secrete high levels of these chemoattractants (e.g., SDF-1–directed metastasis into the bone marrow microenvironment and lymph nodes; ref. 4).

By using small-molecule antagonists to the S1P receptor and shRNA-based receptor knockdown experiments, we have shown that, as reported for other cell types, S1PR1 and S1PR2 play an important role in modulating RMS cell migration and adhesion. Although the C1P receptor has not yet been identified, our migration studies in the presence of S1P antagonists and signaling studies in the presence of specific signaling inhibitors (Fig. 2B and D) have confirmed that the C1P receptor is most likely a G protein–coupled, pertussis toxin–sensitive receptor that is distinct from S1P receptors (15).

A basic clinical problem is the recurrence of metastatic tumors after radio/chemotherapy (18, 48). The reason for this is the presence of therapy-resistant tumor cells that survive at the primary tumor site or in already established sites of micro-metastases. On the other hand, it is known that radio/chemotherapy delivers a toxic insult to the tissues that may result in induction of a prometastatic microenvironment (18, 19). In support of this notion, we have already shown that 2 important RMS prometastatic factors, SDF-1 and HGF/SF, are upregulated at the mRNA level in irradiated bone marrow (44). In this current report, we show that radio/chemotherapy may also induce bioactive lipids in the bone marrow microenvironment as well as in other tissues. Thus, the induction of a prometastatic microenvironment in peripheral tissues may create permissive conditions for tumor cells that survive treatment to lodge and expand, and S1P could here play an important role.

There are several strategies under development for modulating the S1P–S1PR1 receptor axis to inhibit in vivo cancer expansion, including sphingosine kinase inhibitors (49), anti-S1P blocking antibodies (50), and the novel oligonucleotide-based Spiegelmer NOX-S93. In our work to better address the role of radio/chemotherapy-induced S1P, we have used NOX-S93 to block the chemotactic S1P level in circulating blood and peripheral tissues in irradiated mice. We found that S1P released from irradiated or chemotherapy-damaged tissues was efficiently blocked by this chemical entity leading to inhibition of RMS cell migration mediated by the S1P–S1PR1 axis. The effect on bone marrow metastasis was most prominent probably due to the fact that S1P levels increase much stronger in bone marrow than in lung and liver where other chemotactic modulators may play a more important role. We envision that this inhibition of metastasis could be even more efficient when combining anti-S1P Spiegelmers with similar molecules targeted against C1P and SDF-1, and we are currently testing appropriate compounds in in vivo models.

In summary, our data for the first time show that S1P and C1P already present at physiologic concentrations in peripheral blood or lymph induce prometastatic phenotype of RMS cells. We also show that both of these bioactive lipids become upregulated in tissues exposed to radio/chemotherapy, and thus contribute to a prometastatic microenvironment in several organs including bone marrow (Fig. 6C). The radiotherapy-induced spread of RMS cells can be efficiently inhibited in vivo after neutralizing circulating S1P, and compounds such as the anti-S1P Spiegelmer NOX-S93 could play an important clinical role in preventing RMS metastasis. Finally, we are aware that our data conducted with established RMS cell lines, needs further conformation with cells isolated from primary RMS tumors.

Disclosure of Potential Conflicts of Interest

M.Z. Ratajczak has commercial research grant from NOXXON Inc. No potential conflicts of interest were disclosed by the other authors.

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