J Neuropathol Exp Neurol
Vol. 64, No. 8
August 2005
pp. 695–705

ORIGINAL ARTICLE

Sphingosine Kinase-1 Expression Correlates With Poor Survival of Patients With Glioblastoma Multiforme: Roles of Sphingosine Kinase Isoforms in Growth of Glioblastoma Cell Lines

James R. Van Brocklyn, PhD, Catherine A. Jackson, BS, Dennis K. Pearl, PhD, Mark S. Kotur, BS, Pamela J. Snyder, BS, and Thomas W. Prior, PhD

Abstract

Sphingosine-1-phosphate is a bioactive lipid that is mitogenic for human glioma cell lines by signaling through its G protein-coupled receptors. We investigated the role of sphingosine-1-phosphate receptors and the enzymes that form sphingosine-1-phosphate, sphingosine kinase (SphK)-1, and -2 in human astrocytomas. Astrocytomas of various histologic grades expressed three types of sphingosine-1-phosphate receptors, $S1P_1$, $S1P_2$, and $S1P_3$; however, no significant correlation with histologic grade or patient survival was detected. Expression of SphK1, but not SphK2, in human astrocytoma grade 4 (glioblastoma multiforme) tissue correlated with short patient survival. Patients whose tumors had low SphK1 expression survived a median 357 days, whereas those with high levels of SphK1 survived a median 102 days. Decreasing SphK1 expression using RNA interference or pharmacologic inhibition of SphK significantly decreased the rate of proliferation of U-1242 MG and U-87 MG glioblastoma cell lines. Surprisingly, RNA interference to knockdown SphK2 expression inhibited glioblastoma cell proliferation more potently than did SphK1 knockdown. SphK knockdown also prevented cells from exiting G_1 phase of the cell cycle and marginally increased apoptosis. Thus, SphK isoforms may be major contributors to growth of glioblastoma cells in vitro and to aggressive behavior of glioblastoma multiforme.

Key Words: Astrocytoma, Glioblastoma multiforme, Glioma, Lipids, Sphingosine kinase, Sphingosine-1-phosphate.

INTRODUCTION

Sphingosine-1-phosphate (S1P) is a bioactive lipid that has been shown to regulate numerous biologic activities, including cell proliferation, survival, migration, and differentiation (1). S1P is mitogenic for a variety of cell lines, including fibroblasts, smooth muscle cells, and endothelial cells (2). Numerous studies have shown that S1P prevents apoptosis induced by a variety of means, including serum withdrawal, Fas, and ceramide (reviewed in 3). We have previously shown that S1P is mitogenic for (4), and enhances motility and invasiveness of cell lines derived from glioblastoma multiforme (GBM) tissue (5).

Although S1P may exert some of its effects by acting intracellularly, many cellular responses to S1P are mediated through several members of the endothelial differentiation gene (EDG) family of G protein-coupled receptors (6), which were recently renamed S1P receptors (7). S1P binds specifically and with high affinity to activate the receptors $S1P_1$/EDG-1, $S1P_2$/EDG-5, $S1P_3$/EDG-3, $S1P_4$/EDG-6, and $S1P_5$/EDG-8 (8–10). We have recently shown that GBM cell lines and GBM tissue commonly express $S1P_1$, $S1P_2$, and $S1P_3$ (4, 5).

S1P is produced by the enzyme sphingosine kinase (SphK), which is activated by a variety of stimuli, including growth factors (11), immunoglobulin receptors (12, 13), and various G protein-coupled receptors (3). Two SphK isotypes, SphK1 and SphK2, have been cloned (14–16). Although overexpression of SphK2 has recently been shown to induce apoptosis (17), SphK1 overexpression enhances cell survival and increases cell proliferation (18). SphK1 has been implicated in several forms of cancer. SphK1 overexpression transforms NIH 3T3 fibroblasts, suggesting a potential oncogenic function for this enzyme (19). Overexpression of SphK1 in MCF-7 breast cancer cells caused enhanced proliferation, decreased apoptosis, and led to formation of larger tumors in nude mice in an estrogen-dependent manner (20). Moreover, a dominant-negative form of SphK1 inhibited estrogen-mediated mitogenic signaling in MCF-7 cells and decreased tumor formation in nude mice (21). SphK has also been shown to mediate VEGF-induced Ras activation in bladder cancer cells by favoring inactivation of Ras-GAP (22). Furthermore, overexpression of SphK1 in several tumor types, compared with matched normal tissue, suggests that this enzyme may play a role in a wide variety of tumor types (23), and SphK inhibitors decreased proliferation and increased apoptosis of a panel of cancer cell lines (23). However, to our knowledge, this is the first study to examine the role of SphK in primary brain tumors.

From the Division of Neuropathology (JRVB, CAJ, MSK), Department of Pathology (PJS, TWP), and Department of Statistics (DKP), The Ohio State University, Columbus, Ohio.

Send correspondence and reprint requests to: James R. Van Brocklyn, PhD, Division of Neuropathology, Department of Pathology, The Ohio State University, 4164 Graves Hall, 333 W. 10th Ave., Columbus, OH 43210; E-mail: van-brocklyn.1@osu.edu

This work was supported by grant R01 NS41517 from the National Institute of Neurological Disorders and Stroke (NINDS) to JRVB.

In this study, we examined the expression of S1P receptors and SphK isoforms in human astrocytoma tissues and their correlation with histologic grade and patient survival. We also determined the role of SphK1 and SphK2 in proliferation of GBM cell lines. We show that astrocytoma tissue of all four grades express the three S1P receptors $S1P_1$, $S1P_2$, and $S1P_3$, although no correlation with histologic grade or patient survival was found. However, SphK1 expression levels in GBM tissue correlate inversely with survival, and SphK1 and SphK2 contribute to in vitro growth of GBM cell lines.

MATERIALS AND METHODS

Tumor Specimens

Human astrocytoma tissue was provided by the Glioma Tissue Bank in the Division of Neuropathology at The Ohio State University. Use of this tissue for this project was approved by the Biomedical Sciences Institutional Review Board (IRB) of The Ohio State University. Because these tissues were from an archival source, informed consent was waived by the IRB. Diagnosis and grade of each tumor in this bank is a consensus arrived at by either three or four neuropathologists. The criteria for diagnosis and grading and method of review have been previously described (24). Recurrent tumors were excluded from the study to avoid possible effects of treatment on gene expression. The demographics of the patient population in this study were similar to those in the overall population of patients with GBM (25). The median age at diagnosis was 57 years, with the first quartile being 35 years and the third quartile being 65 years. There were four pediatric patients. The gender of the patients was 58% male. All patients with GBM were dead at the time of the study. The overall median survival was 270 days, with the first quartile being 100 days and the third quartile being 530 days.

Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from tissue or cultured cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, followed by treatment with DNase1 (Ambion, Austin, TX) for 20 minutes at 37°C. The RNA was then further purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was created using the Superscript II First Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Control reactions were run without addition of reverse transcriptase (RT). Polymerase chain reaction (PCR) reactions (50 μL) were setup with 5 μL cDNA (equivalent to 125 ng input RNA), 25 μL 2X Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 2.5 μL Assays-on-Demand primers/probe mix, 2.5 μL 18S rRNA internal control, and 15 μL nuclease-free water. Real-time PCR analysis was performed using Applied Biosystems ABI PRISM 7700 Sequence Detection System for 40 cycles with an annealing and extension temperature of 60°C. Threshold values for amplification were set at the lowest point providing exponential increase in product. The cycle number at this threshold was designated C_T. Negative control samples, without template or with RNA not having been reverse transcribed, yielded C_Ts of 38 to 40. Positive expression in samples was taken as C_T below 36. Data was obtained using Sequence Detection System 1.7a software and exported to Microsoft Excel worksheets for analysis. Expression was normalized to rRNA as an internal control within each sample by subtracting the C_T for 18S rRNA amplification from the C_T for amplification of the gene of interest to obtain ΔC_T. A single stock of cDNA from U-373 MG cells was frozen in aliquots and run as a control with each experiment. Values for S1P receptor expression in tumors were then scaled to the $S1P_1$ receptor expression in U-373 MG cDNA by subtracting the ΔC_T for U-373-$S1P_1$ from the ΔC_T for each gene of interest to obtain $\Delta\Delta C_T$. Negative values of $\Delta\Delta C_T$ indicate higher expression, whereas positive values indicate lower expression. The fold difference in comparison to U-373-$S1P_1$ was determined as 2 to the power of the absolute value of the $\Delta\Delta C_T$ as described (26). The efficiencies of all S1P receptor amplicons were determined to be approximately equal by the method of Livak et al (26) and therefore valid for comparison. SphK1 and SphK2 expression were normalized to U-373 MG SphK1 and SphK2 expression, respectively, as described previously for S1P receptors.

The 18S rRNA primers and probe (Applied Biosystems) were designed to accession number X03205 and amplify bases 450 to 636. Sequences of Applied Biosystems Assays-on-Demand gene expression assays are proprietary; however, RefSeq accession numbers for genes, location of binding sites for fluorescent probes, and amplicons, approximately 50 to 150 basepairs surrounding probe location, are provided as follows: $S1P_1$ (catalog HS00173499 m1) based on RefSeq NM 001400. Probe was located at base 83, exon1/exon2 boundary. $S1P_2$ (catalog HS00244677 s1) based on RefSeq NM005226, probe location 968. $S1P_3$ (catalog Hs00245464 s1) based on RefSeq NM004230, probe location 623, SphK1 (catalog Hs00184211 m1) based on RefSeq NM021972, probe location at exon 5/6 boundary, base 802, SphK2 (catalog Hs00219999 m1) based on RefSeq NM020126, probe location at exon 3/4 boundary, base 875.

Cell Culture

U-87 MG, U-373 MG, A172, and M059K cell lines were obtained from ATCC. U-1242 MG cells were a gift from Dr. Allan Yates of The Ohio State University. U-87 MG (27), U-1242 MG (28), U-373 MG (29), M059K (30), and A172 cells (31) were all derived from tumors diagnosed as GBM. All cell lines were maintained in Eagle's minimum essential medium containing 10% fetal bovine serum (FBS), nonessential amino acids, and sodium pyruvate (all media from Mediatech, Herndon, VA). Cells were grown at 37°C in 95% air, 5% CO_2. Cultures were passaged once per week at a ratio of 1:12.

Sphingosine Kinase Assay

Sphingosine kinase activity was measured as described (14) with minor modifications. Briefly, cells were scraped into sphingosine kinase buffer (200 mM Tris, pH 7.4, 20% glycerol, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 1 mM EDTA, 10 μg/mL leupeptin and aprotinin, 1 mM phenylmethylsulfonylfluoride, 15 mM NaF, 1 mM sodium orthovanadate, 40 mM

β -glycerophosphate, and 0.5 mM deoxypyridoxine) and lysed by freeze/thawing seven times. Equal amounts of protein were assayed in the presence of 50 μM sphingosine (Avanti Polar Lipids, Alabaster, AL) and 1 mM ATP containing 10 μCi [γ-^{32}P]ATP for 1 hour at 37°C. Labeled S1P was separated by thin-layer chromatography (TLC) on Silica gel G60 plates with $CHCl_3$/acetone/MeOH/acetic acid/water (10/4/3/2/1) and visualized by autoradiography. Radioactive spots were scraped from TLC plates and quantified by scintillation counting.

RNA Interference

Predesigned small interfering RNA (siRNA) oligonucleotides to SphK1 and SphK2 and random negative control siRNA (Silencer Negative control siRNA 2, catalog 4613) were purchased from Ambion. The negative control siRNA has no significant homology to known human, mouse, or rat genes. Sequences for the SphK specific siRNAs are as follows: SphK1 (pre-designed siRNA ID #1181, sense 5′-GGCUGAAAUCUCCUUCACGtt-3′, antisense 5′-CGUGAAGGAGAUUUCAGCCtc-3′), SphK2 (pre-designed siRNA ID 1677, sense 5′-GGGUAGUGCCUGAUCAAUGtt-3′, antisense 5′-CAUUGAUCAGGCACUACCCtc-3′). Cells were plated at 15,000 cells/cm^2 for M059K and U-1242MG and 20,000 cells/cm^2 for U-87 MG, and siRNA oligonucleotides were transfected into cells using siPORT-lipid (Ambion) according to the manufacturer's instructions. After 4 hours, medium was changed to normal growth medium and cells were incubated for the indicated time.

Measurement of S1P Levels

S1P was assayed as described (32) with minor modifications. Briefly, cells transfected with control siRNA or siRNA specific for SphK1 or SphK2 were washed with cold phosphate-buffered saline (PBS) and resuspended in 1 mL 25 mM HCl in MeOH. S1P was extracted into the aqueous phase with 1 mL $CHCl_3$, 1 M NaCl, and 100 μL of 3 N NaOH. Hydrolysis buffer (450 μL of 200 mM Tris-HCl pH 7.5, 1.2 mM glycine pH 9.0, 75 mM $MgCl_2$) was added along with 3 μL alkaline phosphatase (Type VII-T, Sigma) and reactions were incubated 60 minutes at 37°C to dephosphorylate S1P. The reaction was terminated and sphingosine extracted by addition of $CHCl_3$ and 50 μL concentrated HCl. Solvent was evaporated under nitrogen and sphingosine was redissolved in 175 μL of sphingosine kinase buffer plus 5 μL of 10% triton by vortexing and brief sonication. Sphingosine was then rephosphorylated back to S1P by adding 10 μL recombinant SphK1 obtained from lysates of cells overexpressing SphK1 and 1 mM ATP containing 10 μCi [γ-^{32}P]ATP for 1 hour at 37°C. Labeled S1P was separated by thin-layer chromatography and quantified as described previously for SphK assay. S1P standards (0–100 pmol) were processed in parallel with samples and S1P from cells was quantified by comparison to a standard curve.

Cell Growth Analysis

U-1242 MG and U-87 MG cells were plated in 60-mm dishes and transfected with random control siRNA or siRNA to SphK1 or SphK2 as described previously. The following day, cells were removed by trypsinization and replated into 24 well plates. On each subsequent day, cells from four wells were trypsinized and counted using a Coulter Z2 Particle Count and Size Analyzer. Cells were fed with complete medium every 2 days. For SphK inhibitor experiments, cells were plated at 10,000 cells per well in 24 well plates, and, the following day, SphK inhibitor, 2-(*p*-Hydroxyanilino)-4-(*p*-chlorophenyl) thiazole, (Calbiochem, San Diego, CA) (23) was dissolved in dimethylsulfoxide (DMSO) and diluted 1:2,000 in complete medium before addition to cells to obtain a final concentration of 1 μg/mL (3.3 μM). This inhibitor was found not to inhibit other kinases tested up to 60 μM (23). Control wells received DMSO vehicle at the same concentration.

Measurement of Apoptosis

Cells were transfected with siRNA as described previously. Three days later, cells were harvested and stained with annexin-V-FITC and 7-AAD DNA dye (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions, and fluorescence was measured by using a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). Events of interest were isolated by virtue of their forward- versus sidescatter light properties and assigned to a plot of annexin-V v. 7-AAD. Data were generated by acquiring 10,000 gated events and analyzed using Cell Quest software (Becton-Dickinson). Cells that stain positive for annexin-V, but negative for 7-AAD, have lost asymmetry of plasma membrane phosphatidylserine, but still retain membrane integrity and are thus considered to be in the early phases of apoptosis.

Cell Cycle Analysis

Cells were harvested by mild trypsinization, washed twice with ice cold PBS, resuspended in 70% ethanol, and stored at −20°C. All samples were fixed for a minimum of 24 hours before processing. Samples were washed twice in PBS, resuspended in PBS + 0.1% Triton X-100, and 2 μL RNase cocktail (1 Unit RNase A, 40 Units RNase T1, Ambion) was added. Cells were stained by addition of 5 μg/mL propidium iodide. Samples were allowed to sit overnight at 4°C and read on a FACSCalibur flow cytometer. Cell cycle data was obtained by isolating the single cell events and excluding the aggregates by comparing the pulse width versus area of the fluorescent signal at 675 nm. Data shown represent 20,000 gated events of the 675-nm linear fluorescent area signal. Cell cycle data was generated by using Modfit software (Verity Software, Topsham, ME).

Statistical Analysis

Differences between tissues from GBMs and pilocytic astrocytomas in receptor expression levels were examined for significance on a log scale relative to the U-373 MG cell line and as a percentage of total S1P receptor expression. The prognostic value of SphK1 expression levels in GBM subjects was examined using the Cox proportional hazards model controlling for the age of the patient, assuming a linear (per year of age) increase in the hazard rate beginning at age 50. SphK1 was entered into the Cox models both categorically (below 0.65 vs. above 0.65) and quantitatively on a log scale.

RESULTS

Expression of S1P Receptors in Human Astrocytomas

We previously showed that S1P is both mitogenic for and stimulates motility of human GBM cells in culture by signaling through its G protein-coupled receptors (4, 5). The GBM cell lines used in these studies express various levels of three of the S1P receptors, $S1P_1$, $S1P_2$, and $S1P_3$. In addition, RT-PCR analysis of human GBM tissues demonstrated that these tumors express $S1P_1$, $S1P_2$, and $S1P_3$, but not $S1P_4$ and $S1P_5$ receptors (4). To determine whether expression levels of S1P receptors might play a role in the malignant behavior of human astrocytomas, we quantitatively measured S1P receptor expression in tissue samples from astrocytomas of different histologic grades using real-time RT-PCR. Our results confirmed that $S1P_1$, $S1P_2$, and $S1P_3$ are expressed in GBM and pilocytic astrocytoma tissues at similar levels (Table 1). Expression of all three receptors was also detected in tissues from a limited number of astrocytomas (grade 2) and anaplastic astrocytomas (grade 3) at similar levels (data not shown). Expression levels of all three receptors were highly variable among individual tumors. However, no significant correlation of S1P receptor expression with survival was found for patients with GBM (data not shown). We previously suggested that in glioma cell lines, the relative expression levels of $S1P_1$, $S1P_2$, and $S1P_3$ may indicate how cells will respond to S1P with mitogenesis and/or movement (5). However, when expression level of each S1P receptor is expressed as a percentage of total S1P receptors in that tumor, no significant difference among astrocytomas of different histologic grades was found, (Table 1), and there was no significant correlation of S1P receptor percentages with survival of patients with GBM (data not shown).

Sphingosine Kinase-1 Expression Correlates With Survival of Patients With Glioblastoma Multiforme

We were also interested in determining expression levels of sphingosine kinase (the enzyme that makes S1P) in human astrocytomas. Because SphK1 is known to stimulate cell proliferation as well as prevent apoptosis and has been implicated in other types of cancer (25–28), we measured expression of SphK1 in human astrocytomas using real-time PCR. SphK1 was expressed at various levels by all tumors examined. No significant difference in the level of SphK1 expression was seen between GBM (grade 4) and pilocytic astrocytomas (grade 1) (median levels of 0.24 and 0.19 and means ± standard deviation of 3.44 ± 18.07 and 0.34 ± 0.38, respectively). However, the high variability in SphK1 expression among GBMs led us to examine the relevance of SphK1 expression level to survival within this group. Interestingly, survival analysis of 48 patients with GBM indicated that a strong correlation of SphK1 expression with survival exists (Fig. 1). Patients with GBM whose tumors showed high levels of SphK1 expression had a median survival time of 102 days, whereas those with low levels of SphK1 expression had a median survival of 357 days. The survival difference between high and low SphK1 expressers was significant overall ($p < 0.0001$) and after accounting for the age of the patient ($p = 0.003$). Analyzing the effect of SphK1 quantitatively (on a log scale) found the significance of its effect on survival reduced ($p = 0.04$ after adjusting for age). However, an analysis of residuals from this model found that there appeared to be a threshold level of SphK1 expression above which patient survival is decreased. For example, entering log(SphK1) in the model quantitatively only when SphK1 is > 0.65, which divides the patient population into the lower two thirds and upper one third of SphK1 expression values, produces a highly significant relationship ($p = 0.002$). However, because this form was suggested by the data, it will need to be verified in an independent experiment.

SphK2 expression was also analyzed in 41 of the same GBM samples used for SphK1 analysis. SphK2 was expressed at detectable levels in the majority of these tumors; however,

TABLE 1. Expression Levels of S1P Receptors in Astrocytoma Tissue

Receptor	Pilocytic Astrocytoma (grade 1) (n = 20)	Glioblastoma Multiforme (grade 4) (n = 48)
$S1P_1$*	2.32 ± 2.83 (1.28)	3.67 ± 7.73 (1.35)
$S1P_1$ (%)†	50.17 ± 17.63 (50.1)	47.26 ± 22.97 (46.65)
$S1P_2$	0.61 ± 0.48 (0.48)	1.18 ± 2.38 (0.43)
$S1P_2$ (%)	19.56 ± 13.1 (19.7)	19.30 ± 15.04 (16.15)
$S1P_3$	1.34 ± 1.31 (0.92)	2.67 ± 6.23 (0.89)
$S1P_3$ (%)	30.27 ± 16.00 (30.95)	33.44 ± 22.52 (30.70)

Values are mean ± standard deviation; numbers in parentheses give the medians. Differences between the two diagnostic groups were not significant for any of the three receptors ($p > 0.14$ for each of the comparisons).

*, S1P receptor expression was measured by real-time polymerase chain reaction analysis as described in Materials and Methods. Data are relative levels normalized to U-373 MG cell line.

†, Percentages of total S1P receptor expression.

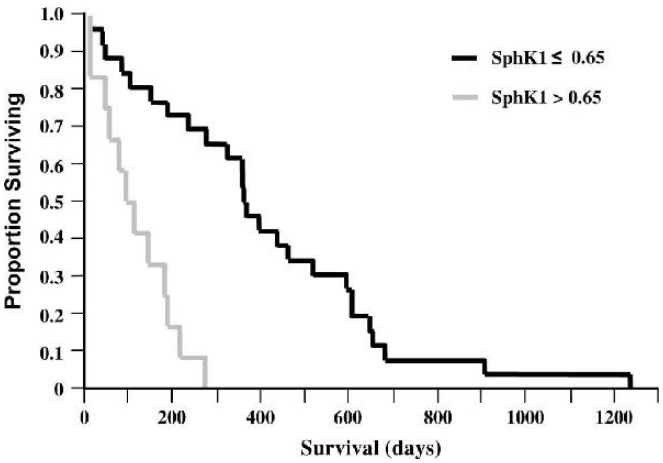

FIGURE 1. Survival analysis of patients with glioblastoma multiforme. Tumors were divided into the lower two thirds (≤ 0.65) versus the upper third (> 0.65) of sphingosine kinase (SphK)1 expression level as determined by quantitative real-time polymerase chain reaction analysis. The difference is statistically significant ($p = 0.002$, $n = 48$) after correcting for patient age.

its expression did not significantly correlate with GBM patient survival ($p = 0.95$). In addition, no significance of the ratio of SphK1 to SphK2 expression with patient survival was detected (data not shown).

Sphingosine Kinase in Human Glioblastoma Cell Lines

We next examined sphingosine kinase activity levels and expression of SphK1 and SphK2 in five human GBM cell lines. As shown in Fig. 2, the cell lines tested exhibited different levels of sphingosine kinase activity. U-87 MG had the highest level of sphingosine kinase activity, followed by M059K > U-1242MG = A172 > U-373 MG. Real-time RT-PCR analysis of RNA isolated from these cell lines confirmed that mRNA encoding SphK1 and SphK2 were expressed at various levels (Fig. 2B). Regression analysis showed that SphK activity correlated with expression of SphK1 in these cell lines. SphK1 expression shows a significant positive correlation with SphK activity (rank correlation = 0.88), with $p < 0.001$. SphK2 expression showed a slight negative correlation with SphK activity (rank correlation = –0.6), with $p < 0.04$. Control real-time PCR experiments using plasmid templates confirmed that SphK1 and SphK2 primers were specific for their respective isoforms (Fig. 2B, inset). Thus, SphK1 may be responsible for the majority of the sphingosine kinase activity in these cells. Because SphK1 expression levels correlated with survival for patients with GBM (Fig. 1), we used three of these cell lines with various levels of SphK1 expression and SphK activity, U-87 MG, M059K, and U-1242 MG, for further experiments.

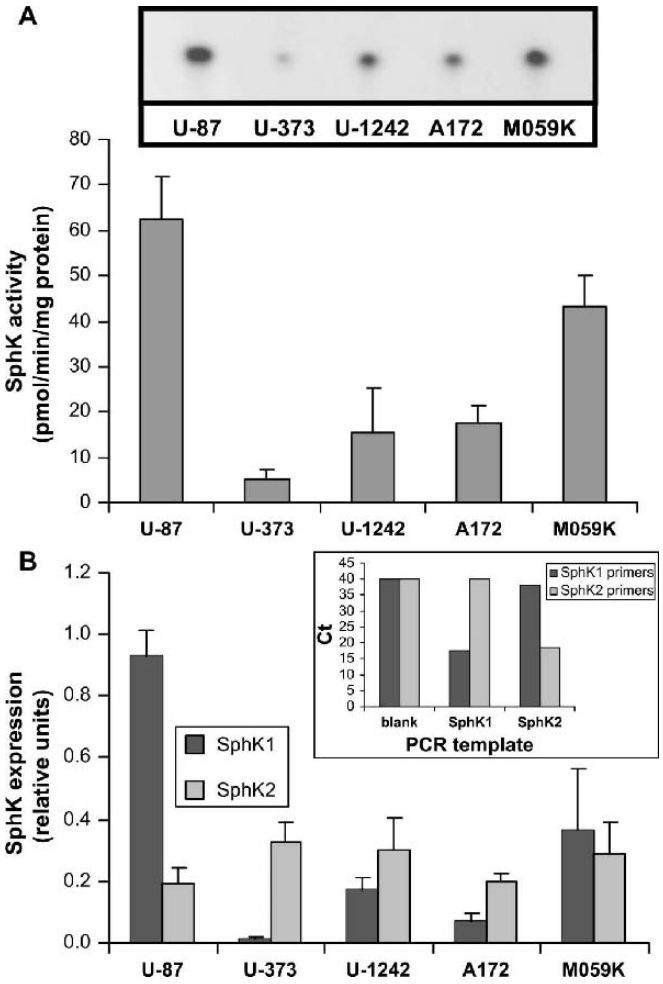

FIGURE 2. Sphingosine kinase activity in various glioblastoma multiforme (GBM) cell lines and correlation with expression of SphK1 and SphK2. **(A)** Cell lysates from five GBM cell lines were analyzed for SphK activity as described in Materials and Methods. Data are means ± standard deviations of three independent determinations. The inset shows a typical thin layer chromatography plate. **(B)** SphK1 and SphK2 expression levels were quantitated using real-time quantitative reverse transcriptase–polymerase chain reaction. Results are means ± standard deviations of three independent determinations. Values are normalized to expression of SphK1 in U-87 MG cells. The inset shows amplification from plasmids containing the SphK1 and SphK2 cDNAs.

To determine the role of SphK in GBM cells, we used RNA interference to knockdown expression levels of SphK1 and SphK2. Transfection of siRNA oligonucleotides targeting SphK1 decreased SphK1 expression, as determined by real-time PCR analysis, by approximately 60% to 70% while having no significant effect on SphK2 expression (Fig. 3A). Conversely, siRNA oligonucleotides to SphK2 decreased SphK2 expression approximately 50% to 60%, but had no effect on SphK1 expression. Measurement of SphK activity revealed that knockdown of SphK1 expression decreased SphK activity in all three cell lines comparably to the reduction in SphK1 expression (Fig. 3B). In contrast, SphK2 knockdown had no detectable effect on overall SphK activity in these three cell lines.

Although the SphK assay used is not isoform-specific, the conditions used for substrate presentation could favor SphK1 activity over SphK2 (16). To verify that the SphK activity seen in these cells was primarily the result of SphK1, we also performed the SphK assay using buffer conditions that favor SphK2 activity over SphK1, as has recently been done to distinguish activity as a result of the two isoforms in tissues from SphK1 null and wild-type mice (33). Using the same samples shown in Fig. 3B, we obtained identical results regardless of the method of substrate presentation (data not shown). Together, these results indicate that the majority of SphK activity present in these cells is the result of expression of SphK1.

To verify that SphK1 knockdown led to decreased S1P levels, S1P was assayed in U-87 MG and U-1242 MG cells after transfection with random control siRNA or siRNA to SphK1 or SphK2. As shown in Fig. 3C, knockdown of SphK1 decreased S1P levels in both cell lines. SphK2 knockdown had no effect on S1P levels in U-87 MG cells while decreasing S1P levels in U-1242 MG to a lesser extent than SphK1 knockdown.

The Role of SphK Isoforms in Glioblastoma Cell Growth

S1P stimulates proliferation of several GBM cell lines (4). Therefore, we next examined the effect of RNA interference for SphK1 on the growth of human GBM cell lines.

Knockdown of SphK1 expression significantly decreased cell proliferation ($p < 0.05$ by Student t test) of both U-87 MG and U-1242 MG cells between 4 and 5 days after transfection with siRNA (Fig. 4A, B, respectively). Although SphK1 knockdown decreased growth rate of M059K cells in several individual experiments, the effect was not statistically significant when independent datasets were combined (Fig. 4C). To verify knockdown of SphK1 in these cells, analysis of SphK activity at each time point in the growth curve was performed for U-87 MG cells. Figure 4D shows that SphK activity was decreased by SphK1 siRNA, relative to control siRNA-treated cells, at each time point to 5 days after transfection. Interestingly, SphK activity decreased with increased time and continued growth of U-87 MG cells in both control and SphK1 knockdown cells. However, at each day, the decreased cell growth of the SphK1 knockdown cells relative to the control cells at that time point reflects the lower SphK1 activity in the SphK1 knockdown cells relative to the control cells on that given day. Similar SphK activity profiles were seen in control and SphK1 knockdown U-1242 MG and M059K cells (data not shown), which have different basal levels of SphK activity and SphK1 expression (Fig. 2). Thus, regardless of whether GBM cells express high or moderate levels of SphK1, the SphK1 that is expressed positively contributes to cell proliferation.

Because SphK2 has been shown to be proapoptotic on overexpression (17), we also determined the effect of SphK2 knockdown on cell growth. Surprisingly, as shown in Figures 4E and 4F, knockdown of SphK2 strongly decreased growth of U-87 MG and U-1242 MG cells. Thus, although SphK1 accounts for the majority of measurable SphK activity in these cells, SphK2 also contributes to GBM cell proliferation.

We next attempted to verify the importance of SphK for GBM cell proliferation using a recently available pharmacologic inhibitor of SphK (23). The SphK inhibitor decreased SphK activity (Fig. 5A) to a similar extent to the decrease seen in RNA interference experiments (compare with Fig. 3B). This inhibitor caused a decrease in the rate of cellular proliferation in U-87 MG and U-1242 MG cell lines (Fig. 5B, C), which was comparable to that seen with RNA interference (Fig. 4A, B). Proliferation of M059K cells was also decreased using this inhibitor (Fig. 5D).

Effect of SphK Knockdown on Glioblastoma Cell Apoptosis

SphK1 and SphK2 have both been shown to regulate apoptosis. SphK1 has an antiapoptotic effect in a variety of cell

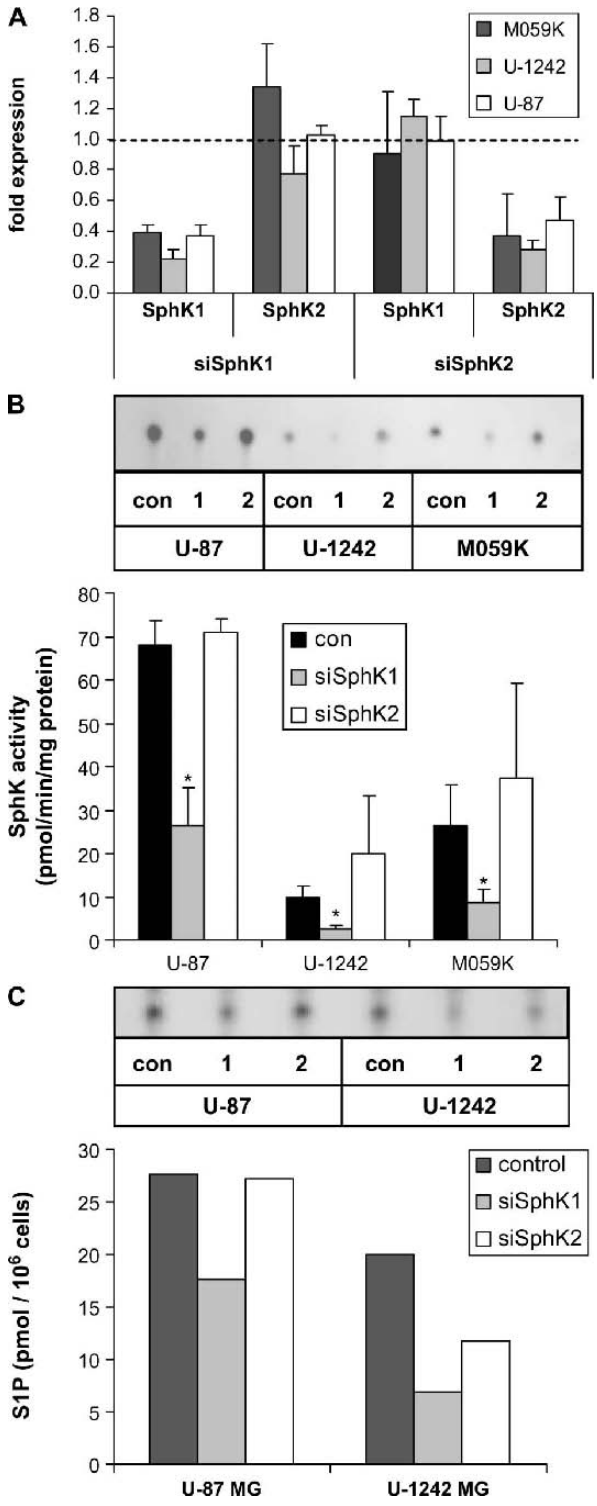

FIGURE 3. Knockdown of SphK1 and SphK2 by RNA interference in glioblastoma multiforme cells. Cells were transfected with control siRNA oligonucleotides or siRNA to SphK1 (siSphK1) or SphK2 (siSphK2), and 2 days later **(A),** expression of SphK1 and SphK2 was measured using real-time polymerase chain reaction analysis. Data are means ± standard deviations of three independent experiments and are expressed as fold expression relative to cells transfected with control siRNA. **(B)** SphK activity was measured in siRNA-transfected cells. Data are means ± standard deviations of three independent experiments. The asterisks (*) indicate statistically significant difference from control by Student *t*-test, $p < 0.05$. The image shows a typical thin-layer chromatogram. con, siRNA control; 1, siSphK1-transfected; 2, siSphK2-transfected cells. **(C)** S1P levels were measured in U-87 MG and U-1242 MG cells transfected with siRNA 2 days after transfection as described in Materials and Methods. Two independent experiments provided similar results.

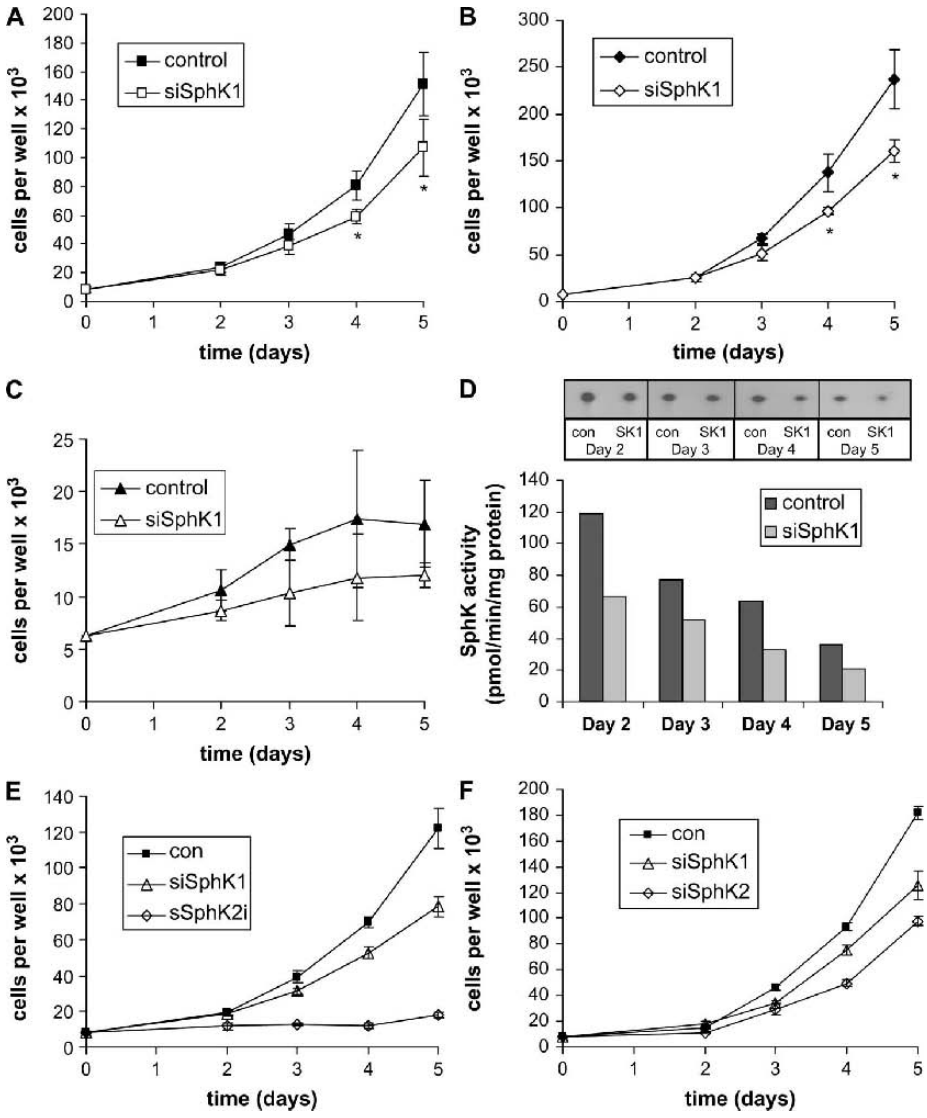

FIGURE 4. Effect of SphK knockdown on growth of glioblastoma multiforme cells. U-87 MG **(A)**, U-1242 MG **(B)**, and M059K **(C)** cells were transfected with siRNA and growth was measured by growth curve analysis as described in Materials and Methods. Data are means ± standard deviations of data from three independent experiments. The asterisks (*) indicate statistically significant difference from control cells by Student *t* test ($p < 0.05$). **(D)** SphK activity was measured in cells allied to each time point of one of the three growth curve experiments for U-87 MG cells. The image shows a typical thin-layer chromatogram. Two independent experiments gave similar results. **(E)** U-87 MG and **(F)** U-1242 MG cells were transfected with siRNA and cell growth was measured. Data are means ± standard deviations of four replicates. All SphK1 and SphK2 knockdown data points at day 3 and beyond were significantly different from the control at the respective day by Student *t*-test ($p < 0.05$). Similar results were obtained in two independent experiments.

systems (3), whereas SphK2 has been shown to induce apoptosis when overexpressed in fibroblasts or PC12 cells (17). Because knockdown of either gene by RNA interference decreased growth of GBM cell lines, it was of interest to determine the effect of SphK knockdown on GBM cell survival. As shown in Figure 6, knockdown of either SphK1 or SphK2 caused a small increase in apoptosis of U-87 MG cells 3 days after siRNA transfection. SphK2 knockdown caused slightly higher levels of apoptosis than did SphK1 knockdown. No significant increase in apoptosis of U-1242 MG cells was seen. The percentage of viable cells, defined as being negative for both the 7-AAD DNA dye and annexin-V staining, was between 84% and 95% for all samples (data not shown).

Effect of SphK Knockdown on Entry Into the Cell Cycle

We next determined whether SphK knockdown decreased the rate of proliferation by affecting entry of cells into the cell cycle. Figure 7 shows that after serum-starvation for 24 hours, SphK1 or SphK2 knockdown caused an increased percentage of U-87 MG cells in G_0/G_1 phase (61% for control vs. 87% for SphK1 and 91% for SphK2 knockdown) and proportionately decreased cell numbers in S and G_2/M phases. After serum stimulation, a high proportion of control cells were found in S phase (39%) and G_2/M (29%), whereas significantly fewer cells entered the cell cycle in either SphK knockdown. In agreement with the more potent effect of SphK2 knockdown on cell growth (Fig. 4), the percentage of cells in S and G_2/M phases was lower in serum-stimulated SphK2 knockdown cells than in SphK1 knockdown cells (8% in S and 5% in G_2/M; and 18% in S and 10% in G_2/M, respectively).

DISCUSSION

Glioblastoma multiforme is the most common primary tumor of the central nervous system occurring in adults and

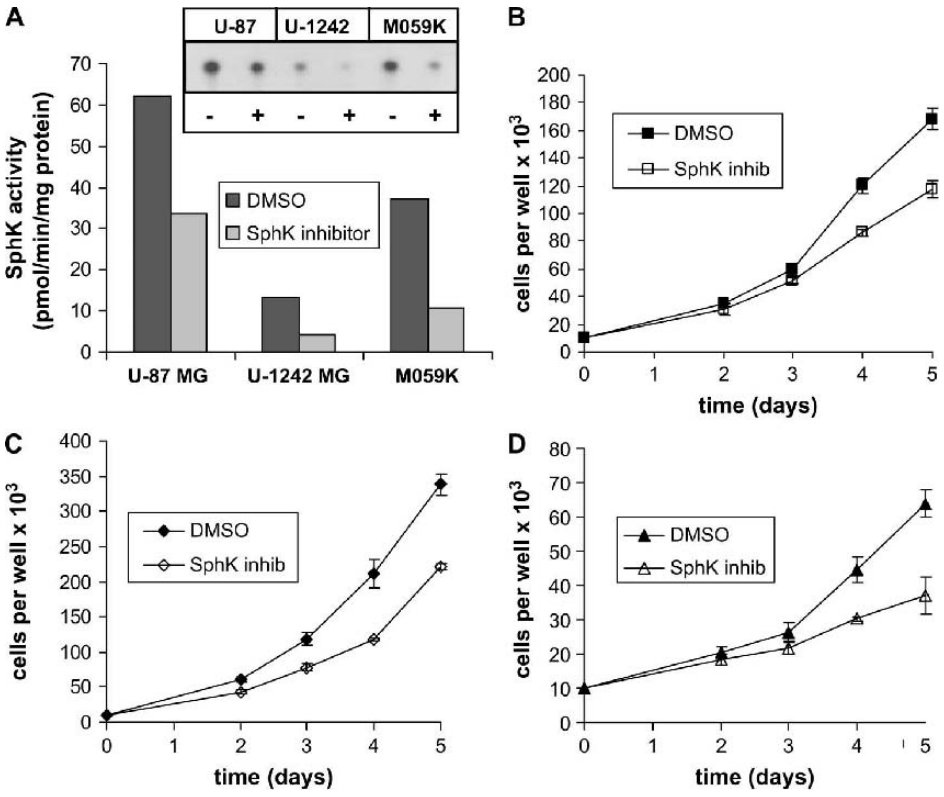

FIGURE 5. Effect of SphK inhibition on growth of glioblastoma multiforme cells. **(A)** Cells were pretreated for 24 hours with SphK inhibitor or DMSO vehicle control. Cells were lysed and SphK activity was measured as described in Materials and Methods. Growth of U-87 MG **(B)**, U-1242 MG **(C)**, or M059K **(D)** cells was measured described in Materials and Methods. Data are means ± standard deviations of four replicates. All data points at day 3 and beyond were significantly different from the control at the respective day by Student *t*-test ($p < 0.05$). Similar results were obtained in two independent experiments.

clinically is very aggressive, with a 1-year survival rate of approximately 29% and a 5-year survival rate of approximately 3% (25). Several genes commonly overexpressed or mutated in GBM have been identified, including amplification of the EGF receptor and loss of the tumor suppressors PTEN and TP53 (34). Although loss of heterozygosity for PTEN has been shown to be a poor prognostic indicator, the relevance of other common genetic alterations to prognosis is controversial (35). Therefore, it would be useful to find additional prognostic indicators for patients with GBM. In this study, we show that expression level of SphK1 correlates with poor survival for patients with GBM.

Two isotypes of SphK have been cloned thus far, both of which appear to be expressed in a wide variety of tissues at various levels, and there is evidence that other SphK isoforms may exist (3). The GBM cell lines examined in this study all expressed both SphK isoforms; however, our data argue that SphK1 accounts for the majority of SphK activity measured in these cells for several reasons: 1) the expression level of SphK1 in human GBM cell lines correlates positively with the total SphK activity measured in these cell lines; 2) SphK2 expression showed only a slight negative correlation with SphK activity; and 3) knockdown of SphK1 decreased SphK activity, whereas SphK2 knockdown did not, even when SphK was assayed using conditions that favor SphK2 activity.

Nevertheless, we show that knockdown of either SphK1 or SphK2 significantly decreased growth of GBM cells in culture. In U-1242 MG cells, SphK2 knockdown was slightly more effective than SphK1 knockdown, whereas proliferation of U-87 MG cells was almost completely prevented by SphK2 knockdown. Two separate processes, cell division and cell death, can affect the accumulation of cells in culture, and SphK has been shown to regulate both of these processes. Measurements of apoptotic cells showed only a slight increase

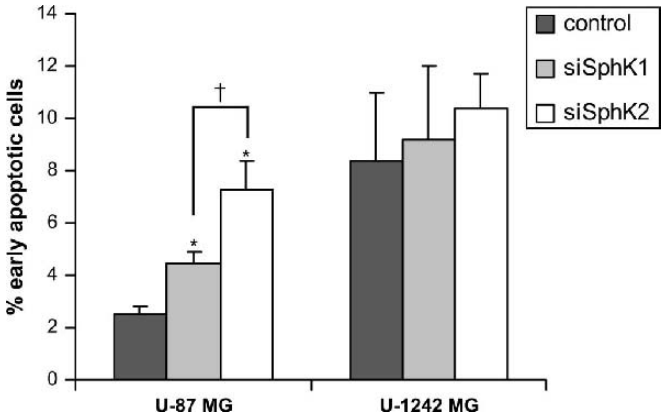

FIGURE 6. Effect of SphK knockdown on glioblastoma multiforme cell apoptosis. U-87 MG and U-1242 MG cells were transfected with siRNA oligonucleotides as described previously. Three days later, apoptotic cells were assessed as described in Materials and Methods. Data are means ± standard deviations of triplicate determinations. The asterisks (*) indicate statistically significant difference from control by Student *t*-test ($p < 0.02$). The dagger (†) indicates statistically significant difference of siSphK2- from siSphK1-transfected cells by Student *t*-test ($p < 0.04$). Three independent experiments provided similar results.

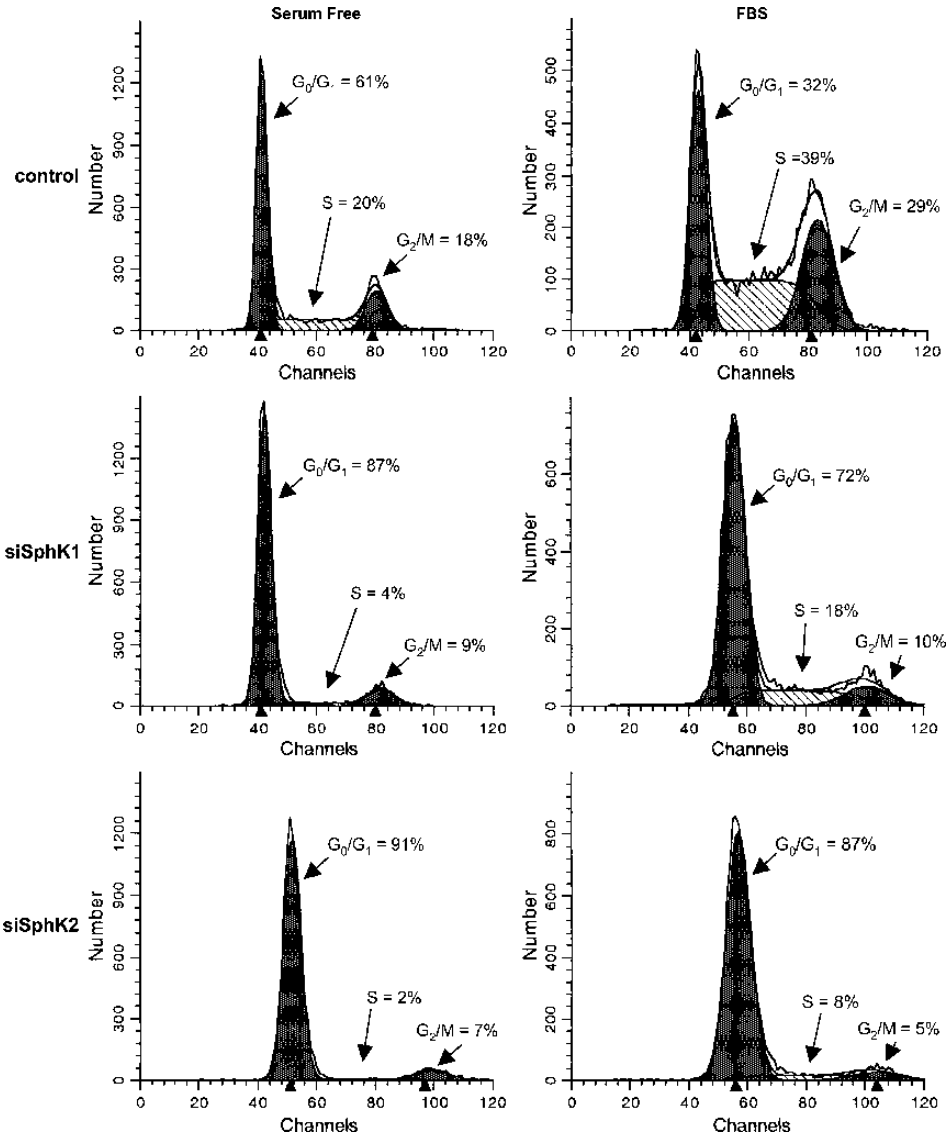

FIGURE 7. Effect of SphK knockdown on cell cycle progression. U-87 MG cells were transfected with siRNA oligonucleotides as described previously. Cells were serum-starved for 24 hours after which one well in each condition was harvested and another well in each condition was stimulated with medium containing 10% fetal bovine serum for an additional 24 hours. Cell cycle distribution was analyzed as described in Materials and Methods. Two independent experiments provided similar results.

in apoptosis of U-87 MG cells after knockdown of either SphK isoform and no significant effect on apoptosis in U-1242 MG cells. However, SphK knockdown strongly inhibited entry of U-87 MG cells into the cell cycle after serum-starvation and subsequent readdition of serum. Moreover, the greater effect of SphK2 knockdown compared with SphK1 knockdown on cell cycle entry corresponds to the stronger inhibition of cell accumulation by SphK2 knockdown seen in growth curve experiments. Thus, it is likely that decreased growth of GBM cells in culture by SphK knockdown is the result of inhibition of the cell cycle rather than enhanced apoptosis, although apoptosis may slightly contribute to this effect, particularly for U-87 MG cells.

One recent study showed that with overexpression of SphK1 tagged with a myristoylation sequence to target it, the plasma membrane resulted in decreased cell proliferation by delaying entry into the cell cycle (36). The authors contributed this difference to altered subcellular localization, because overexpression of wild-type SphK1 had the opposite effect, stimulating proliferation. Although we did not investigate subcellular localization of SphK1 in GBM cells, it is likely that localization was not altered as we knocked down endogenously expressed SphK1 rather than overexpressing a foreign construct.

The decreased cell proliferation and slightly enhanced apoptosis in response to SphK2 knockdown was unexpected because SphK2 has recently been shown to be proapoptotic on overexpression (17). The reason for this discrepancy in our results with those of Spiegel et al is unclear; however, it is possible that the apoptotic effect of SphK2 may require high levels of expression as seen in their overexpression experiments, whereas the lower level of endogenous expression seen in the GBM cells that we examined functions to maintain cell proliferation. In this regard, the proapoptotic effect of SphK2 required its putative BH3 domain, but did not require S1P receptors (17). Thus, it remains possible that SphK2 could

have a proapoptotic effect through its BH3 domain, and prosurvival and growth effects through S1P production. Although the majority of SphK activity and S1P production in GBM cells appears to be the result of SphK1, SphK2 may contribute a small but important pool of S1P, possibly through different subcellular localization.

We have previously shown that S1P is mitogenic for approximately 50% of human glioma cell lines (4). This response occurs at nanomolar concentrations of S1P, consistent with the affinities of S1P for its receptors, suggesting that S1P-induced proliferation may be mediated by cell surface S1P receptors rather than intracellularly. Moreover, this response is partially inhibited by pertussis toxin, indicating that S1P G protein-coupled receptors are involved. Nevertheless, we found in this study that, although S1P receptors are commonly expressed in astrocytomas, their expression levels do not correlate with histologic grade or with patient survival. Two possibilities could explain this. First, it is possible that SphK1 enhances growth of GBM cells through production of S1P that acts intracellularly to stimulate proliferation and enhance cell survival, and that cell surface receptors are not involved. Consistent with this idea, overexpression of SphK1 in mouse embryonic fibroblasts devoid of functional S1P receptors promoted cell growth and survival (37). On the other hand, it also remains possible that although S1P receptors are commonly present in astrocytomas, in the absence of high levels of SphK1 expression, insufficient S1P is generated and/or released to stimulate them enough to enhance cell proliferation. Higher levels of SphK1 expression could create sufficient S1P that autocrine stimulation of S1P receptors could reach significant levels. It should be noted that these possibilities are not mutually exclusive, because S1P could be acting both intracellularly and in an autocrine manner. We have noted that SphK1 and SphK2 knockdown cells contain lower levels of activated ERK1 and ERK2 (data not shown), and thus pathways downstream of S1P appear to be affected in these cells. Therefore, future studies will be aimed at evaluating the involvement of S1P receptors and several S1P-regulated signaling pathways in the downstream effects of SphK isoforms in GBM cells.

In summary, we have shown that high SphK1 expression levels correlate strongly with shorter survival for patients with GBM and that SphK1 may enhance the malignancy of a subset of these tumors by increasing cell proliferation. Although SphK2 expression did not correlate with histologic grade, it may contribute positively to astrocytoma cell proliferation. Further studies will be necessary to verify our statistical model and to determine the role of SphK1 and SphK2 in astrocytoma tumor growth in animal models. Measurement of SphK1 expression level may be useful as a prognostic indicator. Moreover, the development of specific inhibitors of SphK isoforms could provide useful therapeutic tools to treat this devastating malignancy.

ACKNOWLEDGMENTS

The authors thank Dr. Allan Yates and Dr. Hany Saqr for helpful discussions and critical reading of the manuscript.

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