Sphingosine-1-Phosphate Is a Crucial Signal for Migration of Retina Müller Glial Cells

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Purpose. Migration of Müller glial cells is enhanced in proliferative retinopathies, but the mechanisms involved are ill defined. Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid synthesized by sphingosine kinase (SphK), which promotes proliferation, migration, and inflammation, acting as an intracellular mediator and activating a family of membrane receptors (S1PRs). We investigated whether S1P regulated glial migration.

Methods. Müller glial cell cultures from rat retinas were supplemented with 5 μM S1P, and migration was evaluated by scratch-wound assays. Cultures were treated with SphK inhibitor 2 (SphKI 2), a SphK1 inhibitor, or with W146 and BML-241, S1P1 and S1P3 antagonists, respectively, to investigate whether Müller glial cells synthesized S1P and S1P-activated S1PRs to stimulate migration. The effects of LY294002, U0126, and SB203580, which are phosphatidylinositol-3 kinase (PI3K), extracellular signal regulated kinase/mitogen-activated protein kinase (ERK/MAPK), and p38 MAPK inhibitors, respectively, on glial migration were determined.

Results. Sphingosine-1-phosphate addition prompted the formation of lamellipodia and enhanced glial migration. SphKI 2 almost completely prevented glial migration in controls; BML-241 inhibited this migration both in controls and in S1P-supplemented cultures, whereas W146 had no significant effect. Pretreatment with LY294002 and U0126 abrogated glial migration; SB203580 decreased it partially, although not significantly.

Conclusions. Our results suggest that Müller glial cells synthesize S1P, which signals through S1P3 and the PI3K and ERK/MAPK pathways to induce glial migration. As a whole, our data point to a central role for S1P in controlling glial cell motility. Because deregulation of this process is involved in several retinal pathologies, S1P signaling emerges as a potential tool for treating these diseases.

Keywords: sphingosine-1-phosphate receptor, Müller glial cells, cell migration

Müller glial cells, the major glial cell type in the retina, have crucial roles in preserving normal retina functionality. They provide structural, trophic, and metabolic support, maintaining homeostasis of retinal extracellular medium (ions, neurotransmitters) and limiting the spread of excitatory neurotransmitters.[1–3] In contrast with retinal neurons, which are highly sensitive to changes in their environment, Müller glial cells are amazingly resistant and able to survive to most retinal injuries.[4,5] These cells become activated under most pathologic conditions of the retina, as part of a retinal reaction to pathogenic stimuli called "gliosis," which involves rapid and diverse responses. Müller glial cells increase their expression of several filamentous proteins and release different trophic factors, such as fibroblast growth factor and ciliary neurotrophic factor.[4,6,7] Gliosis can be neuroprotective or detrimental for neurons; for example, release of trophic factors protects neurons but also induces neovascularization.[8] In healthy retinas, Müller glial cells have a limited capacity to proliferate and migrate. However, during gliosis, they dedifferentiate, reenter the cell cycle, and increase their proliferation and migration. These processes lead to scar formation, which affects neuro-regeneration, and are characteristic of proliferative retinopathies.[7] The molecular details of these mechanisms are still ill defined, and their thorough understanding is essential for developing effective therapeutic strategies to control gliosis.

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid involved in the regulation of key cellular processes, such as cell survival, proliferation, differentiation, and inflammation,[9–11] and promotes migration of several cell types. Sphingosine-1-phosphate is synthesized by two different sphingosine kinases (SphK), SphK1 and SphK2, and acts both as an intracellular second messenger and as a ligand for a family of five G-protein coupled membrane receptors (S1PRs), named S1P1 to S1P5.[11,12] Sphingosine-1-phosphate is secreted from the cells by specific transporters to signal through its receptors in a paracrine or autocrine manner, named "inside-out" signaling.[11] Although there is still little information concerning S1P functions in the retina, it emerges as a key player in retinal pathologies, which can be either deleterious or neuroprotective.[13] Sphingosine-1-phosphate promotes proliferation and profibrotic and proinflammatory responses in pigment epithelium cells[14,15] and participates in retinal and choroidal neovascularization.[16] Sphingosine-1-phosphate levels increase in retina and serum in lipopolysaccharide-induced inflammation.[17] Sphingosine-1-phosphate also contributes to neuronal

protection. It has been reported to protect photoreceptors from retinal detachment-induced apoptosis[18] and to increase the release of taurine by Müller glial cells, which is neuroprotective.[19] We demonstrated that S1P promotes photoreceptor survival in culture and is a critical regulator of the proliferation of photoreceptor progenitors and of their differentiation.[20] Trophic factors such as glial-derived neurotrophic factor and docosahexaenoic acid (DHA) upregulate the levels of SphK1 to increase S1P synthesis, which then acts as a mediator of their effects in photoreceptors.[20]

The similarity between the opposite effects of S1P and the characteristics of gliotic Müller glial cells led us to hypothesize that S1P might be a mediator of gliosis. We investigated whether S1P stimulated migration of retina Müller glial cells in culture and the signaling pathways involved in this effect. Our results demonstrate for the first time, to our knowledge, that S1P induced glial migration by activating S1P3 and the phosphatidylinositol-3 kinase (PI3K) and extracellular signal regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling pathways. Inhibiting synthesis of S1P or blocking S1P3 activation completely inhibited glial motility, suggesting a central role for S1P in the migration of Müller glial cells.

Materials and Methods

Materials

Three-day-old albino Wistar rats bred in our own colony were used in all the experiments. All proceedings concerning animal use were done in accordance with the guidelines published in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Plastic 35-mm-diameter culture dishes were purchased from Cellstar Greiner Bio-One (GBO; Buenos Aires, Argentina). Fetal calf serum was from Natocor (Córdoba, Argentina). Dulbecco's modified Eagle's medium (DMEM) and type 2 collagenase were from GIBCO Life Technologies (Grand Island, NY, USA). Trypsin, trypsin inhibitor, gentamycin, 4′,6-diamidino-2-phenylindole (DAPI), W146 hydrate, SB203580, U0126, mouse monoclonal antivimentin and antitubulin antibodies, and paraformaldehyde were from Sigma-Aldrich Corp. (St. Louis, MO, USA). Sphingosine-1- phosphate was from Calbiochem (Merck Millipore; Buenos Aires, Argentina). Phalloidin was from Molecular Probes, Invitrogen (Buenos Aires, Argentina), and LY294002 was from ENZO Life Sciences (Miami, FL, USA). Goat polyclonal anti–Iba-1 and mouse monoclonal antiglutamine synthetase antibodies were from Abcam (Cambridge, MA, USA). Mouse monoclonal antiglial fibrillary acidic protein (GFAP) was from Cell Signaling Technologies (Beverly, MA, USA), and mouse monoclonal anti–cellular retinaldehyde-binding protein (CRALBP) was from Genetex (Irvine, CA, USA). Mouse monoclonal antibromodeoxyuridine (BrdU) (clonG3G4) was from Developmental Studies Hybridoma Bank (developed under the auspices of the National Institute of Child Health and Human Development (NICHD) and maintained by the University of Iowa (Iowa City, IA, USA). Rabbit polyclonal anti-S1P1 and -S1P3 antibodies were from Abcam and Santa Cruz (Santa Cruz, CA, USA), respectively, and a generous gift from Nawajes Mandal (Oklahoma State University, Oklahoma City, OK, USA). Cy2- and Cy3- secondary antibodies were from Jackson ImmunoResearch (West Grove, PA, USA). BML-241 was from ENZO Life Sciences and SphK inhibitor 2 was from Cayman Chemical (Ann Arbor, MI, USA). Quick-ZOl was obtained from Kalium Technologies (Bernal, Buenos Aires, Argentina), and enzyme Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) was from Promega (Madison, WI, USA). Primers for PCR were purchased from Biodynamics (Buenos Aires, Argentina). All other reagents used were analytical grade.

Retinal Cultures

Purified cultures of Müller glial cells were prepared using protocols previously described.[21] Briefly, eyes from 3-day-old rat pups were excised and incubated overnight in DMEM at room temperature and then treated with trypsin (1 mg/mL) and type 2 collagenase (2 mg/mL). Retinas were then dissected, chopped into small pieces, and seeded in culture medium supplemented with 10% fetal calf serum (FCS). The medium was routinely replaced every 3 to 4 days. Pure Müller glial cells were used after 12 to 14 days, when they became confluent.

Addition of S1P

An S1P stock solution (0.5 mg/mL) was prepared in methanol: water (95:5). Aliquots were then evaporated under a stream of dry nitrogen, resuspended in a bovine serum albumin (BSA) solution in DMEM (4 mg/mL), and heated at 40°C to 50°C for 30 minutes with occasional vortexing and sonication to allow solubilization. A 5 μM S1P concentration (final concentration in culture) was used. The same volume of the solution used as vehicle was added to controls.

Glial Cell Migration

Glial cell migration was evaluated by performing scratch wound assays. Pure Müller glial cultures were incubated in FCS-containing media until confluent and then changed to DMEM. After 24 hours, several parallel straight lesions were done on the cell monolayer, with a sterile 200-μL plastic tip,[22] producing 1-mm-wide scratches devoid of cells. The culture media were replaced with fresh DMEM, and cultures were supplemented with 5 μM S1P, with vehicle, or with different inhibitors or antagonists. Cells were fixed after 10 or 18 hours, and cell migration was determined by phase microscopy and by visualizing the actin cytoskeleton with phalloidin. In initial experiments, four stages of migration were defined: stage 0, no movement; stage 1, fields with cells extending lamellipodia; stage 2, fields with cells partially covering the scratch; stage 3, cells completely covering the scratch. In later experiments, the fold change in the amount of migrating cells, compared with control conditions, was determined. A total number of 20 fields per dish were observed.

To discern between migration and cell proliferation, we evaluated BrdU uptake in Müller glial cells by incubating the cultures with 20 μM BrdU (final concentration in culture) for 10 hours. Cells were then fixed for 1 hour, treated with 2 N HCl for 30 minutes for DNA denaturalization, and neutralized with 0.1 M boric acid. Bromodeoxyuridine uptake was determined using mouse monoclonal anti-BrdU and Cy2 anti-mouse, as primary and secondary antibodies, respectively.

Involvement of S1P1 and S1P3

The presence of S1P1 and S1P3 was determined by immunocytochemistry and RT-PCR after fixation or lysis of the cultures. For immunocytochemical analysis, once fixed, Müller glial cells were labeled with either anti-S1P1 or anti-S1P3 polyclonal antibodies and with DAPI or phalloidin, respectively.

The S1P1 and S1P3 mRNAs were analyzed by RT-PCR. Total RNA from cells isolated from pure glial cell cultures and from the brains of postnatal day 10 rats, as a positive control, was

extracted using Quick-ZOl (Kalium Technologies) according to the manufacturer's instructions. cDNA was made with enzyme M-MLV RT (Promega) using random hexamer primers. The primers used in this study were as follows: S1P1 forward, AGCTTCGTCCCGCTTGAG and reverse, TTACAGCAAGGCC AGGTCAG; and S1P3 forward, CCCAGATGAGCCTTGCAGAA and reverse, CTCAACAGTCCACGAGAGGG.

To evaluate whether S1P activated S1P1 and/or S1P3 to regulate glial cell migration, pure glial cultures were incubated in DMEM for 24 hours and, after making the scratch, treated either with W146, a S1P1 antagonist,[23] or with BML-241, a S1P3 antagonist,[24,25] or with their vehicles. Working solutions (1 mg/mL) were prepared in dimethyl sulfoxide (DMSO) (W146) or ethanol (BML-241) and then added at a 10-μM concentration to the cultures. Cultures were supplemented with S1P or with vehicle 1 hour later, and glial migration was evaluated 17 hours later.

Inhibition of S1P Synthesis

To investigate whether Müller glial cells synthesized S1P to induce their migration in the absence of exogenous S1P, pure glial cultures were incubated in DMEM for 24 hours before making the scratch. Immediately after, they were treated with 30 μM SphK inhibitor 2, a highly specific inhibitor of SphK1.[26] Glial migration was determined 18 hours later.

Intracellular Pathways Activated by S1P

The intracellular pathways activated by S1P to stimulate glial cell migration were explored. After making the scratch, pure glial cultures were treated with either 50 μM LY294002, a highly selective inhibitor of PI3 kinase; with 10 μM U0126, a selective inhibitor of MEK; with 1 μM SB203580, a p38 MAPK inhibitor; or with their vehicles. Stock solutions of LY294002, U0126, and SB203580 were prepared in DMSO, while working solutions were diluted in DMEM. One hour later, cultures were supplemented with S1P or with its vehicle and incubated for 9 or 17 hours. Cell migration was evaluated by the scratch wound assay, as described above.

Immunocytochemical Methods

Cultures were fixed for 1 hour with 4% paraformaldehyde in phosphate-buffered saline, followed by permeation with Triton X-100 (0.1%) for 15 minutes. In every culture condition, Müller glial cells were identified by their morphology and by immunocytochemistry, using antivimentin, anti-GFAP, antiglutamine synthetase, and anti-CRALBP antibodies. Microglial cells were identified by immunocytochemistry, using an anti–Iba-1 polyclonal antibody.

Cultures were analyzed by phase contrast and epifluorescence microscopy, using a Nikon Eclipse E600 microscope with a C-C Phase Contrast Turret Condenser and a Y-FL Epi-Fluorescence Attachment or with a laser scanning confocal microscope (Leica DMIRE2) with a 63× water objective. Images were collected and processed with LCS software (Leica) and Photoshop 8.0 (Adobe Systems, San Jose, CA, USA).

Statistical Analysis

For migration studies, 20 fields per sample, randomly chosen, were analyzed in each case. Each value represents the average ± SD of at least three experiments, with three to four dishes for each condition. Statistical significance was determined by Student's one-tailed *t*-test or by ANOVA followed by Tukey's test.

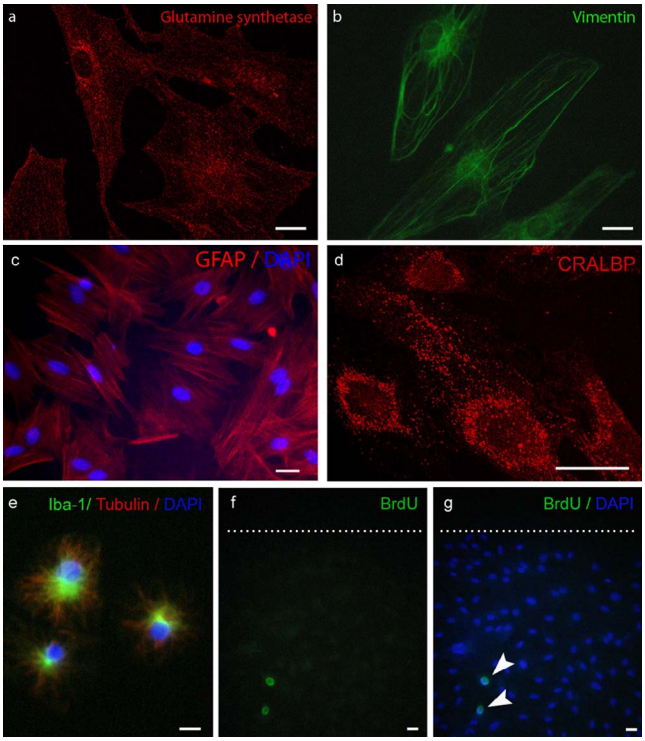

Figure 1. Characterization of Müller glial cell cultures. Photomicrographs show 14-day cultures of glial cells, showing cells expressing characteristic markers of Müller glial cells, such as glutamine synthetase (**a**), vimentin (**b**), GFAP (**c**), CRALBP (**d**) and of microglia, such as Iba-1 ([**e**], *green*) and BrdU uptake ([**f**, **g**], *green*). Nuclei were stained with DAPI ([**c**, **e**, **g**], *blue*). Microglial cells were scarcely found in the cultures and in addition to showing Iba-1 labeling (*green* in [**e**]), they were easily identified by their small and ramified cytoskeleton, labeled with beta tubulin ([**e**], *red*). To discern between cell migration and proliferation, the amount of cells on the scratch that took up BrdU after 10 hours was determined (**f**, **g**). Very few BrdU-positive cells were observed ([**f**, **g**], *arrowheads* in [**g**]), and none of them were on the scar (*dotted line* in [**f**, **g**]). *Scale bars*: 20 μm.

Results

Sphingosine-1-Phosphate Promoted Migration of Müller Glial Cells

To evaluate the effect of S1P on glial migration, we prepared pure Müller glial cultures and confirmed the identity of Müller glial cells by their expression of glial markers such as vimentin, glutamine synthetase, GFAP, and CRALBP (Figs. 1a–d). Microglial cells were present in negligible amounts (less than 0.5%) in the cultures and were easily distinguished by their small cell body, usually bearing multiple thin, highly branched processes, and by their expression of the calcium-binding protein Iba-1 (Fig. 1e).

Scratch wound assays revealed that, after 10 hours, little migration of vimentin-labeled Müller glial cells was observed in control cultures, with most of them showing no visible changes in their actin cytoskeleton (Figs. 2a, 2c, 2e). In contrast, in S1P-supplemented cultures, Müller glial cells were already present on the scratch at this same time (Fig. 2b). Sphingosine-1-phosphate prompted Müller glial cells to rapidly reorganize their actin cytoskeleton, leading to the extension of lamellipodia and filopodia (Figs. 2d, 2f, arrowheads) to advance over the scratch and start to migrate. At this time, few BrdU-positive cells were observed, and none

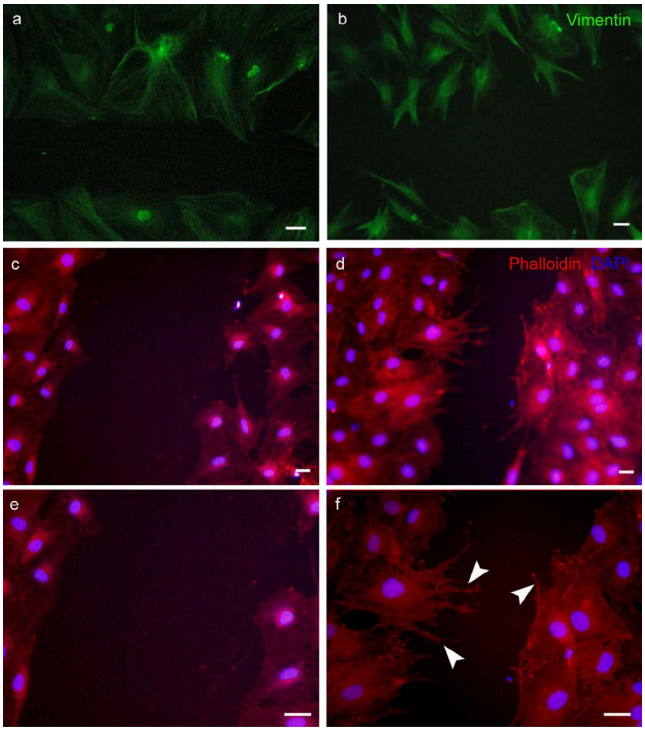

FIGURE 2. Sphingosine-1-phosphate induced migration and lamellipodia formation in Müller glial cells. Glial cultures were incubated in 10% fetal calf serum for 14 days and changed to DMEM for 24 hours before making the scratches. Fresh DMEM was then added, and cultures were supplemented with vehicle (controls, [**a**, **c**, **e**]) or with 5 μM S1P (**b**, **d**, **f**) and cultured for 10 hours. Fluorescence photomicrographs show migration of vimentin- (**a**, **b**) and phalloidin- (**c**–**f**) labeled glial cells in controls and S1P-supplemented cultures and their nuclei, stained with DAPI (**c**–**f**). Sphingosine-1-phosphate addition induced the migration of vimentin-positive glial cells, which advanced over the scratch (**b**) and rapid rearrangements of their actin cytoskeleton (**d**, **f**), leading to filopodia formation (*arrowheads* in [**f**]), which were almost absent in controls (**c**, **e**). *Scale bars*: 20 μm.

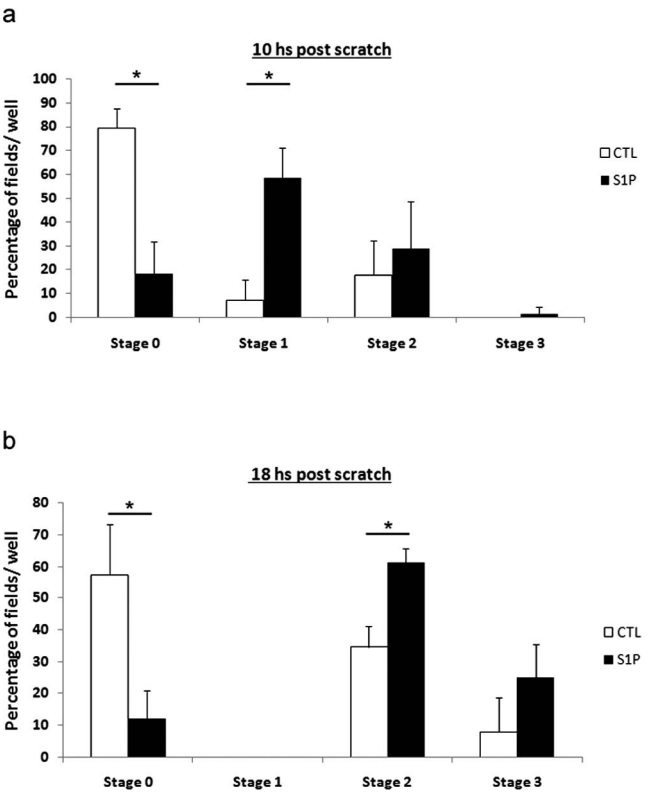

FIGURE 3. Sphingosine-1-phosphate promoted migration of Müller glial cells. The percentage of fields displaying Müller glial cells at different stages of migration in S1P- (*black bars*) or vehicle (CTL, *white bars*)-supplemented cultures, after 10 (**a**) and 18 (**b**) hours of making the scratch was determined by counting phalloidin-labeled glial cells at different stages, defined as follows: stage 0, no movement; stage 1, fields with cells extending lamellipodia; stage 2, fields with cells partially covering the scratch; stage 3, cells completely covering the scratch. *Bars* are means ± SD. Note that S1P treatment decreased the percentage of fields with Müller glial cells in stage 0 and incremented the percentage of fields showing advanced stages of migration compared with controls, at both times analyzed. *Significant differences compared to controls ($P < 0.05$).

were observed on the scratch (Figs. 1f, 1g), implying that the contribution of proliferating cells to those observed on the scratch was negligible. When we evaluated the different stages of migration after 10 hours, in controls, almost 80% of the fields showed Müller glial cells in stage 0, that is, showing no movement and no cytoskeleton alterations. Approximately 6% and 16% of the fields had cells in stages 1 and 2, extending lamellipodia or migrating, respectively (Fig. 3a). Noteworthy, in S1P-supplemented cultures, only 17% of the fields had Müller glial cells in stage 0; in around 57% and 26% of them, cells were in stages 1 and 2, respectively. Sphingosine-1-phosphate–treated cultures already showed a few fields with cells completely covering the scratch, which were absent in controls. Glial migration in controls increased after 18 hours; the percentage of fields with no glial migration decreased to almost 57%, and approximately 34% and 9% of them showed cells at stages 2 and 3, respectively (Fig. 3b). However, after 18 hours, glial migration in S1P-supplemented cultures was significantly higher than in controls; the percentage of fields showing no migration was less than 12%, and cells had migrated over the scratch in approximately 61% of the fields and had completely covered the scratch in almost 27% of the fields (Fig. 3b). Hence, S1P remarkably stimulated migration of Müller glial cells.

Inhibition of SphK1 Blocked Glial Cell Migration

Since even in the absence of exogenous factors, substantial migration of Müller glial cells was observed after 18 hours (Fig. 3b), we investigated whether these cells synthesized S1P to induce their migration. After 18 hours, vimentin-labeled Müller glial cells in controls extended their lamellipodia or migrated over the scratch (Figs. 4a, 4c). Inhibiting SphK1, which is responsible for S1P synthesis, by adding 30 μM SphK inhibitor 2 (SphKI 2), almost completely abrogated formation of lamellipodia and filopodia and prevented glial migration (Figs. 4b, 4d). Quantitative analysis showed that SphKI 2 significantly reduced glial migration, to approximately 20% of that observed in controls (Fig. 4e). This implies that endogenous synthesis of S1P was required for motility of Müller glial cells in basal conditions.

Sphingosine-1-Phosphate Activated S1P3 to Induce Glial Migration

Sphingosine-1-phosphate primarily signals through its five S1PRs, of which S1P1 and S1P3 are known to stimulate cell migration.[27] Immunocytochemical studies revealed that cultured Müller glial cells expressed S1P1 and S1P3 after 14 days

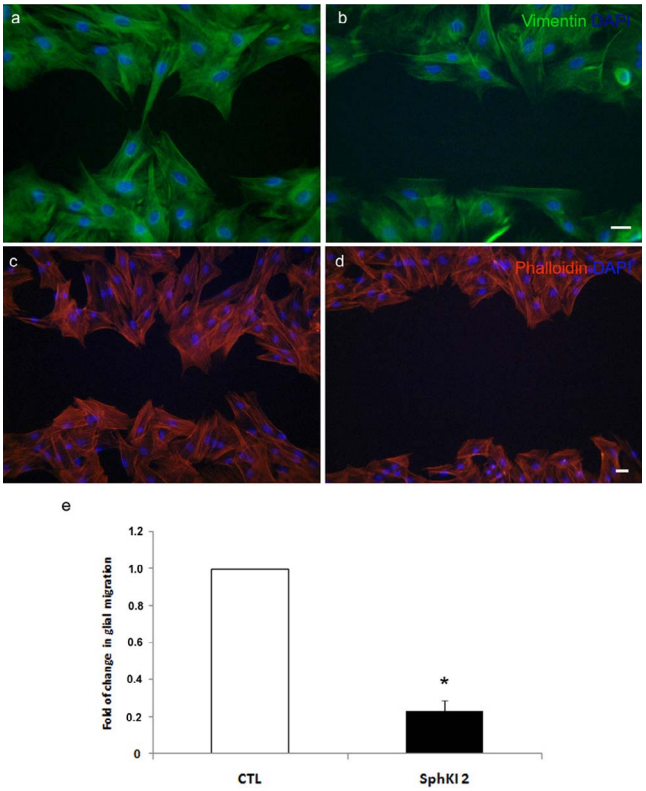

FIGURE 4. Inhibiting S1P synthesis blocked glial cell migration. Glial cultures were supplemented with vehicle (CTL) or with SphK inhibitor 2 (SphKI 2) immediately after making the scratch. Fluorescence photomicrographs show control (**a**, **c**) and SphKI 2–treated cultures (**b**, **d**), showing vimentin- (**a**, **b**) and phalloidin- (**c**, **d**) labeled glial cells 18 hours after making the scratch. Note that inhibition of the endogenous synthesis of S1P with SphKI 2 (**b**, **d**) significantly reduced glial cell migration compared with controls (**a**, **c**). Nuclei were labeled with DAPI (*blue* in [**a**–**d**]). *Scale bars*: 20 μm. *Bars* (**e**) show the fold-change (means ± SD) in glial migration in SphKI 2–treated cultures (*black bar*) with respect to controls (*white bar*). *Significant differences compared with controls ($P < 0.05$).

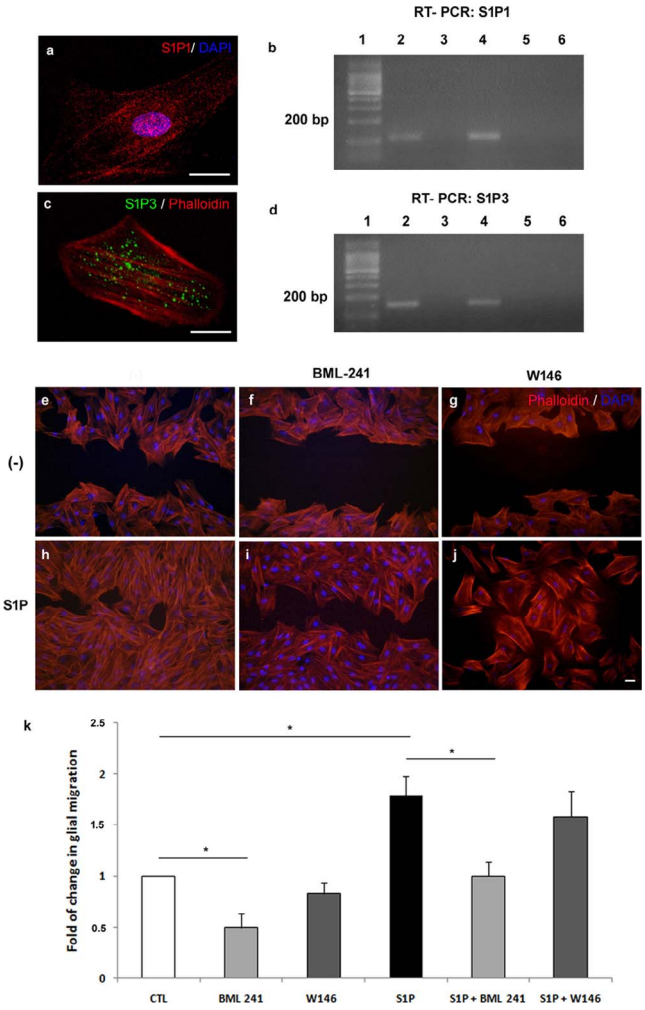

FIGURE 5. Sphingosine-1-phosphate activated S1P3 to induce glial cell migration. Confocal fluorescence photomicrographs showing expression of S1P1 (**a**) and S1P3 (**c**) nuclei stained with DAPI (**a**) and actin cytoskeleton, labeled with phalloidin (**c**). Electrophoresis of S1P1 (**b**) and S1P3 (**d**) cDNAs after RT-PCR, showing 106- and 146-bp bands for S1P1 and S1P3, respectively, in samples from 14-day glial cultures (lane 2) and PN10 rat brain (lane 4), which were absent in their respective controls: mRNA from glial cultures (lane 3) and rat brain (lane 5) lacking RT and distilled water (negative control for the RT-PCR technique, lane 6). Epifluorescence (**e**–**j**) photomicrographs show Müller glial cells labeled with phalloidin (*red*) to visualize their actin cytoskeleton in control (BSA, [**e**–**g**]) and S1P-supplemented cultures (**h**–**j**), with no pretreatment (**e**, **h**), and preincubated with BML-241, a S1P3 antagonist (**f**, **i**) and with W146, a S1P1 antagonist (**g**, **j**). Nuclei were stained with DAPI (*blue*). Migration was analyzed 18 hours after making the scratch. Note that preincubation with BML-241 markedly reduced glial migration both in controls (**f**) and in S1P-supplemented cultures (**i**), whereas glial cells partially covered the scratch in cultures treated with W146 and S1P (**j**). *Scale bars*: 20 μm. *Bars* (**k**) show the fold-change (means ± SD) in glial migration compared with CTL (*white bar*) of cultures treated with S1P (*black bar*) and with BML-241 (*light gray bar*) or W146 (*dark gray bar*), without or with S1P. *Significant differences compared with the respective control condition, indicated with the *black line* ($P < 0.05$).

in vitro (Figs. 5a, 5c), and this expression was confirmed by PCR (Figs. 5b, 5d). To investigate whether S1P1 and S1P3 activation participated in glial migration, we treated glial cultures with W146 and BML-241, which are S1P1 and S1P3 antagonists, respectively, before S1P addition. Migration of Müller glial cells was evident in controls after 18 hours, reduced by half by BML-241, and slightly, but not significantly, affected by W146 (Figs. 5e–g). Sphingosine-1-phosphate increased the motility of Müller glial cells, which almost completely covered the scratch, and nearly doubled the amount of migrating cells compared with controls (Figs. 5h, 5k). Sphingosine-1-phosphate–induced glial migration was markedly reduced by pretreatment with BML-241 to values similar to those of S1P-lacking controls (Figs. 5i, 5k), but it showed no significant reduction by W146 addition (Figs. 5j, 5k). This suggests that activation of S1P3 by both endogenously synthesized and exogenously added S1P was required to induce glial migration, whereas S1P1 was not involved in this effect.

Intracellular Pathways Activated by S1P

Sphingosine-1-phosphate binding to S1PRs leads to the downstream activation of several intracellular pathways.[11,27] To investigate the pathways activated by S1P to stimulate glial migration, we treated glial cultures with LY92004, a PI3K inhibitor; with U0126, a MEK inhibitor; or with SB203580, a p38 MAPK inhibitor. While in S1P-supplemented cultures, Müller glial cells extended lamellipodia and migrated to partially cover the scratch after 10 hours, pretreatment with LY294002 or U0126 before S1P addition abrogated glial

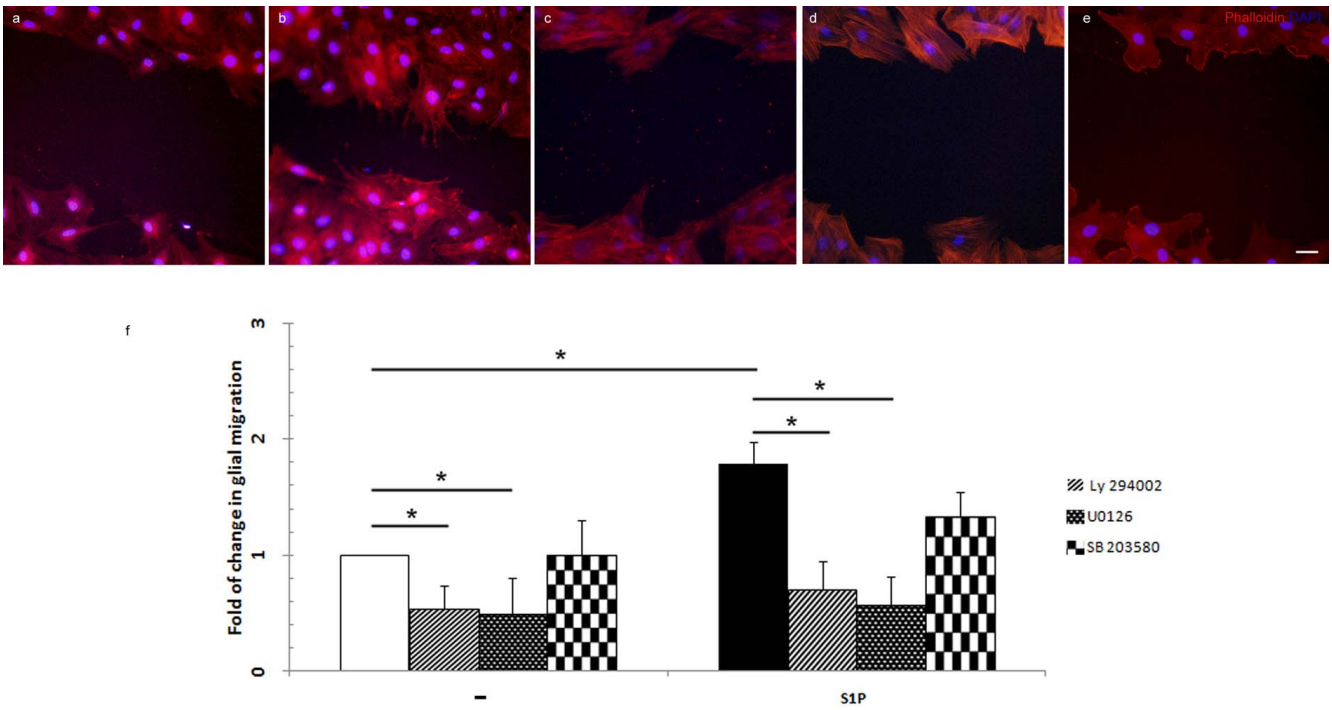

FIGURE 6. Sphingosine-1-phosphate activated different signaling pathways to induce glial migration. Fluorescence photomicrographs show the actin cytoskeleton (labeled with phalloidin) and nuclei (stained with DAPI) of control (**a**), and S1P-supplemented cultures (**b**–**e**) preincubated with LY294002 (**c**), U0126 (**d**), or SB203580 (**e**). After 10 hours, Müller glial cells in S1P-supplemented cultures showed lamellipodia and a reduced scratch width (**b**), compared with controls (**a**), whereas preincubation with LY294002, U0126, or SB203580 decreased glial migration. *Scale bars*: 20 μm. *Bars* (**f**) show the fold-change (means ± SD) in glial migration compared with controls treated only with vehicle (*white bar*) of cultures treated with vehicle (–) and LY294002, U0126, or SB203580 and cultures treated with S1P (*black bar*), and S1P and LY294002, U0126, or SB203580. *Significant differences compared with the respective control condition, indicated with the *black line* ($P < 0.05$).

migration (Figs. 6a–d). Quantitative analysis after 18 hours showed that LY294002 and U0126 significantly reduced glial migration both in cultures without or with S1P to almost half of the migration observed in controls (Fig. 6f). Pretreatment with SB203580 reduced S1P-induced migration, although this reduction was not statistically significant (Figs. 6e, 6f).

DISCUSSION

The molecular mechanisms controlling migration of Müller glial cells in the retina, a key event in retinal gliosis, are still poorly understood. This work shows a central role for S1P in glial motility. Our results show for the first time that synthesis of S1P was required for glial migration and that an increase in S1P levels, by S1P exogenous addition, markedly enhanced this migration. They also suggest that S1P signaled through S1P3, activating the PI3K and the ERK/MAPK pathways to stimulate glial cell motility.

Different environmental insults induce phenotypic and functional changes in Müller glial cells, triggering a gliotic response characterized by their rapid proliferation and migration[28] involved in the development of retina diseases such as proliferative retinopathies.[29] Sphingosine-1-phosphate is a well-known chemoattractant and a modulator of migration in many cell types.[10,30] In the retina, pigment epithelial cells have been shown to secrete and respond to S1P,[15] but little is known concerning S1P effects on Müller glial cells. Sphingosine-1-phosphate was shown to increase Ca^{2+} release in cultured Müller glia from guinea pig retinas, but its reported effects on migration were nonsignificant.[31] Our data show for the first time that S1P is crucial for glial motility. Migration of Müller glial cells in controls was completely prevented when SphK1 activity was inhibited, implying that synthesis of S1P was essential for glial motility. Increasing S1P levels, through S1P addition to glial cultures, remarkably stimulated glial migration. Sphingosine-1-phosphate addition induced rapid changes in the actin cytoskeleton, leading to the formation of prominent lamellipodia and filopodia; this is consistent with findings that S1P promotes motility by regulating cytoskeleton rearrangements. Our data imply that Müller glial cells synthesize and release S1P to regulate its migration and respond to an increase in S1P levels by increasing this migration. Many of the external stimuli and growth factors that enhance motility in several cell types promote activation of SphK, stimulating an increase in the synthesis and release of S1P.[27,32,33] Similarly, different environmental insults that trigger gliosis in proliferative diabetic and vitreoretinopathies, retinal detachment, and retinal injuries[1,7,29] might lead to an increased synthesis and release of S1P in Müller glial cells to promote glial migration.

Sphingosine-1-phosphate can act as an intracellular messenger and as a ligand for a family of five S1P membrane receptors. Sphingosine-1-phosphate receptors couple to different subsets of heterotrimeric G proteins, thus having different roles on cell migration; S1P1 and S1P3 are known to promote cell migration, while S1P2 inhibits it.[34–36] Our data show the expression of S1P1 and S1P3 in Müller glial cells. However, while an antagonist to S1P3 prevented glial migration, both in controls and in cultures supplemented with S1P, a S1P1 antagonist had no significant effect on this migration. Sphingosine-1-phosphate signaling through S1PRs leads to the

activation of the Rac and Rho family of small G proteins, which then activate downstream pathways, leading to the formation of lamellipodia and stress fibers and ultimately to cell detachment, thus promoting motility in many cellular systems.[37] Sphingosine-1-phosphate signaling pathways are cell type and context dependent. Sphingosine-1-phosphate activation of S1P1 and S1P3, and the subsequent activation of Rac, leads to actin polymerization and lamellipodia formation in breast cell invasion and vascular formation,[38,39] whereas S1P controls lamellipodia formation and cell migration in HEK cells acting both by inside-out signaling on S1P1 and as an intracellular messenger.[35] Our work implies that activation of S1P3, and not that of S1P1, is involved in S1P stimulation of glial migration, underscoring the relevance of the S1P-S1P3 axis in controlling glial motility. Noteworthy, proinflammatory conditions in cultured astrocytes induce upregulation of both SphK1 and S1P3.[40] Together, our findings that inhibiting S1P synthesis or blocking S1P3 activation abrogated glial motility suggest that Müller glial cells synthesize and release S1P, which then stimulates glial migration by inside-out signaling on S1P3.

Coupling of S1PRs to different heterotrimeric G proteins, controls cell motility by the downstream activation of multiple signaling pathways. In human primary fibroblast-like synoviocytes, S1P-induced cell motility is regulated by the p38 MAPK, ERK, and Rho pathways,[41] while in vascular smooth muscle and endothelial cells S1P activates the PI3K/Akt and ERK/MAPK.[42] Activation of S1P1 by S1P stimulates Rac in a PI3K-dependent manner, and Akt phosphorylation of S1P1 has been proposed to be crucial in transducing S1P signaling leading to lamellipodia formation.[43,44] The S1P-S1P3 axis controls activation of the ERK/MAPK signaling pathway and astrocyte migration,[40] and S1P signaling through S1P1 and S1P3 activates the same pathway to induce migration of bone marrow-derived mesenchymal stem cells.[25] In cultured Müller glial cells, inhibiting the PI3K or the ERK/MAPK pathways prevented glial migration, both in cultures lacking S1P or in those supplemented with it, whereas the p38 MAPK pathway seemed to contribute slightly to glial motility. These results suggest that S1P signaling through S1P3 leads to the activation of the PI3K and the ERK/MAPK signaling pathways to stimulate glial migration.

In conclusion, our work suggests a central role for the S1P/S1P3 axis in the induction of Müller glial cell migration in the retina. Because this migration is involved in proliferative retinopathies that can severely impair retinal function, targeting the S1P signaling pathway emerges as a potential clinical tool for treating these diseases.

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