Bone Marrow-Derived Mesenchymal Stem Cells Differentiate to Hepatic Myofibroblasts by Transforming Growth Factor- β 1 via Sphingosine Kinase/Sphingosine 1-Phosphate (S1P)/S1P Receptor Axis

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Sphingosine kinase (SphK) is involved in numerous biological processes, including cell growth, proliferation, and differentiation. However, whether SphK participates in the differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) to myofibroblasts has been unknown. In a carbon tetrachloride-treated mouse model, SphK1 was expressed in BMSCs in damaged liver. Furthermore, mRNA expression of both SphK1 and transforming growth factor β 1 (TGF- β 1) was significantly increased after liver injury, with a positive correlation between them. The SphK inhibitor SKI significantly blocked BMSC differentiation to myofibroblasts during liver injury (the proportion of BMSC-derived myofibroblasts decreased markedly, compared with no SKI treatment) and attenuated the extent of liver fibrosis. Using primary mouse BMSCs, we demonstrated that TGF- β 1 induced BMSC differentiation to myofibroblasts, accompanied by the up-regulation of SphK1 and modulation of sphingosine 1-phosphate (S1P) receptor (S1PR) expression. Notably, pharmacological or siRNA-mediated inhibition of SphK1 abrogated the prodifferentiating effect of TGF- β 1. Moreover, using either S1PR subtype-specific antagonists or specific siRNAs, we found that the prodifferentiating effect of TGF- β 1 was mediated by S1PR₁ and S1PR₃. These data suggest that SphK1 activation by TGF- β 1 leads to differentiation of BMSCs to myofibroblasts mediated by S1PR₁ and S1PR₃ up-regulation, thus providing new information on the mechanisms by which TGF- β 1 gives rise to

fibrosis and opening new perspectives for pharmacological treatment of liver fibrosis. (Am J Pathol 2012, 181: 85–97; <http://dx.doi.org/10.1016/j.ajpath.2012.03.014>)

Mesenchymal stem cells (MSCs) are a heterogeneous population of cells with the potential for multilineage differentiation. They have been identified and isolated from various tissues, including blood, adipose tissue, trabecular bone, muscle, and dermis.¹ Under appropriate culture conditions, bone marrow-derived MSCs (BMSCs) are capable of differentiating to a variety of mesenchymal tissues, including bone, cartilage, muscle, ligament, tendon, adipose tissue, and stroma.^{2,3} Their multipotency, easy isolation, and ready availability make MSCs particularly suited for tissue engineering and clinical applications. Thus, it is desirable to identify at the molecular level the regulatory mechanisms underlying the differentiation of MSCs.

Myofibroblasts play a central role in the pathogenesis of liver fibrosis. *De novo* formation of differentiated myofibroblasts is thought to be primarily responsible for excessive extracellular matrix production; thus, elucidating the mechanisms of myofibroblast activation seems indispensable for designing rational therapeutic strategies to inhibit the fibrogenic process that leads to cirrhosis.^{4,5} A high proportion of myofibroblasts in the fibrotic liver are of BMSC origin.^{6–8} In a previous study, we demonstrated

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that BMSCs can migrate to the damaged liver, mediated by endogenous sphingosine 1-phosphate (S1P), which can produce a concentration gradient between liver and bone marrow after liver injury, and that BMSCs differentiate to myofibroblast-like cells expressing α -smooth muscle actin (α -SMA) in mouse fibrotic liver.⁹ However, which factor mediates differentiation of BMSCs to myofibroblasts and the molecular mechanisms governing this process are not fully understood.

Sphingosine kinase (SphK) catalyzes the phosphorylation of sphingosine to generate the bioactive lipid S1P.¹⁰ The two distinct isoforms of mammalian SphK (SphK1 and SphK2) differ in sequence, catalytic properties, localization, and function.¹¹ SphK1 is activated by various factors, including ganglioside GM1 (GM1), G-protein coupled receptors, small GTPases, tyrosine kinase receptors, proinflammatory cytokines, immunoglobulin receptors, and calcium and protein kinase activators, and is involved in numerous biological processes, including cell growth, proliferation, differentiation, apoptosis, motility, and cytoskeletal rearrangement.^{12–15} SphK1 activation translocates from the cytosol to the plasma membrane, leading to S1P formation, which then activates a set of five G-protein-coupled receptors referred to as S1P receptor (S1PR) types 1 to 5 (S1PR_{1–5}), leading to regulation of numerous downstream signaling components.¹⁶ It is becoming increasingly clear that the expression pattern of S1PRs is critical for the final effect elicited by the bioactive lipid in some cell types, given that individual receptor subtypes are coupled to different signaling cascades and are consequently implicated in the regulation of a variety of biological responses.^{17,18}

Transforming growth factor- β 1 (TGF- β 1) is a member of a large family of growth factors involved in numerous biological processes, including cell proliferation, differentiation, embryonic development, carcinogenesis, immune function, inflammation, and wound healing.^{19–22} Some of the effects elicited by TGF- β 1 are transmitted by the pathway initiated by enhancement of SphK. The SphK/S1P/S1PR pathway is critically implicated in the mechanism by which TGF- β 1 elicits invasive behavior of esophageal cancer cells,²³ antiapoptotic action in mesoangioblasts,²⁴ and prodifferentiating effect in various types of fibroblasts^{25–27} and myoblasts.²⁸ In particular, up-regulation of SphK1 has been found to be critical for TGF- β 1-induced transcriptional regulation of the tissue inhibitor of metalloproteinases 1 (TIMP-1), which inhibits degradation of extracellular matrix in fibroblasts.²⁵ Moreover, recent reports have shown that SphK1 and S1PRs play a role in TGF- β 1-dependent extracellular matrix remodeling and myofibroblast differentiation of lung fibroblasts and myoblasts and in collagen production by cardiac fibroblasts.^{26–28} The well-established prodifferentiating effect of TGF- β 1, together with the emerging role of the SphK/S1P/S1PR axis as the TGF- β 1-regulated signaling pathway, spurred us to investigate whether TGF- β 1 could induce differentiation of BMSCs to myofibroblasts and whether SphK1 and S1PRs participate in its biological action.

With the present study, we demonstrate for the first time that TGF- β 1 induces differentiation of BMSCs to myofibroblasts via up-regulation of SphK1 and modifica-

tion of S1PR expression. Importantly, S1PR₁ and S1PR₃, which are up-regulated on TGF- β 1 challenge, seem to be critically implicated in the differentiation of BMSCs to myofibroblasts. *In vivo* administration of SphK inhibitor significantly blocked differentiation of BMSCs to myofibroblasts during liver injury, and thus attenuated the extent of liver fibrosis. These results represent the first experimental evidence that induction of the SphK/S1P/S1PR axis is exploited by TGF- β 1 to induce BMSC differentiation. These results may open new perspectives for pharmacological treatment of liver fibrosis.

Materials and Methods

Chemicals and Reagents

Antibodies against α -SMA and SphK1 were from Sigma-Aldrich (St. Louis, MO) and ECM Biosciences (Versailles, KY), respectively. Anti-CD105 and anti-CD166 antibodies used for flow cytometry analysis were from AbD Serotec (Kidlington, UK) and BD Biosciences (San Jose, CA), respectively. TGF- β 1 was from PeproTech (London, UK), and GM1 was from Sigma-Aldrich. The sphingosine kinase inhibitor SKI [2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole] and *N,N*-dimethylsphingosine (DMS) were from Calbiochem (Bad Soden, Germany) and Biomol (Hamburg, Germany), respectively. The specific S1PR₁/S1PR₃ antagonist VPC23019 and the specific S1PR₁ antagonist W146 were from Avanti Polar Lipids (Alabaster, AL). The specific S1PR₂ antagonist JTE-013 and the S1PR₁ agonist SEW2871 were from Cayman Chemical (Ann Arbor, MI). PCR reagents were from Applied Biosystems (Life Technologies, Foster City, CA). Alkaline phosphatase type VII-T, *o*-phthalaldehyde, and other common reagents were from Sigma-Aldrich.

Isolation of Whole Bone Marrow Cells and BMSCs

ICR mice aged 3 weeks (Laboratory Animal Center, Capital Medical University) were sacrificed by cervical dislocation. Whole bone marrow cells were extracted from the tibias and femurs of mice by flushing with Gibco culture medium (Life Technologies, Grand Island, NY) using a 25-gauge needle. The cells were then passed through 70-mm nylon mesh and were washed three times with PBS containing 2% fetal bovine serum (Biochrom, Berlin, Germany). BMSCs were isolated from bone marrow of enhanced green fluorescent protein (EGFP) transgenic ICR mice or wild-type mice, and were cultured as described previously.⁹ Characterization of BMSCs was performed by flow cytometry analysis.⁹ All animal experiments were performed according to guidelines of the Ethics Committee of Capital Medical University.

Mouse Models

ICR mice aged 6 weeks received intraperitoneal injections of 1 μ L/g body weight of a carbon tetrachloride/olive oil (CCl₄/OO) mixture, 1:9 v/v, twice per week. The mice were sacrificed at 3 days or at 2, 4, 6, or 8 weeks of CCl₄

treatment ($n = 7$ per group), always on the day after the last injection. Liver tissue was collected for real-time RT-PCR and high-performance liquid chromatography (HPLC) analysis.

Another group of mice received lethal irradiation (8 Gy), and then immediately received transplantation by a tail-vein injection of 1.2×10^6 enriched EGFP-positive BMSCs and 1.08×10^7 EGFP-negative whole bone marrow cells. Four weeks later, mice received intraperitoneal injections of CCl_4 twice per week for 4 weeks. SKI (10 mg/kg body weight) or saline was administered the day before CCl_4 or OO treatment ($n = 7$ per group). Liver tissue was collected for real-time RT-PCR, for H&E and Sirius Red staining, and for immunofluorescence analysis.

Immunofluorescence

Liver samples were fixed in 4% paraformaldehyde and embedded in Tissue Tek optimal cutting temperature compound (Sakura Finetek USA, Torrance, CA). Frozen sections (7 μm thick) were used for immunofluorescence staining. Sections were blocked with 2% bovine serum albumin in PBS for 1 hour and then incubated with anti-SphK1 polyclonal antibody (1:100) diluted in PBS. Cy3-conjugated AffiniPure goat anti-rabbit IgG antibody (1:500) was used as a secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The sections were covered with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing DAPI and were observed under a confocal microscope (Leica TCS SP5 with LAS AF version 2.5 software; Leica Microsystems, Wetzlar, Germany). For negative controls, sections were processed the same way, except that incubation with the primary antibody was omitted.

Immunofluorescent detection of α -SMA in liver tissue was performed using a Vector M.O.M. (mouse-on-mouse) immunodetection kit (Vector Laboratories) and a 1:1000 dilution of a monoclonal antibody to α -SMA. The proportion of BMSC-derived myofibroblasts was calculated as the number of both EGFP-positive and α -SMA-positive cells divided by number of α -SMA-positive cells, using Image-Pro Plus version 6.0 software (Media Cybernetics, Silver Spring, MD).

Cultured BMSCs with or without treatments were fixed in 4% paraformaldehyde in PBS for 30 minutes. Then cells were washed twice with PBS, permeabilized in 0.5% Triton X-100 in PBS for 15 minutes, blocked with 2% BSA for 1 hour, and then incubated with anti- α -SMA monoclonal antibody (1:2000), followed by secondary antibody conjugated with Cy3 (1:1000; Jackson ImmunoResearch Laboratories).

Real-Time RT-PCR

Total RNA was extracted from frozen liver specimens or cultured BMSCs with or without treatments, using an RNeasy kit (Qiagen, Hilden, Germany). Real-time RT-PCR was performed with an ABI Prism 7300 sequence-detecting system (Life Technologies, Foster City, CA), as described previously.²⁹ Primers (MWG Biotech, Ebersberg, Germany) used for real-time RT-PCR were as follows: 18S rRNA, sense, 5'-GTAACCCGTTGAACCCCAATT-3', and anti-sense, 5'-CCATCCAATCGGTAGTAGCG-3'; α -SMA, sense, 5'-ATGCTCCCAGGGCTGTTTT-3', and anti-sense, 5'-TTC-

CAACCATTACTCCCTGATGT-3'; SphK1, sense, 5'-TGT-CACCCATGAACCTGCTGTCCCTGCACA-3', and anti-sense, 5'-AGAAGGCACTGGCTCCAGAGGAACAAG-3'; SphK2, sense, 5'-ACAGAACCATGCCCGTGAG-3', and anti-sense, 5'-AGGTCAACACCGACAACCTG-3'; procollagen $\alpha 1(\text{I})$ [Col $\alpha 1(\text{I})$], sense, 5'-AGGGCGAGTGCTGTGCTT T-3', and anti-sense, 5'-CCCTCGACTCCCTACATCTTCTGA-3'; procollagen $\alpha 1(\text{III})$ [Col $\alpha 1(\text{III})$], sense, 5'-TGAAACCCCAGCAAAA-CAAAA-3', and anti-sense, 5'-TCACCTGCACTGGTTGATA-AGATTAA-3'; S1PR₁, sense, 5'-ACTTTGCGAGTGAGCTG-3', and anti-sense, 5'-AGTGAGCCTTCAGTTACAGC-3'; S1PR₂, sense, 5'-TTCTGGAGGGTAACACAGTGGT-3', and anti-sense, 5'-ACACCCCTTTGTATCAAGTGGCA-3'; S1PR₃, sense, 5'-TGGTGTGCGGCTGTCTAGTCAA-3', and anti-sense, 5'-CACAGCAAGCAGACCTCCAGA-3'; and TGF- $\beta 1$, sense, 5'-TGCGCTTGCAAGATTAAAA-3', and anti-sense, 5'-TCAC-TGGAGTTGTACGGCAG-3'.

Quantitative Analysis of Liver Fibrosis

Liver tissues were fixed in PBS containing 4% paraformaldehyde for 24 hours and then were embedded in paraffin. Sections (6 μm thick) were stained with Sirius Red for collagen visualization and with H&E for liver histology, as described previously.³⁰ The fibrotic area was assessed by means of computer-assisted image analysis with Leica Qwin V3 software. The expressed percentage of fibrotic or inflammatory area was calculated based on the mean value of 15 randomly selected areas per sample.

Western Blot Analysis

Western blot analysis of α -SMA and SphK1 was performed with 20 μg of protein extract, obtained as described previously,³¹ using mouse monoclonal antibody and rabbit polyclonal antibody to α -SMA (1:1000) and SphK1 (1:1000), respectively. Peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG antibody (1:5000; Jackson ImmunoResearch Laboratories) was used as a secondary antibody. Protein expression was visualized by using an enhanced chemiluminescence assay kit according to the manufacturer's instructions (Amersham ECL Plus; GE Healthcare Life Sciences, Arlington Heights, IL). The bands were quantified using GeneSnap and GeneTools software from PerkinElmer (Waltham, MA), and results were normalized relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (rabbit monoclonal anti-GAPDH antibody, 1:1000; Sigma-Aldrich) or tubulin (rabbit monoclonal anti-tubulin antibody, 1:1000, Epitomics, Burlingame, CA) expression to correct for variations in protein loading and transfer.

RNA Interference

The siRNA sequences specifically targeting mouse SphK1, S1PR₁, S1PR₂, or S1PR₃ were synthesized by Dharmacon: L-040671-00, L-051684-00, L-063765-00, and L-040959-00, respectively (Thermo Scientific, Lafayette, CO). At 40% to 50% confluency, BMSCs were prepared in 60-mm dishes. Transient transfection of siRNA (40 nmol/L) was performed by using Invitrogen Lipofectamine RNAiMAX

(Life Technologies, Carlsbad, CA), as recommended by the manufacturer. Control cells were treated with 40 nmol/L RNAi negative control duplexes (scrambled siRNA). After 48 hours, cells were used to perform the differentiation assay.

Measurement of S1P by HPLC Analysis

After 24 hours stimulation of TGF- β 1 or GM1, both BMSCs and their supernatants were collected. Extraction of S1P and sample analysis were performed as described previously.³²

Statistical Analysis

Data are expressed as means $\pm$ SEM and were analyzed by Student's *t*-test or analysis of variance when appropriate. *P* < 0.05 was considered significant.

Results

SphK Is Involved in the Differentiation of BMSCs to Myofibroblasts in Mouse Fibrotic Liver

We first reconstituted bone marrow in the irradiated mice by transplantation of the genetic EGFP-labeled BMSCs. Liver fibrosis was then induced by the administration of CCl₄ for 4 weeks, and expression of α -SMA, a marker for myofibroblasts, was examined in the damaged liver by immunofluorescence analysis. In OO-treated liver, α -SMA was expressed only in the vascular area, and there were only a few, scattered EGFP-positive cells (BMSCs) in the liver. No colocalization was observed between α -SMA-positive and EGFP-positive cells (Figure 1A). After CCl₄ treatment, however, there was strong immunoreactivity for α -SMA in the fibrotic areas, with distribution similar to that of EGFP-positive cells (Figure 1B). Confocal microscopy images showed that these EGFP-positive cells (BMSCs) had a myofibroblast phenotype (Figure 1C). We then determined the proportion of BMSC-derived myofibroblasts and found that a large proportion (69%) of α -SMA-positive cells in the fibrotic areas were also positive for EGFP, suggesting that BMSCs could differentiate to myofibroblasts during liver fibrosis (Figure 1E). We also quantitated the numbers of myofibroblasts of non-bone marrow origin (including hepatic stellate cells); the percentage was only 31%. Given the significant contribution of BMSCs to the myofibroblast lineage, we focused on the myofibroblasts derived from BMSC differentiation.

To prove fully that BMSCs actually leave the bone marrow and then engraft the liver, we used flow cytometry analysis to explore whether the EGFP-positive cells in the blood of chimeric mice were MSCs. We first characterized these EGFP-positive cells and found that more EGFP-positive cells were detected in the blood of chimeric animals treated with CCl₄, compared with the OO-treated control (see Supplemental Figure S1A at <http://ajp.amjpathol.org>). Moreover, we used the typical MSC marker proteins, CD166 and CD105, to confirm the phenotype of MSCs in mice treated with OO or CCl₄. Some of

the EGFP-positive cells circulating in the blood of chimeric animals were also positive for CD166 (see Supplemental Figure S1B at <http://ajp.amjpathol.org>) or CD105 (see Supplemental Figure S1C at <http://ajp.amjpathol.org>). These results suggest that, after liver injury, these MSCs actually leave the bone marrow, enter the circulation, and then traffic to the liver (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

SphK is reported to play a pivotal role in the differentiation of many types of cells.^{25–28} To investigate whether SphK/S1P/S1PR signaling is involved in BMSC differentiation, we first examined the mRNA expression of SphK1 in CCl₄-induced liver fibrosis models. SphK1 mRNA level increased drastically from 3 days after CCl₄ treatment, and continued to increase in a way correlated with the progression of liver fibrosis, with a maximal increase (26.5-fold) at 56 days after CCl₄ treatment (Figure 2A). Correspondingly, the production of S1P on SphK1 activation was also increased from 3 to 42 days after CCl₄ administration (Figure 2B).

Our results demonstrated that BMSCs differentiated to myofibroblasts in mouse liver fibrosis (Figure 1, B and C). To study the molecular mechanism underlying BMSC differentiation, we next examined whether SphK1 is expressed in BMSCs in mouse fibrotic liver by immunofluorescence analysis. SphK1 was expressed primarily in the vascular area (Figure 2D), which is in accord with previous studies.³³ In addition, the fibrotic liver displayed strong immunoreactivity for SphK1 in the fibrotic septa. EGFP-positive cells (ie, BMSCs) distributed in a pattern similar to that of SphK1 staining along the fibrotic septa. Confocal microscope images showed that significant numbers of EGFP-positive cells in the fibrotic area were also positive for SphK1 (Figure 2E), suggesting that SphK1 was probably involved in BMSC differentiation.

To determine the role of SphK in BMSC differentiation *in vivo*, we used a pharmacological inhibitor of SphK, SKI, to investigate whether SphK is involved in the differentiation of BMSCs to myofibroblasts in CCl₄-induced liver injury. The SKI significantly blocked differentiation of BMSCs to myofibroblasts during liver injury (Figure 1D), because the proportion of BMSC-derived myofibroblasts decreased markedly compared with that in the liver without SKI treatment (from 69% to 20%) (Figure 1E).

We then speculated that S1P, the product of SphK1, may be involved in the differentiation of BMSCs to myofibroblasts. After S1P stimulation, we detected mRNA expression of α -SMA and of the major components of the extracellular matrix, Col α 1(I) and Col α 1(III), in BMSCs. Unexpectedly, there were no increases in these mRNA levels after S1P stimulation (Figure 2C). This result suggests that S1P does not directly induce differentiation of BMSCs to myofibroblasts.

SphK1 mRNA and Protein Expression Is Up-Regulated by TGF- β 1 in BMSCs

TGF- β 1 has been reported to be the crucial cytokine inducing the transformation of many types of cells to myofibroblasts.^{26–28} In a previous study we showed that

Figure 1. BMSCs differentiate to myofibroblasts during liver injury, which can be blocked by SphK inhibitor (SKI). ICR mice were lethally irradiated and received transplants of enriched EGFP-positive BMSCs and EGFP-negative whole bone marrow cells. Liver fibrosis was then induced by administration of carbon tetrachloride (CCl₄) for 4 weeks. **A–D:** Control mice received olive oil (OO) treatment (**A**). Saline solution (**B** and **C**) or SKI (10 mg/kg body weight) (**D**) was administered the day before CCl₄ or OO treatment (*n* = 7 per group). Representative images of α -SMA (red), EGFP (green), and merged fields from immunofluorescence analysis to track myofibroblasts of BMSC origin after 4 weeks of CCl₄ treatment without (–) or with (+) SKI administration; DAPI (blue) was used to visualize nuclei. **Arrows** in **A** indicate the cells positive for α -SMA and in **B** and **D** indicate the cells both positive for α -SMA and EGFP. **E:** The proportion of myofibroblasts from BMSCs was evaluated using Image-Pro Plus software. Data are expressed as means $\pm$ SEM. **P* < 0.05 versus CCl₄-treated alone. Scale bars: 50 μ m (**A**, **B**, and **D**); 5 μ m (**C**).

TGF- β 1 induces differentiation of BMSCs to myofibroblasts by stimulating the mRNA expression of α -SMA, Col α 1(I), and Col α 1(III).⁹ In the present study, we observed marked up-regulation of TGF- β 1 mRNA expression in liver tissue after CCl₄ treatment (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). To investigate the molecular mechanism underlying BMSC differentiation, we first analyzed the correlation between TGF- β 1 and SphK1 mRNA in liver. A straight-line fit was obtained in regression analysis between them, with correlation coefficient *r* = 0.895 (*P* < 0.001) (Figure 3A), further suggesting that SphK1 highly expressed in fibrotic liver may play a role in the differentiation of BMSCs to myofibroblasts induced by TGF- β 1.

Reports have indicated that SphK expression can be increased by TGF- β 1 stimulation in fibroblasts and myoblasts,^{26–28} but whether TGF- β 1 regulates SphK in BMSCs had not been investigated previously. Real-time

RT-PCR analysis revealed a pronounced increase in SphK1 mRNA expression stimulated by TGF- β 1 in a dose-dependent manner, with a maximal increase at a concentration of 10 ng/mL at 24 hours (Figure 3, B and C), in parallel with the maximal up-regulation of α -SMA, Col α 1(I), and Col α 1(III) (see Supplemental Figure S3 at <http://ajp.amjpathol.org>). We next examined the effects of TGF- β 1 on SphK1 protein expression by Western blot. TGF- β 1 treatment markedly up-regulated SphK1 protein levels at 24 hours, with a maximal increase at 48 hours (Figure 3D); in controls (0 hour), levels were barely detectable. The elevated levels of SphK1 protein persisted for at least 72 hours. In contrast, SphK2 expression levels were not affected by TGF- β 1 treatment (data not shown). Together, these data demonstrate that SphK1 mRNA and protein levels are increased by TGF- β 1 in BMSCs, suggesting that SphK1 might play a key role in the biological effects of TGF- β 1.

Figure 2. SphK1 is expressed in BMSCs in damaged liver. Total RNA was extracted from liver in mice treated with CCl_4 or OO ($n = 7$ per group). **A:** mRNA expression was quantified using real-time RT-PCR. **B:** S1P production was analyzed by HPLC. **C:** mRNA expression of α -SMA, Col $\alpha 1(\text{I})$, and Col $\alpha 1(\text{III})$ in confluent serum-starved BMSCs in response to exogenous S1P. **D** and **E:** Representative images from immunofluorescence analysis to track the expression of SphK1 (red) in BMSCs (EGFP, green) after 4 weeks of CCl_4 treatment. **Arrowheads** indicate expression of SphK1 in the vascular area and **arrows** indicate cells positive for both SphK1 and EGFP. DAPI (blue) was used to visualize nuclei. Data are expressed as means $\pm$ SEM. Scale bars: 100 μm (**D**); 20 μm (**E**).

TGF- $\beta 1$ -Induced Differentiation of BMSCs Is Dependent on SphK1

Having identified the ability of TGF- $\beta 1$ to regulate SphK1 expression in BMSCs, we next evaluated whether the

TGF- $\beta 1$ -stimulated BMSC differentiation involves the activation of SphK1. First, we confirmed that TGF- $\beta 1$ treatment induced a significant increase of α -SMA, Col $\alpha 1(\text{I})$, and Col $\alpha 1(\text{III})$, with a maximal response at 24 hours (see Supplemental Figure S3 at <http://ajp.amjpathol.org>). We

Figure 3. TGF- $\beta 1$ stimulates SphK1 mRNA and protein expression in BMSCs. **A:** Correlation between SphK1 and TGF- $\beta 1$ mRNA in liver tissue. **B:** SphK1 mRNA expression in BMSCs treated with five concentrations of TGF- $\beta 1$ (0 to 10 ng/mL) for 24 hours. **C:** SphK1 mRNA expression in BMSCs treated with 10 ng/mL TGF- $\beta 1$ for 0 to 80 hours. **D:** Cell lysates were prepared and subjected to Western blot analysis. The intensity of each band with SphK1 antibody was quantified and normalized to that with anti- β -tubulin antibody. All results were confirmed in three independent experiments. Data are expressed as means $\pm$ SEM. * $P < 0.05$ versus untreated control cells.

Figure 4. SphK1 is involved in TGF- β 1-induced differentiation of BMSCs. BMSCs were pretreated or not for 1 hour with 5 μ mol/L DMS or 10 μ mol/L SKI or were transfected with SphK1 siRNA; 24 hours of TGF- β 1 treatment followed. **A:** mRNA expression of α -SMA, Col α 1(I), and Col α 1(III) in response to TGF- β 1 in the presence of DMS, SKI, or SphK1 siRNA. **B:** Cells were transfected with SCR siRNA or SphK1 for 48 hours. SphK1 mRNA was then evaluated by real-time RT-PCR. **C–E:** α -SMA protein expression in response to TGF- β 1 in the presence of DMS (**C**), SKI (**D**), and SphK1 siRNA (**E**). **F:** Immunofluorescence analysis of α -SMA (red) in BMSCs without (–) or with (+) TGF- β 1 treatment. All results were confirmed in three independent experiments. Data are expressed as means $\pm$ SEM. * P < 0.05 versus untreated control cells; † P < 0.05 versus TGF- β 1-treated cells alone. Scale bar = 100 μ m.

used two different pharmacological inhibitors of SphK, DMS and SKI. Enhanced mRNA expression of α -SMA, Col α 1(I), and Col α 1(III) induced by TGF- β 1 was blunted by preincubation with 5 μ mol/L DMS or 10 μ mol/L SKI for 1 hour before TGF- β 1 stimulation (Figure 4A). Neither SphK inhibitor alone had any effect on cellular viability (data not shown) or the expression of α -SMA, Col α 1(I), and Col α 1(III) (Figure 4A). To further assess the involve-

ment of SphK1 in the biological response evoked by TGF- β 1 in BMSCs, we used siRNA technology to knock down SphK1. First, we confirmed that SphK1 siRNA down-regulated SphK1 mRNA expression, by 86% (Figure 4B). In accord with the results for SphK inhibitors, TGF- β 1-induced α -SMA, Col α 1(I), and Col α 1(III) mRNA expression was significantly reduced in SphK1-knock-down cells, whereas scrambled siRNA had no effect

Figure 5. GM1 induces BMSC differentiation. **A:** SphK1 mRNA expression in BMSCs treated with four concentrations of GM1 (0 to 1 μmol/L) for 24 hours. **B:** SphK1 and tubulin protein expression by Western blot in BMSCs treated with the four concentrations of GM1. **C:** mRNA expression of α-SMA, Colα1(I), and Colα1(III) in BMSCs treated with the four concentrations of GM1 for 24 hours. **D:** Immunofluorescence analysis of α-SMA (red) in BMSCs. All results were confirmed in three independent experiments. Data are expressed as means ± SEM. **P* < 0.05 versus untreated control cells. Scale bar = 100 μm.

(Figure 4A). In accord, α-SMA protein expression was down-regulated by DMS (Figure 4C), SKI treatment (Figure 4D), and SphK1 siRNA transfection (Figure 4E). These results provide further support for the key role of SphK1 in BMSC differentiation brought about by TGF-β1 and strongly suggest that SphK1 contributes to TGF-β1-induced differentiation of BMSCs to myofibroblasts.

To further assess the involvement of SphK1 in BMSC differentiation, we performed immunofluorescent staining for α-SMA to reveal morphological changes in BMSCs. Under normal conditions, isolated BMSCs showed very weak expression of α-SMA. Treatment of TGF-β1 resulted in the appearance of numerous bundles of actin microfilaments and markedly enhanced the expression of α-SMA in BMSCs; however, when BMSCs were treated with SphK inhibitors or transfected with SphK1 siRNA, the ability of TGF-β1 to enhance α-SMA was markedly reduced (Figure 4F).

GM1, a SphK Activator, Exerts a Mimic Effect on BMSC Differentiation as TGF-β1

To further determine whether SphK1 mediates TGF-β1-induced differentiation of BMSCs, we investigated the effects of GM1, a SphK activator,¹⁵ on BMSC differentiation. As predicted, both SphK1 mRNA (Figure 5A) and SphK1 protein (Figure 5B) levels in BMSCs were elevated after GM1 stimulation. In analogy to TGF-β1, GM1 also resulted in a significant increase in α-SMA, Colα1(I), and Colα1(III) mRNA expression in a dose-dependent manner (Figure 5C). Immunofluorescent staining also showed that GM1 increased the expression of α-SMA (Figure 5D). Thus, GM1 may be used to mimic the biological effects of TGF-β1 on BMSC differentiation, further confirming that TGF-β1 induces differentiation of BMSCs to myofibroblasts via the activation of SphK1.

S1PR₁ and S1PR₃ Are Required for the Differentiation of BMSCs Induced by TGF-β1 or GM1

Activated SphK1 translocates from the cytosol to the plasma membrane, leading to S1P formation, which then activates S1PRs.¹¹ We therefore first determined whether TGF-β1 or GM1 stimulation resulted in corresponding increases of S1P. Levels of S1P were tested by HPLC in

confluent serum-starved cells treated with TGF-β1 or GM1 for 24 hours. S1P levels in BMSCs and their supernatants were significantly increased, by approximately 1.9-fold, 1.5-fold, and 3.5-fold after the 10 ng/mL TGF-β1 treatment, the 0.1 μmol/L GM1 treatment, and the 1 μmol/L GM1 treatment, respectively (Figure 6A).

Next, we examined whether the prodifferentiating action exerted by TGF-β1 was S1PR-mediated. The expression of α-SMA, Colα1(I), and Colα1(III) was evaluated in BMSCs challenged with TGF-β1 in the absence or presence of 10 μmol/L JTE-013 (a specific antagonist of S1PR₂), 1 μmol/L VPC23019 (a specific S1PR₁/S1PR₃ antagonist), or 10 μmol/L W146 (a specific S1PR₁ antagonist) (Figure 6B). The increased content of α-SMA, Colα1(I), and Colα1(III) after TGF-β1 treatment was partially abrogated when S1PR₁/S1PR₃ was blocked, but was not affected by inhibition of S1PR₂ (Figure 6B), suggesting a role of S1PR₁/S1PR₃ but not S1PR₂ in TGF-β1-induced response. Furthermore, differentiation of BMSCs induced by TGF-β1 was partially abrogated when S1PR₁ was blocked by its specific antagonist, W146 (Figure 6B). Next, we used an agonist of S1PR₁ (SEW2871) to further investigate the effect of S1PR₁ on BMSC differentiation. This S1PR₁ agonist also increased the expression of α-SMA, Colα1(I), and Colα1(III) (Figure 6C).

To further test this hypothesis, individual S1PRs were knocked down by specific siRNAs. First, we confirmed that S1PR₁, S1PR₂, or S1PR₃ siRNA down-regulated each mRNA expression (by 87%, 86%, and 75%, respectively). S1PR₁ or S1PR₃ siRNA strongly attenuated the enhancement of α-SMA and Colα1(I) mRNA expression elicited by TGF-β1 and abrogated the increase of Colα1(III) mRNA expression. Conversely, the down-regulation of S1PR₂ by specific siRNA did not alter the prodifferentiating effect of the cytokine (Figure 6D). Altogether, these data suggest that S1PR₁ and S1PR₃ are required for the differentiation of BMSCs induced by TGF-β1.

To further investigate the role of S1PRs in BMSC differentiation, BMSCs were preincubated with inhibitors or transfected with specific siRNAs of S1PRs, to test for the responsiveness to GM1. Consistent with the findings for TGF-β1 treatment, knockdown of S1PR₁ or S1PR₃ blunted the increase of α-SMA, Colα1(I), and Colα1(III) mRNA expression induced by GM1, whereas knockdown of S1PR₂ had no effect (Figure 6E). These results indicate that S1PR₁ and S1PR₃ are essential for GM1-induced

Figure 6. Role of S1PRs in TGF-β1-induced or GM1-induced differentiation of BMSCs. **A:** S1P levels in BMSCs after treatment with TGF-β1 or GM1. **B:** BMSCs were treated without (–) or with (+) 1 μmol/L VPC23019, 1 μmol/L W146, or 10 μmol/L JTE-013 for 1 hour before TGF-β1 challenge (10 ng/mL) for 24 hours. mRNA expression of α-SMA, Colα1(I), and Colα1(III) was analyzed by real-time RT-PCR. **C:** The mRNA expression of α-SMA, Colα1(I), and Colα1(III) in response to SEW2871. **D:** BMSCs, transfected with SCR or S1PR siRNAs and incubated in the absence (–) or presence (+) of 10 ng/mL TGF-β1 for the last 24 hours of transfection, were evaluated for mRNA expression of α-SMA, Colα1(I), and Colα1(III). **E:** Effects of S1PR inhibitors or specific siRNAs on the mRNA expression of α-SMA, Colα1(I), and Colα1(III) in response to GM1. All results were confirmed in three independent experiments. Data are expressed as means ± SEM. **P* < 0.05 versus untreated control cells; †*P* < 0.05 versus TGF-β1-treated or GM1-treated cells alone.

BMSC differentiation, further supporting the critical role of S1PRs in TGF-β1-induced MSC differentiation.

TGF-β1 or GM1, But Not Exogenous S1P, Modifies S1PR Expression Pattern

To gain further insight into the mechanism by which TGF-β1 exploits the SphK/S1P/S1PR axis to exert its prodifferentiating effect in BMSCs, we examined whether cell treatment with the cytokine affected S1PR expression pattern. Real-time RT-PCR analysis showed that incubation of BMSCs for 24 hours in the presence of TGF-β1 apparently up-regulated the expression of S1PR₁ and S1PR₃ (Figure 7A). Similar results were obtained after GM1 stimulation (Figure 7B). Exogenous S1P, however, did not affect S1PR expression levels (Figure 7C). Given

that TGF-β1 or GM1 could induce BMSC differentiation, whereas exogenous S1P had no direct effect on it, these findings further confirm that up-regulation of S1PR₁ and S1PR₃ on TGF-β1 or GM1 challenge is essential for the differentiation of BMSCs to myofibroblasts.

Blockage of SphK Attenuates CCl₄-Induced Liver Fibrosis

Finally, we analyzed the effects of the SphK inhibitor SKI on the underlying liver fibrosis process. Hepatic collagen deposition was evaluated by morphometric analysis with Sirius Red staining and was quantified by digital image analysis. Collagen deposition was markedly attenuated after SKI administration (Figure 8, A and C). Moreover, H&E-stained sections showed a significant decrease in

Figure 7. TGF-β1 or GM1, but not exogenous S1P, modifies S1PR expression pattern. mRNA expression of S1PRs after TGF-β1 (A), GM1 (B), or exogenous S1P (C) stimulation for 24 hours was analyzed by real-time RT-PCR. **P* < 0.05 versus control.

liver injury after SKI administration in CCl₄-treated mice (Figure 8B). We also examined the effect of SKI on the fibrosis markers α-SMA, Colα1(I), and Colα1(III) in CCl₄-induced mouse liver fibrosis. After 4 weeks of administration of CCl₄, mRNA expressions of α-SMA, Colα1(I), and Colα1(III) in liver tissue were markedly up-regulated; however, SKI administration resulted in a significant drop in mRNA expression of α-SMA (Figure 8D), Colα1(I) (Figure 8E), and Colα1(III) (Figure 8F), compared with control. These results demonstrate that blockage of SphK attenuates CCl₄-induced liver fibrosis.

Discussion

Understanding the regulatory mechanisms at the molecular level underlying BMSC differentiation is a prerequi-

site for optimizing mesenchymal stem cell-based clinical applications. Here, we have described a novel signaling pathway, triggered by TGF-β1 in BMSCs via modulation of SphK1 and S1PR_{1/3}, that is critically implicated in the differentiation of BMSCs to myofibroblasts.

A key finding of the present study is that SphK1 is implicated in the prodifferentiating action of TGF-β1 on BMSCs. First, we examined the *in vivo* expression of SphK1 by immunofluorescence analysis in CCl₄-induced liver fibrosis models and found that SphK1 is expressed in BMSCs in the damaged liver. Together with the drastic increase in SphK1 mRNA level after liver injury, these results suggest that SphK1 is highly involved in the differentiation of BMSCs to myofibroblasts. We demonstrated for the first time *in vitro* that TGF-β1-directed regulation of SphK1 is involved in the up-regulation of

Figure 8. The effect of SphK blockade on CCl₄-induced liver fibrosis in mice. **A:** Representative images of Sirius Red staining in the absence (-) or presence (+) of the SphK inhibitor SKI after CCl₄ administration. **Inset:** Sirius Red staining for OO-treated liver. **B:** Representative H&E-stained liver sections in the absence (-) or presence (+) of SKI after CCl₄ administration. **Inset:** H&E staining for OO-treated livers. **C:** For quantitative analysis of liver fibrosis, 15 randomly selected fields were quantitated for each mouse using the Leica Qwin V3 software. Relative hepatic levels of α-SMA (D), Colα1(I) (E), and Colα1(III) (F) were determined by real-time RT-PCR. Data are expressed as means ± SEM. **P* < 0.05 versus control. †*P* < 0.05 versus CCl₄-treated alone. Scale bar = 50 μm (main images and insets).

myofibroblast marker proteins during BMSC differentiation. When TGF- β 1-stimulated increase in SphK1 expression is inhibited with siRNA or a SphK inhibitor, the mRNA expression of α -SMA, Col α 1(I), and Col α 1(III) and the protein expression of α -SMA stimulated by TGF- β 1 are abrogated. These results are in agreement with recent reports that highlight a pivotal role of TGF- β 1-directed SphK1 up-regulation in mediating the prodifferentiating action in different types of fibroblasts and myocytes,^{25–28} and suggests that endogenous S1P production is necessary for BMSC differentiation induced by TGF- β 1.

Importantly, to evaluate the role of SphK1 in BMSC differentiation *in vivo*, we performed an animal experiment by administration of a SphK inhibitor, SKI. We characterized the EGFP-positive cells by staining for α -SMA, with confocal analysis, and determined the proportion of BMSC-derived myofibroblasts. Our results show that SKI significantly blocks BMSC differentiation to myofibroblasts during liver injury, because the proportion of BMSC-derived myofibroblasts decreased markedly, compared with that in the liver without SKI treatment, thus attenuating the extent of liver fibrosis. A recent report indicated that bone marrow-derived cells do not contribute significantly to liver fibrosis.³⁴ In the present study, however, we do observe BMSC-derived myofibroblasts in the injured liver. In addition, reports from several experimental and human studies indicate that bone marrow-derived cells can differentiate to hepatic stellate cells, myofibroblasts, and fibrocytes, and may participate in liver fibrosis.^{8,35}

Although the exact origin of myofibroblasts in liver injury and fibrosis is yet to be determined, it is often presumed that they derive from local activation of residential fibroblasts, hepatic stellate cells, or epithelial-mesenchymal transition. Consistent with previous reports, we confirm that BMSCs contribute to liver fibrosis during CCl₄-induced liver fibrosis. Thus, the profibrotic potential of BMSCs should not be underestimated, in particular when BSMCs are used as therapy for fibrosis or cirrhosis.

GM1 is a ganglioside that activates sphingosine kinase to increase intracellular levels of S1P.³⁶ We used GM1 as a tool to indirectly confirm the important role of SphK1 and S1PRs in BMSC differentiation induced by TGF- β 1. Our data suggest that GM1 is an important, previously unrecognized mediator of liver fibrosis through both direct and indirect effects on BMSC differentiation, and that GM1 markedly increases mRNA and protein expression of SphK1 and stimulates endogenous S1P production in BMSCs. Moreover, like TGF- β 1, GM1 modulates the expression of S1PRs and induces BMSC differentiation via up-regulation of S1PR₁ and S1PR₃, which is in accord with a report that, in mouse cardiomyocytes, S1P generated by GM1 treatment must be exported from the cell and couple with its cognate receptor to exert the biological effects.³⁷ These data support the hypothesis that TGF- β 1 stimulates SphK1 expression, leading to production of endogenous S1P, which is then released and acts on S1PR₁ and S1PR₃ to exert final prodifferentiating effects. Although the reason why GM1 increased S1PR₁ and S1PR₃ expression but decreased S1PR₂ expression is not yet clear, GM1 could be used as a positive control

to further demonstrate the role of SphK1. To our knowledge, this is the first time that GM1 has been shown to mimic TGF- β 1 signaling and to have a direct role in BMSC differentiation, suggesting crosstalk between TGF- β 1 and S1P signaling pathways in BMSCs.

Another novel finding of the present study is that up-regulation of S1PR₁ and S1PR₃ is essential for the differentiation of BMSCs to myofibroblasts. We found that exogenous S1P does not directly induce BMSC differentiation. In contrast, endogenous S1P, generated by TGF- β 1 or GM1, modulates the expression of S1PRs and induces BMSC differentiation. A robust prodifferentiating effect of S1P has been identified in other cellular contexts, including fibroblasts, myoblasts, and retinal pigmented epithelial cells.^{27,38–41} A possible explanation for this discrepancy is the different effects of S1P on its receptors. In the present study, exogenous S1P treatment did not affect the expression of S1PRs; whereas endogenous S1P modified S1PR expression pattern by up-regulation of S1PR₁ and S1PR₃, which were shown to be involved in the final prodifferentiating effects. Further studies are needed to elucidate the exact mechanism of the different effects of exogenous versus endogenous S1P on S1PRs.

The S1PR subtypes are expressed in distinct combinations in different cell types to produce an appropriate biological action. The functional response of each cell to S1P varies, depending on the S1PR repertoire. We previously showed that S1PR₁, S1PR₂, and S1PR₃ are abundant in mouse BMSCs.⁹ In the present study, we analyzed the early intracellular signaling events of BMSC differentiation triggered by TGF- β 1. Our results show that S1PR₁ and S1PR₃ are up-regulated after TGF- β 1 or GM1 stimulation and that their inhibition by selective antagonists or knockdown by specific siRNAs blunts the increase of α -SMA, Col α 1(I), and Col α 1(III) induced by TGF- β 1 or GM1, suggesting the critical role of S1PR₁ and S1PR₃ in the regulation of BMSC differentiation. In addition, we found that the S1PR₁ agonist SEW2871 can mimic the effects of TGF- β 1 by increasing the expression of α -SMA, Col α 1(I), and Col α 1(III). These results are in accord with several published reports, which together support a key role of S1PRs in transmitting the prodifferentiating action of TGF- β 1. For example, S1PR₂ and S1PR₃ were previously identified as downstream of TGF- β 1 regulation of α -SMA expression in lung fibroblasts.²⁶ Furthermore, knockdown of S1PR₂ using siRNA completely suppressed TGF- β 1-stimulated collagen production and differentiation of cardiac fibroblasts to myofibroblasts.²⁷ More recently, Cencetti et al²⁸ reported that S1PR₃ plays a key role in the TGF- β 1-induced differentiation of myocytes to myofibroblasts. Taken together, our findings suggest that up-regulation of S1PR₁ and S1PR₃ by TGF- β 1 or GM1 is required for the differentiation of BMSCs to myofibroblasts. It is possible that TGF- β 1 regulates the differentiation of BMSCs to myofibroblasts by remodeling expression of S1PRs.

The mechanism by which TGF- β 1-related molecules elicit their multiple biological effects is quite complex and has not yet been fully elucidated. Although TGF- β 1 is known to induce these different responses through the same receptor complex and Smad proteins, over the last

few years it has been shown that the cytokine can activate several non-Smad signaling pathways in a cell-type-specific fashion.^{42–46} The Rho/Rho kinase pathway is one of the important pathways for TGF- β 1-induced response in addition to Smad signaling. This pathway is known to be involved in the fibrotic process that affects multiple different tissues^{47–49} and is necessary for mature myofibroblasts.^{26–28} For example, Rho/Rho kinase was involved in S1PR₃-dependent up-regulation of fibrosis marker expression brought about by TGF- β 1 in the differentiation of myocytes to myofibroblasts.²⁸ Furthermore, Rho kinase was also implicated in the mechanism by which SphK1 regulates differentiation of lung fibroblasts mediated by TGF- β 1.²⁶ Consistent with these findings, Gellings Lowe et al²⁷ demonstrated that S1P-stimulated myofibroblast transformation and collagen production are dependent on activation of Rho kinase. In addition, connective tissue growth factor (CTGF) was also shown to be a downstream mediator of TGF- β 1,⁵⁰ and IGFBP3 has been implicated in S1P pathway.⁵¹ However, whether Rho signals or other signaling pathways are implicated in BMSC differentiation remains to be investigated. Here, we have focused on the relationship between the SphK/S1P/S1PR axis and TGF- β 1 during BMSC differentiation to myofibroblasts. Further insight into crosstalk between the Smad, Rho, CTGF, and/or IGFBP3 pathways and the SphK pathway is important for understanding BMSC differentiation induced by TGF- β 1.

In conclusion, with this study we have identified the unique ability of TGF- β 1 to exploit S1P signaling to differentiate BMSCs to myofibroblasts by simultaneously affecting SphK1 and S1PR expression pattern. We thus provide new clues to the molecular mechanisms underlying the differentiation of BMSCs elicited by the cytokine, which may open new perspectives for pharmacological treatment of liver fibrosis.

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