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Intracellular Sphingosine 1-Phosphate Contributes to Collagen Expression of Hepatic Myofibroblasts in Human Liver Fibrosis Independent of Its Receptors

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Sphingosine 1-phosphate (S1P) is involved in multiple pathological processes, including fibrogenesis. S1P participates in mouse liver fibrogenesis via a paracrine manner. Herein, we investigated the involvement of S1P in human liver fibrosis. Human fibrotic samples were obtained from livers of patients undergoing liver transplantation. Expression of sphingosine kinase (SphK1), collagen (Col) $\alpha 1(I)$, Col $\alpha 1(III)$, α -smooth muscle actin, and p-Smad2/3 was characterized by immunofluorescence, real-time RT-PCR, high-content analysis, or Western blot analysis in the fibrotic liver, human bone marrow–derived mesenchymal stem cells, and human hepatogenic profibrotic cells. The effect of SphK1 was assessed using siSphK1 or SphK-specific inhibitor. SphK1, which was expressed in human fibrotic liver myofibroblasts, could be detected in human bone marrow–derived mesenchymal stem cells or human hepatogenic profibrotic cells activated by transforming growth factor $\beta 1$ (TGF- $\beta 1$). TGF- $\beta 1$ evoked the activation of SphK1, increased intracellular S1P, and up-regulated expression of SphK1, Col $\alpha 1(I)$, and Col $\alpha 1(III)$ in a TGF- β receptor–dependent manner. TGF- $\beta 1$ induced expression of Col $\alpha 1(I)$ and Col $\alpha 1(III)$ via SphK1, which was mediated by intracellular S1P, independent of S1P receptors. TGF- $\beta 1$ evoked nuclear translocation of p-Smad2 and p-Smad3 in TGF- β receptor–dependent, but SphK1-independent, manner. In conclusion, intracellular S1P plays a crucial role in the TGF- $\beta 1$ –induced expression of Col $\alpha 1(I)$ and Col $\alpha 1(III)$, which is required for human fibrosis development. S1P exerts its effects in S1P receptor–independent manner. (*Am J Pathol* 2015, 185: 387–398; <http://dx.doi.org/10.1016/j.ajpath.2014.09.023>)

Sphingosine 1-phosphate (S1P) is a bioactive lipid that regulates vital physiological and pathological actions.¹ There is much evidence demonstrating that S1P is a pivotal regulator of fibrosis diseases, including cardiac fibrosis, pulmonary fibrosis, renal fibrosis, systemic sclerosis, and liver fibrosis.^{2–6} It has been recently reported that S1P participates in mouse hepatic myofibroblast activation and rat hepatocyte apoptosis, which contribute to liver fibrosis.^{7,8} Under normal conditions, S1P is produced by the phosphorylation of sphingosine, which is catalyzed by sphingosine kinase (SphK). S1P can function as an intracellular second messenger. It has been demonstrated that S1P binds and alters the functions of several intracellular proteins, such as histone deacetylases, tumor necrosis factor (TNF) receptor–associated factor 2, and ceramide synthase

2, which influence diverse biological actions.^{9–11} S1P can also be secreted into the extracellular space, providing access to S1P receptors (S1PRs) on the cell surface, thereby allowing paracrine signaling of S1P.¹² After binding to S1PRs, S1P activates diverse downstream signaling pathways and induces diverse cellular responses.^{7,13}

Liver fibrosis is characterized by the excessive deposition of collagen (Col), especially Col $\alpha 1(I)$ and Col $\alpha 1(III)$.^{14,15} Myofibroblasts are the crucial collagen-producing cells during liver fibrosis.¹⁶ Comprehensive sources contribute to myofibroblasts

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in liver, including hepatic stellate cells (HSCs), portal fibroblasts, bone marrow–derived mesenchymal stem cells (BMSCs), and epithelial mesenchymal transition from hepatocytes and cholangiocytes.¹⁵ HSCs were separated from fat-storing cells from outgrowth of liver explants.^{17,18} But, in general, these cells are a mixture of HSCs, portal myofibroblasts, and fibroblasts. We named these cells as hepatogenic profibrotic cells (HPCs). HPCs and BMSCs are thought to be the main sources of hepatic myofibroblasts.^{19–21} Thus, it is interesting to understand the regulatory mechanisms underlying the collagen production in BMSCs and HPCs. It has been proved that the synthesis of collagen is highly regulated by different factors and cytokines at various levels,²² and transforming growth factor (TGF)- β 1 is a central mediator of collagen production.^{23–26} TGF- β 1 usually exerts its functions by canonical TGF- β receptor (TGF β R)/Smad pathway.²⁷ TGF- β binds to its receptor TGF β R, finally resulting in the phosphorylation of Smad2/3, which then associate with Smad4 and translocate into the nucleus to control transcriptional gene expression in chondrocytes, fibroblasts, or hepatocytes, resulting in extracellular matrix production or experimental liver fibrosis.^{28–31}

Previously, we have shown that S1P is crucial in liver fibrosis,^{7,32–36} and the mode of action of S1P is diverse because of the differences in various species. It has been reported that S1P participates in the differentiation of mouse BMSCs to myofibroblasts via S1PR₁ and S1PR₃ up-regulation.⁷ But, in human BMSCs (hMSCs), collagen expression is regulated negatively by S1PR₁ and S1PR₃.³⁷ These results elucidate the disparity between human and animal experiments, and strongly suggest the necessity of verifying the experiments performed in animal models in a human context. Previously, we found that TGF- β 1 induces activation of mouse BMSCs via SphK1 up-regulation, and this process is S1PR dependent.⁷ But, the operating mechanisms in human cells are still unclear. To explore the molecular mechanisms of human liver fibrosis, it is necessary to study the mode of action of S1P in the expression of collagen in primary human HPCs (hHPCs) and hMSCs.

Herein, we demonstrate, for the first time, that the production of Col α 1(I) and Col α 1(III) is increased by both activation and up-regulation of SphK1, which elevates intracellular S1P in hMSCs and hHPCs after TGF- β 1 treatment. In addition, TGF- β 1–induced expression of SphK1 and collagen is TGF β R dependent. Moreover, phosphorylations of Smad2 or Smad3 are SphK1 independent. More important, these results represent the first experimental evidence that S1P participates in the production of collagens in an S1PR-independent manner, and unravel a novel mode of action of intracellular S1P in the process of human liver fibrogenesis.

Materials and Methods

Human Liver Specimen and Serum

Snap-frozen surgical liver resections from 21 patients (13 men and 8 women; mean age, 56 years; age range, 42 to 69 years)

were studied retrospectively. Normal liver samples were collected from five patients undergoing hepatic resection for colorectal metastasis. Fibrotic samples (fibrosis stage: F2 to F4) were obtained from 16 livers of patients undergoing liver transplantation. Fibrosis was consecutive to chronic hepatitis C virus ($n = 4$) or hepatitis B virus ($n = 10$) infections, and alcohol-induced liver disease ($n = 2$). Normal serum samples were collected from 16 healthy volunteers (10 men and 6 women; mean age, 46 years; age range, 36 to 58 years), and fibrotic serum samples were obtained from 17 patients (11 men and 6 women; mean age, 49 years; age range, 40 to 65 years) with liver fibrosis. All tissues were obtained with donor consent and the approval of the Capital Medical University (Beijing, China) Ethics Committee (2011SY08).

Cell Isolation and Culture

hMSCs (Cyagen, Sunnyvale, CA) were cultured as previously described.³⁷ hMSCs of passage 6 to passage 10 were used in the experiments per the user manual recommendations. hHPCs were obtained and cultured by outgrowth of explants prepared from surgical specimens of normal livers, as previously described.³⁸ This procedure was performed in accordance with ethical regulations imposed by the Chinese legislation. Cells were used between the fourth and ninth passage for experiments, without any noticeable differences in results observed with cells obtained from various passages or from various livers. Unless otherwise indicated, cells prepared for experiments were cultured in medium without serum for 24 hours and treated with TGF- β 1 (PeproTech, London, UK), TGF β R1 inhibitor (LY364947; Calbiochem, La Jolla, CA), *N,N*-dimethylsphingosine (DMS; Biomol, Hamburg, Germany), S1P and dihydro-S1P (H₂S1P; Biomol, Tebu, France), W146 (Avanti Polar Lipids, Alabaster, AL), and JTE-013 and CAY10444 (Cayman Chemical, Ann Arbor, MI).

Immunofluorescence

Immunofluorescence of cultured hMSCs or hHPCs was performed as described.³⁶ Cells were incubated with antibodies of anti-Col α 1(I), Col α 1(III), SphK1 (dilution 1:50; Santa Cruz Biotechnology, Santa Cruz, CA), p-Smad2 (dilution 1:50; Cell Signaling, Beverly, MA), p-Smad3 (dilution 1:50; Santa Cruz Biotechnology), or α -smooth muscle actin (α -SMA; dilution 1:1000; Sigma, St. Louis, MO), followed by secondary antibodies conjugated with Cy3 or fluorescein isothiocyanate (diluted 1:100; Jackson Immunoresearch, West Grove, PA).

Double staining of SphK1 and α -SMA was performed in human liver sections. Immunofluorescence was performed as described.⁷ Sections were first incubated with anti-SphK1 antibody described above, and incubated with Cy3-conjugated AffiniPure donkey anti-goat IgG antibody (diluted 1:100; Jackson Immunoresearch, West Grove, PA) as a secondary antibody. Next, sections were incubated with anti- α -SMA

antibody described above, and incubated with fluorescein isothiocyanate–conjugated AffiniPure goat anti-mouse IgG antibody (diluted 1:100) as a secondary antibody. Sections or cells were incubated without primary antibodies as negative control.

High Content Analysis

hMSCs ($n = 5000$) and hHPCs ($n = 6000$) were plated in the wells of 96-well plates (Corning, NY), attached overnight, and then treated with chemicals. Immunofluorescence staining for Col $\alpha 1$ (I), Col $\alpha 1$ (III), p-Smad2, or p-Smad3 was performed as described above. The plates were imaged on Thermo Scientific CellInsight personal cell imaging platform (Cellomics, Inc., Thermo Fisher Scientific Inc., Waltham, MA). High content analysis was performed as described previously.³⁷

Real-Time RT-PCR

Extraction of total RNA and real-time RT-PCR was performed as described previously.³² Primers were as follows: 18S rRNA, 5'-GTAACCCGTTGAACCCATT-3' (sense) and 5'-CCATCCAATCGGTAGTAGCG-3' (anti-sense); Col $\alpha 1$ (I), 5'-AGGTCCCCCTGGAAAGAA-3' (sense) and 5'-AATCC-TCGAGCACCCTGA-3' (anti-sense); Col $\alpha 1$ (III), 5'-AGCT-GGAAAGAGTGGTGACAG-3' (sense) and 5'-CCTTGAG-GACCAGGAGCAC-3' (anti-sense); S1PR₁, 5'-TCTGCGG-GAAGGGAGTATGT-3' (sense) and 5'-CGATGGCGAG-GAGACTGAA-3' (anti-sense); S1PR₂, 5'-TGCCCGCCTT-CAGCAT-3' (sense) and 5'-AAAAAGTAGTGGGCTT-TGTAGAGGAT-3' (anti-sense); and S1PR₃, 5'-TCTCAG-CCTTCATCCATTAACCTCTAC-3' (sense) and 5'-AGG-GAGCCTTATGTCATACCACAA-3' (anti-sense). Probe (Life Technologies, Foster City, CA) used for real-time RT-PCR was as follows: SphK1: Hs00184211_m1.

Western Blot Analysis

Western blot analysis was performed as described.⁷ The blots were incubated with primary antibodies against SphK1 or p-SphK1 (dilution 1:1000; ECM Biosciences, Versailles, KY) or Col $\alpha 1$ (I) or Col $\alpha 1$ (III) (dilution 1:500; Santa Cruz Biotechnology). Results were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (rabbit anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody, dilution 1:1000; Sigma) or tubulin (rabbit monoclonal anti-tubulin antibody, dilution 1:1000; Epitomics, Burlingame, CA) expression to correct for variations in protein loading and transfer.

RNA Interference

The siRNA sequences specifically targeting human SphK1, S1PR₁, S1PR₂, S1PR₃, or negative control siRNA were synthesized (S16957, S4448, S17766, S4455, or 4390843, respectively; Ambion, Austin, TX). RNA interferences of hMSCs or hHPCs were performed as described.⁷

TGF- $\beta 1$ Enzyme-Linked Immunosorbent Assay

TGF- $\beta 1$ concentrations in human serum were determined using commercially specific enzyme-linked immunosorbent assay and were processed according to the manufacturer's specifications (Promega, Charbonnières, France).

Measurement of S1P by High-Performance Liquid Chromatographic Analysis

hMSCs and hHPCs, with or without stimulation of 10 ng/mL TGF- $\beta 1$, were collected. The preparation and analysis of sample were performed as described.³⁵ Sample analysis was performed using Agilent 1200 high-performance liquid chromatographic equipment (Agilent Technologies, Wilmington, DE).

Statistical Analysis

All results were confirmed in at least three independent experiments. Data were expressed as means $\pm$ SEM. Statistical analyses were performed by Student's *t*-test or analysis of variance when appropriate. Statistical significance was defined as $P < 0.05$.

Results

Human Hepatic Myofibroblasts Express SphK1 during Liver Fibrosis

It is well established that myofibroblasts are the principal effector cells in fibrotic liver.¹⁵ In liver tissue sections of patients with liver fibrosis, there was strong immunoreactivity for α -SMA, a marker for myofibroblasts, in the fibrotic areas (Figure 1B). Double-immunofluorescence staining for SphK1 and α -SMA showed that the numbers of α -SMA–positive cells in the fibrotic areas were significantly positive for SphK1 (Figure 1B). We also detected the basal expression of SphK1 and α -SMA in normal human liver tissue sections (Figure 1A); there was basal expression of SphK1 and α -SMA in the portal area, in the vascular endothelial cells (SphK1), and in smooth muscle cells (α -SMA), respectively.

TGF- $\beta 1$ is a well-known profibrotic mediator that plays an important role in activation of myofibroblasts.¹⁵ In serum of patients with liver fibrosis, the level of TGF- $\beta 1$ was increased by approximately 1.6-fold (Figure 1C). Hence, we performed immunofluorescence of α -SMA and SphK1 in TGF- $\beta 1$ –stimulated hMSCs or hHPCs, the main sources of human myofibroblasts, *in vitro*. There was strong expression of both α -SMA and SphK1 in these activated myofibroblasts (Figure 1D). These results showed that SphK1 probably participated in activation of myofibroblasts.

The Activation and Expression of SphK1 Are Evoked by TGF- $\beta 1$ in hMSCs and hHPCs

TGF- $\beta 1$ has been shown to elevate expression of SphK1 in many cell types.^{2,5,7,39} To explore the compliance of SphK1

Figure 1 Sphingosine kinase (SphK1) is expressed in human fibrotic livers, activated human bone marrow–derived mesenchymal stem cells (hMSCs), or human hepatogenic profibrotic cells (hHPCs). Representative images of immunofluorescence analysis to track the expressions of SphK1 and α -smooth muscle actin (α -SMA) in normal human liver (**A**) and patient fibrotic liver (**B**). **C**: Transforming growth factor β 1 (TGF- β 1) level in serum. **D**: Immunofluorescence analysis of SphK1 and α -SMA in activated hMSCs and hHPCs. DAPI was used to visualize nuclei (blue). * P < 0.05 versus normal group. Scale bars: 50 μ m (**A**, and **B**, top panels); 5 μ m (**B**, bottom panels); 25 μ m (**D**).

to TGF- β 1 in hMSCs and hHPCs, mRNA and protein levels of SphK1 were evaluated by real-time RT-PCR and Western blot analysis. Cells were treated with increasing concentrations of TGF- β 1 (1, 2, 5, 7.5, or 10 ng/mL), which induced a dose-dependent increase in the mRNA expression of SphK1 (Figure 2A). SphK1 mRNA expression increased in a time-dependent manner at 10 ng/mL TGF- β 1, with a maximal increase at 24 hours in both hMSCs and hHPCs (Figure 2B). On the basis of these experiments, stimulation by 10 ng/mL TGF- β 1 for 24 hours was adopted in the following experiments, unless otherwise stated. Likewise, Western blot analysis revealed that TGF- β 1 markedly up-regulated total protein level of SphK1 at 24 hours in the two cell types (Figure 2C). SphK1 activity is regulated by its phosphorylation.⁴⁰ Consequently, we used Western blot analysis to examine the level of activated SphK1 and p-SphK1 in hMSCs or hHPCs treated with TGF- β 1. TGF- β 1 markedly elevated the level of p-SphK1, with a significant increase

after 15 minutes (Figure 2D). Because S1P was the downstream metabolite of SphK1,⁴¹ we examined whether the concentration of S1P was affected by TGF- β 1. High-performance liquid chromatographic analysis revealed that the level of S1P was significantly increased by approximately 1.6- or 1.8-fold in hMSCs and hHPCs stimulated by TGF- β 1, respectively (Figure 2E).

Together, these data demonstrated that activity and expression of SphK1 were elevated by TGF- β 1 in both hMSCs and hHPCs.

SphK1 Plays a Vital Role in TGF- β 1–Induced Expression of Collagens in hMSCs and hHPCs

Because deposition of collagen was an important feature in the fibrogenic functions of myofibroblasts, we assessed the ability of TGF- β 1 to stimulate collagen production. We first used high content analysis to measure protein levels of

Figure 2 Transforming growth factor β 1 (TGF- β 1) stimulates sphingosine kinase (SphK1) mRNA and protein expression in human bone marrow–derived mesenchymal stem cells (hMSCs) or human hepatogenic profibrotic cells (hHPCs). **A:** mRNA expression of SphK1 in hMSCs or hHPCs treated with different concentrations of TGF- β 1 for 24 hours. **B:** mRNA expression of SphK1 in hMSCs or hHPCs treated with 10 ng/mL TGF- β 1 and collected at different times. **C:** SphK1 protein expression in hMSCs or hHPCs treated with 10 ng/mL TGF- β 1. **D:** p-SphK1 protein in hMSCs or hHPCs after TGF- β 1 treatment. **E:** Sphingosine 1-phosphate (S1P) level in hMSCs or hHPCs, measured by high-performance liquid chromatography. All results were confirmed in at least three independent experiments. * $P < 0.05$ versus untreated control (Ctrl) cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

collagens in hMSCs and hHPCs stimulated by TGF- β 1 or vehicle. Immunofluorescence showed that slight immunoreactivities for Col α 1(I) and Col α 1(III) were detected in both untreated hMSCs and hHPCs, respectively, whereas strong immunoreactivities for Col α 1(I) and Col α 1(III) were detected in both hMSCs and hHPCs stimulated by TGF- β 1, respectively (Figure 3A). Furthermore, mature collagen, which was secreted into the medium, was also detected by immunofluorescence (Figure 3A). Mean immunofluorescence intensity, which was tested by high content analysis and reflected collagen protein level, demonstrated a pronounced increase in response to TGF- β 1 (Figure 3B). Western blot analyses were also used to verify the protein levels of Col α 1(I) and Col α 1(III) stimulated by TGF- β 1, which showed an obvious increase in the protein expression of Col α 1(I) and Col α 1(III) in hMSCs and hHPCs, respectively (Figure 3C). We then used real-time RT-PCR to measure mRNA levels of Col α 1(I) and Col α 1(III) in hMSCs and hHPCs stimulated by TGF- β 1, respectively. TGF- β 1 induced a dose-dependent increase in the mRNA expression of Col α 1(I) or Col α 1(III), with a maximal increase at 10 ng/mL (Figure 3D). These results revealed that TGF- β 1 prompted human hepatic myofibroblasts producing more collagens.

To verify the effects of SphK1 on the productions of Col α 1(I) and Col α 1(III) of activated human hepatic myofibroblasts, we used siRNA against SphK1 or a specific SphK inhibitor, DMS, in hMSCs and hHPCs. After knockdown of SphK1, SphK1 mRNA expression was inhibited by 82% and 84% in hMSCs and hHPCs, respectively (Figure 4A). Meanwhile, SphK1 protein expression was reduced after knockdown of SphK1, measured by Western blot analysis (Figure 4B). Moreover, TGF- β 1–induced protein expression of Col α 1(I) and Col α 1(III), which was tested by Western blot (Figure 4B) or high content analysis (Figure 4C), was markedly inhibited in SphK1 knockdown cells. In addition, TGF- β 1–induced mRNA expression of Col α 1(I) and Col α 1(III), which was tested by real-time RT-PCR, was also significantly reduced in SphK1 knockdown cells, whereas scrambled siRNA had no effect (Figure 4D). In accord with these results, TGF- β 1–stimulated protein and mRNA expression of Col α 1(I) and Col α 1(III) was also blocked by 5 μ mol/L DMS (Figure 4, E–G). These results indicated that TGF- β 1–induced up-regulation of collagen was SphK1 dependent.

Figure 3 Collagen (Col) is up-regulated in transforming growth factor $\beta 1$ (TGF- $\beta 1$)-stimulated human bone marrow–derived mesenchymal stem cells (hMSCs) or human hepatogenic profibrotic cells (hHPCs). **A:** Immunofluorescence analysis of Col $\alpha 1$ (I) and Col $\alpha 1$ (III) in hMSCs or hHPCs. DAPI was used to visualize nuclei (blue). The **arrowheads** indicate mature collagen, which was secreted into the medium. **B:** Immunofluorescence intensity analysis of Col $\alpha 1$ (I) and Col $\alpha 1$ (III) in response to TGF- $\beta 1$ in hMSCs or hHPCs. **C:** Western blot analyses of Col $\alpha 1$ (I) and Col $\alpha 1$ (III) in response to TGF- $\beta 1$ in hMSCs or hHPCs. **D:** mRNA expression of Col $\alpha 1$ (I) and Col $\alpha 1$ (III) treated with the different concentrations of TGF- $\beta 1$ for 24 hours. All results were confirmed in at least three independent experiments. * $P < 0.05$ versus untreated control cells. Scale bar = 25 μ m (**A**).

TGF- $\beta 1$ –Induced Expression of Collagens Is Mediated by Intracellular S1P, Independent of S1PRs

Furthermore, we explored the mode of action of S1P on collagen expression of hepatic myofibroblasts. Initially, 1 μ mol/L exogenous S1P, which mainly functions via binding to S1PRs, had no detectable effect on the production of Col $\alpha 1$ (I) and Col $\alpha 1$ (III). However, 10 μ mol/L exogenous S1P, which distinctly increases intracellular S1P level,^{10,42} enhanced the mRNA expression of Col $\alpha 1$ (I) and Col $\alpha 1$ (III) (Figure 5A). The above results suggested that TGF- $\beta 1$ –induced collagen expression was mediated by intracellular S1P. To confirm our speculation, H₂S1P, a structural analog of S1P that binds and activates S1P receptors with no

intracellular effects,³¹ was used in hMSCs and hHPCs. Results showed that neither 1 nor 10 μ mol/L exogenous H₂S1P potentiated the mRNA expression of Col $\alpha 1$ (I) and Col $\alpha 1$ (III) (Figure 5B). These results further attested that up-regulation of collagen expression was mediated by intracellular S1P. Moreover, 0.5 μ mol/L W146 (a specific antagonist of S1PR₁), 5 μ mol/L JTE013 (a specific antagonist of S1PR₂), and 5 μ mol/L CAY10444 (a specific antagonist of S1PR₃) were used corporately to further verify the mode of action of S1P in hMSCs and hHPCs. Combined application of S1PR antagonists did not inhibit TGF- $\beta 1$ –induced collagen production (Figure 5C). To further confirm our conclusion, siS1PR₁₋₃ were used in the tests. After knock-down of S1PR₁₋₃, S1PR₁₋₃ mRNA expression was inhibited

Figure 4 Sphingosine kinase (SphK1) is involved in transforming growth factor β 1 (TGF- β 1)-induced collagen (Col) production in human bone marrow–derived mesenchymal stem cells (hMSCs) and human hepatogenic profibrotic cells (hHPCs). Cells were transfected with Scrambled (SCR) siRNA or SphK1 siRNA for 48 hours. **A**: mRNA expression of SphK1 was then evaluated by real-time RT-PCR. **B**: Protein expression of SphK1, Col α 1(I), and Col α 1(III) in response to TGF- β 1 in the presence of SphK1 siRNA, evaluated by Western blot analyses. **C** and **D**: mRNA and protein expression of Col α 1(I) and Col α 1(III) in response to TGF- β 1 in the presence of SphK1 siRNA measured by real-time RT-PCR and high content analysis. **E**: Protein expression of Col α 1(I) and Col α 1(III) in response to TGF- β 1 in the presence of *N,N*-dimethylsphingosine (DMS), evaluated by Western blot analysis. **F** and **G**: Protein and mRNA expression of Col α 1(I) and Col α 1(III) in response to TGF- β 1 in the presence of DMS measured by high content analysis and real-time RT-PCR. All results were confirmed in at least three independent experiments. * P < 0.05 versus untreated control cells; † P < 0.05 versus TGF- β 1–treated cells alone.

by 72%, 80%, and 78% in hMSCs (Figure 5D) and by 88%, 70%, and 76% in hHPCs (Figure 5E), respectively. TGF- β 1–induced mRNA expression of Col α 1(I) and Col α 1(III), which was tested by real-time RT-PCR, was not significantly

reduced in S1PR₁₋₃ knockdown cells (Figure 5F). These results corporately confirmed that TGF- β 1–induced collagen expression was mediated by intracellular S1P, independent of S1PRs.

Figure 5 Intracellular sphingosine 1-phosphate (S1P) mediates collagen (Col) production of human bone marrow–derived mesenchymal stem cells (hMSCs) and human hepatogenic profibrotic cells (hHPCs) induced by transforming growth factor β1 (TGF-β1), independent of S1P receptors (S1PRs). mRNA expressions of Col α1(I) and Col α1(III) in response to three concentrations of S1P (**A**) and dihydro-S1P (H₂S1P; **B**) in hMSCs and hHPCs. **C**: hMSCs or hHPCs were pretreated with 0.5 μmol/L W146 (W), 5 μmol/L JTE013 (J), and 5 μmol/L CAY10444 (C), for 1 hour before 10 ng/mL TGF-β1 for 24 hours. mRNA expression of Col α1(I) and Col α1(III) in response to TGF-β1 in the presence of S1PR antagonists. **D** and **E**: Cells were transfected with SCR siRNA or S1PR₁₋₃ siRNAs for 48 hours. mRNA expression of S1PR₁₋₃ was then evaluated by real-time RT-PCR. **F**: mRNA expression of Col α1(I) and Col α1(III) in response to TGF-β1 in the presence of S1PR₁₋₃ siRNAs. All results were confirmed in at least three independent experiments. **P* < 0.05 versus untreated control cells.

SphK1/S1P and TGF-β1/TGFβR/Smad Signaling Pathways Function Independently in hMSCs and hHPCs

To deeply explore the mechanism underlying the activation of SphK1 and up-regulation of collagens induced by TGF-β1 in hMSCs and hHPCs, TGFβR1 inhibitor was used. TGF-β1–induced mRNA expression of SphK1 could be reversed by TGFβR1 inhibitor, LY364947, in hMSCs and hHPCs (Figure 6A). In addition, TGF-β1–induced mRNA

expression of Col α1(I) and Col α1(III) was also inhibited by LY364947 in the two cell types (Figure 6B). These results showed that TGF-β1 activated SphK1 and up-regulated collagen expression via TGFβR in hMSCs and hHPCs. As downstream proteins of TGF-β, Smad2 and Smad3 can be phosphorylated and transferred to nucleus, where p-Smad2 and p-Smad3 exert biological functions. To evaluate phosphorylation and nuclear accumulation of Smad2 or Smad3, nuclear/cytoplasmic ratios for p-Smad2 or

Figure 6 Sphingosine kinase (SphK1)/sphingosine 1-phosphate (S1P) and transforming growth factor β 1 (TGF- β 1)/TGF β receptor (TGF β R)/Smad signaling pathways function independently in human hepatic myofibroblasts. mRNA expression of SphK1 (**A**), and collagen (Col) α 1(I) and Col α 1(III) (**B**) in response to TGF- β 1 in the presence of LY364947. **C**: Ratios of nuclear/cytoplasmic p-Smad2/3 in human bone marrow-derived mesenchymal stem cells (hMSCs) in response to TGF- β 1. **D**: The representative images of immunostaining analysis for nuclear translocations of p-Smad2/3 in response to TGF- β 1 in the presence of DMS or LY364947. **E**: Scheme of human hepatic myofibroblast activation. TGF- β 1 activates SphK1 via TGF β R, resulting in increased level of intracellular S1P, which affects the production of collagen in an S1P receptor (S1PR)—independent manner. On the other hand, TGF- β 1 phosphorylates Smad2/3 and stimulates nuclear translocations of p-Smad2/3 via TGF β R, which cannot be affected by SphK1. Scale bar = 25 μ m. All results were confirmed in at least three independent experiments. * P < 0.05 versus untreated control cells; † P < 0.05 versus TGF- β 1-treated cells alone. DMS, dimethyl-sphingosine; hHPC, human hepatogenic profibrotic cell.

p-Smad3 in hMSCs were detected by high content analysis. Results showed that TGF- β 1 markedly enhanced phosphorylation and nuclear accumulation of p-Smad2 and p-Smad3, with a maximal increase at 30 minutes (Figure 6C). We then used DMS and LY364947 to further examine the effects of SphK1 and TGF- β 1 on Smad signaling. As immunofluorescence showed, TGF- β 1-induced nuclear translocations of p-Smad2 and p-Smad3 were not affected by DMS, but were inhibited by LY36497 (Figure 6D). These results suggested that TGF- β 1/TGF β R/Smad signaling functions independent of SphK1 in human hepatic myofibroblasts.

Discussion

Activation of fibrogenic cells and their collagen expression are the critical events in the progression of liver fibrosis. The results presented showed that activity and expression of SphK1 were enhanced in human hepatic fibrogenesis. In addition, we identified a novel SphK1-dependent mechanism by which TGF- β 1 mediated collagen expression of human hepatic myofibroblasts. Most of all, we demonstrated, for the first time, that TGF- β 1 induced production of collagens in a SphK1/S1P-dependent, but S1PR-independent, manner in hMSCs and hHPCs.

Many molecules have been reported to stimulate collagen synthesis during fibrogenesis, such as TGF- β , platelet-derived growth factor, interleukin, TNF- α , and connective tissue growth factor.¹⁵ SphK1 can be activated by most of these factors.⁴³ During the process of hepatic fibrogenesis, TGF- β and platelet-derived growth factor are key factors that mediate SphK1 activation.^{14,44} But, these growth factors mediate hepatic fibrogenesis via different mechanisms. Platelet-derived growth factor mainly functions as a potent mitogen of myofibroblasts, whereas TGF- β primarily stimulates collagen production in myofibroblasts.⁴⁵ Moreover, TGF- β is considered to be the most powerful mediator of HSC activation *in vitro* and *in vivo*.^{14,44} Hence, we used TGF- β 1 to study the molecular mechanism underlying the collagen expression of hHPCs and hMSCs. It has been shown that TGF- β 1 can evoke up-regulation of SphK1 in various cells,^{2,5,7,39} but the mechanism has not been well elaborated. Herein, we demonstrated, for the first time, that SphK1 could be regulated by TGF- β 1 in a biphasic manner in hMSCs and hHPCs. The first phase of activation was rapid (minutes) and transient, via post-translational modification, which increased enzymatic activity. The second phase of regulation lasted for 24 hours, in which total protein expression of SphK1 was elevated.

S1P can act on S1PRs on the cell surface or intracellular target sites. Our previous research revealed that S1P played a pivotal role in differentiation of mouse BMSCs in a paracrine manner, specifically S1PR_{1/3} dependent.⁷ However, the present research in hMSCs and hHPCs showed that TGF- β 1-induced collagen expression was

mediated by intracellular S1P, independent of S1PRs. Exogenous S1P in high concentration increased its intracellular level and elevated the production of collagens in hMSCs and hHPCs, whereas H₂S1P or S1P in low concentration had no effect. Moreover, TGF- β 1-induced collagen production was not affected by S1PR₁₋₃ antagonists or siS1PR₁₋₃, which further confirmed that TGF- β 1-stimulated production of collagens is S1PR independent.

The differences between humans and animals can be observed in many experiments. For example, our previous research showed that S1PR₁₋₃ levels were expressed in hHSCs,³⁴ whereas there is no S1PR₃ expression in rat HSCs.⁴⁶ In addition, S1PR₁ and S1PR₃ were strongly induced in human fibrotic samples, whereas expression of S1PR₂ was massively decreased.³⁴ But, in mouse fibrotic liver, only high expression of S1PR₃ could be detected.^{7,33} Thus, we explored the mode of action of S1P in human liver samples and human primary cells.

In recent years, there are many studies about the intracellular target proteins of S1P.^{47,48} The first identified intracellular target protein is ceramide synthase 2.¹¹ It has been shown that S1P, produced by SphK2 in the nucleus, specifically binds to histone deacetylases 1/2, inhibits their activities, and, consequently, modifies gene transcription.⁹ The p21-activated protein kinase 1 and prohibitin-2 are also deemed to be intracellular targets of S1P.^{49,50} Another study shows that S1P can directly bind to cytosolic TNF receptor-associated factor 2, which is an important factor in the NF- κ B pathway.¹⁰ Interestingly, several of these intracellular targets of S1P have been proved to be associated with fibrosis.⁴² For example, TNF receptor-associated factor 2 is elevated in keloid fibroblasts, which are relative to skin fibroblasts and may be involved in the up-regulation of anti-apoptotic genes.⁵¹ TNF receptor-associated factor 2 is also connected with the CD40-stimulated production of cytokines and chemoattractants in HSCs.⁵² However, the mechanism underlying intracellular S1P participating in liver fibrosis is scarce and should be valued in further research.

In conclusion, our data provide evidence for a new paradigm for TGF- β -evoked non-Smad signaling. In this scheme (Figure 6E), TGF- β 1 activates SphK1 via TGF β R, resulting in an increased level of intracellular S1P, which affects the production of collagen in an S1PR-independent manner. On the other hand, TGF- β 1 phosphorylates Smad2/3 and stimulates nuclear translocations of p-Smad2/3 via TGF β R, which cannot be affected by SphK1. More important, our studies suggest that intracellular S1P is an important mediator that participates in progression of fibrosis during human liver diseases.

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