Research Article

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# Sphingosine 1-phosphate (S1P)/S1P receptors are involved in human liver fibrosis by action on hepatic myofibroblasts motility

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**Background & Aims**: Directed migration of hepatic myofibroblasts (hMFs) contributes to the development of liver fibrosis. However, the signals regulating the motility of these cells are incompletely understood. We have recently shown that sphingosine 1-phosphate (S1P) and S1P receptors (S1PRs) are involved in mouse liver fibrogenesis. Here, we investigated the role of S1P/S1PRs signals in human liver fibrosis involving motility of human hMFs.

**Methods**: S1P level in the liver was examined by high-performance liquid chromatography. Expression of S1PRs was characterized, in biopsy specimens of human liver and cultured hMFs, by immunofluorescence and real-time RT-PCR or Western blot analysis. Cell migration was determined in Boyden chambers, by using the selective S1P receptor agonist or antagonist and silencing of S1PRs expression with small interfering RNA.

**Results**: S1P level in the human fibrotic liver was increased through up-regulation of sphingosine kinase (SphK), irrespective of the etiology of fibrosis. S1P receptors type 1, 2, and 3 ($S1P_{1,2,3}$) were expressed in human hMFs *in vivo* and *in vitro*. Interestingly, $S1P_{1,3}$ were strongly induced in human fibrotic samples, whereas expression of $S1P_2$ was massively decreased. S1P exerted a powerful migratory action on human hMFs. Furthermore, the effect of S1P was mimicked by SEW2871 (an $S1P_1$ agonist), and blocked by suramin (an $S1P_3$ antagonist) and by silencing $S1P_{1,3}$ expression. In contrast, JTE-013 (an $S1P_2$ antagonist) and silencing of $S1P_2$ expression enhanced S1P-induced migration.

**Conclusions**: SphK/S1P/S1PRs signaling axis plays an important role in human liver fibrosis and is involved in the directed migration of human hMFs into the damaged areas.



Keywords: Liver fibrosis; Cell migration; Sphingosine 1-phosphate; Sphingosine 1-phosphate receptors; Hepatic myofibroblasts.

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*Abbreviations:* hMFs, hepatic myofibroblasts; S1P, sphingosine 1-phosphate; $S1P_{1-5}$, S1P receptor type 1–5; S1PRs, S1P receptors; SphK, sphingosine kinase; SphK1 and 2, SphK type 1 and 2; PDGF, platelet-derived growth factor; siRNA, small interfering RNA; α-SMA, smooth muscle-actin.

## Introduction

Liver fibrosis is the common response to chronic liver injury and is characterized by excessive deposition of collagen and other components of the extracellular matrix. Hepatic myofibroblasts (hMFs) play a central role in the development of liver fibrosis during chronic liver diseases [1–4]. Several lines of evidence indicate that during the fibrogenic process, the directed migration of hMFs enhances their accumulation at the sites of tissue repair, where they proliferate, secrete proinflammatory cytokines, and chemokines, and synthetise large amounts of matrix components [5]. Therefore, understanding the mechanisms of hMFs motility is important for designing therapeutic strategies aimed at the reduction of liver fibrosis. Previous studies have shown that platelet-derived growth factor (PDGF) and monocyte chemotactic protein-1 are potent stimulators of chemotaxis for hMFs or activated hepatic stellate cells [6–9]. However, the picture of the extracellular cues, which are recognized to be able to regulate hMFs motility, is far to be completed.

Sphingosine 1-phosphate (S1P), a pleiotropic lysophospholipid mediator, is converted primarily from sphingosine by sphingosine kinase (SphK) and stimulates multiple signaling pathways resulting in a wide variety of important cellular processes, including cell proliferation, differentiation, motility, and survival [10–12]. There are two mammalian isoforms of this lipid, termed SphK types 1 and 2 (SphK1 and SphK2) [13]. They differ in sequence, catalytic properties, localization, and functions [14]. The concentration of S1P in cells is normally low and tightly regulated by the equilibrium between its formation, catalyzed by SphK, and its degradation, catalyzed by S1P lyase and S1P phosphatase [15]. Of note, most of the characterized actions of S1P are mediated through a family of five G protein-coupled receptors named $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ [13]. These S1P receptors (S1PRs) couple to distinct heterotrimeric G-proteins and regulate diverse downstream signaling properties and ultimately various cell behaviors [13].

One of the broader functions of extracellular S1P is the regulation of cell motility [16–21]. Recently, it has been demonstrated that S1P plays a critical role in lymphocyte egress from secondary lymphoid tissues and the thymus [22–24].

We have recently shown that, following acute and/or chronic liver injury induced by carbon tetrachloride injection or bile duct ligation (BDL) in mice, S1P levels in liver tissue and serum were

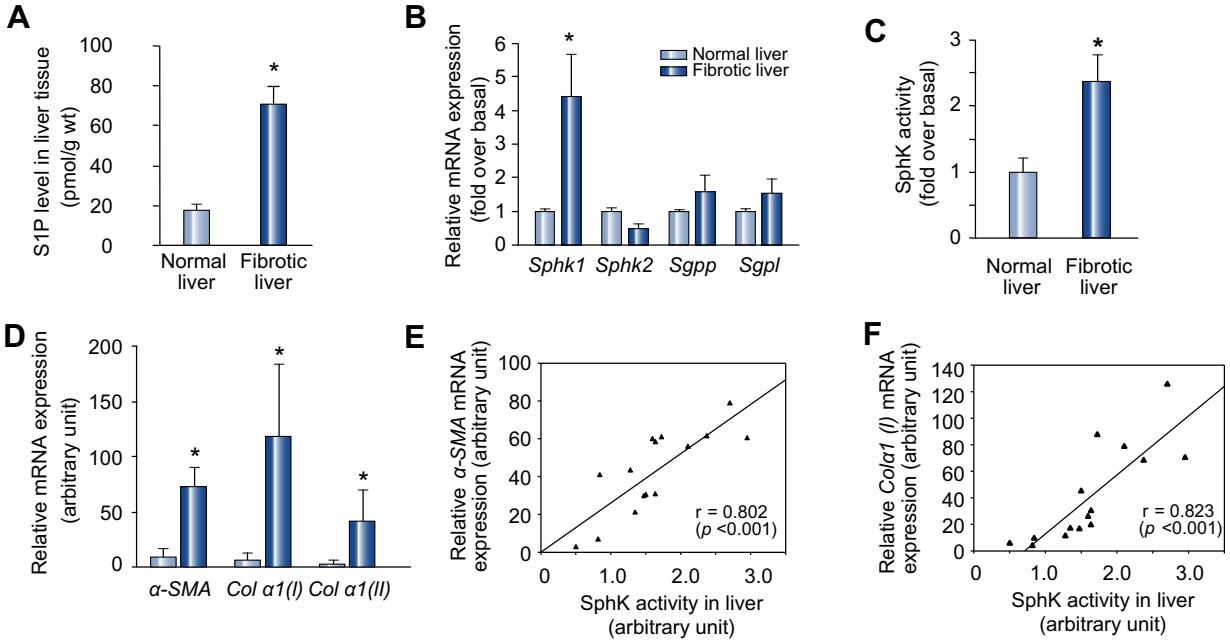


**Fig. 1. Induction of SphK/S1P in human liver fibrosis.** (A) S1P level in liver tissue, measured by high-performance liquid chromatography. (B) Relative hepatic levels of *SphK1*, *SphK2*, S1P phosphatase (*Sgpp*), and S1P lyase (*Sgpl*) mRNA, measured by real-time RT-PCR. (C) SphK activity in human liver tissue. (D) Expression of α-*SMA*, procollagen α1(I) (*Col α1(I)*) and procollagen α1(III) (*Col α1(III)*) mRNA in the liver. The correlation between expression of α-*SMA* (E) or *Col α1(I)* (F) and SphK activity in human liver. Data are presented as the means ± SEM. *$p$ <0.05, compared with the normal liver.

significantly increased, suggesting that S1P may play a role in hepatic fibrogenesis. Furthermore, our findings revealed that S1P/$S1P_3$ signaling mediated the migration of bone marrow-derived cells to the damaged liver [25,26]. Thus, studies conducted by our group and by other investigators have indicated that S1P is one of the most important bioactive lysophospholipids and a very good candidate for the induction of cell motility [16–24], which led us to put forward the possibility that S1P may induce hMFs migration into the damaged areas.

In this study, we explored the S1P system in the human fibrotic liver and investigated the possible role of S1P in the process of liver fibrosis. We showed that SphK/S1P/S1PRs signaling axis was involved in human liver fibrosis. Furthermore, we analyzed the effect of S1P on human hMFs motility and characterized the involvement of specific S1PRs, unraveling a novel function of S1P in the process of human liver fibrogenesis.

Understanding the pathophysiological role of SphK/S1P/S1PRs will potentially provide new therapies to control a variety of diseases, including liver fibrosis and cirrhosis.

## Materials and methods

*Materials*

DMEM was from Invitrogen (Grand Island, NY). Fetal bovine serum was from Biochrom (Berlin, Germany). PCR reagents were from Applied Biosystems (Foster City, CA). S1P and dihydro-S1P ($H_2S1P$) were from Biomol (Tebu, France). SEW2871 and JTE-013 were from Cayman Chemical (Ann Arbor, MI). Suramin, bovine serum albumin, and other common reagents were from Sigma (St. Louis, MO).

*Human specimen*

We retrospectively studied snap-frozen surgical liver resections from 21 patients (13 men, 8 women; mean age, 56 y; range, 42–69 y). Normal liver samples were collected from 5 patients undergoing hepatic resection for colorectal metastasis ($n$ = 5). Fibrotic samples (fibrosis stage: F2-4) 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). Informed consent was obtained for each subject. This study was undertaken in accordance with ethical regulations imposed by the Chinese legislation.

*Cell isolation and culture*

Human hMFs were obtained by outgrowth of explants prepared from surgical specimens of normal livers, as previously described [4]. This procedure was performed in accordance with ethical regulations imposed by the Chinese legislation. Cells were cultured as previously described [4] and were used between the fourth and ninth passage.

*Measurement of S1P by high-performance liquid chromatography analysis*

Extraction of S1P from human liver tissue and sample analysis were carried out as described previously by Min et al. [27] with minor modifications [25]. Detailed information is available in Supplementary materials and methods.

*Measurement of sphingosine kinase activity*

Sphingosine kinase activity from human liver samples was performed as described previously [28]. In brief, the kinase assay was performed by mixing 100 μg protein extracts with 10 μl of 1 mM sphingosine and 10 μl of [$\gamma$-$^{32}$P]ATP (10 μCi, 20 mM) containing 200 mM $MgCl_2$. After incubation for 30 min at 37 °C, the [$\gamma$-$^{32}$P]ATP-labeled S1P was extracted with 240 μl chloroform and analyzed by thin layer chromatography developed in butanol/acetic acid/water (3:1:1, v/v/v), followed by autoradiography.