Sphingosine 1-phosphate regulates regeneration and fibrosis after liver injury via sphingosine 1-phosphate receptor 2

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Abstract Sphingosine 1-phosphate (SIP), a bioactive lipid mediator, stimulates proliferation and contractility in hepatic stellate cells, the principal matrix-producing cells in the liver, and inhibits proliferation via SIP receptor 2 (SIP2) in hepatocytes in rats in vitro. A potential role of SIP and SIP2 in liver regeneration and fibrosis was examined in SIP2-deficient mice. Nuclear 5-bromo-2′-deoxy-uridine labeling, proliferating cell nuclear antigen (PCNA) staining in hepatocytes, and the ratio of liver weight to body weight were enhanced at 48 h in SIP2-deficient mice after a single carbon tetrachloride (CCl4) injection. After dimethylnitrosamine (DMN) administration with a lethal dose, PCNA staining in hepatocytes was enhanced at 48 h and survival rate was higher in SIP2-deficient mice. Serum aminotransferase level was unaltered in SIP2-deficient mice. Nuclear 5-bromo-2′-deoxy-uridine labeling, proliferating cell nuclear antigen (PCNA) staining in hepatocytes, and the ratio of liver weight to body weight were enhanced at 48 h in SIP2-deficient mice after a single carbon tetrachloride (CCl4) injection. After dimethylnitrosamine (DMN) administration with a lethal dose, PCNA staining in hepatocytes was enhanced at 48 h and survival rate was higher in SIP2-deficient mice. Serum aminotransferase level was unaltered in those mice compared with wild-type mice in both CCL4 and DMN-induced liver injury, suggesting that SIP2 inactivation accelerated regeneration not as a response to enhanced liver damage. After chronic CCL4 administration, fibrosis was less apparent, with reduced expression of smooth-muscle α-actin-positive cells in the livers of SIP2-deficient mice. Thus, SIP plays a significant role in regeneration and fibrosis after liver injury via SIP2—Ikeda, H., N. Watanabe, I. Ishii, T. Shimosawa, Y. Kume, T. Tomiya, Y. Inoue, T. Nishikawa, N. Ohtomo, Y. Tanoue, S. Itsuka, R. Fujita, M. Omata, J. Chun, and Y. Yatomi. Sphingosine 1-phosphate regulates regeneration and fibrosis after liver injury via sphingosine 1-phosphate receptor 2. J. Lipid Res. 2009. 50: 556–564.

Supplementary key words liver regeneration • liver fibrosis • hepatocyte • hepatic stellate cell • hepatic myofibroblast

Sphingosine 1-phosphate (SIP), which elicits a wide variety of cell responses (1), has emerged as a novel lipid intracellular mediator. SIP was shown to act as an intracellular second messenger of platelet-derived growth factor and serum in their mitogenic actions in cultured fibroblasts (2, 3), and furthermore, intracellular levels of SIP and ceramide were reported to determine cell survival or death (4, 5). However, evidence indicating that SIP also acts as an extracellular mediator has been reported; some of the diverse effects of SIP, such as stimulation of cell proliferation or contractility, are known to be sensitive to pertussis toxin (6) or ADP-ribosyltransferase C3 from Clostridium botulinum (7, 8), suggesting that SIP may activate a receptor coupled to G protein(s). Indeed, recent investigation has revealed that SIP acts through at least five high-affinity G protein-coupled receptors referred to as SIP1–5 (9, 10). Regarding the source of SIP in vivo, it is shown to be stored in platelets (11), and recent data using conditional knockouts of sphingosine kinases support release of SIP from erythrocytes (12, 13). These findings suggest that SIP has normal in vivo roles as well as potentially pathophysiological roles as a circulating paracrine mediator, a view further supported by the phenotypes of SIP receptor mutants (10, 14, 15).

SIP receptors are also expressed in the liver (14). To investigate the function of SIP in liver pathophysiology, we have determined the effect of SIP on liver cells in culture. We first demonstrated that SIP stimulates proliferation and contractility in rat hepatic stellate cells in culture; the stimulation of proliferation is pertussis toxin-sensitive,
and the stimulation of contractility is C3 exotoxin-sensitive (16). This stimulatory effect of S1P on contractility in those cells was found to be via S1P2 with Rho activation (17). On the other hand, we revealed that S1P inhibits proliferation in cultured rat hepatocytes. With the use of a specific antagonist to S1P2 and C3 exotoxin, this inhibitory effect of S1P on hepatocyte proliferation in culture involved Rho activation via S1P2 (18). Furthermore, the administration of S1P in 70% hepatectomized rats indeed reduces a response of hepatocytes to synthesize DNA (18).

Irrespective of the insults, such as viruses, alcohol abuse, or drugs, the regenerative and wound-healing responses generally occur in the liver after the injury, and the persistence of these responses may result in liver fibrosis (19, 20). Hepatocytes play a major role in liver regeneration (19) as do hepatic stellate cells in the wound-healing response, and hence, liver fibrosis (20). The enhanced proliferation and contractility of hepatic stellate cells are among the main features of liver fibrosis (21, 22). Although our previous evidence was obtained mainly by in vitro studies and indicated a pharmacologically inhibitory effect of S1P on liver regeneration in vivo, it raised the possibility that S1P, and potentially S1P2, might play a pathophysiological role in the liver after the injury. Thus, we planned to extend our study using S1P2-deficient mice to clarify this point.

In this context, Serrier-Lanneau et al. (23) recently reported that the wound-healing response to acute liver injury elicited by carbon tetrachloride (CCl4) was reduced in S1P2−/− mice with reduced accumulation of hepatic myofibroblasts and that hepatic myofibroblasts isolated from S1P2−/− mice did not proliferate in response to S1P. There is controversy as to whether hepatic myofibroblasts are distinct from hepatic stellate cells. Hepatic myofibroblasts have been studied as the cells developed from hepatic stellate cells by transdifferentiation (24–27). In contrast, hepatic myofibroblasts have been reported to belong to a cell population different from hepatic stellate cells (28). Whatever the case, hepatic myofibroblasts have been assumed to be a principle matrix-producing cell of the diseased liver (29). On the other hand, the regenerative response after a single injection of CCl4 in S1P2−/− mice was not different from that in wild-type mice, suggesting that S1P2 inac-
vation did not affect hepatocyte regeneration (23). With these findings, we aimed to perform more-detailed examination of hepatocyte regeneration in acute liver injury elicited by dimethylnitrosamine (DMN) in addition to CCl₄ and to determine whether reduced wound-healing response could lead to reduced liver fibrosis after chronic CCl₄ administration in S1P₂⁻/⁻ mice.

MATERIALS AND METHODS

Animals

Heterozygous S1P₂⁺/⁻ mice were originally generated by our group on the (129/Sv × 129/)F₁ (an embryonic stem cell origin)/C57BL/6N (a blastocyst origin) mixed background (30). In this study, they were backcrossed onto the inbred C57BL/6N strain (Clea Japan, Tokyo, Japan) for 7–8 generations to achieve >99.2% genetic homogeneity, and the obtained S1P₂⁺/⁻ mice were bred to produce S1P₂⁻/⁻ mice. Deletion of S1P₂ in these S1P₂⁻/⁻ mice has been repeatedly confirmed by the complete absence of s1p₂ gene expression in tissues (e.g., adult lung, spleen, brain, heart, cochlea, and embryonic fibroblasts) in which s1p₂ is normally expressed (15, 30, 31) as well as the specific appearance or disappearance of some S1P-mediated cellular signaling/systemic effects (15, 30–32). Age-matched wild-type C57BL/6N mice were used as controls. They were fed a standard pelleted diet and water ad libitum under normal laboratory conditions of 12 h light/dark cycles. All animals received humane care, and the research was conducted in conformity with Public Health Service policy on the humane care and use of laboratory animals. The experimental protocol was approved by the Animal Research Committee of the University of Tokyo and followed National Institutes of Health guidelines for the care and use of laboratory animals.

CCl₄ treatment

To assess the responses to a single CCl₄ administration, male and female wild-type and S1P₂⁻/⁻ mice, 8–10 weeks of age, were bred to produce S1P₂⁻/⁻ mice. Deletion of S1P₂ in these S1P₂⁻/⁻ mice has been repeatedly confirmed by the complete absence of s1p₂ gene expression in tissues (e.g., adult lung, spleen, brain, heart, cochlea, and embryonic fibroblasts) in which s1p₂ is normally expressed (15, 30, 31) as well as the specific appearance or disappearance of some S1P-mediated cellular signaling/systemic effects (15, 30–32). Age-matched wild-type C57BL/6N mice were used as controls. They were fed a standard pelleted diet and water ad libitum under normal laboratory conditions of 12 h light/dark cycles. All animals received humane care, and the research was conducted in conformity with Public Health Service policy on the humane care and use of laboratory animals. The experimental protocol was approved by the Animal Research Committee of the University of Tokyo and followed National Institutes of Health guidelines for the care and use of laboratory animals.

**Fig. 2.** The ratio of liver weight to body weight, body weight loss, and liver function tests in wild-type and S1P₂⁻/⁻ mice in acute liver injury induced by CCl₄. A single injection of CCl₄ was performed in wild-type (WT) and S1P₂⁻/⁻ mice. The ratio of liver weight to body weight was measured in untreated wild-type and S1P₂⁻/⁻ mice from 24 to 96 h at euthanization after CCl₄ injection (A). The alteration of body weight was determined by comparison of body weights before CCl₄ injection with those at 24, 48, 72, and 96 h after CCl₄ injection (B). Serum concentrations of alanine aminotransferase (ALT) (C) and alkaline phosphatase (ALP) (D) were measured in wild-type and S1P₂⁻/⁻ mice at 24, 48, 72, and 96 h after CCl₄ injection. Columns and bars represent means ± SEM of four animals. The asterisk indicates a significant difference from wild type in Student’s t-test (P < 0.05).
injected intraperitoneally with 0.5 ml/kg body weight of CCl₄ dissolved in the same amount of olive oil (1:1) (23), in which liver injury, assessed by serum aminotransferase level, was nongender specific. To develop liver fibrosis, female wild-type and S1P₂⁻/⁻ mice, 8 weeks of age, were injected intraperitoneally with 1 ml/kg body weight of CCl₄ dissolved in the same amount of olive oil (1:1), twice a week for 4 weeks (33, 34). Control mice received injection of the carrier (olive oil) alone. Each group consisted of four to five mice. For the experiment on chronic CCl₄ treatment, the livers were harvested at 4 days after the end of the administration.

**DMN treatment**

Male and female wild-type and S1P₂⁻/⁻ mice, 8–10 weeks of age, were injected intraperitoneally with 15 mg/kg body weight DMN dissolved in HBSS (0.3%) (GIBCO) (35), in which liver injury, assessed by serum aminotransferase level, was nongender specific.

**Measurement of nuclear labeling by 5-bromo-2′-deoxy-uridine**

One hour before euthanization, 50 mg/4 ml/kg body weight of 5-bromo-2′-deoxy-uridine (BrdU) (Roche Molecular Biochemicals, Mannheim, Germany) was injected intraperitoneally, and then the mice were euthanized at 24, 48, 72, and 96 h after CCl₄ administration. The liver was excised, immediately fixed in 10% formalin, and embedded in paraffin. The nuclear labeling was measured using BrdU labeling and detection kit II (Roche Molecular Biochemicals). The number of BrdU-positive hepatocytes was determined as the mean of five random areas at 400-fold magnification in each section.

**Immunohistochemical analysis of proliferating cell nuclear antigen**

Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was performed in liver tissue using a PCNA staining kit (Zymed Laboratories) in accordance with the protocol specified by the manufacturer. The ratio of PCNA-positive hepatocytes to all hepatocytes was determined with five random areas at 400-fold magnification in each section in CCl₄-treated mice and with ten random areas in DMN-treated mice, because submassive hemorrhagic necrosis was focally found in DMN-treated livers as previously described (36). The percentage of PCNA-positive nonparenchymal cells was determined with five random areas at 400-fold magnification in each section in CCl₄-treated mice.

**Measurement of liver function**

The serum levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined using an automated analyzer (Hitachi 7170; Hitachi Instruments Service Co., Ltd., Tokyo, Japan).

**Histological analysis of fibrosis**

Tissue sections (4 μm thick) of liver specimens fixed in formalin and embedded in paraffin were analyzed with Masson’s trichrome staining (37). The microscopic fields (five per each slide) were blindly selected and captured with the aid of the Nikon Digital Camera DXM1200 (NIKON, Japan). A fibrous portion stained in blue was extracted, and the extent of liver fibrosis was quantified by the technique reported previously (38) by calculating the area of fibrosis/area of section using image analysis software of the public domain Scion Image (Scion Corporation).

**Immunohistochemical analysis of smooth-muscle α-actin**

Immunohistochemical analysis of smooth-muscle α-actin was performed in liver tissue fixed in formalin and embedded in paraffin using a Vector M.O.M. immunodetection kit (Vector Laboratories, Burlingame, CA) in accordance with the protocol specified by the manufacturer, with a 1:5 dilution of mouse monoclonal antibody to smooth-muscle α-actin (Sigma). The quantitation of the positive staining area for smooth-muscle α-actin was performed blindly on four or five liver fragments per animal using the computer software of the public domain Scion Image developed by the Scion Corporation.

**Statistical analysis**

Data are represented as mean ± SEM. An ANOVA followed by Tukey’s honest significance differences test (Tukey’s HSD) or Student’s t-test were used to determine whether significant differences existed between groups. Fisher’s exact test was performed when appropriate. Differences were considered to be significant at P values of <0.05.

**RESULTS**

**Regenerative response of hepatocytes to CCl₄-induced acute liver injury was enhanced in S1P₂⁻/⁻ mice**

After a single CCl₄ administration, DNA synthesis of hepatocytes determined by BrdU labeling peaked at 48 h in

![Fig. 3.](image-url)
both wild-type and S1P2<sup>−/−</sup> mice (Fig. 1). As depicted in Fig. 1, BrdU-positive hepatocytes seemed more apparent in S1P2<sup>−/−</sup> mice than in wild-type mice (Fig. 1A) at 48 h after CCl<sub>4</sub> administration, and in fact, the number of BrdU-positive hepatocytes at 48 h after CCl<sub>4</sub> administration in S1P2<sup>−/−</sup> mice was significantly increased by 1.8-fold compared with wild-type mice (Fig. 1B). On the other hand, BrdU-positive hepatocytes were not found in both wild-type and S1P2<sup>−/−</sup> mice treated with olive oil alone. We next performed immunohistochemical analysis of PCNA in wild-type and S1P2<sup>−/−</sup> livers after a single CCl<sub>4</sub> administration. As demonstrated in Fig. 1, PCNA-positive hepatocytes were more apparent in S1P2<sup>−/−</sup> mice than in wild-type mice (Fig. 1C) at 48 h after CCl<sub>4</sub> administration. The number of PCNA-positive hepatocytes significantly increased by 1.7-fold in S1P2<sup>−/−</sup> mice compared with wild-type mice (Fig. 1D).

**Fig. 4.** The ratio of liver weight to body weight, body weight loss, liver function test, and survival rate in wild-type and S1P2<sup>−/−</sup> mice in acute liver injury induced by DMN. A single injection of DMN was performed in wild-type (WT) and S1P2<sup>−/−</sup> mice. The ratio of liver weight to body weight (A), body weight loss (B), and serum concentration of ALT (C) and ALP (D) were measured at 48 h after DMN injection. Columns and bars represent means ± SEM of four animals. Survival rate was also determined in wild-type (n = 17) and S1P2<sup>−/−</sup> (n = 16) mice up to 120 h after DMN injection (E). Significantly higher survival rate in S1P2<sup>−/−</sup> mice was determined at 96 and 120 h after DMN injection in Fisher’s exact test (P < 0.05).
On the other hand, it is of note that PCNA-positive nonparenchymal cells were less apparent in S1P2−/− mice compared with wild-type mice (Fig. 1C). The percentage of PCNA-positive nonparenchymal cells in S1P2−/− mice was reduced to 62% of that in wild-type mice (Fig. 1E). In both wild-type and S1P2−/− mice treated with olive oil alone, PCNA-positive cells were not determined. These results suggest that the regenerative response after CCl4 administration is enhanced in hepatocytes, but reduced in nonparenchymal cells in S1P2−/− mice.

We then analyzed both liver weight and body weight at euthanization after a single injection of CCl4. The ratio of liver weight to body weight was significantly higher in S1P2−/− mice than in wild-type mice at 48 h after CCl4 administration, but not different between wild-type and S1P2−/− mice at 24, 72, and 96 h (Fig. 2A). Both wild-type and S1P2−/− mice lost their weight body weight maximally at 24 h after CCl4 administration and then gradually gained weight up to 96 h, as shown in Fig. 2B. The alterations of body weight after CCl4 administration were not different between wild-type and S1P2−/− mice. These results suggest that the regenerative response in the liver after CCl4 administration is enhanced in S1P2−/− mice.

To examine the extent of liver damage elicited by CCl4 administration, serum levels of ALT and ALP were measured up to 96 h after treatment. Serum ALT level peaked at 24 h after CCl4 administration and was not significantly different between wild-type and S1P2−/− mice (Fig. 2C), as previously reported (23). Furthermore, serum level of ALP was not different between wild-type and S1P2−/− mice (Fig. 2D). These results suggest that the extent of liver damage caused by a single injection of CCl4 was not different in S1P2−/− mice compared with wild-type mice.

**Enhanced regenerative response of hepatocytes to DMN-induced acute liver injury led to increased survival rate in S1P2−/− mice**

To determine whether the enhanced hepatocyte regeneration in S1P2−/− mice could be the specific phenomenon in CCl4-induced acute liver injury, the regenerative response of hepatocytes in S1P2−/− mice was also examined in DMN-induced acute liver injury (35). In this experiment, BrdU labeling was not suitable for determining the proliferative response, because significant amounts of ascites were found in both wild-type and S1P2−/− mice in which BrdU was administered intraperitoneally. Thus, we determined the proliferative response by PCNA staining. At 48 h after DMN administration, PCNA-positive hepatocytes were more apparent in S1P2−/− mice than in wild-type mice (Fig. 3A). The percentage of PCNA-positive hepatocytes increased by 1.5-fold in S1P2−/− mice compared with wild-type mice (Fig. 3B). On the other hand, PCNA-positive hepatocytes were not found in both wild-type and S1P2−/− mice treated with vehicle alone. This result suggests that the regenerative response is also enhanced after DMN administration in hepatocytes in S1P2−/− mice. At the same time point, the ratio of liver weight to body weight was not different between wild-type and S1P2−/− mice (Fig. 4A), and the trend of more body weight loss in wild-type mice was noted (P = 0.05116) (Fig. 4B). Serum levels of ALT (Fig. 4C) and ALP (Fig. 4D) were not different between wild-type and S1P2−/− mice at the same time point, suggesting that the extent of DMN-induced liver damage was not different in S1P2−/− mice compared with wild-type mice.

Although we next planned to determine the regenerative response in hepatocytes in wild-type and S1P2−/− mice beyond 48 h after DMN administration, a substantial number of wild-type mice were found to be dead at 72 h after DMN administration. Thus, our experiment was changed to determine survival rate of wild-type and S1P2−/− mice with DMN intoxication. As shown in Fig. 4E, survival rate was significantly higher in S1P2−/− mice compared with wild-type mice at 96 to 120 h after DMN injection. Thus, increased regenerative response in hepatocytes after DMN intoxication may lead to increased survival rate in S1P2−/− mice.

**Liver fibrosis induced by chronic CCl4 administration was reduced in S1P2−/− mice**

Next, the role of S1P2 receptor signaling in liver fibrosis was examined in chronic liver injury elicited by CCl4. In wild-type mice, chronic administration of CCl4 for 4 weeks caused an extensive accumulation of extracellular matrices.

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**Fig. 5.** Liver fibrosis in wild-type and S1P2−/− mice after chronic CCl4 administration. Wild-type (WT) and S1P2−/− mice were treated with CCl4 for 4 weeks. Representative liver histology with Masson’s trichrome staining of wild-type and S1P2−/− mice is shown (A). Bar = 100 μm. Arrows indicate the formation of fibrotic septa. The extent of liver fibrosis in CCl4- and olive oil (vehicle)-treated wild-type and S1P2−/− mice was quantitated by calculating the ratio of the area of fibrosis to the area of section using Scion Image (B). Columns and bars represent means ± SEM of five animals. The asterisk indicates a significant difference between CCl4-treated wild-type and S1P2−/− mice in ANOVA followed by Tukey’s honestly significant differences (Tukey’s HSD) (P < 0.05).
with bridging fibrosis in the liver, whereas in S1P2−/− mice, such dense fibrotic septum development with nodule formation was not found (Fig. 5A). Quantitative analysis of fibrosis development revealed that the mean fibrotic area in the livers in wild-type mice was significantly larger than that in S1P2−/− mice treated with CCl4. The extent of liver fibrosis in S1P2−/− mice was approximately 40% that of wild-type mice (Fig. 5B). On the other hand, liver histology in S1P2−/− mice administered olive oil alone, i.e., without CCl4 treatment, was essentially unaltered compared with that of wild-type mice administered identically (Fig. 5B). In addition, serum ALT levels at the time of euthanization after chronic CCl4 administration for 4 weeks were essentially unaltered in wild-type and S1P2−/− mice; 58 ± 6 IU/l (n = 5) and 51 ± 30 IU/l (n = 5), respectively. These results suggest that S1P2 inactivation causes less liver fibrosis in response to chronic CCl4 administration.

We examined the ratio of liver weight to body weight at euthanization after chronic CCl4 administration. The trend of higher ratio of liver weight to body weight in S1P2−/− mice than in wild-type mice was noted (P = 0.1475) (Fig. 6A); 3.3% in wild-type and 4.8% in S1P2−/− mice. On the other hand, in mice treated with olive oil alone, the ratio of liver weight to body weight was not different with or without S1P2 inactivation (data not shown). Alterations of body weight in wild-type and S1P2−/− mice with chronic CCl4 administration were then examined by analysis of body weights at the first administration of CCl4 and at euthanization after chronic CCl4 administration. Repeated CCl4 administration for 4 weeks caused no increase in body weight in wild-type mice, but a 25.6% increase in S1P2−/− mice (Fig. 6B). This body weight gain in S1P2−/− mice after chronic CCl4 administration was comparable to that in both wild-type and S1P2−/− mice treated with olive oil alone for 4 weeks. Accordingly, only wild-type mice treated with CCl4 for 4 weeks had much less weight gain. These data may be compatible with the finding that CCl4-induced liver fibrosis was abrogated in S1P2−/− mice.

Both hepatic stellate cells and hepatic myofibroblasts are thought to be the principal matrix-producing cells with smooth-muscle α-actin expression, although controversy still exists as to whether hepatic myofibroblasts are distinct from hepatic stellate cells (28, 39). Thus, smooth-muscle α-actin expression in the liver is considered to be a marker of...
the matrix-producing cells in the liver. Chronic CCl₄ administration caused a significant accumulation of smooth-muscle α-actin-positive cells in wild-type livers, whereas it was less apparent in S1P₂⁻/⁻ livers (Fig. 7A). Quantitative analysis revealed that the smooth-muscle α-actin-positive cell area was reduced by 78% in S1P₂⁻/⁻ livers compared with wild-type livers (Fig. 7B). Thus, these results suggest that S1P₂ inactivation leads to decreased accumulation of the matrix-producing cells in the liver, namely hepatic stellate cells and/or hepatic myofibroblasts, in CCl₄-induced liver fibrosis.

**DISCUSSION**

In the current study, liver regeneration was enhanced in S1P₂⁻/⁻ mice compared with wild-type mice after acute liver injury induced by a single injection of CCl₄ or DMN, suggesting that SIP may be involved in liver regeneration, possibly as an inhibitory regulator via S1P₂. This was determined by the increased nuclear labeling with BrdU and immunohistochemical staining of PCNA in hepatocytes and by the increased ratio of liver weight to body weight in S1P₂⁻/⁻ mice compared with wild-type mice in CCl₄-induced acute liver injury. On the other hand, in DMN-induced acute liver injury, increased liver regeneration was determined by the increased immunohistochemical staining of PCNA in hepatocytes in S1P₂⁻/⁻ mice compared with wild-type mice, although the enhanced ratio of liver weight to body weight was not found in S1P₂⁻/⁻ mice. Because submassive hemorrhagic necrosis is characteristic of DMN-intoxicated liver (36), the resultant congestion in the liver may affect the exact weight determination of liver parenchyma. Furthermore, higher survival rate after DMN intoxication with a lethal dose (35) in S1P₂⁻/⁻ mice compared with wild-type mice was determined. This may be explained by increased liver regeneration, and the S1P₂ antagonist merits consideration as a therapeutic agent for liver failure caused by impaired liver regeneration. Because the liver damage evaluated by liver function tests was not different between wild-type and S1P₂⁻/⁻ mice in both CCl₄- and DMN-induced liver injury, the enhanced liver regeneration in S1P₂⁻/⁻ mice may not be due to a response to the enhanced liver damage. Collectively, the current evidence has suggested that SIP plays a physiological role in liver regeneration via S1P₂ as a negative regulator.

A recent study by Serrier-Lanneau et al. (23) reported that the regenerative response after a single injection of CCl₄ in S1P₂⁻/⁻ mice was not different from that in wild-type mice, where liver regeneration was assessed by Western blot analysis of PCNA expression in the liver. In our study, immunohistochemical analysis of PCNA revealed that PCNA-positive hepatocytes were increased in S1P₂⁻/⁻ livers by 1.7-fold compared with wild-type livers at 48 h after CCl₄ administration. Another interesting finding was that PCNA-positive nonparenchymal cells were found in significant numbers in wild-type livers, but were much less apparent in S1P₂⁻/⁻ livers, which may be in line with the finding by Serrier-Lanneau et al. (23) that hepatic myofibroblasts from S1P₂⁻/⁻ livers were less regenerative compared with those from wild-type livers after a single CCl₄ injection. It is likely that Western blot analysis of PCNA expression in the whole-liver extracts determined regeneration in both nonparenchymal cells and hepatocytes. We would also assume that a less than 2-fold increase may occasionally be difficult to detect by Western blot analysis, inasmuch as our Western blot analysis did not reveal significant differences in PCNA expression between wild-type and S1P₂⁻/⁻ livers (data not shown).

On the other hand, liver fibrosis induced by chronic CCl₄ administration was less apparent in S1P₂⁻/⁻ mice than in wild-type mice. The reduced body weight loss in S1P₂⁻/⁻ mice after chronic CCl₄ administration may be compatible with the reduction of CCl₄-induced liver fibrosis in S1P₂⁻/⁻ mice. Such evidence indicates that S1P₂ inactivation leads to reduced liver fibrosis induced by CCl₄, suggesting that SIP and S1P may play a role in the development of liver fibrosis.

The reduction of liver fibrosis in S1P₂ inactivation may not be explained by the alteration in the extent of liver damage by CCl₄, because the liver damage was essentially the same in wild-type and S1P₂⁻/⁻ mice. Instead, the reduced accumulation of smooth-muscle α-actin-positive cells in the livers of S1P₂⁻/⁻ mice treated with CCl₄ for 4 weeks may be a key to the reduced liver fibrosis in those mice, because smooth-muscle α-actin-positive cells in the liver are assumed to be the principal matrix-producing cells of the diseased liver, namely hepatic stellate cells and hepatic myofibroblasts (29).

We previously showed abundant mRNA expression of S1P₁ and S1P₂ in both hepatocytes (18) and hepatic stellate cells (16) in rats. On the other hand, increased mRNA expression of S1P₂ and S1P₃ and unaltered mRNA expression of S1P₁ in the liver after a single CCl₄ injection were reported in mice (23). Although our current evidence suggests that S1P₂ may play a significant role in the response to liver injury, the significance of abundant expression of S1P₁ in liver cells and upregulation of S1P₂ expression in the liver after injury remains to be examined.

Recent evidence suggests that SIP receptors regulate important physiological functions of the vascular system, such as vascular morphogenesis and maturation, cardiac function, vascular permeability, and tumor angiogenesis (40–43). Our current evidence suggests that S1P₂ may also play a significant role in liver pathophysiology.

**REFERENCES**


