Biological activities of novel lipid mediator sphingosine 1-phosphate in rat hepatic stellate cells

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Sphingomyelin, a ubiquitous membrane component of eukaryotic cells, has been assumed to play a role in the maintenance of membrane structure (16, 17). A sphingomyelin metabolite, ceramide, was first shown to act as an intracellular second messenger to regulate apoptosis (6, 29, 34). Recently, sphingosine 1-phosphate (S-1-P), a lipid mediator shown to be a ligand for G protein-coupled receptors (AGRs), endothelial differentiation gene (EDG)1 (23, 27, 47), EDG3 (1, 28), and AGR16/EDG5 (15), indicating that S-1-P works extracellularly as well as intracellularly to evoke biological responses (38). Interestingly, S-1-P is abundantly stored in platelets (42) and released on their activation (43). These observations suggest that S-1-P may have some functions in vivo.

Platelet consumption occurs under various pathological conditions including liver diseases. Cirrhosis is complicated by accelerated platelet destruction (3, 37). Platelets accumulate in the sinusoidal and perisinusoidal Disse spaces in acute liver injury induced by injection of lipopolysaccharides in mice (7, 8). Hepatic stellate cells (HSCs), located in the Disse space, play an important role in wound healing and possible subsequent fibrogenesis (12). Thus we wondered whether S-1-P might affect the functions of HSCs in liver injury associated with platelet consumption.

On liver injury and the process of fibrogenesis, HSCs are known to undergo activation (13) characterized by proliferation (9), contractility (33), enhanced production of extracellular matrices (14, 24), and expression of smooth muscle α-actin (32). Activation of HSCs is known also to occur when the cells are cultured on noncoated plastic (14, 32). In this study, the effects of S-1-P on HSCs in both early and late phases of activation in culture and the expression of receptors for S-1-P in HSCs were investigated.

Materials and Methods

Animals. Male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) fed a standard pelleted diet and water ad libitum were used in all experiments. All animals received humane care in compliance with the institution’s guidelines and the National Institutes of Health guidelines.

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Cell isolation and culture. HSCs were isolated from rats (weighing 300–400 g) using a metrizamide (Sigma Chemical, St. Louis, MO) gradient centrifugation, as previously described (20). The isolated cells were seeded on noncoated plastic tissue culture dishes (Falcon, Lincoln Park, NJ) at a starting density of 1–4 × 10^5 cells/cm^2 and cultured in DMEM (Nissui Pharmaceutical, Tokyo, Japan) containing 10% FCS (GIBCO, Grand Island, NY). The confluent cells (days 7–10 of primary culture) were subcultured as previously described (31). For subcultured cells, experiments were performed between days 8 and 11.

Measurement of DNA synthesis. HSCs cultured for 3 days or subcultured HSCs were incubated in DMEM plus 0.5% FCS containing various concentrations of S-1-P (Bioulom Research Laboratories, Plymouth Meeting, PA) with or without 10 ng/ml platelet-derived growth factor-BB (PDGF-BB; R&D Systems, Minneapolis, MN) for 24 h or in 10 μM S-1-P for various periods and labeled with 5 μCi/ml [methyl-3H]thymidine (20 Ci/mmol; New England Nuclear, Boston, MA) in the presence or absence of 10 μM hydroxyurea for the last 2 h of incubation. DNA synthesis was determined as previously described (29). To examine the effect of pertussis toxin (PTX; Clostridium botulinum) on DNA synthesis, HSCs were incubated with 100 ng/ml PTX for 16 h, and then the test materials were added to the medium.

Measurement of cell number. Subcultured HSCs were placed in DMEM plus 0.5% FCS containing 10 μM S-1-P. At 3 days after the addition of S-1-P, the cell numbers were determined after trypsinization.

Measurement of mitogen-activated protein kinase activity. Subcultured HSCs were placed in DMEM containing 0.5% FCS for 18 h and then DMEM without serum for 6 h. The cells were incubated with 10 μM S-1-P for various periods, lysed in ice-cold lysis buffer (10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM diethiothreitol, 1 mM orthovandate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml apotinin, pH 7.4), and centrifuged at 25,000 g for 20 min at 4°C. Mitogen-activated protein kinase (MAPK) activity in the supernatant was measured using the p42/p44 MAPK enzyme assay system (Amersham) as previously described (39).

Measurement of contraction of collagen lattices containing HSCs. Contraction of collagen lattices containing HSCs cultured for 3 days or subcultured HSCs was measured as described previously (4) with some modifications (33). Briefly, a mixture of 4 parts collagen type I solution from porcine tendon (Wako, Osaka, Japan), 1 part 10× minimal essential medium (Nissui), and 1 part 0.2 M HEPES (resulting in collagen concentration of 1.2 mg/ml) was made at 4°C and added to the wells. The cells were then inoculated to the collagen lattices (5 × 10^5 cells/cm^2 for freshly isolated cells and 1 × 10^5 cells/cm^2 for subcultured cells). After cell attachment for 72 h the cells were placed in serum-free medium for 6 h, and then the various concentrations of S-1-P were added to the medium. At various periods, the lattices were detached by gentle circumferential dislodgment using a 200-g pipette tip. Contraction was measured as change in the lattice area. To examine the effect of ADP-ribosyltransferase C3 from Clostridium botulinum (C3 exotoxin; Wako), the cells were preincubated with 0.2–2 μg/ml C3 exotoxin for 6 h, and then the test materials were added to the medium.

Measurement of collagen and noncollagenous protein synthesis. HSCs cultured for 3 days or subcultured HSCs were incubated with DMEM containing 0.5% FCS, 0.1 mM ascorbic acid, 0.5 mM β-aminopropionitrile, and 10 μM S-1-P for 24 h. The cells were labeled with 20 μCi/ml [3H]proline (25–55 Ci/mmol; New England Nuclear) for the last 6 h of incubation. Collagen and noncollagenous protein synthesis were measured by collagenase digestion assay as previously described (20).

Measurement of smooth muscle α-actin expression. HSCs cultured for 3 days or subcultured HSCs were incubated with DMEM containing 0.5% FCS and 10 μM S-1-P for 24 h. The medium was discarded, and the cells were suspended in 0.5% SDS-60 mM Tris, pH 6.8, boiled for 5 min, and then disrupted by sonication. Identification and quantitation of smooth muscle α-actin were performed by immunoblotting as described previously (20).

Preparation of cell membranes of HSCs. Subcultured HSCs were removed from the culture dishes and resuspended in ice-cold 20 mM Tris·HCl, pH 7.5. The cells were disrupted using a Dounce homogenizer. Large pieces of cell debris were removed by centrifugation of the homogenate at 1,000 g for 15 min at 4°C. The resulting supernatant was centrifuged at 40,000 g for 15 min at 4°C. The pellet was resuspended in 20 mM Tris·HCl, pH 7.5, and used as the cell membrane preparation (36).

Measurement of S-1-P binding to cell membranes of HSCs. [3H]-S-1-P was prepared by ATP-dependent phosphorylation of [3H]sphingosine (15–30 Ci/mmol; New England Nuclear) catalyzed by sphingosine kinase obtained from human platelets (44). The cell membranes were incubated at 60 μg/ml in 500 μl of 20 mM Tris·HCl, pH 7.5, containing [3H]-S-1-P for 3 h at 4°C. [3H]-S-1-P bound to the membranes was separated from free radioligand by gel filtration, using Sephadex G-25 columns (PD-10; Pharmacia) (36). The column eluates were collected, and the radioactivity was measured in a scintillation counter. Nonspecific binding was defined as the amount of radioactivity bound to the cells in the presence of 50 μM unlabeled S-1-P. Specific binding was calculated by subtracting the nonspecific binding from the total binding.

Induction of hepatic fibrosis in rats. Fibroitic liver was induced in rats (weighing 200–250 g) by subcutaneous injection of 0.5 ml/kg body wt of CCl4 as a 20% (vol/vol) solution in olive oil twice a week. HSCs were isolated at 8 wk of treatment.

Isolation of RNA. Total RNA was isolated by the guanidinium thiocyanate-phenol-chloroform extraction method (5).

Preparation of complementary DNA probe. With the use of a random-primer synthesis method (11), the complementary DNA fragments of rat EDG1 (27), human EDG3 (28), rat AGR16 (17), and human glyceraldehyde-3-phosphate dehydrogenase (Clontech Laboratories, Palo Alto, CA) were radio-labeled with [α-32P]dCTP (Amersham) up to a specific activity of 1 × 10^9 cpm/mg DNA.

Northern blot. Ten micrograms of total RNA were subjected to electrophoresis through agarose gels containing formaldehyde and transferred onto nylon filters, and the filters were prehybridized and hybridized as previously described (20).

Statistical analysis. Paired data were analyzed by Student's t-test.

RESULTS

Effect of S-1-P on proliferation of HSCs. S-1-P increased DNA synthesis by subcultured HSCs but did not affect that by HSCs cultured for 3 days. The enhancement by S-1-P of DNA synthesis by subcultured HSCs was more prominent in low-density cell cultures (1 × 10^4 cells/cm^2; ~25% confluence) than in high-density cell cultures (4 × 10^5 cells/cm^2; 100% confluence; data not shown). Figure 1A demonstrates the
kinetics of the DNA synthesis after addition of 10 μM S-1-P in low-density cell cultures of subcultured HSCs. Maximal enhancement of DNA synthesis was observed at 24 h after the addition of S-1-P. Enhancement of DNA synthesis by S-1-P in low-density cell cultures of subcultured HSCs was dose related up to 10 μM (Fig. 1B). When subcultured HSCs at low density were incubated with or without 10 μM S-1-P for 3 days, S-1-P significantly increased the cell number compared with the nontreated control (Fig. 2), indicating that an increase in [3H]thymidine incorporation was accompanied by an increase in cell count.

Figure 3 depicts the effect of PTX on the mitogenic action of S-1-P in HSCs. Preincubation with 100 ng/ml PTX for 16 h significantly decreased the mitogenic action of S-1-P on subcultured HSCs at low density. However, PTX did not affect DNA synthesis by HSCs not treated with S-1-P.

Figure 4 demonstrates the effect of S-1-P on MAPK activity of subcultured HSCs at low density. S-1-P at 10 μM increased the MAPK activity to fourfold that of the untreated control, reaching a maximal level 3 min after the addition of S-1-P and declining thereafter.

The effects of S-1-P on DNA synthesis by HSCs in the presence or absence of PDGF-BB are shown in Fig. 5. The effect of 10 μM S-1-P on DNA synthesis by subcultured HSCs was additive with that of 10 ng/ml PDGF-BB.

Effects of S-1-P on contraction of collagen lattices containing HSCs. The effect of S-1-P on contraction of HSC-populated collagen lattices is demonstrated in Figs. 6 and 7. S-1-P enhanced the contraction of collagen lattices containing subcultured HSCs for 3 days (Fig. 6A) and subcultured HSCs (Fig. 6B) in a dose-related manner up to 10 μM. The preincubation of C3 exotoxin dose-relatedly reduced S-1-P-mediated enhancement of the contraction of collagen lattices containing subcultured HSCs, as shown in Fig. 7.

Effect of S-1-P on collagen synthesis and smooth muscle α-actin expression of HSCs. S-1-P had no effects on collagen synthesis or smooth muscle α-actin expression in HSCs cultured for 3 days or subcultured HSCs, irrespective of cell density (data not shown).

mRNA expression of receptors for S-1-P in HSCs. In HSCs cultured for 3 days and subcultured HSCs,
EDG1 and AGR16 mRNAs were detected as shown in Fig. 8, but EDG3 mRNA was not detectable (data not shown). EDG1 mRNA levels were higher in HSCs cultured for 3 days than in subcultured HSCs, whereas AGR16 mRNA levels were not different between cultured and subcultured HSCs (Fig. 8).

mRNA expression of receptors for S-1-P in HSCs as fresh isolates is demonstrated in Fig. 9. EDG1 mRNA was detected in HSCs isolated from normal rats but was minimal in HSCs from rats treated with CCl4. In contrast, AGR16 mRNA was detected to the same extent in HSCs isolated from both normal rats and rats treated with CCl4. EDG3 mRNA was not detectable in either group of HSCs (data not shown).

**Binding of S-1-P to cell membranes of HSCs.** To examine whether the observed distinct responsiveness to S-1-P in proliferation of subcultured HSCs in low-density cultures was due to the presence of factors in the culture medium, HSCs were incubated with various concentrations of S-1-P. The effects of S-1-P on DNA synthesis and contraction of collagen lattices containing HSCs were measured. The results are shown in Figs. 4-7.

**Fig. 4.** Effect of S-1-P on mitogen-activated protein kinase (MAPK) activity in subcultured HSCs. Subcultured HSCs at low density (1 × 10⁶ cells/cm²) were incubated with 10 μM S-1-P for various times and then in DMEM containing 0.5% FCS for 18 h and then in DMEM without serum for 6 h. The cells were lysed and centrifuged at 25,000 g. MAPK activity in the supernatant was measured using the p42/p44 MAPK enzyme assay system. The representative result of 3 experiments is shown. Bars represent means ± SD of triplicate determinations. Changes were significant (P < 0.05 or higher degree of significance) at 1, 3, and 5 min.

**Fig. 5.** Effect of S-1-P on DNA synthesis by subcultured HSCs. Subcultured HSCs at low density (1 × 10⁶ cells/cm²; 25% confluence) were incubated with 10 μM S-1-P and 10 ng/ml PDGF-BB for 24 h and labeled with 5 μCi/ml [methyl-³H]thymidine for the last 2 h. The radioactivity in the trichloroacetic acid-precipitable materials in the cells was expressed as DNA synthesis. The results are expressed as fold increase of the nontreated control. Bars represent means ± SD of 3 experiments.

**Fig. 6.** Effect of S-1-P on contraction of collagen lattices containing HSCs. Freshly isolated HSCs (A) or subcultured HSCs (B) were inoculated to the collagen lattices, and after cell attachment for 72 h the cells were placed in serum-free medium for 6 h. The various concentrations of S-1-P were then added to the medium. At various times, the lattices were detached by gentle circumferential dislodgment using a 200-μl micropipette tip. Contraction was measured as change in the lattice area. Bars represent means ± SD of 3 experiments. Control, cells not treated with S-1-P. Changes were significant (P < 0.05 or higher degree of significance) at 2 and 6 h for 0.1, 1, and 10 μM S-1-P.

**Fig. 7.** Effect of C3 exotoxin and S-1-P on contraction of collagen lattices containing subcultured HSCs. Subcultured HSCs were inoculated to the collagen lattices, and after cell attachment for 72 h the cells were placed in serum-free medium with or without 0.2–2 μg/ml C3 exotoxin for 6 h. Ten micromolar S-1-P was added to the medium. At various time, the lattices were detached by gentle circumferential dislodgment using a 200-μl micropipette tip. Contraction was measured as change in the lattice area. Bars represent means ± SD of 3 experiments. Control, cells untreated with S-1-P. Changes were significant (P < 0.05 or higher degree of significance) at 2 and 6 h for 0.2 and 2 μg/ml C3 exotoxin compared with S-1-P alone.
and high-density cell cultures might be explained by the distinct binding of S-1-P to the cells, the binding of \(^{[3}H]\)S-1-P was measured in cell membranes isolated from the cells in both conditions. The specific binding of \(^{[3}H]\)S-1-P to the cell membranes of low-density cell cultures was not different from that of high-density cell cultures, as shown in Table 1, indicating that the density-dependent mitogenic effect of S-1-P on subcultured HSCs is not caused by the difference in binding of S-1-P. Consistently, EDG1 and AGR16 mRNA levels in subcultured HSCs in low-density cell cultures were not different from those in high-density cell cultures (data not shown).

**DISCUSSION**

In the current study, S-1-P increased the proliferation of culture-activated HSCs in a PTX-sensitive, MAPK-dependent and cell density-dependent manner but did not increase the proliferation of HSCs in early culture. S-1-P enhanced the contraction of HSC-populated collagen lattices in a C3 exotoxin-sensitive manner, irrespective of the state of cell activation. Among the receptors for S-1-P, mRNAs of EDG1 and AGR16, but not of EDG3, were detected in HSCs. EDG1 mRNA levels were downregulated in the process of activation in culture on noncoated plastic or in vivo induced by CCl\(_4\) treatment. In contrast, AGR16 mRNA levels were unchanged by activation.

PDGF-BB is a well-known mitogen for HSCs (31). Because S-1-P was shown to be an intracellular second messenger of PDGF in cultured fibroblasts (29), a possible relation between the signaling pathways of S-1-P and PDGF-BB was examined. As shown in Fig. 5, the effects of 10 \(\mu\)M S-1-P and 10 ng/ml PDGF-BB were additive. Both doses are maximal to exert mitogenic action (10, 31). Thus the signaling pathway of S-1-P is distinct from that of PDGF-BB in HSCs. Consistent with this, the sensitivities to PTX of the mitogenic actions of PDGF and S-1-P are distinct; in the presence of PTX, the mitogenic action of PDGF is shown to be partially reduced (26), whereas that of S-1-P is completely abolished (Fig. 3).

S-1-P enhanced the contraction of collagen lattices containing HSCs, irrespective of the state of activation (Fig. 6), and C3 exotoxin, a specific inactivator of Rho, reduced the S-1-P-mediated enhancement of contraction of HSC-populated collagen lattices (Fig. 7). Recent

**Table 1. Binding of S-1-P to cell membranes of HSCs**

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<tr>
<th>Cell Density, cells/cm(^2)</th>
<th>Specific S-1-P Binding, fmol/µg protein</th>
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<tr>
<td>(1 \times 10^4)</td>
<td>0.29 ± 0.06</td>
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<tr>
<td>(4 \times 10^4)</td>
<td>0.32 ± 0.03</td>
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Binding data are shown as means ± SD of 3 experiments. Cell membranes were prepared from subcultured hepatic stellate cells (HSCs) grown to the indicated cell densities, which corresponded to 25 and 100% confluency. The cell membranes at 60 µg/ml were incubated in 500 µl of 20 mM Tris · HCl, pH 7.5, containing [\(^{3}H\)]sphingosine 1-phosphate (S-1-P).
S-1-P could lose its mitogenic activity. It is worthwhile to assume that S-1-P stimulates the contractility of HSCs through the activation of Rho. Determination of cellular contraction using collagen lattices was introduced by Bell and colleagues (4) and has been validated as a model of cellular contraction in a variety of cells including HSCs (33). However, it was recently pointed out that this method may not allow differentiation between active contraction and passive changes in tension (35). In fact, proliferation or cell spreading would contribute to changes in lattice area. Because the enhancement of contraction of collagen lattices containing HSCs was evident as early as 30 min after the addition of S-1-P to the culture medium (Fig. 6), proliferation could not contribute to changes in lattice area. Although the possible contribution of cell spreading to enhanced contraction of collagen lattices remains to be investigated, our results imply that S-1-P might modulate blood flow via sinusoidal constriction, considering that HSCs surround the sinusoid, extending their processes into collagen fibers (21, 41).

When $^{14}$C-labeled S-1-P was injected in mice, the major part was distributed into the liver (30). This observation may suggest that $I$ the receptor(s) and/or the transporter(s) of S-1-P are highly expressed in the liver and that $2$ the liver is one of the major target organs of S-1-P. Consistently, mRNA expressions of EDG1 and AGR16 in mouse liver were reported (46). Similarly in HSCs, EDG1 and AGR16 mRNAs were detected (Figs. 8 and 9). The critical question is why multiple receptors exist for S-1-P. Controversy exists as to whether these receptors might couple to distinct signaling pathways regulating specific biological responses or might couple to similar signaling pathways in a redundant manner (2). Because EDG1 mRNA levels were lower in fully activated HSCs, which are responsive to S-1-P to proliferate, it is likely that AGR16 could be involved in the stimulation of proliferation. Recent observations report that AGR16 may be involved in Rho-mediated action stimulated by S-1-P (15, 40). Thus it would be reasonable to assume that AGR16 might also be involved in the stimulation of contraction of HSCs. These matters may be clarified with the discovery of subtype-specific antagonists to each receptor.

The S-1-P levels in human plasma and serum were reported to be $\sim 200$ and $500$ nM, respectively (45). Therefore, where platelet activation or destruction occurs, the S-1-P level could be in the range exerting its effects found in this study. Considering that HSCs are capable of production of extracellular matrices and modulation of blood flow in sinusoids, our results suggest that S-1-P may play a role in the wound healing process after liver injury by stimulating proliferation and contraction of HSCs. Interestingly, the mitogenic effect of S-1-P on activated HSCs was more evident in sparse cell cultures. This evidence might imply that after proliferation of HSCs on wound healing in vivo S-1-P could lose its mitogenic activity. It is worthwhile to investigate the pathophysiological significance of S-1-P in the liver diseases.

REFERENCES


