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Sphingosine-1-Phosphate Mediates Fibrosis in Orbital Fibroblasts in Graves' Orbitopathy

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<TABLE OF CONTENTS>

LIST OF FIGURES

ABSTRACT

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Purpose: To investigate the effect of sphingosine-1-phosphate (S1P) on fibrosis in orbital fibroblasts in Graves' orbitopathy (GO).

Methods: Orbital fibroblasts were cultured from orbital adipose/connective tissues of patients with GO and healthy control subjects. Effects of treatment with transforming growth factor (TGF)-β and cigarette smoke extract (CSE) on S1P receptor (S1PR) messenger RNAs (mRNA) were evaluated by real-time polymerase chain reaction. To evaluate the role of S1P in fibrosis, cells were pretreated with W146 (S1PR1 antagonist), JTE013 (S1PR2 antagonist), FTY720 (S1PR1 modulator), or 5C (sphingosine kinase-1 blocker) for 1 h before stimulation with TGF-β, CSE, or interleukin (IL)-1β. Expression of fibrosis-related proteins—collagen Iα, fibronectin, and α-smooth muscle actin (SMA)—and tissue remodeling-related proteins—matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinase (TIMP)-1—was then evaluated by Western blotting.

Results: Expression levels of S1PR mRNAs in GO orbital fibroblasts increased upon TGF-β and CSE treatment. Treatment with S1PR blockers and 5C inhibited TGF-β and CSE-induced expression of collagen Iα, fibronectin, and α-SMA as well as IL-1β-induced expression of MMP-1, MMP-2, MMP-9, and TIMP-1. Exogenous S1P treatment without pro-fibrotic stimulants upregulated collagen Iα, fibronectin, α-SMA, MMP-1, MMP-2, MMP-9, and TIMP-1

expression in a dose-dependent manner.

Conclusion: Blocking of S1PR activity and inhibition of S1P synthesis led to decreased expression of fibrosis and tissue remodeling-related proteins in primary cultures of orbital fibroblasts derived from patients with GO. Thus, modulation of S1P activity might have therapeutic potential in the suppression of fibrosis in GO.

Key words: fibrosis, Graves' orbitopathy, orbital fibroblast, sphingosine-1-phosphate

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I. INTRODUCTION

Graves' orbitopathy (GO), an autoimmune component of Graves' disease, occurs in up to 50% of patients with Graves' disease.[1] It is characterized by inflammation and swelling of orbital tissue, with fibrosis and adipogenesis being predominant features.[2–4] Current evidence indicates orbital fibroblasts as the key effector cells in GO. Although it is widely known that inflammation and adipogenesis are key participants in the pathogenesis of GO, little is known about its fibrogenic mechanism.

Three major classes of lipids—glycerolipids, sphingolipids, and sterols—constitute the double-layered surface membrane of all eukaryotic cells.[5] It was previously believed that lipids play exclusive roles in energy metabolism and membrane composition. Recent studies, however, have elucidated the concept of 'bioactive lipids' and identified the biologically active sphingosine-1-phosphate (S1P), which regulates diverse cellular processes including cytoskeletal rearrangement, cell survival and migration, and inflammation.[5,6] Cellular concentrations of S1P are largely controlled through S1P synthesis by phosphorylation of sphingosine catalyzed by sphingosine kinase (SphK) and, to a lesser extent, by S1P degradation catalyzed by S1P phosphatase or S1P lyase.[7] Of the two isoforms of SphK—SphK1 and SphK2—the latter has approximately 10-fold lower specific activity than

SphK1.[7] Synthesis of S1P occurs intracellularly in organelles and on the inner leaflet of the plasma membrane. It acts as an intracellular second messenger, mediating calcium homeostasis and apoptosis.[5,8] Additionally, S1P is secreted extracellularly through the adenosine triphosphate-binding cassette transporter superfamily and reaches the outer leaflet of the plasma membrane,[6] where it binds to S1P receptors (S1PRs) and acts as an autocrine and paracrine modulator.[6,9,10] Although S1P has a dual mechanism of action as an intracellular second and extracellular first messenger, its greatest effect is exerted extracellularly through binding with S1PRs.[11] The five S1PRs, S1PR1–5, exhibit selective tissue expression, which is crucial for their biological function, and employ well-known G-protein coupled receptor intracellular signaling pathways to mediate their specific effect.[6,9,12]

Emerging evidence indicates that S1P acts on several types of target cells and is engaged in fibrogenic processes in the liver,[13,14] kidney,[15] lung,[16] and cardiac muscles.[17] Occurrence of SphK and S1PR has also been reported in retinal pigment epithelium (RPE) and conjunctival and corneal fibroblasts.[18] In RPE, S1P engages in proliferation and pro-fibrotic protein expression.[18] In a previous study, anti-S1P monoclonal antibodies inhibited sub-retinal fibrosis in a murine model of laser-induced choroidal neovascularization.[19]

We have previously demonstrated the expression S1PRs in GO orbital fibroblasts as well as the role of S1P in the differentiation of orbital adipocytes.[20] In the present study, we aimed to determine whether S1P mediates fibrogenic processes in GO orbital fibroblasts and whether blocking of S1P and S1PRs decreases fibrosis in GO.

II. MATERIALS AND METHODS

1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS),

penicillin, and gentamycin were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Recombinant human transforming growth factor (TGF)-β and interleukin (IL)-1β were purchased from R&D System (Minneapolis, UT, USA). Sphingosine-1-phosphate, FTY720—an S1PR1 modulator that causes internalization of S1PR1—and 5C—an SphK1 blocker—were purchased from Sigma-Aldrich (St. Louise, MO, USA). W146, a trifluoroacetate salt that acts as an S1PR1 antagonist, and JTE013, an S1PR2 antagonist, were purchased from Cayman Chemical (Ann Arbor, MI, USA). Cigarette smoke extract (CSE) was freshly prepared within an hour of each experiment from commercially available filtered cigarettes (Marlboro 20 class A cigarettes [8.0 mg tar; 0.7 mg nicotine]; Philip Morris Korea, Inc., Seoul, Korea) as described in our previous study.[20]

2. Cell culture protocols

Orbital adipose/connective tissue explants were obtained as surgical waste during decompression surgery from eight patients with GO, and normal control tissues were harvested during eyelid or orbital surgery from eight individuals with no history or clinical evidence of thyroid disease or GO. All eight patients with GO had achieved stable euthyroidism at the time of surgery, and their clinical activity scores at the time of surgery were less than 4. Additionally, none of the patients with GO had received steroid treatment or radiotherapy for at least 3 months before surgery. For control orbital tissues, we tried to match the clinical characteristics of non-GO patients and patients with GO; however, because of the limited number of donors, it was not possible to achieve perfect matching. The institutional review board of the Severance Hospital, Yonsei University College of Medicine, Seoul, Korea, approved the study, and written informed consent was obtained from all participants after explanation of the nature and possible consequences of the study. This study followed the tenets of the Declaration of Helsinki.

Primary cultures of orbital fibroblasts were established as described in our previous study.[20] Briefly, minced tissue was placed directly in 1:1 DMEM:F12 medium with 20% FBS and antibiotics. Upon growth of fibroblasts from the explants, monolayers were passaged serially with trypsin/ ethylenediaminetetraacetic acid solution, and cultures were maintained in DMEM with 10% FBS and antibiotics. Cells between the second and fifth passages were used for analysis.

3. Real-time polymerase chain reaction

Expression levels of S1PR1–3 were evaluated by real-time polymerase chain reaction (PCR). RNA isolation and real-time PCR were performed as described previously.[20] All PCR reactions were performed in triplicate, and all samples were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels. Expression levels were determined by the 2-ΔΔCt method as fold change of threshold cycle (Ct) value relative to the control. Amplification bands were quantified by densitometry and normalized against corresponding GAPDH bands to control for PCR variability. The primers used for amplification were as follows: human S1PR1, 5´-TGC GGG AAG GGA GTA TGT TT-3´ (forward) and 5´-CCA TCC CCA CCA CAC TCA AC-3´ (reverse); human S1PR2, 5´-GCC TCT CTA CGC CAA GCA TTA-3´ (forward) and 5´-TTG AGC GGA CCA CGC AGT A-3´ (reverse); human S1PR3, 5´-TGA TTG TGG TGA GCG TCT TCA-3´ (forward) and 5´-GGC CAC ATC AAT GAG GAA GAG-3´ (reverse); and GAPDH 5´-GCC AAG GTC ATC CAT GAC AAC-3´ (forward) and 5´-GTC CAC CAC CCT GTT GCT GTA-3´ (reverse).

4. Western blotting

Western blot analysis of SphK1, collagen Iα, fibronectin, α-smooth muscle actin

(SMA), matrix metalloproteinase (MMP)-1, MMP-2, MMP-7, MMP-9, and tissue inhibitor of metalloproteinase (TIMP)-1 was performed as described previously.[20] The relative quantity of protein in each immunoreactive band was quantified by densitometry and normalized to the quantity of β-actin in the same sample. Anti-SphK1, anti-fibronectin, anti-MMP-7, and anti-TIMP-1 antibodies were purchased from Abcam (Cambridge, UK), anti-collagen Iα antibody from Pierce (Rockford, IL, USA), anti-α-SMA antibody from Dako Corporation (Carpinteria, CA, USA), anti-MMP-2 and anti-MMP-9 antibodies from Cell Signaling Technology (Beverly, MA, USA), and anti-MMP-1 and anti-β-actin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

5. Transfection with short interfering RNA (siRNA)

siRNA of SphK1 and negative control siRNA were purchased from Santa Cruz Biotechnology. Approximately 80% confluent orbital fibroblasts from patients with GO were prepared in 100-mm plates. Negative control siRNA or SphK1 siRNA was transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. After transfection, cells were incubated with or without 5 ng/ml TGF-β for 24 h.

6. Statistical analysis

All experiments were performed with cells from at least three different samples, with the samples being assayed in duplicate or triplicate each time. For statistical analysis of the Western blotting and real time-PCR results, mean values and standard deviations were calculated for normalized measurements of each protein or mRNA from at least three different samples. Differences in evaluated variables between the experimental and control groups were assessed by the Student's t-test or Wilcoxon rank-sum test using the SPSS program for Windows, version 16 (SPSS, Chicago, IL, USA). Values of $P < 0.05$ were considered significant.

III. RESULTS

1. Effect of TGF-β and CSE treatment on S1PR1–3 mRNA expression in orbital fibroblasts

To determine the effect of TGF-β and CSE treatment on S1PR1–3 mRNA expression, orbital fibroblasts derived from individuals with and without GO were treated with TGF-β (5 ng/ml) or CSE (2.5%) for 16 h. mRNA expression levels of S1PR1–3 were compared between treated and untreated cells by real-time PCR analysis. Although TGF-β induced a marked increase in S1PR1 mRNA expression in both GO and non-GO orbital fibroblasts, the increment in non-GO cells was lower compared to that in GO cells. Expression of S1PR2 mRNA in TGF-β-treated GO cells was also significantly increased (Figure 1A). Treatment with CSE resulted in a marked increase of S1PR1–3 expression in both GO and non-GO orbital fibroblasts, with the degree of induction in GO cells being higher compared to that in non-GO cells (Figure 1B).

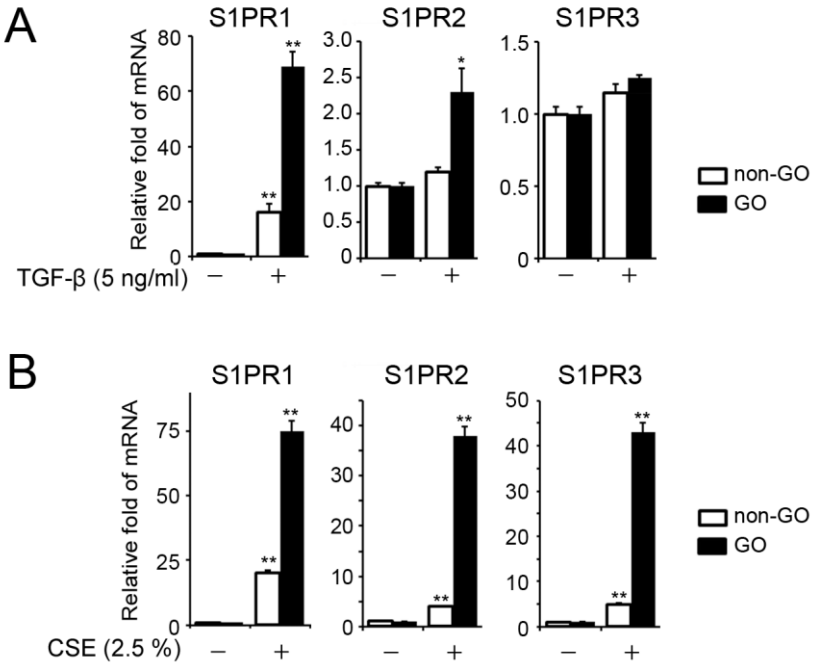

Figure 1. Effect of TGF-β and CSE treatment on mRNA expression of S1PR1–3 in orbital fibroblasts. Orbital fibroblasts of individuals with (black columns) and without (white columns) GO were treated with 5 ng/ml TGF-β or 2.5% CSE for 16 h. mRNA expression levels of S1PR1–3 in (A) TGF-β- and (B) CSE-treated cells were compared with those in untreated cells by real-time PCR. Data in the columns indicate the mean relative fold of mRNA levels ± SD of three experiments (*P < 0.05 and **P < 0.01 versus untreated control cells). CSE = cigarette smoke extract; GO = Graves' orbitopathy; mRNA = messenger RNA; PCR = polymerase chain reaction; S1PR = sphingosine-1-phosphate receptor; SD = standard deviation; TGF = transforming growth factor.

2. S1PR and SphK1 Blockers Attenuate Pro-Fibrotic Proteins in GO Orbital Fibroblasts

Expression levels of pro-fibrotic proteins—collagen Iα, fibronectin, and α-SMA—in TGF-β-stimulated GO orbital fibroblasts with or without pretreatment with S1PR (W146, JTE013, or FTY720) or SphK1 (5C) blockers were evaluated by Western blotting as described previously.[21–24] While TGF-β-induced expression of collagen Iα and fibronectin was downregulated by pretreatment with FTY720 or 5C, TGF-β-induced α-SMA expression was downregulated by pretreatment with W146, JTE013, FTY720, or 5C (Figure 2A). Upon investigating the effect of S1PR and SphK1 blockers on oxidative stress-induced pro-fibrotic protein expression using CSE, CSE-induced expression of collagen Iα, fibronectin, and α-SMA was found to be downregulated by pretreatment with W146, JTE013, FTY720, and 5C (Figure 2B). In addition, the expression levels of pro-fibrotic proteins in non-stimulated GO orbital fibroblasts with or without pretreatment with S1PR (W146, JTE013, or FTY720) or SphK1 (5C) blockers were evaluated, the results of which are presented in Figure 3A.

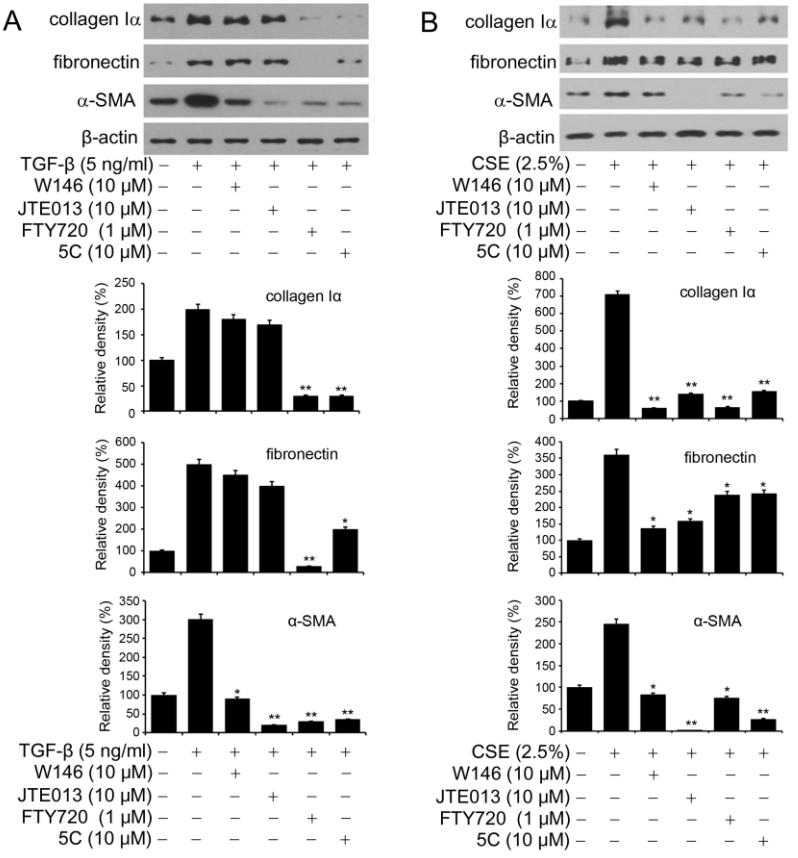

Figure 2. Effect of S1PR and SphK1 blockers on expression of TGF-β- or CSE-induced pro-fibrotic proteins in orbital fibroblasts in GO. Confluent orbital fibroblasts derived from individuals with GO were either untreated or pretreated with 10 μM W146, 10 μM JTE013, 1 μM FTY720, or 10 μM 5C for 1 h prior to treatment with TGF-β (5 ng/ml) or CSE (2.5%) for 24 h. Collagen-Iα, fibronectin, and α-SMA levels in (A) TGF-β- and (B) CSE-treated cultured cells were assayed by Western blotting. Data in the columns indicate the mean relative density ratios ± SD of three experiments (*P < 0.05 and **P < 0.01 versus TGF-β or CSE-treated cells without pretreatment). CSE = cigarette smoke extract; GO = Graves' orbitopathy; S1PR = sphingosine-1-phosphate receptor; SphK1 = sphingosine kinase 1; SD = standard deviation; SMA = smooth muscle actin; TGF = transforming growth factor.

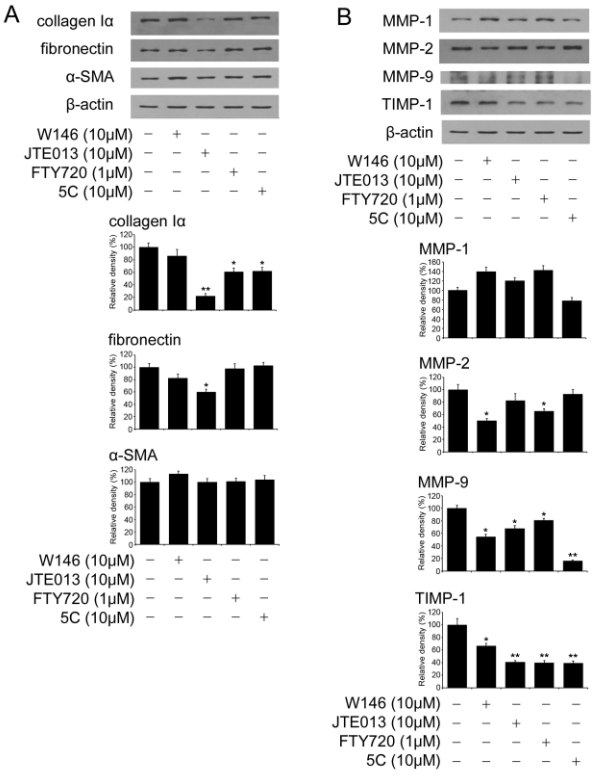

Figure 3. Effect of S1PR and SphK1 blockers on expression of pro-fibrotic and tissue-remodeling proteins in non-stimulated orbital fibroblasts in GO. Confluent orbital fibroblasts derived from individuals with GO were either untreated or treated with 10 μM W146, 10 μM JTE013, 1 μM FTY720, or 10 μM 5C for 24 h. Expression levels of (A) pro-fibrotic proteins (collagen-Iα, fibronectin, and α-SMA) and (B) tissue-remodeling proteins (MMP-1, MMP-2, MMP-9, and TIMP-1) in cultured cells were assayed by Western blotting. Data in the columns indicate the mean relative density ratios ± SD of three experiments (*P < 0.05 and **P < 0.01 versus untreated control cells). GO = Graves' orbitopathy; MMP = matrix metalloproteinase; S1PR = sphingosine-1-phosphate receptor; SphK1 = sphingosine kinase 1; SD = standard deviation; SMA = smooth muscle actin; TIMP = tissue inhibitor of metalloproteinase.

3. Silencing of SphK1 expression by siRNA-mediated inhibition of TGF-β-induced pro-fibrotic protein expression

The levels of TGF-β-induced collagen Iα, fibronectin, and α-SMA expression in GO orbital fibroblasts transfected with SphK1-targeted siRNA were compared with those in negative control siRNA-transfected cells. Western blotting findings revealed that transfection with SphK1 siRNA resulted in downregulation of SphK1. In addition, TGF-β-induced collagen Iα and α-SMA expression levels were significantly decreased in SphK1-knockdown cells (Figure 4).

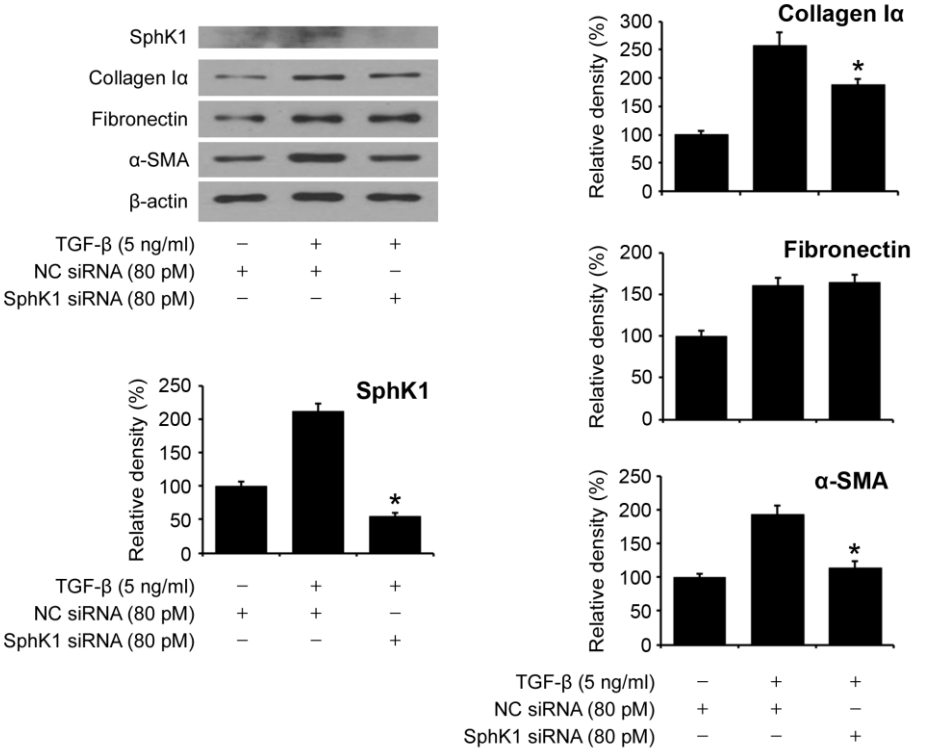

Figure 4. Effect of siRNA-mediated Sphk1 knockdown on TGF-β-induced expression of pro-fibrotic proteins. Approximately 80% confluent orbital fibroblasts from patients with GO were prepared in 100-mm plates. Transfection of NC or SphK1 siRNA was performed with Lipofectamine 2000 in accordance with the manufacturer's instructions. After transfection, cells were incubated with or without 5 ng/ml TGF-β for 24 h. Expression levels of collagen-Iα, fibronectin, and α-SMA were then evaluated by Western blotting. Data in the columns indicate the mean relative density ratios ± SD of three experiments (*P < 0.05 versus NC siRNA transfected TGF-β-stimulated cells). GO = Graves' orbitopathy; NC = negative control; SD = standard deviation; siRNA = short interfering RNA; SMA = smooth muscle actin; SphK1 = sphingosine kinase 1; TGF = transforming growth factor.

4. Exogenous S1P promotes pro-fibrotic and tissue-remodeling protein expression in GO orbital fibroblasts

Collagen Iα, fibronectin, and α-SMA expression levels in GO orbital fibroblasts treated with varying concentration of S1P were evaluated by Western blotting. Exogenous treatment with S1P resulted in dose-dependent increases in collagen Iα, fibronectin, and α-SMA expression (Figure 5A).

To investigate the role of S1P in tissue remodeling in GO, MMP-1, MMP-2, MMP-9, MMP-7, and TIMP-1 expression levels in GO orbital fibroblasts treated with different concentrations of S1P were evaluated by Western blotting. Expression levels of MMP-1, MMP-2, MMP-9, and TIMP-1 were found to be significantly increased upon treatment with 5 and 10 μM of S1P (Figure 5B; Figure 6A).

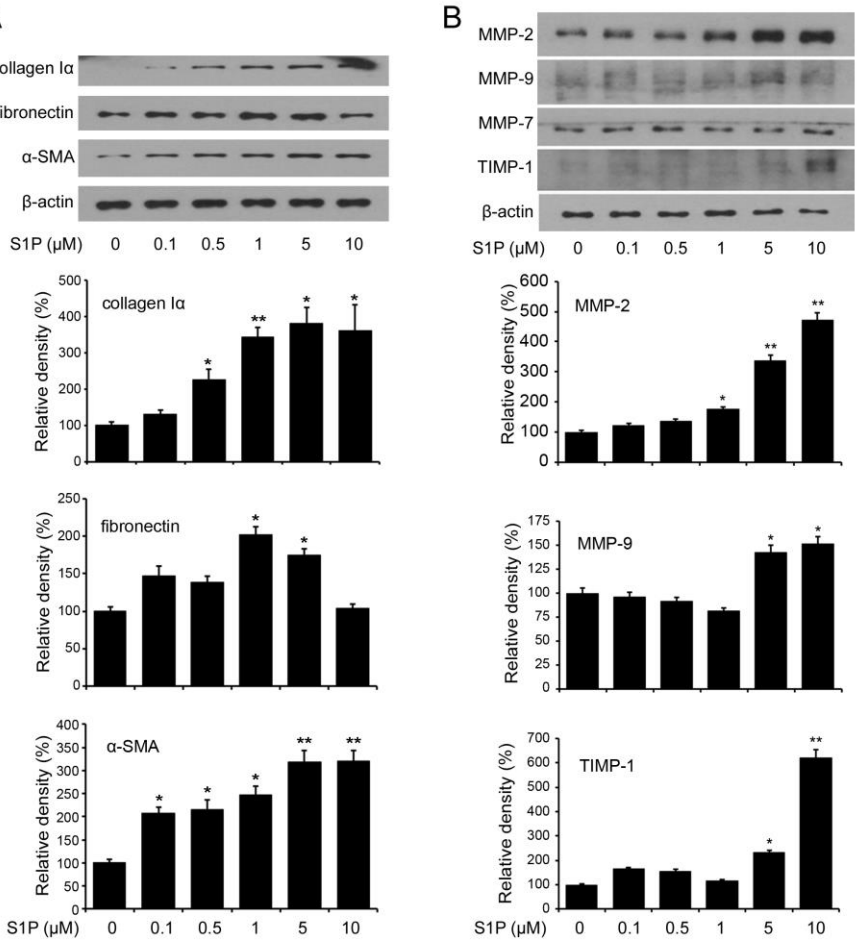

Figure 5. Effect of S1P on collagen Iα, MMP-2, MMP-9, and TIMP-1 expression in orbital fibroblasts in GO. Confluent orbital fibroblasts derived from individuals with GO were treated with different concentrations of S1P (0–10 μM) for 16 h. Expression levels of (A) collagen Iα, fibronectin, and α-SMA and (B) MMP-2, MMP-9, MMP-7, and TIMP-1 were then evaluated by Western blotting. Data in the columns indicate the mean relative density ratios ± SD of three experiments (*P < 0.05 and **P<0.01 versus untreated control cells). GO = Graves' orbitopathy; MMP = matrix metalloproteinase; S1P = sphingosine-1-phosphate; SD = standard deviation; SMA = smooth muscle actin; TIMP = tissue inhibitor of metalloproteinase.

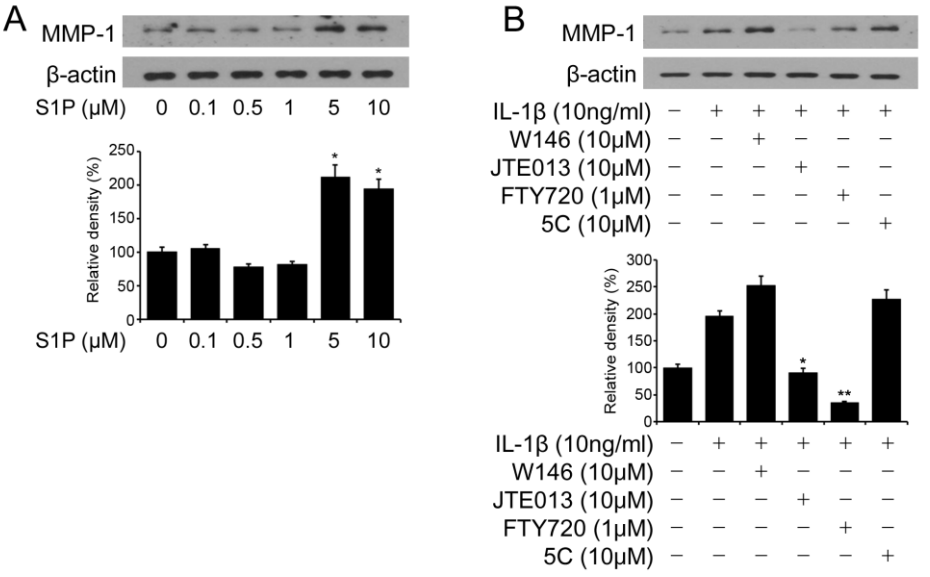

Figure 6. Effect of exogenous S1P on MMP-1 expression in orbital fibroblasts in GO. (A) Confluent orbital fibroblasts derived from individuals with GO were treated with different concentrations of S1P (0–10 μM) for 16 h. Expression levels MMP-1 were then evaluated by Western blotting. Data in the columns indicate the mean relative density ratios ± SD of three experiments (*P < 0.05 versus untreated control cells). (B) Confluent orbital fibroblasts derived from individuals with GO were either untreated or pretreated with 10 μM W146, 10 μM JTE013, 1 μM FTY720, or 10 μM 5C for 1 h prior to treatment with IL-1β (10 ng/ml, 24 h). Expression levels of MMP-1 were then evaluated by Western blotting. Data in the columns indicate the mean relative density ratios ± SD of three experiments (*P < 0.05 and **P < 0.01 versus IL-1β-treated cells without pretreatment). GO = Graves' orbitopathy; IL = interleukin; MMP = matrix metalloproteinase; S1P = sphingosine-1-phosphate; SD = standard deviation.

5. S1PR and SphK1 blockers attenuate tissue remodeling proteins in GO orbital fibroblasts

Expression levels of MMP-1, MMP-2, MMP-9, MMP-7, and TIMP-1 in IL-1β-stimulated GO orbital fibroblasts with or without pretreatment with W146, JTE013, FTY720, or 5C were evaluated by Western blotting. S1PR inhibitors and 5C inhibited the IL-1β-induced expression of MMP-1, MMP-2, MMP-9 and TIMP-1, although their specific actions of the inhibitors were varied (Figure 7; Figure 6B). W146 inhibited IL-1β-induced expression of MMP-2, MMP-9, and TIMP-1; JTE013 inhibited IL-1β-induced expression of MMP-1, MMP-9, and TIMP-1; FTY720 inhibited IL-1β-induced expression of MMP-1, MMP-2, MMP-9, and TIMP-1; and 5C inhibited IL-1β-induced expression of MMP-2 and TIMP-1. Pretreatment with S1PR blockers or 5C had no effect on MMP-7 expression. In addition, the expression levels of tissue-remodeling proteins in non-stimulated GO orbital fibroblasts with or without pretreatment with W146, JTE013, FTY720, or 5C were evaluated, the results of which are presented in Figure 3B.

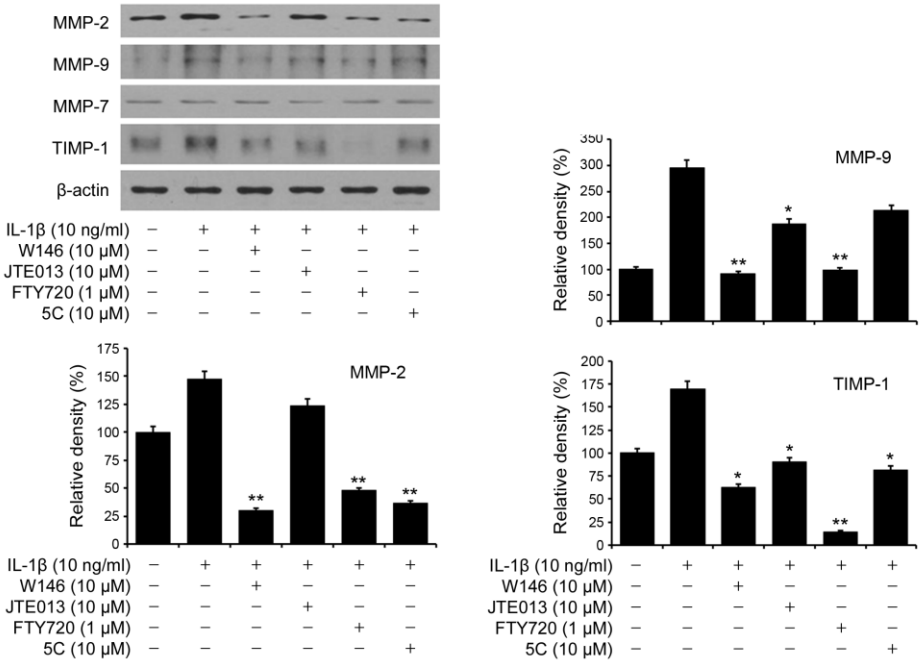

Figure 7. Effect of S1PR and SphK1 blockers on expression of IL-1β-induced tissue remodeling proteins in orbital fibroblasts in GO. Confluent orbital fibroblasts derived from individuals with GO were either untreated or pretreated with 10 μM W146, 10 μM JTE013, 1 μM FTY720, or 10 μM 5C for 1 h prior to treatment with IL-1β (10 ng/ml, 24 h). Expression levels of MMP-2, MMP-9, MMP-7, and TIMP-1 were then evaluated by Western blotting. Data in the columns indicate the mean relative density ratios ± SD of three experiments (*P < 0.05 and **P < 0.01 versus IL-1β-treated cells without pretreatment). GO = Graves' orbitopathy; IL = interleukin; MMP = matrix metalloproteinase; S1PR = sphingosine-1-phosphate receptor; SphK1 = sphingosine kinase 1; SD = standard deviation; TIMP = tissue inhibitor of metalloproteinase.

IV. DISCUSSION

Although pathogenesis of GO involves inflammation, adipogenesis, and fibrosis, glucocorticoids remain the mainstay of medical treatment for GO. Glucocorticoids are primarily effective in active inflammatory conditions and have a limited role in the treatment of fibrosis of orbital connective tissues.[25] Here, we investigated the role of S1P, a potential therapeutic target, as a mediator of fibrosis in GO.

As a central mediator of fibrotic diseases, TGF-β induces fibroblasts to synthesize extracellular matrix (ECM).[26] It regulates the expression of pro-fibrotic proteins—collagen I, collagen IV, connective tissue growth factor, and fibronectin—through the Smad and rho/rho-associated protein kinase pathways.[10,26] Additionally, there is evidence of cross talk between S1PRs and TGF-β-induced fibrosis.[27] Not only does TGF-β regulate S1P production through modification of SphK expression and activity in fibrotic lesions, but it also modifies S1PR expression.[10,11] For example, TGF-β1 upregulates SphK1 expression in murine myoblasts in an Smad-dependent manner and concomitantly modifies S1PR expression by downregulating S1PR1 and upregulating S1PR3 mRNA expression.[28] In this report, we have demonstrated that TGF-β stimulates S1PR1–2 mRNA. Our data indicate a connection between S1P and TGF-β signaling in orbital fibroblasts.

The pro-fibrotic potential of fibroblasts is characterized by their capability to synthesize ECM components such as the collagen family of proteins and fibronectin. α-SMA is a marker that directs the differentiation of fibroblasts into myofibroblasts, which are key effector cells in fibrogenesis.[29] The downregulation of TGF-β-induced collagen Iα and fibronectin expression by FTY720 and 5C in the present study indicates that the S1P/S1PR1 pathway plays a role in mediating TGF-β-induced ECM synthesis. Additionally, given that FTY720, 5C, and JTE013 exhibited an inhibitory effect on TGF-β-induced

α-SMA expression, both S1P/S1PR1 and S1P/S1PR2 pathways seem to serve as mediators of TGF-β-induced myofibroblast differentiation. In this study, FTY720 and W146 exhibited different results in the experiment on S1PR1 inhibition, as demonstrated in Figure 2A. W146 is a specific S1PR1 antagonist, competitive for S1P.[30] On the other hand, FTY720 causes persistent activation of S1PR1, followed by internalization and downregulation of the receptor protein.[31] The inconsistencies in results between the two compounds might be due to their different mechanisms of action.

Cigarette smoking is the most important risk factor for the development and deterioration of GO; smoking activates pathways associated with adipogenesis, inflammation, and fibrosis.[32–34] Treatment with CSE induces oxidative stress in orbital fibroblasts by generating reactive oxygen species.[32,35] Oxidative stress influences S1P expression — moderate levels of oxidative stress increase S1P expression by activation of SphK1, and excessive oxidative stress decreases S1P expression by inducing SphK1 degradation.[36] Treatment with CSE also increases S1PR2–5 mRNA expression as well as SphK1–2 expression in THP-1 macrophages.[37,38] Given that CSE treatment increased S1PR1–3 mRNA expression in GO and non-GO orbital fibroblasts (Figures 1B), the S1P/S1PR pathway seems to play a role in response to oxidative stress in GO orbital fibroblasts. Additionally, since W146, JTE013, FTY720, and 5C inhibited CSE-induced fibrosis-related protein expression, the S1P/S1PR1–2 pathway might be a part of the mechanism underlying the pro-fibrotic effect of smoking in GO. Moreover, exogenous S1P treatment of GO orbital fibroblasts without TGF-β or CSE stimulation resulted in upregulation of collagen Iα, fibronectin, and α-SMA expression. Therefore, S1P also seems to have a direct effect on fibrosis in GO orbital fibroblasts, without stimulation by TGF-β or CSE.

Remodeling of ECM as well as excessive ECM production are important factors in the process of tissue fibrosis. Remodeling of ECM is largely effected

by the activities of proteinases that can degrade matrix, such as MMPs and their inhibitor, TIMP.[39,40] There is excellent evidence in both human liver disease and animal models indicating that hepatic fibrosis is potentially reversible.[41] Decline in TIMP levels, which tips the overall MMP–TIMP balance towards MMP, results in increased matrix degradation and net degradation of scar tissue.[42,43] However, very few studies have investigated tissue remodeling in GO. Han et al. reported that IL-1β-treatment of orbital fibroblasts increases TIMP-1 expression, thus disrupting the balance between MMPs and TIMP.[44] Additionally, treatment with antioxidants decreases the levels of MMPs and TIMP-1.[34,45] The results of the present study demonstrated that IL-1β increases MMP-2, MMP-9, and TIMP-1 expression in GO orbital fibroblasts, which is concordant with the findings of previous studies.[34,46] Since treatment with W146, JTE013, FTY720, and 5C resulted in the downregulation of IL-1β-induced expression of MMP-1, MMP-2, MMP-9, and TIMP-1, we believe that S1P might act as a mediator of inflammation-induced tissue remodeling. Additionally, since exogenous S1P treatment increased the expression of MMP-1, MMP-2, MMP-9, and TIMP-1, we assume that S1P also has a direct effect on stimulation of tissue remodeling. Matrix metalloproteinase-1, also known as interstitial collagenase, degrades fibrillar collagen types I, II, and III, which are major components of the ECM.[39] However, to date, expression of tissue remodeling proteins in GO orbital fibroblasts has only been reported in relation to TIMP-1, MMP-2, and MMP-9.[34,44,45] The present results demonstrate that IL-1β stimulation can induce MMP-1 expression in GO orbital fibroblasts and that S1P is involved in this induction process. It is possible that S1P-mediated upregulation or S1PR blocker-mediated suppression of collagen Iα and MMP-1 expression are related; however, this aspect has not been investigated in this study. Further confirmation of related pathways is necessary. Moreover, MMP expression varies depending on the organ, and while some MMPs are indeed anti-fibrotic, others might have pro-fibrotic functions.[40] For example, in the kidney, MMP-9

has pro-fibrotic activity, while MMP-2 has an anti-fibrotic function.[47,48] Therefore, detailed studies on elucidating the function of MMPs in orbital fibroblasts are required.

Orbital fibroblasts can be divided into two subsets on the basis of surface expression of Thy-1, a surface glycoprotein.[49] Each subset responds to different extracellular stimuli and differentiates into distinct cell types.[46,50] Only Thy-1+ orbital fibroblasts are capable of differentiation into myofibroblasts after TGF-β treatment, and only Thy1- cells differentiate into lipofibroblasts.[50] In the present study, although we did not sort orbital fibroblasts on the basis of Thy-1 expression, our findings demonstrated that S1P plays a role in TGF-β-induced myofibroblast differentiation, which is exclusive to Thy-1+ cells. Moreover, we have demonstrated in our previous report that S1P is also involved in the adipogenesis of GO orbital fibroblasts, which is exclusive to Thy-1- cells.[20] Therefore, S1P appears to play a role in the pathogenesis of GO in both Thy-1+ and Thy-1- orbital fibroblasts.

Orbital tissues of patients with GO are continuously exposed to pro-inflammatory and oxidative stress.[1,51] Although cultured orbital fibroblasts derived from individuals with and without GO often exhibit phenotypic differences, primary cultures of orbital fibroblasts might not directly reflect the in vivo inflammatory or fibrotic conditions. Because of this limitation, it is inevitable that pro-fibrotic or pro-inflammatory conditions are induced with stimulants to observe the effects of certain chemicals. In the present study, some of the evaluated S1PR blockers inhibited the expression of pro-fibrotic and tissue-remodeling proteins in non-stimulated cells. However, in most cases, the inhibitory effects of S1P blockers in TGF-β-, CSE-, or IL-1β-stimulated conditions were more prominent than those in the non-stimulated condition.

V. CONCLUSION

In conclusion, our results provide evidence indicating that S1P plays an important role in orbital tissue fibrosis in GO through induction of fibrosis- and tissue remodeling-related protein expression. These findings indicate that S1P blocking agents might have therapeutic potential in the suppression of fibrosis in GO.

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ABSTRACT (IN KOREAN)

갑상샘눈병증 환자의 안와 섬유모세포에서 섬유화에 대한 sphingosine-1-phosphate의 역할

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고 재 상

목적: 갑상샘눈병증의 안와 섬유모세포에서 일어나는 섬유화반응에 sphingosine-1-phosphate (S1P)가 미치는 영향을 알아보고자 하였다.

방법: 갑상샘눈병증 환자 및 건강한 대조군의 안와 지방조직으로부터 안와 섬유모세포를 배양하였다. S1P 수용체 RNA 발현에 대한 TGF-β 및 담배 연기 추출물의 치료 효과를 신시간 중합 효소 연쇄 반응으로 평가하였다. 섬유화에 대한 S1P의 역할을 평가하기 위해 세포를 TGF-β, 담배연기 추출물 혹은 인터루킨-1베타 자극 전 1시간 동안 W146 (S1PR1 길항제), JTE013 (S1PR2 길항제), FTY720 (S1PR1 조절제) 또는 5C (Sphingosine kinase-1 차단제)를 전처치 하였다. 이후 안와 섬유모세포에서 발현되는 섬유화 관련 단백질과 조직 리모델링 관련 단백질들의 발현을 평가하였다.

결과: TGF-β와 담배연기 추출물의 자극에 의해 안와 섬유모세포의 S1P수용체 mRNA의 발현이 증가하였다. S1P수용체 길항제와 5C 처치에 의해 TGF-β와 담배연기 추출물로 유도된 collagen Iα, fibronectin, α-SMA의 발현이 억제되었으며 인터루킨-1베타로 유도된 MMP-1, MMP-2, MMP-9, TIMP-1의 발현 또한 억제되었다.

섬유화 유도 자극 없이 외부에서 S1P를 투여하였을 때에도 collagen Iα, fibronectin, α-SMA, MMP-1, MMP-2, MMP-9, TIMP-1의 발현 증가가 용량 비례적으로 관찰되었다.

결론: 갑상샘눈병증 환자의 안와 조직에서 일차배양한 안와 섬유모세포에서 S1P수용체의 차단과 S1P 생성의 차단은 섬유화 관련 단백질과 조직 리모델링 관련 단백질의 발현을 억제시켰다. 즉, S1P의 활성도를 조절하는 것이 갑상샘눈병증에서 섬유화를 억제하는 측면에서 치료적 효과를 가질 수 있음을 시사한다.

핵심되는 말: 갑상샘눈병증, 안와 섬유모세포, 섬유화, 스핑고신-1-인산