Role of Sphingosine-1-Phosphate in Human Dental Pulp Cells to Form Hard Tissue

（ヒト歯髄細胞の硬組織形成におけるスフィンゴシン−1−リン酸の役割）

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Abstract:

Objectives: Preservation of the dental pulp depends on the stimulation of hard tissue formation within the pulp itself. However, the presence of sphingosine-1-phosphate (S1P) in vivo has attracted considerable attention. S1P reportedly promotes osteoblast differentiation, and is believed to be involved in hard tissue formation. In the present study, we assessed the ability of S1P to induce hard tissue formation in cultured human dental pulp cells (hDPCs).

Methods: hDPCs were cultured from aseptically extracted pulp tissue obtained from the first premolar of 20-year-old patients that required orthodontic treatment. The effect of S1P on hard tissue formation was observed by measuring alkaline phosphatase (ALP) activity and performing alizarin red staining. Additionally, BMP-2 mRNA, and BMP-2 and DSPP protein expression levels were examined to confirm hard tissue formation. We also investigated the expression of the S1P receptor mRNA and changes in intracellular calcium ion concentration ($[Ca^{2+}]i$) dynamics in S1P-stimulated hDPCs.

Result: Stimulation of cultured hDPCs with S1P increased ALP activity and enhanced alizarin red staining. Additionally, S1P stimulation elevated BMP-2 mRNA expression along with BMP-2 and DSPP protein levels. Moreover, mRNA expression of S1P receptors 1-3 was also observed in cultured hDPCs.

Conclusions: S1P stimulation enhances the expression of hard tissue formation markers in cultured hDPCs, suggesting that S1P promotes hard tissue formation. Moreover, we propose that S1P induces an increase in $[Ca^{2+}]i$ levels by facilitating the release of Ca^{2+} from the endoplasmic reticulum via S1P receptors 1-3.

Key words:

Calcification, Human dental pulp cells, Intracellular calcium ion concentration, Sphingosine-1-phosphate

Introduction

The pulp, composed of various cells, plays an important role in tooth function and preservation. Clinically, teeth with pulp loss are prone to root fractures and other problems, and require extraction. Unfortunately, the progression of inflammation in the unique pulp environment, which is surrounded by hard tissues, often leads to pulp loss during extraction. However, in the case of external pathological stimuli to the pulp, reparative dentin formation as a protective response to the induced inflammatory response preserves and safeguards the pulp. Recently, given the potential of the pulp for spontaneous healing, dentin and pulp regenerative therapy has been explored with the aim of functional regeneration, including dentin formation and pulp preservation therapy using calcium hydroxide or mineral trioxide aggregate (MTA) cement in direct pulp lamination[1-3]. Notably, the differentiation of pulp cells leads to the formation of hard tissue and facilitates healing. However, such therapies are associated with complications, including the formation of porous and heterogeneous hard tissue and necrosis in the pulp tissue due to the highly alkaline nature of calcium hydroxide[4-6].

The role of lipid mediators in living organisms has received considerable attention. In particular, sphingolipids, such as sphingosine-1-phosphate (S1P), have been studied for their ability to induce various cellular responses[7,8]. S1P is a complex lipid derived from sphingolipids, consisting of a phosphate-type polar head and a sphingolipid-type hydrophobic tail. S1P is present in the plasma at a concentration of a few μM and exhibits various physiological activities by binding to the five S1P receptors (S1PR1-5) on the cell membrane[9]. These are 7-transmembrane G-protein coupled receptors, and S1P is involved in various signaling pathways through their activation. S1P is mainly targeted to the vascular and immune systems and plays multiple roles as a lipid mediator in the regulation of cell proliferation, differentiation, adhesion, and other cellular functions[10-12]. The effects of S1P and its receptors have been utilized in drug discovery; for example, fingolimod (FTY720) is a novel immunosuppressant drug that targets S1P receptors and has been approved for the treatment of multiple sclerosis in Japan[13,14]. It functions as an antagonist by binding to S1PR after phosphorylation by FTY720P. Additionally, S1P activates bone differentiation signals and enhances bone regeneration by regulating differentiation factors against apical periodontitis-induced apical periodontal tissue

destruction. Matsuzaki et al. reported that S1PR1 and 2 of the five S1P receptors are highly expressed in osteoblasts exhibiting osteoblast differentiation and bone formation-promoting effects[15-17]. Thus, the relationship between S1P and its receptors has been exploited for the treatment of diseases.

Currently, there is growing interest in dentin/pulp regenerative and pulp preservation therapies aiming to promote the formation of robust and better-quality hard tissues for reliable pulp preservation. However, the precise mechanism underlying hard tissue formation remains unknown, which necessitates further clarification. Additionally, S1P, which is abundant in the blood[7,9], plays a significant role in signal transduction and physiological activity in pulp tissue, which is rich in vascular tissue; however, there research on this topic is insufficient. Therefore, in the present study, we investigated the ability of S1P to induce hard tissue formation in cultured human dental pulp cells (hDPCs) to elucidate its function in this process.

Materials and Methods

1. Cell culture

This study was approved by the Ethics Committee of the Nihon University School of Dentistry at Matsudo (No.: EC22-21-20-19-19-003-4).

Cells were cultured, as previously described by Somerman et al.[18]. Briefly, cells from the first premolars extracted for orthodontic reasons from 20-year-old patients who provided informed consent for the study, were used. The pulp was aseptically extracted, rinsed thoroughly with phosphate-buffered saline (PBS Tablets, TaKaRa Bio Inc., Shiga, Japan), cut into approximately 2 mm squares, and placed in a 35 mm cell culture dish. The cells were then cultured in an α-minimum essential medium (α-MEM, GIBCO BRL Life Technologies Ltd., Tokyo, Japan), supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich Co., St. Louis, MO, USA), 10,000 units/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml Fungizone (Antibiotic-Antimycotic (100×), GIBCO BRL Life Technologies Ltd.) at 37°C, 5% CO2, and 95% gas phase, and allowed to grow for six to eight passages, which were then used as hDPCs.

2. Stimulation conditions

S1P (Sphingosine-1-phosphate (d18:1), Cayman Chemical Co., Ann Harbor, MI, USA) was used at different concentrations: 0 (control), 0.1, 1, and 10 μM. To examine the calcification potential of hDPCs, a calcification induction medium containing 2 mM β-glycerophosphate (Sigma-Aldrich Co.) and 50 μg/ml ascorbic acid (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) was used in addition to α-MEM.

3. Treatment of hDPCs with S1P receptor antagonists

To analyze the effect of S1P antagonists on hDPCs, we used selective antagonists for S1PR1 (W146, 1 μM; Cayman Chemical Co.), S1PR2 (JTE013, 1 μM; Cayman Chemical Co.), and S1PR3 (CAY10444, 1 μM; Cayman Chemical Co.).

4. Alkaline phosphatase (ALP) activity

ALP activity was measured, as described previously by Ohshima et al.[19]. hDPCs (1×10^5 cells/35 mm dish) were cultured with various concentrations of S1P. The cells were cultured in calcification induction medium for 1 to 15 days, washed twice with PBS, and then treated with 0.1 mol/l glycine-NaOH buffer and 8 μmol/l p-nitrophenylphosphate as a substrate, at 37°C for 30 min. The reaction was stopped by adding 0.1 mol/l NaOH, and absorbance was measured at 415 nm. The enzyme activity was determined as 1 mUnit (mU) per μmol/l of p-nitrophenol released in 1 min.

5. Alizarin red staining

Calcified nodule formation in hDPCs (1×10^5 cells/35 mm dish) cultured with various concentrations of S1P for up to 21 days was confirmed using alizarin red staining. Briefly, hDPCs were cultured in the calcification induction medium supplemented with S1P at different concentrations. On days 19 and 21 of culture, cells were fixed with 10% formalin solution (FUJIFILM Wako Pure Chemical Corp.) for 30 min, and subsequently washed with distilled water. The cells were then stained with alizarin red solution (PG Research Co., Ltd., Tokyo, Japan) for 30 min, washed with distilled water, and stained for calcified nodules.

6. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from hDPCs (1×10^6 cells/10 cm dish) stimulated with 1 μM S1P for up to 3 h using the RNeasy® Mini Kit (QIAGEN, Hilden, Germany). Real-time RT-PCR was performed using total RNA as the template. First, reverse transcription was carried out using the One Step SYBR® Primescript ™ RT-PCR KIT II Perfect Real Time (TaKaRa Bio Inc.) and real-time PCR analysis was performed using the Thermal Cycler Dice® Real-Time System (TaKaRa Bio Inc.). Primers for BMP-2 and GAPDH (Table 1)[20,21] were used. Reverse transcription was performed at 42°C for 5 min, followed by denaturation at 95°C for 10 s, 50 cycles of denaturation at 95°C for 10 min, and annealing and elongation at 60°C for 30 s in two steps. Quantitative analysis was performed using the 2-ΔΔCT method[22] with GAPDH as a control.

7. Western blotting

After stimulation, hDPCs were treated with Cellytic M Cell (Sigma-Aldrich Co.) Layer containing 100 μM phenyl methyl sulfonyl fluoride, 0.2 mM ethylene glycol tetra-acetic acid (EGTA), and 2 mM ethylenediamine tetra-acetic acid (EDTA). Following protein quantification using the Bradford method[23], sodium dodecyl sulfate (SDS) sample buffer (New England Biolabs Japan Inc., Tokyo, Japan) was added to the protein lysates, which were then boiled for 5 min, and centrifuged at 15,000 rpm for 1 min. The supernatant was then electrophoresed on 7.5% and 12% SDS-polyacrylamide gel electrophoresis gels (Mini-PROTEAN TGX Gels, Bio-Rad Laboratories Inc., CA, USA), and transferred to nitrocellulose membranes (Bio-Rad Laboratories Inc.) using a transcription apparatus (NIHON EIDO Corp., Tokyo, Japan). The membranes were then blocked with skim milk (Becton Dickinson Co., New Jersey, USA) or Block Ace (KAC Co., Ltd., Kyoto, Japan) for 50 min at 26°C, followed by incubation with primary antibodies at 26°C for 2 h with shaking and then with the secondary antibody at 26°C for 90 min with shaking. The following primary antibodies were used: anti-BMP-2 antibody (1/2,000, BS3473, Bioworld Technology Inc., Nanjing, China), anti-DSPP antibody (1/2,000, sc-73632, Santa Cruz Biotechnology Inc., Dallas, TX, USA), or anti-β-actin antibody (1/2,000, 4970S, Cell Signaling Technology Inc., Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1/10,000, 170-6516, Bio-Rad Laboratories Inc.) or HRP-conjugated anti-rabbit IgG antibody (1/10,000, 7074S, Cell Signaling Technology Inc.) was used as the secondary antibody. Chemiluminescence was performed using an ECL prime western blotting detection system (GE HealthCare Technologies Inc., Amersham, UK), and the expression was confirmed by sensitizing the X-ray film (Hyperfilm, GE HealthCare Technologies Inc.). Intensities of specific proteins' bands were measured by Image J (National Institutes of Health, MD, USA) from the image translated using a computer.

8. RT-PCR

Total RNA was extracted from hDPCs (1×10^6 cells/10 cm dish) using RNeasy® Mini (QIAGEN). RT-PCR was performed using QIAGEN® One Step RT-PCR KIT (QIAGEN) on total RNA using DNA

primers for S1PR1, S1PR2, S1PR3, S1PR4, S1PR5, and GAPDH. The primer sequences used for RT-PCR are listed in Table 2[24,25]. The RT-PCR conditions were as follows: cDNA was synthesized in one cycle of 50°C for 30 min and 95°C for 15 min, followed by denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 10 min. The resulting PCR products were then subjected to 1.5% agarose gel electrophoresis, followed by ethidium bromide staining to confirm gene amplification.

9. Measurement of intercellular Ca^{2+} ($[Ca^{2+}]i$).

hDPCs (1×10^6 cells/10 cm dish) were incubated with 2 μM Fura-2/AM (DOJINDO LABORATORIES Kumamoto, Japan) in α-MEM at 37°C for 30 min. The cells were then detached with 0.25% trypsin and 0.02% EDTA, and incubated in Krebs-Ringer-HEPES solution (120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.96 mM NaH_2PO_4, 0.2% glucose, and 0.1% bovine serum albumin), and S1P was added at different concentrations after stirring in $CaCl_2$ and 20 mM HEPES buffer (pH 7.4). Fluorescence at 500 nm was measured using a CAF-110 spectrofluorometer (JASCO Corp., Tokyo, Japan), with excitation at 340 and 380 nm. Intracellular Ca^{2+} was calculated from the ratio of fluorescence intensities, as described previously by Grynkiewicz et al.[26]. Thapsigargin (1 μM, FUJIFILM Wako Pure Chemical Corp.) was used as an inhibitor of Ca^{2+}-ATPase (SERCA) on the endoplasmic reticulum (ER) membrane.

10. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Differences between multiple groups were analyzed by one-way analysis of variance along with the Tukey test. All statistical analyses were performed at 1 and 5% significance levels.

Results

1. Effect of S1P on ALP activity of hDPCs

To evaluate the effect of S1P on hard tissue formation in hDPCs, ALP activity was measured in cells cultured for 1-15 days in calcification induction medium with different S1P concentrations. ALP activity increased with time in the 0.1 and 1 μM S1P-treated groups, peaked on day 7, and then declined. In contrast, the 10 μM group showed significantly lower ALP activity than the control group on all days (Fig. 1A). Notably, on day 7, when ALP activity was at its peak, the 1 μM group exhibited a significant increase in ALP activity when compared with that in the control group. (Fig. 1B).

2. Calcified nodules in hDPCs with S1P stimulation

To examine the effect of S1P on the calcification ability of hDPCs, calcified nodules were stained with alizarin red after 19 and 21 days of culture in the calcification induction medium with varying concentrations of S1P. Cells in the 0.1 and 1 μM SIP-treated groups displayed increased staining when compared to the control group. However, the 10 μM group did not show any notable increase in staining (Fig. 2).

3. Effect of S1P on BMP-2 mRNA expression in hDPCs

The expression of osteogenesis-related genes in S1P-stimulated hDPCs was examined using RT-PCR. BMP-2 mRNA expression increased in a time-dependent manner in hDPCs treated with 1 μM S1P and increased significantly at 1 h post-stimulation when compared with that in the control group (Fig. 3).

4. Effect of S1P on the expression of hard tissue formation-related proteins in hDPCs

BMP-2 and DSPP protein expression in S1P-induced hDPCs was assessed using western blotting. In the 1 μM S1P-treated group, BMP-2 protein expression increased in a time-dependent manner, showing elevated expression at 3 and 6 h after S1P stimulation (Fig. 4A). Similarly, DSPP protein expression in hDPCs increased in a time-dependent manner upon stimulation with 1 μM S1P, with maximum expression on the 1st and 3rd day of stimulation (Fig. 4B).

5. Expression of S1PR mRNA in hDPCs

S1PR mRNA expression in hDPCs cultured in normal medium was determined using semi-quantitative RT-PCR. S1PR1, S1PR2, and S1PR3 mRNA expression levels were notable, whereas S1PR4 and S1PR5 mRNA expression levels were not (Fig. 5).

6. Effect of S1P on $[Ca^{2+}]i$ in hDPCs

We investigated whether S1P induced an increase in $[Ca^{2+}]i$ through S1PR. When 1 μM S1P was administered to hDPCs treated with Fura-2, $[Ca^{2+}]i$ increased, reaching a maximum after approximately 30 s, and then decreased over time (Fig. 6A). Moreover, this effect was concentration-dependent (Fig. 6B). There was no change in the SIP-induced increase in $[Ca^{2+}]i$ when extracellular Ca^{2+} in the Krebs-Ringer-HEPES solution was removed (Fig. 6C). Furthermore, when 1 μM S1P was administered after pre-treatment of cells with thapsigargin, $[Ca^{2+}]i$ did not increase (Fig. 6D).

We further investigated the role of S1PR in hDPCs by measuring $[Ca^{2+}]i$ following treatment with S1PR antagonists. Semi-quantitative RT-PCR analysis showed that hDPCs pretreated for 3 h with 1 μM of W146, JTE013, or CAY10444, which are antagonists for S1PR1-3, respectively, showed significant suppression in the maximum peak value of $[Ca^{2+}]i$ when stimulated with 1 μM S1P, when compared to the control cells that were not treated with the antagonists (Fig. 6E, F).

Discussion

S1P has recently garnered attention for its role in diverse signal transductions mediated by S1PR, which serves as a major signaling target in the vascular and immune systems[9-12]. Fingolimod, a drug that targets S1PR, has been approved for the treatment of multiple sclerosis and is currently in clinical use. The relationship between S1P and S1PR holds substantial importance, as these entities not only govern physiological functions but also play a role in drug discovery and treatment, with ongoing research endeavors[13,14]. However, there have been few reports on the nature of this relationship, specifically with respect to pulp research. Hence, in the present study, we explored the role of S1P on hard tissue formation in hDPCs and changes in $[Ca^{2+}]i$ in the same in the dental pulp.

First, we examined the effect of S1P on hard tissue formation in hDPCs by monitoring changes in ALP activity and calcified nodule formation using alizarin red staining. ALP activity serves as an indicator of hard tissue formation during the early stages of pulp calcification[27,28]. Our results showed that S1P increased ALP activity in the 0.1 and 1 μM S1P-treated hDPCs compared with that in the control group cells. Furthermore, a significant increase in ALP activity was observed on day 7, corresponding to the peak activity period. The formation of calcified nodules was also examined using alizarin red staining; an increase in staining was noted at 19 and 21 days post-S1P stimulation. These outcomes strongly suggest that 0.1 and 1 μM S1P promote the formation of calcified nodules in hDPCs. In contrast, the S1P 10 μM group did not show increased ALP activity or increased staining. Sakamoto et al. reported that in human dental pulp cells, low concentrations of PGE_2 acted to promote ALP activity, while high concentrations inhibited ALP activity[29]. In addition, Adachi et al. reported that in hDPCs, high concentrations of PGE_2 stimulation inhibited hard tissue formation by blocking Smad1/5/8 phosphorylation and BMP signalling[30].

In the present study, the effect of S1P on hard tissue formation showed similar behaviour, suggesting that concentrations deviating from those found in vivo may provide negative feedback on hard tissue formation capacity in hDPCs. Additionally, we investigated the effect of S1P on the expression of hard tissue formation markers in hDPCs and found that the exposure of hDPCs to 1 μM S1P significantly increased BMP-2 mRNA expression 1 h after stimulation when compared with that in the control group.

Subsequent western blotting analysis confirmed increased expression of BMP-2 protein in hDPCs 3 and 6 h after S1P stimulation. Moreover, DSPP protein expression enhanced on days 1 and 3 of stimulation. These findings suggest that S1P acts on hDPCs by activating the signal transduction mechanism for hard tissue formation, thereby increasing gene and protein expression of BMP-2. Increases expression of DSPP, a non-collagenous protein that is closely related to dentin calcification and exists around dentinoblasts and dentin tubules, implies that S1P is strongly involved in hard tissue formation and may positively regulate the ability of dental pulp cells to facilitate the same. Furthermore, Okabe et al. reported that under alkaline conditions, ALP activity is increased by the enhanced BMP-2 gene expression[31]. In the present study, S1P-induced BMP-2 gene expression and increased ALP activity may be closely linked to hard tissue formation.

S1P regulates various physiological activities by binding to S1PR expressed on the cell membrane. Five types of S1P receptors, S1PR1-5, have been discovered to date. S1PR1-3 are widely expressed throughout the body, while S1PR4 and S1PR5 are localized in some tissues, such as lymphoid tissues, lungs, and spleen[9]. To confirm the presence of S1PR in the dental pulp, we examined S1PR mRNA expression in hDPCs cultured under normal conditions and found increased mRNA expression of S1PR1-3 and low mRNA expression of S1PR4 and S1PR5. S1PR1-3, are widely expressed in normal organisms, and a similar trend was observed in hDPCs. In particular, studies have highlighted the involvement of S1PR1 and S1PR2 in hard tissue formation in the alveolar bone[15-17]. These receptors are considered to play a significant role in the formation of bone and hard tissues, implying their crucial involvement in hard tissue formation in the dental pulp.

Elevated $[Ca^{2+}]i$ is involved in various physiological activities[32-35], including in dental pulp cells. Changes in $[Ca^{2+}]i$ are closely related to various signaling mechanisms in the dental pulp. Given that S1P promotes hard tissue formation in hDPCs, we examined its effects on $[Ca^{2+}]i$ dynamics. Upon stimulation with S1P, we observed a concentration-dependent increase in $[Ca^{2+}]i$ in hDPCs. This increase in $[Ca^{2+}]i$ is caused by the binding of extracellular S1P to S1PR on the plasma membrane, depending on the concentration of S1P. Furthermore, to confirm that the binding of S1P to S1PR causes an increase in $[Ca^{2+}]i$, we assessed $[Ca^{2+}]i$ in the presence of S1PR antagonists. Pre-treatment of hDPCs

with the S1PR1-3 antagonists, W146, JTE013, and CAAY10444, respectively, for 3 h, significantly suppressed the peak of $[Ca^{2+}]i$ when compared with that in the control cells.

Increases in $[Ca^{2+}]i$ can be attributed to both the influx of Ca^{2+} from outside the cell and its release from inside the cell, such as the ER, and are thought to be involved in various signaling mechanisms[32,36]. In hDPCs, however, which of the two routes is responsible for the observed S1P-induced increase in $[Ca^{2+}]i$ remains unknown. When $[Ca^{2+}]i$ was measured under conditions where Ca^{2+} in HEPES solution was chelated with 5 mM EGTA, the increase in $[Ca^{2+}]i$ induced by S1P was similar to that observed in the presence of extracellular Ca^{2+}. Furthermore, administration of 1 μM S1P after thapsigargin treatment did not result in an increase in $[Ca^{2+}]i$. Hence, S1P-induced increase in $[Ca^{2+}]i$ was not affected by extracellular Ca^{2+}, and $[Ca^{2+}]i$ did not increase under conditions of suppressed release from the ER. This suggests that S1P acts on the ER to induce changes in $[Ca^{2+}]i$ dynamics. In dentinoblasts, $[Ca^{2+}]i$ is reported to be increased through the activation of G-protein coupled receptors and is involved in induction of dentin formation[37]. Thus, S1P may also promote hard tissue formation in hDPCs through a similar mechanism.

Lipids have become a subject of significant interest due to their roles in various physiological activities. Along with carbohydrates and proteins, they are essential for maintaining life; they are major components of cell membranes in living organisms and exist in the form of lipid bilayers. Cell membrane receptors play a pivotal role in the physiological activities of living organisms. S1P, similar to lysophosphatidic acid, is a lysophospholipid with a single-chain structure, which releases arachidonic acid. It is believed that inflammatory cytokines and stress stimuli are involved in the S1P production pathway, and the produced S1P exerts its physiological effects by binding to S1P receptors expressed on the cell membrane. The relationship between S1P and its receptors has been extensively studied *in vivo*. Recently, S1P was found to be involved in the regulation of bone formation and homeostasis[38-40], thereby making it an intriguing prospect for application in the treatment of autoimmune diseases[13,14]. Thus, the application of S1P and its receptor-targeting mechanism may serve as promising new targets for drug discovery and bioactivity *in vivo*.

In the present study, we investigated the relationship between S1P and its receptors on hard tissue

formation in the dental pulp. The results suggest that S1P may promote hard tissue formation in hDPCs by increasing $[Ca^{2+}]i$ and the expression of hard tissue formation markers, such as DSPP. Based on these findings, S1P can potentially be useful in pulp preservation procedures by improving sealing and reducing the risk of infection. However, the mechanism of hard tissue formation in the dental pulp is intricately regulated, and the mechanism underlying the role of S1P in hard tissue formation requires further elucidation. Additionally, this is a basic study using cultured cells, and further in vivo studies using animal models are needed to determine its suitability for clinical applications.

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Conflicts of Interest

The authors have declared that no COI exists

References

1. Nakashima M and Iohara K. New treatment for dental caries and pulpitis using regenerative dentistry to achieve dental health maintenance and longevity of teeth. Jpn J Conserv Dent 54: 157-161, 2011
2. Fitzgerald M and Heys RJ. A clinical and histological evaluation of conservative pulpal therapy in human teeth. Oper Dent 16: 101-112, 1991
3. Aeinehchi M, Eslami B, Ghanbariha M and Saffar AS. Mineral trioxide aggregate (MTA) and calcium hydroxide as pulp-capping agents in human teeth: A preliminary report. Int Endod J 36: 225-231, 2003
4. Linu S, Lekshmi MS, Varunkumar VS and Sam Joseph VG. Treatment outcome following direct pulp capping using bioceramic materials in mature permanent teeth with carious exposure: A pilot retrospective study. J Endod 43: 1635-1639, 2017
5. Okiji T and Yoshiba K. Reparative dentinogenesis induced by mineral trioxide aggregate: A review from the biological and physicochemical points of view. Int J Dent 2009: Article ID 464280, 2009
6. Ebihara T and Katoh Y. Histopathological study on development of adhesive resinous material containing calcium hydroxide as direct pulp capping agent. Jpn J Conserv Dent 39: 1288-1315, 1996
7. Okada T and Nakamura S. Role of S1P acting both inside and outside the cells. Seikagaku J Jpn Biochem 84: 92-101, 2012
8. Chi H. Sphingosine-1-phosphate and immune regulation: Trafficking and beyond. Trends Pharmacol Sci 32: 16-24, 2011
9. Takuwa Y. Emerging role of sphingosine-1-phosphate in physiology and diseases. JOS 18: 333-340, 2018
10. Fernández-Pisonero I, Dueñas AI, Barreiro O, Montero O, Sánchez-Madrid F and García-Rodríguez C. Lipopolysaccharide and sphingosine-1-phosphate cooperate to induce inflammatory molecules and leukocyte adhesion in endothelial cells. J Immunol 189: 5402-5410, 2012
11. Walter DH, Rochwalsky U, Reinhold J, Seeger F, Aicher A, Urbich C, Spyridopoulos I, Chun J, Brinkmann V, Keul P and Levkau B. Sphingosine-1-phosphate stimulates the functional capacity of progenitor cells by activation of the CXCR4-dependent signaling pathway via the S1P3 receptor.

Arterioscler Thromb Vasc Biol 27: 275-282, 2007

12. Takahashi M. Lipid mediator sphingosine-1-phosphate (S1P): Efficacy of novel S1P receptor agonists in treatment of autoimmune diseases. Shinshu Med J 57: 127-136, 2009

13. Tsai HC and Han MH. Sphingosine-1-phosphate (S1P) and S1P signaling pathway: Therapeutic targets in autoimmunity and inflammation. Drugs 76: 1067-1079, 2016

14. Chiba K. A new therapeutic approach for autoimmune diseases by the sphingosine-1-phosphate receptor modulator, fingolimod (FTY720). Nihon Rinsho Meneki Gakkai Kaishi 32: 92-101, 2009

15. Matsuzaki E, Hirose H, Fujimasa S, Yoshimoto S, Yanagi T, Matsumoto K, Nikaido M, Minakami M, Matsumoto N and Anan H. Sphingosine-1-phosphate receptor 2 agonist induces bone formation in rat apicoectomy and alveolar bone defect model. J Dent Sci 17: 787-794, 2022

16. Matsuzaki E, Minakami M, Matsumoto N and Anan H. Dental regenerative therapy targeting sphingosine-1-phosphate (S1P) signaling pathway in endodontics. Jpn Dent Sci Rev 56: 127-134, 2020

17. Higashi K, Matsuzaki E, Hashimoto Y, Takahashi-Yanaga F, Takano A, Anan H, Hirata M and Nishimura F. Sphingosine-1-phosphate/S1PR2-mediated signaling triggers Smad1/5/8 phosphorylation and thereby induces Runx2 expression in osteoblasts. Bone 93: 1-11, 2016

18. Somerman MJ, Archer SY, Imm GR and Foster RA. A comparative study of human periodontal ligament cells and gingival fibroblasts *in vitro*. J Dent Res 67: 66-70, 1988

19. Ohshima M, Kuwata F, Otsuka K, Saito R, Sato K, Shioji S and Suzuki K. Alkaline phosphatase activities of cultured human periodontal ligament cells. J Nihon Univ Sch Dent 30: 208-217, 1988

20. Fukai J, Watanabe T, Okabe T and Matsushima K. Effect of Ga-Al-As laser irradiation at wavelengths of 660 nm or 810 nm with a constant output on hard tissue forming ability in human pulp. Jpn J Conserv dent 61: 1-9, 2018

21. Hayama T, Kamio N, Okabe T, Muromachi K and Matsushima K. Kallikrein promotes inflammation in human dental pulp cells via protease-activated receptor-1. J Cell Biochem 117: 1522-1528, 2016

22. Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. Methods 25: 402-408, 2001

23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254, 1976

24. Asghar MY, Kemppainen K, Lassila T and Törnquist K. Sphingosine 1-phosphate attenuates MMP2 and MMP9 in human anaplastic thyroid cancer C643 cells: Importance of S1P2. PLoS One 13: e0196992, 2018

25. Kamio N, Hashizume H, Nakao S, Matsushima K and Sugiya H. Plasmin is involved in inflammation via protease-activated receptor-1 activation in human dental pulp. Biochem Pharmacol 75: 1974-1980, 2008

26. Grynkiewicz G, Poenie M and Tsien RY. A new generation of Ca2+ indicators with greatly florescence properties. J Biol Chem 260: 3440-3450, 1985

27. Ishida Y, Komaru K and Oda K. Structure and function of alkaline phosphatases. Japanese J Clin Chem 33: 36-44, 2004

28. Goseki M, Oida S, Nifuji A and Sasaki S. Properties of alkaline phosphatase of the human dental pulp. J Dent Res 69: 909-912, 1990

29. Sakamoto M, Shiozawa A, Matsushima K and Yamazaki M. Stimulation of hard tissue formation on human dental pulp cells by prostaglandin E2. Jpn J Conserv Dent 46: 454-450, 2003

30. Adachi T, Okabe T and Matsushima K. Effects of PGE2 on Smad phosphorylation in human dental pulp cells. Int J Oral-Med Sci 11(4): 268-273, 2013

31. Okabe T, Sakamoto M, Takeuchi H and Matsushima K. Effects of pH on mineralization ability of human dental pulp cells. J Endod 32: 198-201, 2006

32. Ushioda R, Miyamoto A, Inoue M, Watanabe S, Okumura M, Maegawa KI, Uegaki K, Fujii S, Fukuda Y, Umitsu M and Takagi J. Redox-assisted regulation of Ca^{2+} homeostasis in the endoplasmic reticulum by disulfide reductase ERdj5. Proc Natl Acad Sci USA 113: E6055-E6063, 2016

33. Niisato N, Ogata Y, Furuyama S and Sugiya H. Histamine H1 receptor-stimulated Ca^{2+} signaling pathway in human periodontal ligament cells. J Periodontal Res 31: 113-119, 1996

34. Foskett JK, White C, Cheung KH and Mak DO. Inositol trisphosphate receptor Ca^{2+} release channels. Physiol Rev 87: 593-658, 2007

35. Verkhratsky A. Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons. Physiol Rev 85: 201-279, 2005

36. Dupont G. Modeling the intracellular organization of calcium signaling. Wiley Interdiscip Rev Syst Biol Med 6: 227-237, 2014

37. Kimura M, Mochizuki H, Satou R, Iwasaki M, Kokubu E, Kono K, Nomura S, Sakurai T, Kuroda H and Shibukawa Y. Plasma membrane Ca^{2+}-ATPase in rat and human odontoblasts mediates dentin mineralization. Biomolecules 11: 1010, 2021

38. Sato C, Iwasaki T, Kitano S, Tsunemi S and Sano H. Sphingosine 1-phosphate receptor activation enhances BMP-2-induced osteoblast differentiation. Biochem Biophys Res Commun 423: 200-205, 2012

39. Meshcheryakova A, Mechtcheriakova D and Pietschmann P. Sphingosine 1-phosphate signaling in bone remodeling: multifaceted roles and therapeutic potential. Expert Opin Ther Targets 21: 725-737, 2017

40. Grewe JM, Knapstein PR, Donat A, Jiang S, Smit DJ, Xie W and Keller J. The role of sphingosine-1-phosphate in bone remodeling and osteoporosis. Bone Res 10: 34, 2022

Figures

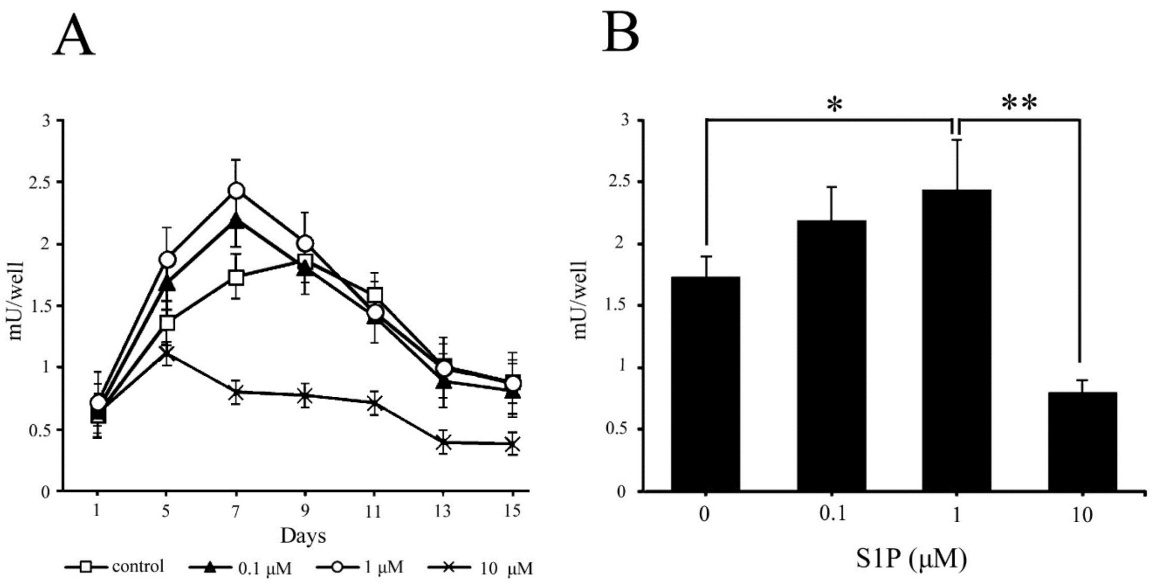

Figure 1. Effect of sphingosine-1-phosphate (S1P) on alkaline phosphatase (ALP) activity in human dental pulp cells (hDPC). (A) Changes in ALP activity on S1P stimulation. hDPC were cultured with various concentrations of S1P for 1-15 days, and ALP activity was subsequently measured (n = 4). (B) Effect of S1P on ALP activity 7 days after S1P stimulation. A significant increase in ALP activity was observed on day 7 in the 1 μM S1P-treated group compared to that in the control group. Data are presented as the mean ± standard deviation (n = 4, *: $p < 0.05$, **: $p < 0.01$).

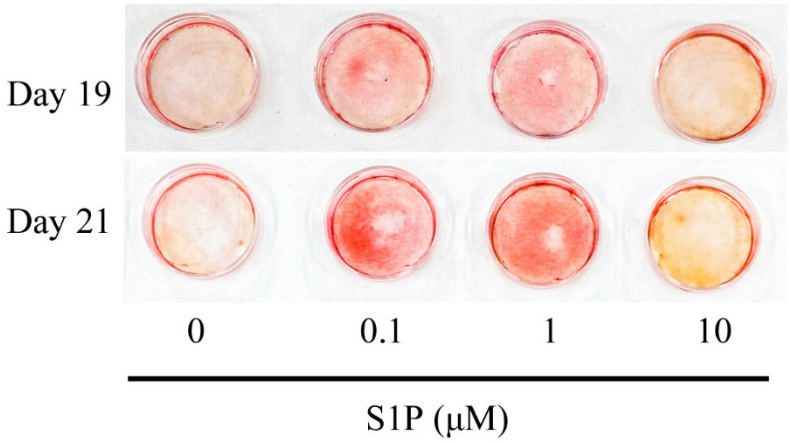

Figure 2. Effect of S1P on calcified nodule formation in hDPC. hDPC were cultured with various concentrations of S1P and alizarin red staining was performed on days 19 and 21 after S1P stimulation.

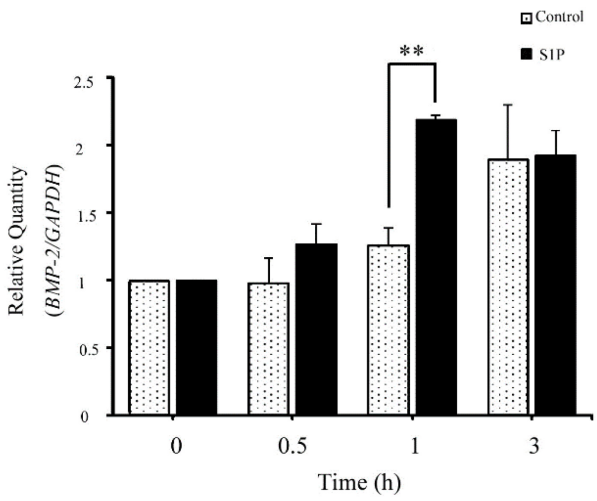

Figure 3. Effect of S1P on *BMP-2* mRNA expression in hDPC. BMP-2 mRNA expression after stimulation of hDPC with 1 μM S1P was analyzed by real-time PCR. Data are presented as the mean ± standard deviation (n = 3, ** : $p < 0.01$).

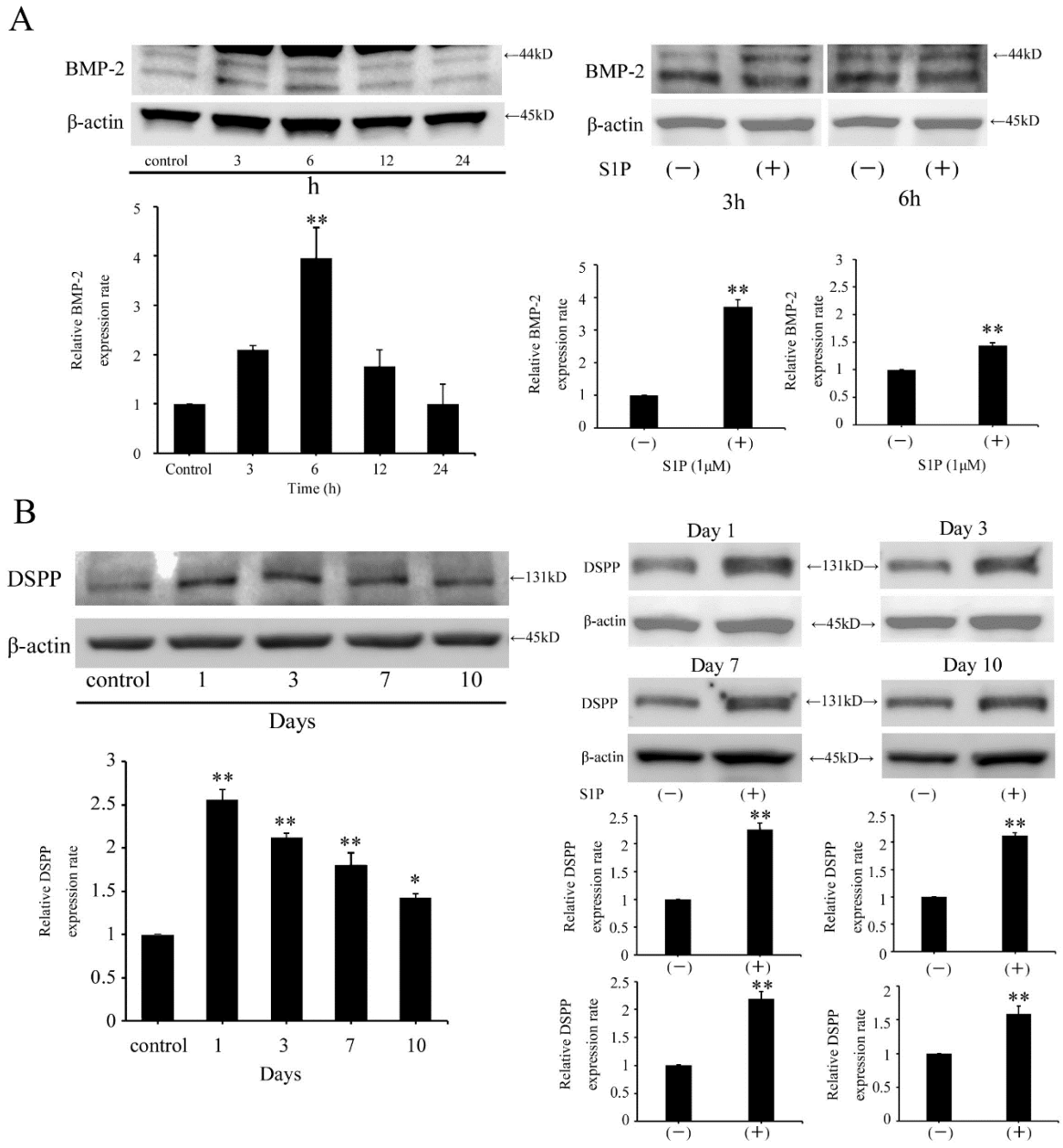

Figure 4. Effect of S1P on the expression of hard tissue formation-related protein. (A) BMP-2 and (B) DSPP protein expression was examined by western blotting following stimulation of hDPC with 1 μM S1P. Data are presented as the mean ± standard deviation. Stimulation was used as the positive control. Values are means ± SD from 3 independent experiments. (n = 3, *: $p < 0.05$, **: $p < 0.01$).

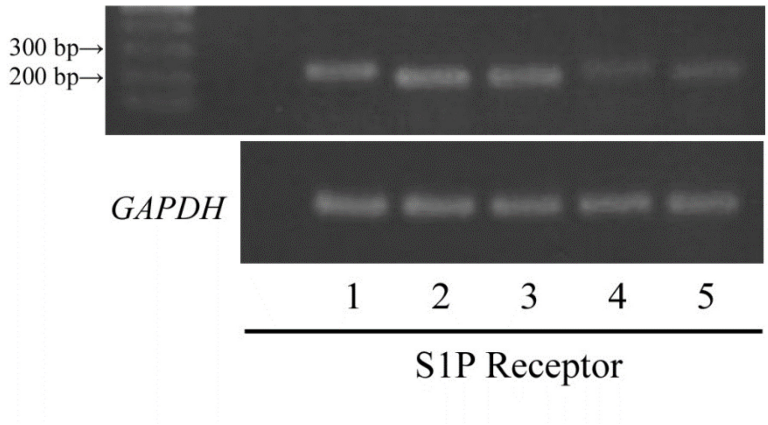

Figure 5. mRNA expression of S1P receptors in hDPC. The mRNA expression of S1P receptors 1-5 in hDPC was analyzed by RT-PCR.

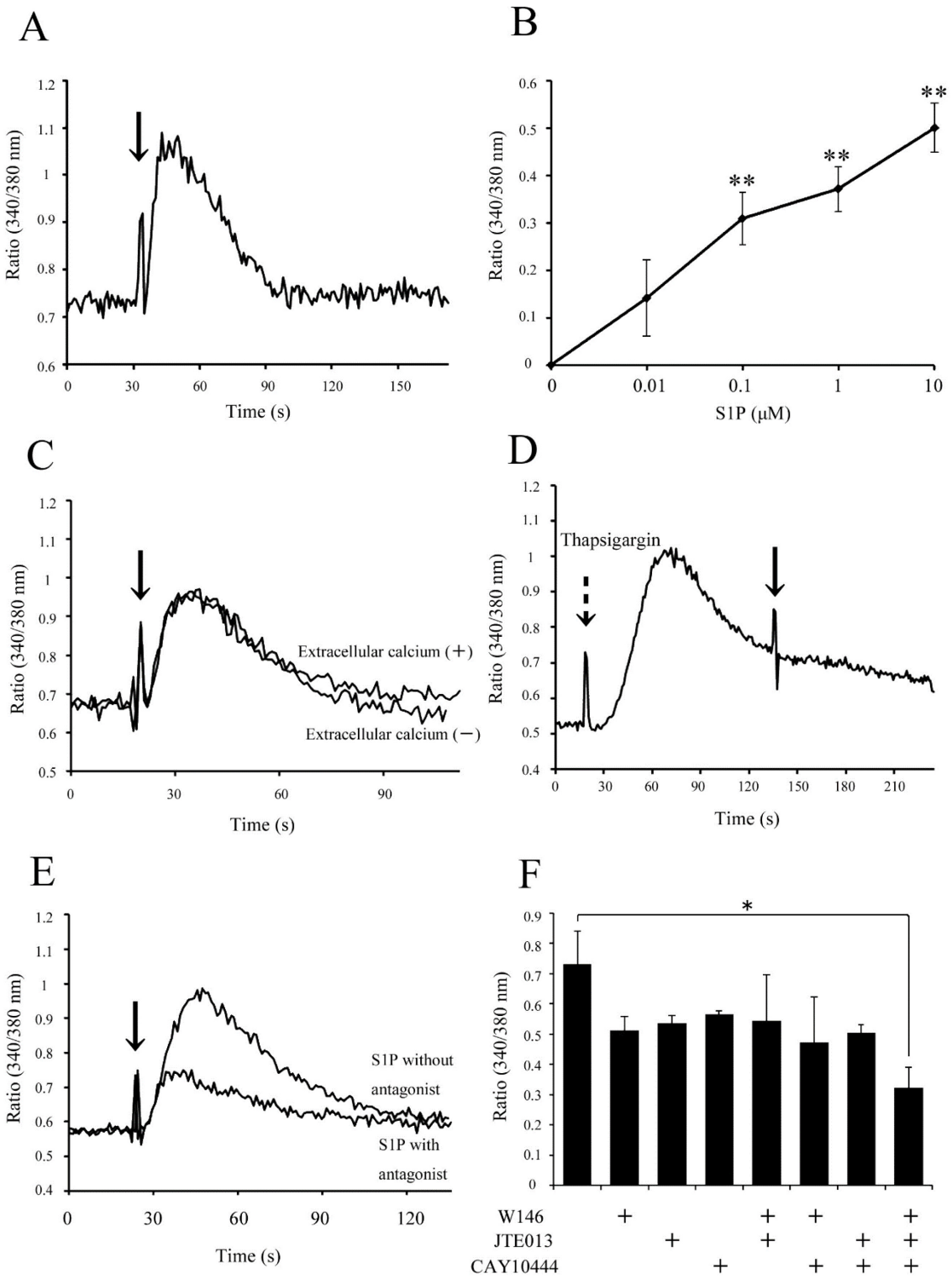
A
Ratio (340/380 nm)
Time (s)
B
Ratio (340/380 nm)
S1P (μM)
**
**
**
C
Ratio (340/380 nm)
Extracellular calcium (+)
Extracellular calcium (−)
Time (s)
D
Thapsigargin
Ratio (340/380 nm)
Time (s)
E
Ratio (340/380 nm)
S1P without antagonist
S1P with antagonist
Time (s)
F
*
Ratio (340/380 nm)
W146
JTE013
CAY10444

Figure 6. Effect of S1P on intracellular calcium ion concentration ($[Ca^{2+}]i$) dynamics in hDPC. (A) To assess the effect of S1P on $[Ca^{2+}]i$ in hDPC, Fura-2 loaded cells were stimulated with 1 μM S1P. (B) To assess the concentration-dependent effects of $[Ca^{2+}]i$ on hDPC, Fura-2-loaded cells were stimulated with various concentrations of S1P and $[Ca^{2+}]i$ was measured. The baseline ratio in control cells (before stimulation) was subtracted from the S1P-induced peak Ca^{2+} value. Data are presented as the mean ± standard deviation (n = 3, **: $p < 0.01$). (C) To assess the effect of S1P on $[Ca^{2+}]i$ in the presence or absence of extracellular Ca^{2+}, Fura-2 loaded cells were stimulated with 1 μM S1P in the presence or absence of extracellular Ca^{2+}. (D) To assess the effect of S1P on $[Ca^{2+}]i$ in hDPC, Fura-2 loaded cells were pretreated with 1 μM thapsigargin and then stimulated with 1 μM S1P. (E) The effect of S1P on $[Ca^{2+}]i$ in the presence or absence of S1P receptor antagonists. Cells pretreated with antagonists for S1P receptors 1-3 for 3 h were stimulated with 1 μM S1P and loaded with Fura-2. (F) Effect of each S1P receptor antagonist on $[Ca^{2+}]i$. Cells pretreated for 3 h with each antagonist against S1P receptor 1-3 were stimulated with 1 μM S1P and loaded with Fura-2 and the $[Ca^{2+}]i$ was assessed. The baseline ratio in control cells (before stimulation) was subtracted from the S1P-induced peak Ca^{2+} value. Data are presented as the mean ± standard deviation. (n = 3, *: $p < 0.05$)

Table 1. Primers for Realtime-PCR

Gane	Primer Sequence	Size (bp)
BMP-2	Forward 5′ -CTGGCTGATCATCTGAACTCCACT-3′ Reverse 5′ -TCGGGACACAGCATGCCTTA-3′	94
GAPDH	Forward 5′ -GCACCGTCAAGGCTGAGAAC-3′ Reverse 5′ -TGGTGAAGACGCCA GTGGA-3′	138

Table 2. Primers for RT-PCR

Gane	Primer Sequence	Size (bp)	Cycle
S1PR1	Forward 5′-GGCTGGAACTGCATCAGTGCG-3′ Reverse 5′-GAGCAGCGCCACATTCTCAGAGC-3′	223	25
S1PR2	Forward 5′-CCGAAACAGCAAGTTCCACT-3′ Reverse 5′-CCAGGAGGCTGAAGACAGAG-3′	197	
S1PR3	Forward 5′-AAGGCTCAGTGGTTCATCGT-3′ Reverse 5′-GCTATTGTTGCTGCTGCTTG-3	201	
S1PR4	Forward 5′-CCTTCAGCCTGCTCTTCACT-3′ Reverse 5′-AAGAGGATGTAGCGCTTGGA-3′	223	
S1PR5	Forward 5′-AGGACTTCGCTTTTGCTCTG-3′ Reverse 5′-TCTAGAATCCACGGGGTCTG-3′	201	
GAPDH	Forward 5′-ATCACCATCTTCCAGGAG-3′ Reverse 5′-ATGGACTGTGGTCATGAG-3	318	