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Original Article

Sphingosine-1-phosphate receptor 2 agonist induces bone formation in rat apicoectomy and alveolar bone defect model

Etsuko Matsuzaki [a,b*], Haruna Hirose [a], Seishiro Fujimasa [a], Shohei Yoshimoto [b,c], Tsukasa Yanagi [d], Kazuma Matsumoto [a], Misaki Nikaido [a], Masahiko Minakami [a], Noriyoshi Matsumoto [a], Hisashi Anan [a]

[a] *Section of Operative Dentistry and Endodontology, Department of Odontology, Fukuoka Dental College, Fukuoka, Japan*
[b] *Oral Medicine Research Center, Fukuoka Dental College, Fukuoka, Japan*
[c] *Section of Pathology, Department of Morphological Biology, Division of Biomedical Sciences, Fukuoka Dental College, Fukuoka, Japan*
[d] *Section of Oral Implantology, Department of Oral Rehabilitation, Fukuoka Dental College, Fukuoka, Japan*

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Abstract *Background/purpose:* Sphingosine-1-phosphate (S1P) is a lipid mediator that exerts its physiological functions in vivo through receptors. In the bone, S1P induces osteoblast differentiation. Here, we investigated the effects of S1P receptor agonists on the expression of osteoblast differentiation markers locally in the bone. Then, a rat apicoectomy and alveolar bone defect model was established to extend S1P applications to endodontics, and the effect of local administration of S1P receptor agonist on postoperative bone formation was examined.
Materials and methods: Sphingosine-1-phosphate receptor (S1PR) 1/S1PR3 agonists, S1PR2 agonists, and their signal-related agents were intraperitoneally administered to mice. Using the mRNA collected from the tibial bone, the expression of osteoblast differentiation markers was evaluated by real-time reverse-transcriptase quantitative polymerase chain reaction. An apicoectomy and alveolar bone defect model was established on the mesial root of the rat mandibular first molar. Bone formation parameters were measured by micro-computed tomography analysis 3 weeks after the procedure.
Results: Intraperitoneal administration of S1PR2 agonist significantly increased the mRNA expression of osteoblast differentiation markers, including alkaline phosphatase (*ALP*),

* Corresponding author. Section of Operative Dentistry and Endodontology, Department of Odontology, Fukuoka Dental College, 2-15-1 Tamura, Sawara-ku, Fukuoka, 814-0193, Japan.
E-mail address: matsuzaki@college.fdcnet.ac.jp (E. Matsuzaki).

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osteopontin (*OPN)*, bone sialoprotein (*BSP*), and osteocalcin, in the local tibial bone of mice. The S1PR2/Rho-associated coiled-coil forming kinase (ROCK) signaling was thought to be involved in the upregulated mRNA expression of *ALP*, *OPN*, and *BSP*. In the rat apical defects, bone formation parameters significantly increased following local administration of S1PR2 agonist.

Conclusion: In the rat apicoectomy and alveolar bone defect model, therapeutic agents targeting S1PR2 agonist are effective against osteogenesis.

Introduction

Sphingosine-1-phosphate (S1P) is a type of phospholipid that activates its own S1P signaling pathway via five types of G protein-coupled receptors (sphingosine-1-phosphate receptor 1—5; S1PR1-S1PR5) and acts as a signaling molecule, including cell differentiation, proliferation, and chemotaxis.[1—3] Drugs targeting S1P receptors have been developed for therapeutic applications such as S1PR1/S1PR3 (fingolimod; FTY720) in multiple sclerosis.[4—6]

In the bone tissue, a new physiological function of S1P was discovered where it controlled the chemotaxis of osteoclast precursor cells and suppressed bone resorption.[7] Hence, the effects of S1P on osteoclast/osteoblast differentiation and the receptor involved in this process are under active research.[8] We have previously reported increased expression of S1PR1 and S1PR2 in the process of osteoblast differentiation, and demonstrated S1P-mediated osteoblastic differentiation through activation of the novel S1PR1/Akt/β-catenin and S1PR2/RhoA/Rho-associated coiled-coil forming kinase (ROCK) signaling pathways.[9,10] We also demonstrated trabecular bone formation in mice following intraperitoneal administration of S1PR1/S1PR3 and S1PR2 agonists.[10] Additionally, the S1PR2 agonist CYM-5520 is known to increase the bone mass and osteoblast count in a mouse osteoporosis model.[11]

Aside from the root canal treatment, surgical endodontic treatment such as apicoectomy for a wide range of alveolar bone defects such as radicular cysts. However, a four-year follow-up period is deemed necessary to determine the prognosis.[12] If the alveolar bone can be promptly repaired after surgery, more teeth can likely be preserved. So far, S1PR1 has been reported to be expressed in rat apical periodontitis lesions where it is known to be involved in bone resorption.[13,14] However, there is no report on the involvement of S1PR2 in endodontics. Therefore, we decided to investigate the effect of S1P receptor agonists on the repair of alveolar bone defects at the apex. The intracellular S1PR1 expression level is known to fluctuate in response to various extracellular stimuli under conditions inducing inflammation.[15,16] Therefore, in this study, we created an animal model of apicoectomy and alveolar bone defect instead of apical periodontitis to evaluate the effect of S1P receptor agonists on bone repair. In addition, we used a previously established model,[10] to examine the S1PR1/S1PR3 agonists and S1PR2 agonists that are more effective against the expression of osteoblast differentiation marker gene in the local mouse tibial bone.

Materials and methods

Reagents

CYM-5520 (Merk KGaA, Darmstadt, Germany) was used as S1PR2 agonist, JTE-013 (Cayman Chemical, Ann Arbor, MI, USA) as S1PR2 inhibitor, and Y27632 (Fujifilm Wako Co., Osaka, Japan) as the ROCK inhibitor downstream of S1PR2 signal. In addition, FTY720 (Cayman Chemical) served as S1PR1/S1PR3 agonist and W146 (Cayman Chemical) as S1PR1 inhibitor.

Systemic administration in mice and RNA extraction

Thirty-six eight-week-old male C57BL/6N mice (CLEA Japan Inc., Tokyo, Japan) were used for the experiment in compliance with ARRIVE Guidelines after obtaining an approval from the animal care committee of Fukuoka Dental College (No. 15022). Mice were once daily intraperitoneally administrated with the following agents for 28 days: CYM-5520 alone, CYM-5520 plus JTE-013 or W146 or Y27632, FTY720 alone, FTY720 plus CYM-5520 or W146, or phosphate-buffered saline (Fujifilm Wako Co.) as a control. After sacrifice, the left tibia was collected and crushed using homogenizer (POLYTRON homogenizer PT3100; Kinematica AG, Malters, Swizerland), an ultrasonic device. Then, RNA was extracted and used for real-time polymerase chain reaction (PCR).

Real-time reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using RNAiso Plus (Takara Bio Inc., Shiga, Japan), and 500 ng of single-stranded cDNA was synthesized using PrimeScript RT Master Mix (Takara Bio Inc.). RT-qPCR was performed on StepOne system (Thermo Fisher Scientific Inc., Waltham, MA, USA) using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific Inc.). PCR products were normalized using *GAPDH* expression. The primers used for RT-qPCR were synthesized based on the sequence obtained from the GenBank database (Table 1).

Table 1 RT-qPCR primers.

GenBank ID	Target gene	Primer sequence forward/reverse	Size of amplified products (bp)
NM_007431	mouse ALP	5'- GTTGCCAAGCTGGGAAGAACAC -3' / 5'- CCCACCCCGCTATTCCAAAC -3'	121
NM_009263	mouse OPN	5'- TACGACCATGAGATTGGCAGTGA -3' / 5'- TATAGGATCTGGGTGCAGGCTGTAA -3'	114
NM_008318	mouse BSP	5'- ATGGAGACGGCGATAGTTCCGAAG -3' / 5'- CGTAGCTAGCTGTTACACCCGAGAG -3'	153
NM_001032298	mouse OCL	5'- GAACAGACTCCGGCGCTA -3' / 5'- AGGGAGGATCAAGTCCCG -3'	227
NM_008084	mouse GAPDH	5'- TGTGTCCGTCGTGGATCTGA-3' / 5'-TTGCTGTTGAAGTCGCAGGAG -3'	150

Rat apicoectomy and alveolar bone defect model

Experiments were conducted by modifying the previously reported methods of Yoneda et al.[17] For the experiment using rats, approval was obtained from the animal care committee of Fukuoka Dental College (No. 18014). The protocol is shown in Fig. 1. Six ten-week-old male Wistar rats (CLEA Japan Inc.) were subjected to general anesthesia with isoflurane inhalation, while local anesthesia with dental xylocaine cartridge containing 1:80,000 epinephrine (Dentsply Sankin, Tokyo, Japan) was applied to the left mandibular first molar. A rubber dam sheet and a rubber dam clamp (YDM, Tokyo, Japan) prepared for rats were used for tooth isolation (Fig. 1A). After opening the pulp

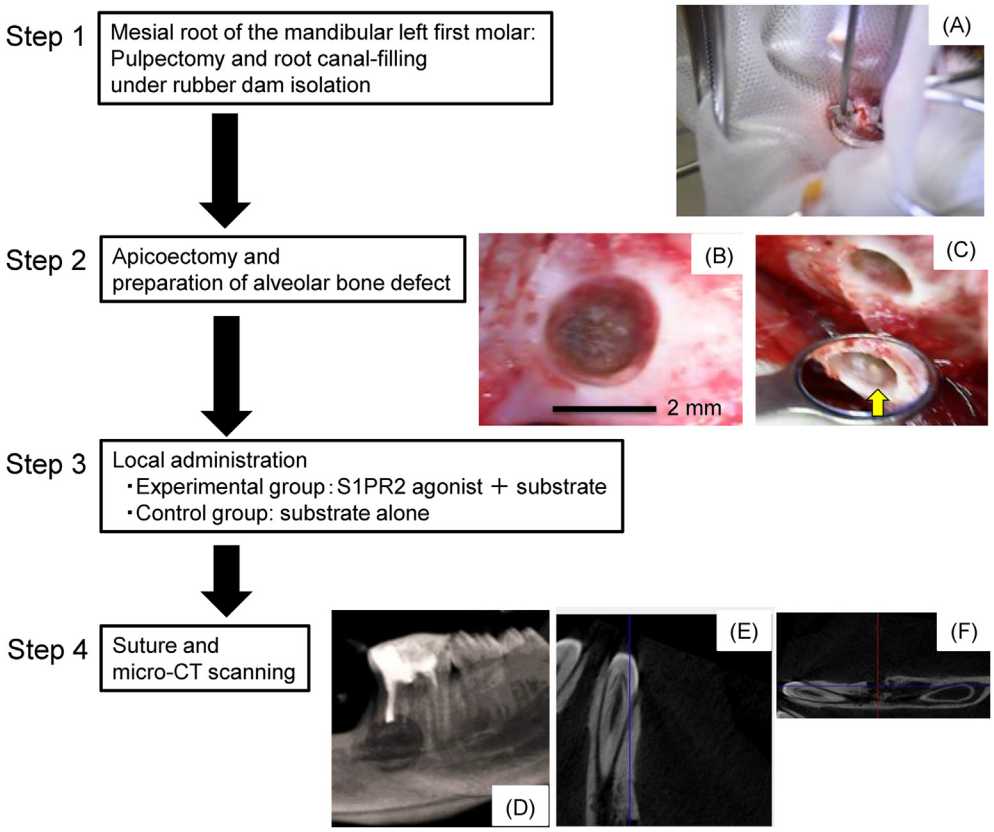

Figure 1 Experimental protocol of rat apicoectomy and alveolar bone defect model development. Steps were carried out in the order of 1–4. (A) After rubber dam isolation with custom-made rubber dam clamp, pulpectomy was performed in the mesial root of the mandibular left first molar. The other three roots were pulp capped with MTA cement. (B) After root canal filling, an alveolar bone defect with a diameter of 2 mm and depth of 1 mm was created at the same site as the mesial root apicoectomy. (C) A micro-mirror was inserted into the bone defect cavity to confirm the root cut surface. Arrow: cross section of the root. Gutta-purcha point was seen. (D to F) Typical micro-CT image in this surgical model (D: sagittal view, E: coronal view, F: axial view).

chamber with a #1/2 dental round bur (Dentsply Maillefer, Ballaigues, Switzerland) and electric engine (VIVAMATE G5; Nakanishi Inc., Tochigi, Japan), pulpectomy of the mesial root was performed using #8-10 K-file (Mani Inc., Tochigi, Japan). A nickel titanium rotary file (Hyflex CM 20/04; Coltene, Langenau, Germany), and a motor with an electrical root canal length measurement function (Tri-Auto ZX2; J. Morita Corp., Tokyo, Japan) was used for root canal preparation. Root canal was cleaned with a 3% sodium hypochlorite aqueous solution (Nippon Shika Yakuhin Co., Ltd., Yamaguchi, Japan) and then dried using sterile paper points (#20; J. Morita Corp.). Then, a gutta-percha point (ISO GT.04 TAPER#20; J. Morita Corp.) and a sealer (Meta-SEAL Soft; Sun Medical, Shiga, Japan) were used to fill the root canal. The other three roots were pulp capped with MTA cement (Pro Root MTA; Dentsply Maillefer). Crown restoration was performed with an Ionosit baseliner (DMG, Hamburg, Germany).

Next, we removed the gingiva around the mesial root apex using a sterile No. 15 surgical blade (Feather Co., Osaka, Japan). We prepared bone defect (Fig. 1B) and performed apicoectomy of the mesial root (Fig. 1C) using a stent standardized to a diameter of 2 mm and a depth of 1 mm with a # 1/2 round bur and fissure bur (J. Morita Corp.). Then, the prepared bone cavity was filled with a mixture of CYM-5520 (0.6 mg/30 μl) and 10 μl atelocollagen (2% KOKEN Atelocollagen implant; Koken Co., Ltd, Tokyo, Japan) as a substrate or atelocollagen alone. The cavity was then sutured with absorbent threads (Coated VICRYL; Johnson & Johnson, New Brunswick, NJ, USA). Rats were euthanized with carbon dioxide asphyxiation 3 weeks after operation, and the mandibular samples were dissected, followed by fixation in 10% paraformaldehyde for 2 days at 4 °C.

Micro-computed tomography (CT) analysis

The micro-architecture of the bone after surgery was evaluated using a Micro-CT system (SkySCAN 1176 micro-CT; Bruker, Billerica, MA, USA). Imaging was performed under following parameters: 35 μm pixel size, 50 kVp/498 μA, angular rotation step of 0.8° (Fig. 1D–F). After reconstructing the 3D data with NRecon software (Bruker), structural indices including bone volume density relative to tissue volume (B.V/T.V.), trabecular number (Tb.N.), and trabecular thickness (Tb.Th.) were calculated using CT-An software (Bruker) and images were obtained with CT-Vol software (Bruker). The analysis was performed on a region of interest (ROI) extracted from time points of bone defect preparation and 3 weeks after surgery. The data obtained in the bone defect region were used to set each axis such that the X and Y axes passed through the center of the bone cavity and the Z axis was parallel to the cross section of the bone. We then set 25 continuous slices with a constant diameter of 2 mm to evaluate calculated values.

Statistics

The results were calculated by means ± standard error (SE). Significance was determined using the Student's *t*-test (GraphPad Prism 5.0, GraphPad Software, San Diego, CA, USA), and $p < 0.05$ was considered to be statistically significant.

Results

mRNA expression of osteoblast differentiation markers in the local bone following intraperitoneal administration of S1P

First, we investigated the effects of intraperitoneal administration of S1P signal-related agents on the mRNA expression of osteoblast differentiation markers in the mouse tibial bone. Fig. 2 shows that S1PR2 signaling affected the mRNA expression of the following osteoblast differentiation marker genes: alkaline phosphatase (*ALP*), osteopontin (*OPN*), bone sialoprotein (*BSP*), and osteocalcin (*OCL*). The mRNA expression levels of *ALP*, *OPN*, *BSP*, and *OCL* were significantly higher after administration of S1PR2 agonist CYM-5520 than that observed after control treatment (Fig. 2A–D). The increase in the mRNA expression of *ALP*, *OPN*, and *BSP* was significantly reduced by the S1PR2 inhibitor JTE-013 (Fig. 2A–C). As we have previously reported the bone formation-inducing effect by S1PR2 signaling via RhoA/ROCK,[10] we examined the effect of the ROCK inhibitor Y27632. Y27632 treatment significantly decreased the upregulated mRNA expression of *ALP*, *OPN*, and *BSP* by CYM-5520. On the other hand, the S1PR1 inhibitor W146 had no effect on CYM-5520-mediated increase in the mRNA expression of *ALP*, *OPN*, and *BSP* (Fig. 2A–C). The significant increase in *OCL* mRNA expression after CYM-5520 treatment had a tendency to decrease by JTE-013. Y27632 treatment had no significant effect on CYM-5520-mediated increase of *OCL* mRNA expression (Fig. 2D).

On the other hand, the mRNA expression of *ALP*, *OPN*, and *BSP* after administration of S1PR1/S1PR3 agonist FTY720 was significantly higher than that observed after control treatment (Fig. 3A–C). The increase in *BSP* mRNA expression was significantly reduced (60.8%) by W146. The reduction rate of BSP mRNA expression treated with JTE-013 was lower (25.1%) than that observed after S1PR1 inhibition, but statistically significant (Fig. 3C). No statistically significant difference was observed in FTY720-mediated *ALP* and *OPN* levels after S1PR1 or S1PR2 inhibition (Fig. 3A and B). In addition, *OCL* mRNA expression increased by 1.66 times after FTY720 treatment, but the difference was not statistically significant as compared with the control. Similarly, no statistically significant difference was observed after S1PR1 or S1PR2 inhibition (Fig. 3D).

Effect of S1PR2 agonist on the defective bone in apicoectomy and alveolar bone defect model

To extend the application of S1P as an inducer of bone formation to the field of endodontics, we examined the effect of local administration of S1P receptor agonist on the alveolar bone defect site after apicoectomy. Because the increase in osteoblast differentiation marker gene expression in the local bone was more evident from S1PR2 signal

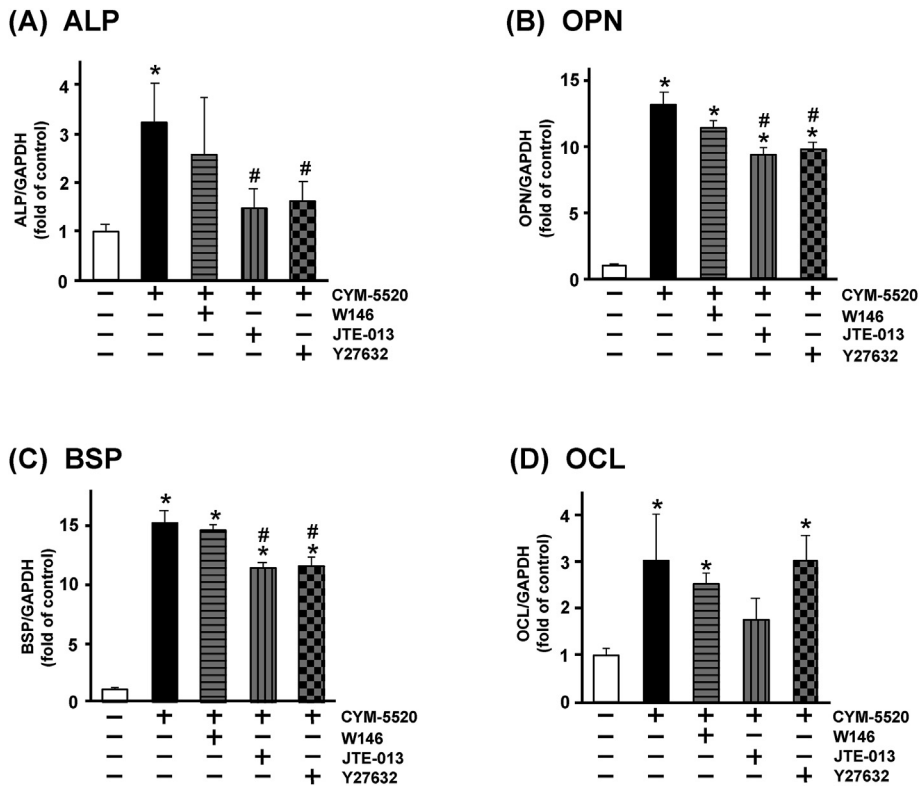

Figure 2 Effect of S1PR2 signal on the mRNA expression of osteoblast differentiation marker genes (A: *ALP*, B: *OPN*, C: *BSP*, D: *OCL*) in mouse tibial bone. CYM-5520 (1 mg/kg; S1PR2 agonist) alone or along with W146 (1 mg/kg; S1PR1 inhibitor), JTE-013 (1 mg/kg; S1PR2 inhibitor), and Y27632 (1 mg/kg; ROCK inhibitor) were intraperitoneally administered once daily for 28 days in the abdominal cavity of 8-week-old male mice. As a control, phosphate-buffered saline was similarly administered. After sacrifice, the left tibia was collected and the RNA was extracted. The mRNA level of each gene was quantified by RT-qPCR. Values are expressed as means ± SE (n = 4). $^{*}p < 0.05$ vs control, $^{\#}p < 0.05$ vs CYM-5520-treated mice by Student's *t*-test.

than from S1PR1 signal (Figs. 2 and 3), we used S1PR2 agonist CYM-5520 for the experiment.

As shown in superimposing micro-CT analysis images (Fig. 4A), bone properties were analyzed. The average increase in B.V/T.V. as well as Tb.N. in the CYM-5520-treated group was significantly higher than that observed in the control group (Fig. 4B and C). CYM-5520 had a tendency to increase Tb.Th (Fig. 4D).

Discussion

We have previously performed intraperitoneal administration of S1PR1/S1PR3 and S1PR2 agonists and analyzed trabecular bone formation in the mouse tibia. We reported that some S1PR1 and S1PR2 signals found in vitro are involved in the induction of bone formation.[10] Therefore, in this study, we investigated the effects of intraperitoneal administration of S1PR1/S1PR3 and S1PR2 agonists on osteoblast differentiation marker gene expression locally in mouse tibial bone. The S1PR2/ROCK signaling was likely involved in the increased mRNA expression of *ALP*, *OPN*, and *BSP*. These results are consistent with the increase in osteoblast differentiation marker expression following exposure to S1P in vitro.[10] On the contrary, neither S1PR1 inhibition nor S1PR2 inhibition had any significant effect on S1PR1/S1PR3 agonist-induced *ALP* and *OPN* mRNA expression. Thus, the involvement of S1PR1 signaling was not clear. These results are not in line with those of bone formation induction mediated by S1PR1 signaling in mouse tibial micro-CT analysis.[10] S1PR2/ROCK signaling in vitro is considered as a pathway independent of S1PR1 signaling.[10] However, intracellular S1PR expression level fluctuates in response to various extracellular stimuli.[15,16] It is possible that S1PR1 and S1PR2 signals act complementarily to control bone formation in the local bone in vivo. In addition, *OCL* mRNA expression was significantly upregulated by S1PR2 agonist, but no statistically significant difference was observed with the S1PR1/S1PR3 agonist treatment; neither S1PR inhibition nor S1PR2 inhibition had any effect. These findings suggest that the regulation of OCL gene expression may be independent of S1P signal. Furthermore, recent studies with OCL-deficient mice, unlike previous reports,[18] revealed the involvement of OCL in the arrangement of apatite crystals and bone strength but not in the regulation of bone mass.[19]

In this study, we created an animal model of surgical endodontic treatment to investigate the clinical application of S1P to alveolar bone repair. In recent years, S1PR1 has drawn attention in the field of endodontics. It was clarified

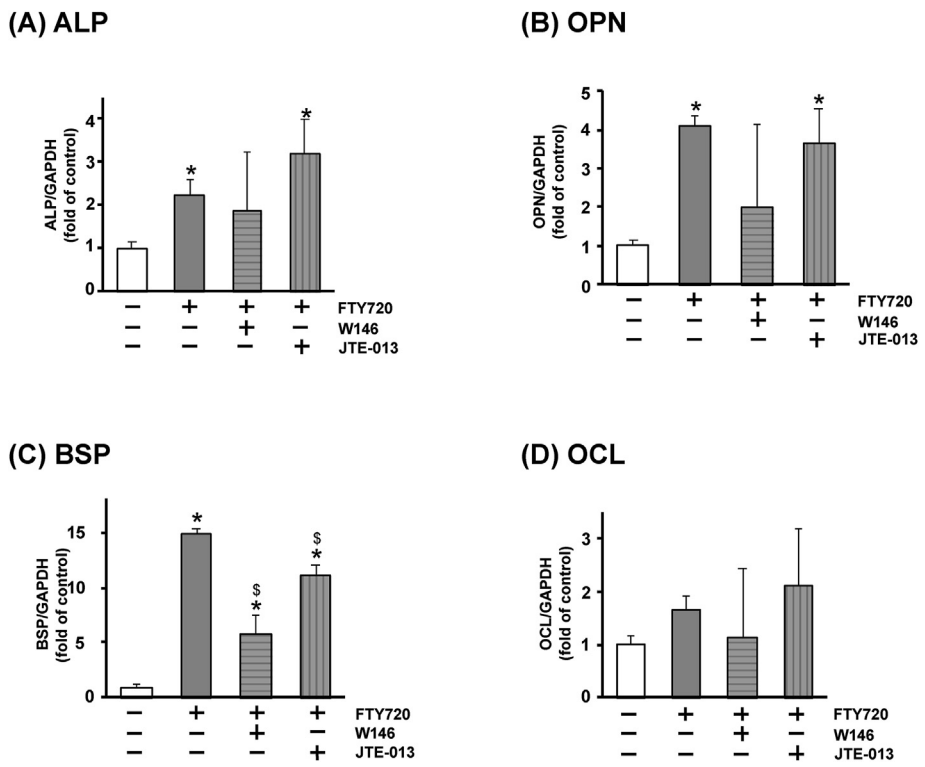

Figure 3 Effect of S1PR1 signal on the mRNA expression of osteoblast differentiation markers (A: *ALP*, B: *OPN*, C: *BSP*, D: *OCL*) in mouse tibial bone. FTY720 (1 mg/kg) alone or with W146 (1 mg/kg) and JTE-013 (1 mg/kg) were intraperitoneally administered once daily for 28 days in the abdominal cavity of 8-week-old male mice. Phosphate-buffered saline was similarly administered as a control. After sacrifice, the left tibia was collected and the RNA was extracted. The mRNA level of each gene was quantified by RT-qPCR and compared with the control. Values are expressed as means ± SE (n = 4). $^{*}p < 0.05$ vs control, $^{\$}p < 0.05$ vs FTY720-treated mice by Student's *t*-test.

that the expression of S1PR1 in the lesions of rat apical periodontitis positively correlated with that of receptor activator of nuclear factor kappa B ligand (RANKL), which induced osteoclast differentiation.[13] In addition, the administration of S1PR1/S1PR3 agonists to this animal model was shown to reduce RANKL expression and suppress bone resorption.[14] These findings suggest that S1PR1 may be involved in bone resorption or expression of inflammatory factors in apical periodontitis. Although there is no report on the relationship between S1PR2 and apical periodontitis, osteopenia associated with decreased osteoprotegerin expression has been observed in S1PR2-deficient mice.[20] Thus, one may suggest that S1PR2 also acts as a coupling factor between osteoclasts and osteoblasts in the apical periodontitis lesion site, similar to S1PR1. In the present study, we evaluated the effect of S1PR2 agonist on the alveolar bone defect site using local administration system. However, osteoclasts and osteoblasts themselves can also produce S1P.[21,22] Thus, it is necessary to consider whether bone formation in the alveolar bone defect site is attributed to the locally produced S1P.

Atelocollagen was used as a substrate for local administration of S1PR2 agonist. As atelocollagen exhibits low antigenicity and high biocompatibility, it is widely applied in medical devices. By placing it in the alveolar bone defect, we expected that it may act as a scaffold along with the blood clot formed at the surgical site, and an activator of functional bone formation.[23] Administration of S1PR2 agonist significantly increased bone formation parameters as compared with control. While bone formation in the control originated from the outermost part of the defect, that is, around the base bone, it was also formed at a site distant from the base bone in S1PR2 agonist treatment group. Thus, it is likely that S1PR2 agonist may have osteoinductive effect. Although there is no report on the osteoinductive effect of S1PR2 agonist, the application of the S1PR2 agonist CYM-5520 to a mouse osteoporosis model by ovariectomy led to an increase in the bone mass and number of osteoblasts owing to its anabolic action,[11] suggesting that the S1PR2 agonist may be an effective agent for osteoporosis-related diseases.

In this study, S1PR2 agonist significantly increased the mRNA expression of osteoblast differentiation markers *ALP*, *OPN*, and *BSP* via S1PR2/ROCK signaling in the local mouse tibial bone. In addition, a significant increase in bone formation parameters was observed in rat apicoectomy and alveolar bone defect model after local administration of S1PR2 agonist. Thus, a therapeutic agent targeting S1PR2 is likely to be effective in mediating osteogenesis after apicoectomy. A more comprehensive study should be carried out in future to view that SIPR2 agonist would accelerate the healing process of apical defects.

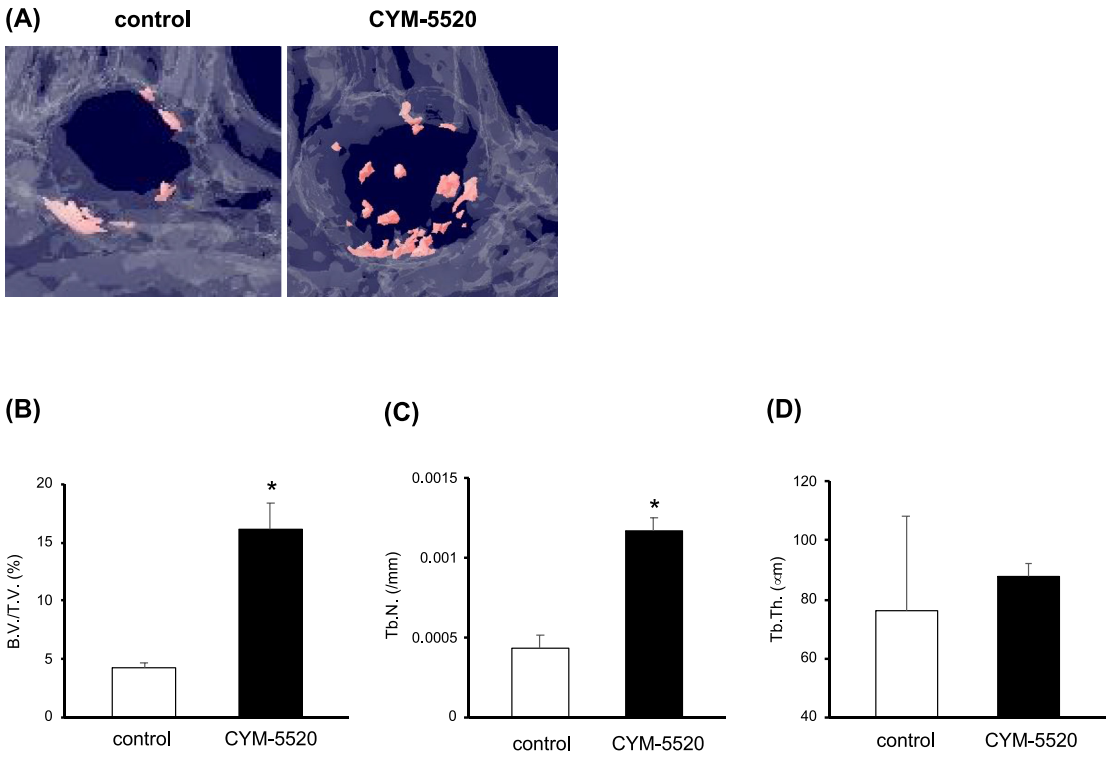

Figure 4 S1PR2 agonist promotes bone formation at the site of rat apicoectomy and alveolar bone defect. (A) Micro-CT images taken immediately after surgery and 3 weeks after surgery were superimposed using CT-Vol, and the new bone-like tissue observed 3 weeks later was shown in pink. The amount of bone formation was set as the value obtained by subtracting the value at the time of bone defect preparation from the value 3 weeks after surgery (left: control, right: CYM-5520). (B to D) Quantified micro-CT data. (B) Bone volume relative to tissue volume (B.V/T.V.), (C) trabecular number (Tb.N.), (D) trabecular thickness (Tb.Th.). Values are expressed as means ± SE (n = 3). *$p < 0.05$ vs control by Student's *t*-test.

Declaration of competing interest

The authors declare no conflict of interest in this study.

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