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Effect of Small-molecule GSK3 Antagonist on Differentiation of Rat Dental Pulp Cells into Odontoblasts

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Abstract. *Background: It has been reported that glycogen synthase kinase 3 (GSK3) antagonist promoted the reparative formation of dentin. The aim of the present study was to evaluate whether treatment schedule of Tidegrusib® (TG), a small-molecule GSK3 antagonist, affected in vitro differentiation of dental pulp cells toward odontoblast-like cells. Materials and Methods: Pulp cells isolated from rat incisors were repeatedly exposed to TG for the first 6 h (intermittent exposure) or the full 48 h (continuous exposure) of each 48-h incubation cycle. Histological analysis of alkaline phosphatase and von Kossa staining were performed. The expression of dentin sialophosphoprotein (Dspp) and osteocalcin (Ocn) mRNA were examined by real-time polymerase chain reaction. Western blotting assays were used to monitor the expression of β-catenin and its phosphorylated form. Results: When pulp cells were intermittently exposed to TG for only the first 6 h of each incubation cycle, pulp cells differentiated into odontoblast-like cells, characterized by an increase in alkaline phosphatase activity, nodule formation, and mRNA expression of Dspp. and Ocn; this did not occur under the continuous exposure. Phosphorylation of β-catenin was enhanced by continuous exposure to TG compared with intermittent exposure. Conclusion: These results suggest that the TG-induced odontoblast-like cell differentiation reflects in vivo reparative dentin formation and depends on the exposure time.*

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Dental pulp is a connective tissue that contains odontoblasts. The pulp responds to external stimuli, such as cavity preparation, excess tooth wear, restorative materials and caries (1). It produces two forms of tertiary dentin called reactionary dentin and reparative dentin (2, 3). Reactionary dentin is formed by primary odontoblasts located at the periphery of the pulp chamber, while reparative dentin is formed by the second generation of odontoblast-like cells that originate from mesenchymal stem cells in the pulp (4).

It is conceivable that signaling molecules expressed by pulp cells play an important role in the healing of pulp during dental repair (5). WNT/β-catenin signaling regulates dentin secretion by odontoblasts by directly affecting the formation of reparative dentin (6). Glycogen synthase kinase 3 (GSK3) is a key cytoplasmic component of WNT/β-catenin signal transduction, and phosphorylates β-catenin and axin, leading to their ubiquitination and degradation in the absence of WNT ligand/receptor binding (7). GSK3 is well known to play important roles in restoring the functional integrity of teeth, preserving their vitality and protecting the pulp from further damage.

Direct pulp capping is performed on teeth that have exposed pulp caused by caries, caries excavation, or traumatic injuries. Calcium hydroxide paste and mineral trioxide aggregate are commonly used capping materials (8) that induce reparative dentin formation by their stimuli. It has recently been reported that the small-molecule GSK3 antagonist, Tidegrusib® (also known as NP-12 or NP031112) (TG), promoted reparative dentin formation and restoration *in vivo* (7). Although TG is a bio-degradable organic material, it induced higher reparative dentin formation compared with non-degradable mineral trioxide aggregate cement that was previously considered the most effective pulp-capping agent (9). Furthermore, TG is under study in clinical trials for treating neurological disorders such as Alzheimer's disease (10, 11).

It has been reported that the organic matrix of dentin is deposited at a rate of 4 μm/day and mineralized in a 12-hour

cycle (12). The effect of parathyroid hormone (PTH) on osteoblast differentiation and signal transduction systems has been reported to be considerably variable, depending on the exposure time *in vitro* (13). Short exposure (6 h) of osteoblastic cells to PTH resulted in several-fold increase in the expression of mRNA for alkaline phosphatase (ALP) and osteocalcin (13). In the present study, we tested the possibility that the intermittent stimulation of rat pulp cells induces differentiation towards odontoblast-like cells and reparative dentin formation more efficiently than does continuous stimulation.

Materials and Methods

Animals. Wistar strain 5-week-old littermate male rats (Saitama Experimental Animals Supply Co., Ltd., Sugito, Saitama, Japan) (chosen due to easy manipulation of anesthetization and removal of mandibles and pulp) were used to prepare pulp cells, according to the methods established by Yokose *et al*. (14), after approval by the intramural Animal Care and Use Committee (no. A1927).

Cell culture. Pulp cells were detached from the dish by trypsinization and inoculated onto 6-well plates (Falcon Labware, Corning, NY, USA) at a density of 10^4 cells/cm^2. Cells at the second passage were used for the experiments. They were cultured in α-modified Eagle's minimum essential medium-containing 10% heat-inactivated calf serum (Thermo Fisher Scientific K.K., Tokyo, Japan), 300 mg/ml β-glycerophosphate (FUJIFILM Wako Pure Chem. Co., Osaka, Japan), 50 mg/ml ascorbic acid (FUJIFILM Wako Pure Chem. Co.), and antibiotics (100 mg/ml of penicillin G and 100 IU/ml of streptomycin) (Thermo Fisher Scientific K.K.).

Experimental protocol for TG exposure. Pulp cells, inoculated at a density of 10^4 cells/cm^2, were cultured for 16 h to allow complete attachment. They were then divided into three groups and treated as follows: (i) Intermittent exposure: cells were exposed to TG (Monmouth Junction, NJ, USA) (50 nM) for the first 6 h of each 48-h incubation cycle, and then cultured in the absence of TG for the remainder of the cycle; (ii) continuous exposure: cells were continuously exposed to TG, with a change of culture medium every 48 h; and (iii) control cells were not exposed at all to TG throughout the experimental period. The 48-h culture cycle was repeated eight times, and then the cells were fixed and stained for ALP on day 20.

Histochemistry. The cells were fixed with 10% neutral buffered formalin, and ALP activity was determined histochemically, using naphthol AS-MX phosphate (substrate) (Sigma-Aldrich Inc. St. Louis, MO, USA), and Fast Red Violet LB Salt (coupler) (Sigma-Aldrich Inc.), as described previously (15). Mineralized nodules were detected by von Kossa's staining and quantified by counting the number of black and brown nodules in each well of 6-well plates (n=4). The mean±SEM were calculated. Statistical treatment was performed with the Mann-Whitney *U*-test. A value of $p<0.05$ was considered statistically significant.

Real-time reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from the pulp cells grown in 6-well plates on days 10 and 20 using RNAiso Plus (Takara Bio Inc., Tokyo, Japan). cDNA was synthesized from total RNA (1 mg) using an RT-PCR kit (Takara Bio Inc.) and random primers. Subsequently, 100 ng cDNA was used as a template for the real-time PCR. Primers were used at 5 mM with 12.5 μl SYBR Green Premix (Takara Biomedical) in a final volume of 25 μl. SYBR Green PCR amplification and real-time fluorescence detection were performed using a Smart Cycler II System (Takara Biomedical). Denaturation was performed at 95˚C for 10 s, followed by annealing/extension (45 cycles at 95˚C for 5 s and 60˚C for 20 s). The sequences of primers (Takara Biomedicals) were: Forward: 5'-CTCAGTTAGTGC CGCTGGAGA-3', reverse: 5'-GAATCGTCGTTAGTGGCGTTG-3' for rat *Dspp*; forward: 5'-AGACTCCGGCGCTACCTCAA-3' and reverse: 5'-CGTCCTGGAAGCCAATGTG-3' for rat *Ocn*; and forward: 5'-TGACAGGATGCAGAAGGAGGA-3' and reverse: 5'-TAGAGCCACCAATCCACACA-3' for rat β-actin (*Actb*). Measurements were undertaken during the extension step at 60°C in each cycle, and the threshold cycle was calculated by the second-derivative method as described previously (14). The target gene-expression level was normalized to that of *Actb* in each sample. After amplification, the melting curve of PCR products was analyzed to differentiate between specific and non-specific PCR products.

Western blot analysis. The cells after the first cycle of exposure to TG were washed with PBS, lysed with RIPA solution (Nacalai Tesque, Kyoto, Japan) and processed for western blot analysis, as described previously (16). The intensity of protein expression was

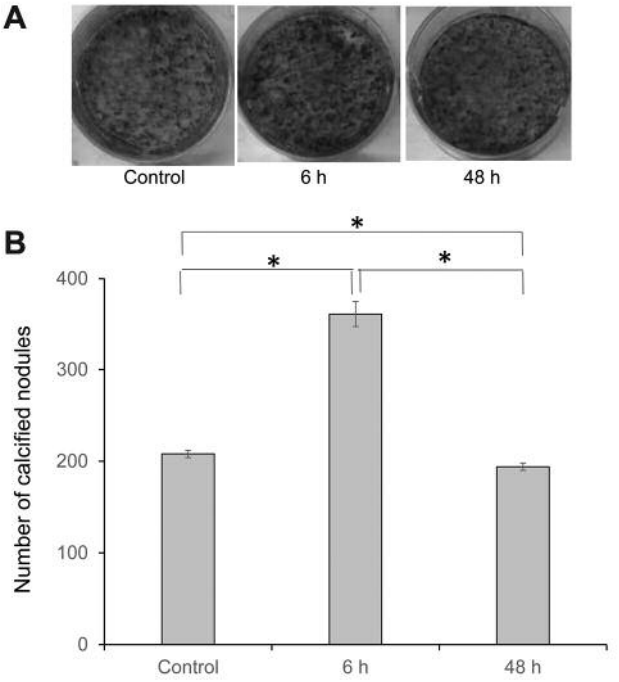

Figure 1. *Stimulated formation of calcified nodules in pulp cells by intermittent exposure to Tidegrusib® (TG). A: Pulp cells were exposed to TG (50 nM) for the first 6 h of each 48-h incubation cycle (intermittent exposure group), or exposed continuously from day 1 to day 17 (the day of termination of the eighth treatment cycle), 3 days before cell harvest) (continuous exposure group), or not exposed for 20 days (control group). Medium changes were performed every 48 h. B: Quantification of the number of calcified nodules from groups as shown in A. Data are the mean±SD of four cultures. *Statistically significant at p<0.05.*

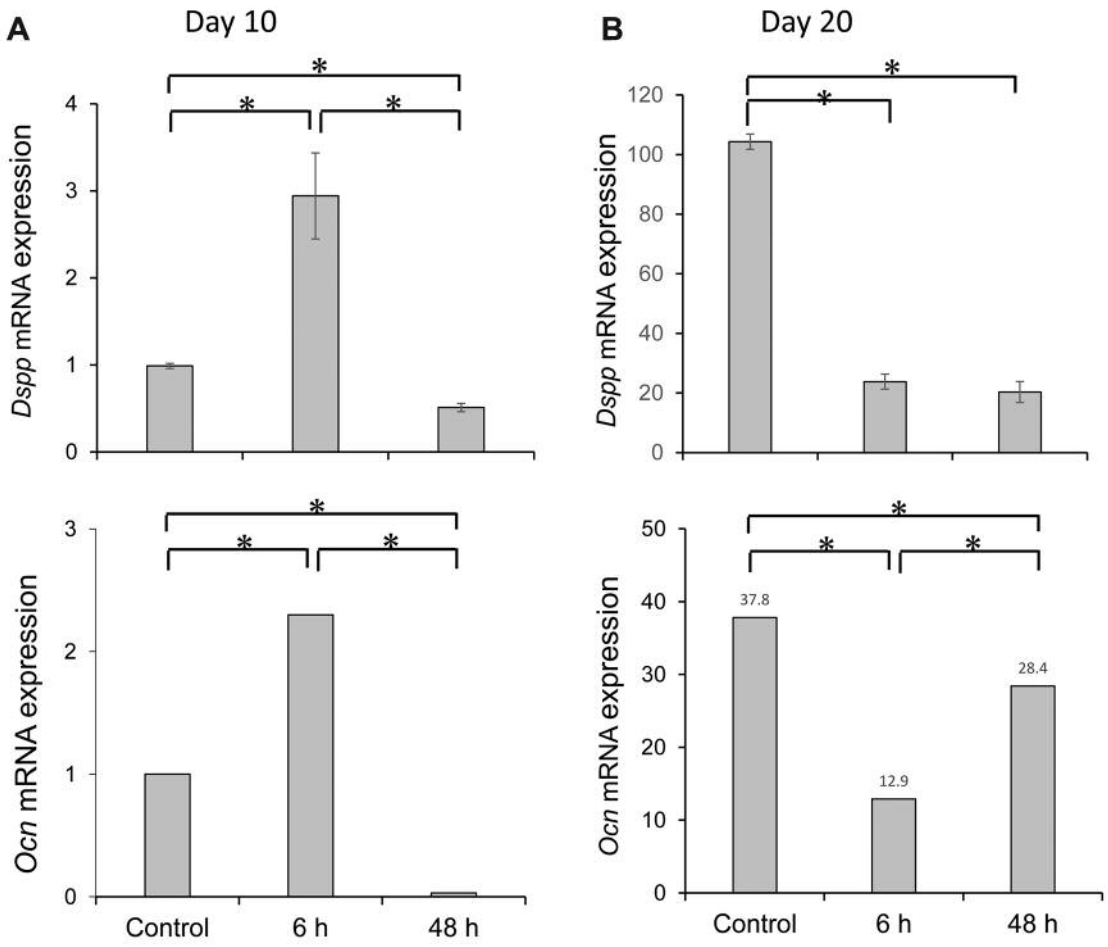

Figure 2. *Effects of intermittent and continuous exposure to Tidegrusib® (TG) on the expression of dentin sialophosphoprotein (Dspp) and osteocalcin (Ocn) mRNA. Pulp cells were exposed to TG (50 nM) for 10 (A) or 20 (B) days intermittently for 6 h or continuously for 48 h, or were not exposed to TG (control), and then Dspp mRNA expression (upper panels) Ocn mRNA expression (lower panels) were quantified by real-time polymerase chain reaction. Each value represents the mean±SD of three independent experiments. Mann-Whitney U-test: *Statistically significant at p<0.05.*

quantified by ImageJ (NIH, Bethesda, MD, USA). As primary antibodies, antibodies against β-catenin (E247; Abcam, Cambridge, UK), phosphorylated β-catenin (Phospho-Ser33; Signalway Antibody LLC, College Park, MD, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology, Inc., Danvers, MA, USA) were used. As a secondary antibody, anti-rabbit IgG (Cell Signaling Technology, Inc.) antibody conjugated with horseradish peroxidase was used. As a positive control, cells that were continuously exposed to LiCl (10 mM) for 48 h in the first cycle were used (17).

Results

Stimulated formation of calcified nodules by intermittent exposure to TG. During incubation for 20 days in α-MEM supplemented with β-glycerophosphate and ascorbic acid, pulp cells spontaneously differentiated into odontoblast-like cells characterized by numerous ALP-positive calcified nodules (Figure 1A). The number of nodules increased in the 6-h TG exposure group (Figure 1A). On the other hand, when pulp cells were treated with TG continuously, the numbers of nodules were slightly but significantly reduced (Figure 1A).

When the number of calcified nodules was quantified, the 6-h intermittent exposure group produced a significantly (p<0.05) higher number of calcified nodues compared with the control and cells exposed continuously to TG (Figure 1B). On the other hand, continuous exposure induced only a slight but significant decrease in the number of nodules compared with the control.

Transient increase of Dspp. and Ocn mRNA expression by intermittent exposure to TG. Real-time PCR analysis demonstrated that intermittent exposure to TG resulted in a

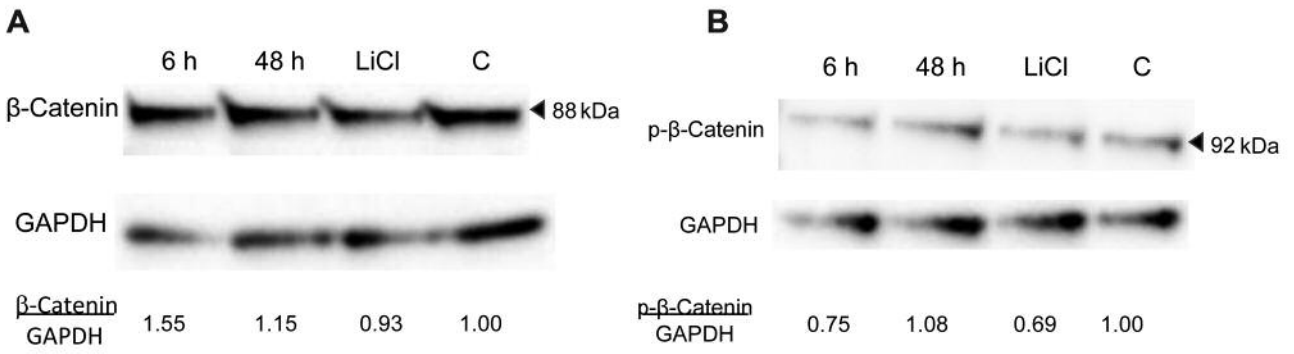

Figure 3. *Western blot analysis of the expression of β-catenin and its phosphorylated form after one cycle of exposure for 6 or 48 h to Tidegrusib® (50 nM). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. LiCl: Cells were continuously exposed to 10 mM LiCl as a positive control; C: control, cells were incubated without TG. The expression of β-catenin and its phosphorylated form relative to that of GAPDH was quantified by ImageJ (NIH), and then expressed as a ratio to that of the control.*

significant ($p<0.05$) increase in *Dspp* and *Ocn* mRNA on day 10 (2.9 and 2.3-fold increase as compared with control, respectively; Figure 2A) but in a reduction on day 20 (Figure 2B). On the other hand, continuous exposure to TG did not induce such increases in *Dspp* and *Ocn* mRNA on days 10 and 20 but led to a reduction in expression compared with the control (Figure 2B).

Effect on β-catenin and its phosphorylated form. Western blot analysis demonstrated that after one 48-h cycle, β-catenin expression was higher in the 6-h exposure than the 48-h exposure and exposure to LiCl (used as a positive control) (Figure 3); expression in the control was almost the same as that of the 48-h exposure (Figure 3A). Phosphorylation of β-catenin was higher with 48-h exposure than in the 6-h exposure, LiCl exposure and control (Figure 3B). This indicates higher degradation of phosphorylated β-catenin with 48-h exposure, that may have reduced its nuclear transport, and the transcription of genes involved in calcification, leading to a decrease of odontoblastic differentiation.

Discussion

The present study demonstrated that short intermittent (6 h) exposure to TG (50 nM) induced odontoblastic differentiation. This conclusion is based on our following findings. (i) Six-hour intermittent exposure to TG significantly increased the formation of calcified nodules on day 20, while continuous 48-h exposure inhibited the formation of the nodules compared with 6-h exposure and the control (Figure 1). (ii) Expression of both *Dspp* and *Ocn* mRNA were increased by 6-h exposure compared with 48-h exposure and the control on day 10 (Figure 2). (iii) Expression of β-catenin and its phosphorylation at Ser33 were enhanced by continuous exposure but not intermittent exposure after 48 h (Figure 3).

It has previously reported that short-term PTH treatment (5 to 30 min) stimulated cell proliferation of osteoblasts more efficiently than long-term treatment (18), possibly due to the selective regulation by cyclic AMP/protein kinase A. The present study demonstrated that continuous exposure to TG induced the phosphorylation of β-catenin. This suggests that phosphorylated β-catenin may be degraded in the proteasome (17); without phosphorylation, β-catenin should be in a monomeric form for translocation to the nucleus to initiate gene transcription (18).

Kadokura *et al*. reported that phosphorylation of β-catenin was inhibited by LiCl in cultured pulp cells (19), consistent with the present finding that phosphorylation of β-catenin was inhibited by continuous exposure to LiCl, as well as by 6-h exposure to TG after the first cycle (Figure 3).

The WNT signaling pathway is one of the important molecular cascades regulating cell fate. This pathway not only plays a crucial role in growth and development but also in the maintenance of the mature skeleton and response to conditions of loading (20). For maintenance of function, WNT/β-catenin signaling may accelerate gene transcription suppressed by the antagonists of WNT receptors including sclerostin, dickkopf-1, -2, and -3, and secreted frizzled-related protein 1 protein (21, 22). Gene expression is regulated by the balance between accelerator and suppressor to maintain homeostasis. The 6 h-exposure to TG may have accelerated β-catenin accumulation in the cytoplasm without switching on the suppressor. On the other hand, continuous 48-h exposure may have accelerated the β-catenin accumulation in the cytoplasm with switching-on of the suppressor, thus leading to the suppression of differentiation of odontoblasts-like cells. Further studies are needed to make clear the mechanism of reparative dentin formation.

In conclusion, the present study suggests that TG has diverse effects on odontoblast-like cell differentiation

depending on the exposure time *in vitro* and reflect part of the *in vivo* action in reparative dentin formation.

Conflicts of Interest

The Authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Authors' Contributions

Y.M. performed all experiments and wrote the article. H.S., S.Y. and N.U. supported the experiments and edited the article.

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