Continuous Administration of Propofol Suppresses Osteoclast Differentiation of RAW264.7 Cells

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( Accepted for publication, December 21, 2020)

Abstract: Propofol is an intravenous anesthetic and used for sedation and general anesthesia in a treatment involving invasion of the bone. Despite the period and dose of propofol being varied depending on the purpose of administration, effects of propofol on bone metabolism are less investigated. Osteoclast progenitor cells fuse with each other to differentiate into osteoclasts in the presence of receptor activator of nuclear factor kappa B (RANK) ligand (RANKL), and osteoclasts play a central role in bone resorption during bone remodeling. In the current study, we examined the effects of both temporary and continuous propofol stimulation on RANKL-induced osteoclastogenesis. RAW264.7 cells were stimulated with 0, 10, 20, or 30 µM propofol for 5 h, 1.5 days, or 4 days in the presence of RANKL. At the end of stimulation for 5 h and 1.5 days, cells were continued to be cultured in medium containing RANKL without propofol until the end of total culture period. The expression of dendritic cell-specific transmembrane protein (DC-STAMP) and osteoclast stimulatory transmembrane protein (OC-STAMP) that are involved in the fusion of cells as well as the expression of LGR and leucine-rich repeat-containing G-protein-coupled receptor 4 (LGR4) could be examined by real-time PCR. The formation of osteoclast-like cells was verified by tartrate-resistant acid phosphatase staining. Propofol stimulation for 1.5 or 4 days suppressed the expression of DC-STAMP, OC-STAMP and RANK, as well as the formation of osteoclast-like cells, whereas the expression of LGR 4 was increased by propofol stimulation for 4 days. These findings suggest that several hours of propofol administration do not affect RANKL-induced osteoclastogenesis. However, several days of propofol exposure may suppress the differentiation of osteoclasts due to decreased expression of DC-STAMP, OC-STAMP and RANK, as well as increased expression of LGR4.

Key words: Propofol, DC-STAMP, OC-STAMP, LGR4, RANKL

Introduction

Propofol is an intravenous anesthetic known to have sedative, hypnotic, amnestic, and antiemetic effects. It broadly suppresses central nerves by acting as an agonist of the γ-aminobutyric acid type A receptor and as an antagonist of the N-methyl-D-aspartate receptor, which are present mainly in the central nervous system1,2). The advantages of propofol include its superior usability and controllability due to rapid onset of action and short half-life, as well as its low accumulation in vivo2,3). However, adverse effects caused by repeated or long-term use of general anesthetics, such as defects in brain development due to central nervous system toxicity, have been indicated in recent years4). Propofol is often used for sedation and general anesthesia in a variety of surgeries, including during extended oral surgery involving invasion of the jawbone. Osteoclasts as well as osteoblasts in the jawbone may be exposed to propofol.

Osteoclasts are multinucleated giant cells that play a central role in bone resorption during bone remodeling. Osteoclast progenitor cells, derived from hematopoietic stem cells of the monocyte/macrophage lineage, fuse with each other to differentiate into osteoclasts5,6). Two cytokines play critical roles in the differentiation process, receptor activator of nuclear factor kappa B (RANK) ligand (RANKL) and osteoprotegerin (OPG). RANKL, produced by osteoblasts or lymphocytes, binds to RANK expressed in osteoclast progenitor cells, which strongly induces osteoclast differentiation and promotes osteoclastic bone resorption7). Dendritic cell-specific transmembrane protein (DC-STAMP) and osteoclast stimulatory transmembrane protein (OC-STAMP) are highly expressed during the differentiation process of osteoclasts and are known to be involved in the fusion of osteoclast progenitor cells8,9). In contrast, osteoblast-produced OPG as a decoy receptor attenuates the action of RANKL/RANK, suppressing osteoclastic bone resorption10). In addition, osteoclasts and osteoclast progenitor cells express leucine-rich repeat-containing G-protein-coupled receptor 4 (LGR4), a RANK receptor with a function different from that of RANKL. While osteoclast formation is promoted by RANKL binding to RANK, the binding of RANKL to LGR4 suppresses the formation of osteoclasts11,12,13). Previous studies on the effects of propofol on osteoclast differentiation have demonstrated that the administration of propofol reduces osteoclast formation with decreased RANKL/OPG ratios from osteoblasts14). Furthermore, direct stimulation of osteoclast progenitor cells with
Propofol promotes osteoclast differentiation by increasing DC-STAMP expression\(^\text{15}\). In these prior studies, propofol stimulation was sustained throughout the cell culture period, despite the period of propofol administration being varied depending on the intended purpose of its use. Accordingly, there has been a lack of consideration regarding the temporary exposure of osteoclasts and osteoblasts to propofol. Therefore, in the current study, we aimed to examine the effects of both temporary and continuous propofol stimulation on RANKL-induced differentiation of osteoclast progenitor cells into osteoclasts.

**Materials and Methods**

**Reagents**

The propofol used for cell stimulation was purchased as a 1% propofol injection solution (Maruishi Co., Osaka, Japan). Penicillin/streptomycin was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from HyClone Laboratories Inc. (Logan, UT, USA). Soluble RANKL, α-minimal essential medium (α-MEM), and phosphate-buffered saline (PBS) were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). The NucleoSpin RNA Extraction Kit, PrimeScript™ RT reagent Kit, and TB Green® Premix Ex Taq™ solution were purchased from Takara Bio Inc. (Otsu, Japan).

**Osteoclast cultures and propofol stimulation**

The murine monocyte/macrophage cell line RAW264.7 (Dainippon Pharmaceutical Co., Osaka, Japan) was used as an osteoclast precursor. It was previously reported that RAW264.7 cells differentiate into osteoclasts in the presence of RANKL without macrophage colony stimulating factor (M-CSF)\(^\text{16-18}\). Cells were seeded into 24- or 96-well plates at a density of 1×10\(^6\) cells/cm\(^2\) and cultured overnight in α-MEM containing 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO\(_2\). The cells were then stimulated by changing to medium containing 50 ng/ml RANKL plus 0 (control), 10, 20, or 30 µM propofol in addition to FBS and antibiotics for 5 h, 1.5 days, or 4 days. At the end of the propofol stimulation, the cells stimulated with propofol for 5 h and 1.5 days were continued to be cultured in medium containing RANKL without propofol until the end of a 4-day total culture period (Fig. 1).

**Tartrate-resistant acid phosphatase (TRAP) staining**

The formation of osteoclast-like cells was verified by tartrate-resistant acid phosphatase (TRAP) staining using a commercial staining kit (Cosmo Bio Co., Ltd., Tokyo, Japan). Images of stained cells were captured using a DIAPHOT inverted microscope (Nikon Corp., Tokyo, Japan) and the micrographs were used to count the number of TRAP activity-positive cells (osteoclast-like cells).

**Quantitative real-time polymerase chain reaction (PCR)**

Total RNA was extracted from RAW264.7 cells stimulated with/without propofol for 5 h, 1.5 days, or 4 days using the NucleoSpin RNA Extraction Kit. Using 1 µg of the extracted RNA, cDNA was synthesized in 20-µl reaction mixtures containing the random primer, dNTP mix, and reverse transcriptase included in the PrimeScript™ RT Kit. The cDNA mixture (2 µl) was subjected to quantitative real-time PCR. The PCR reaction mixture consisted of a 25-µl volume containing 1× RPR buffer, 1.5 mM dNTP mixture, 1× TB green, 1 mM MgCl\(_2\), and 0.25 unit of Ex Taq polymerase included in the TB Green® Premix Ex Taq™ solution, as well as 20 µM of the appropriate forward and reverse primers. The primer sequences were as follows: DC-STAMP Forward: 5′-CTA GCT GGC TGG ACT TCA TCC-3′, DC-STAMP Reverse: 5′-TCA TGC TGT CTA GGA GAC CTC-3′; OC-STAMP Forward: 5′-AGC TGT AGC CTG GGC TCA GAA G-3′, OC-STAMP Reverse: 5′-AGG CTG TGG TAG ATG ACA GTC GTG-3′; LGR4 Forward: 5′-CTG ATT GCC ACC ACG TGG GTT TAA GTA G-3′, LGR4 Reverse: 5′-AGG ACA TTG CCA GTC CAG ATG AG-3′; RANK Forward: 5′-AGG ACA TTG CCA GTC CAG ATG AG-3′; RANK Reverse: 5′-TGG ACA TCA ACA AGG ATA CGA-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Forward: 5′-AAA TGG TGA AGG TCG GTG TG-3′, GAPDH Reverse: 5′-GAG ATG TCT TGA AGG TCG GTG TG-3′. The thermal cycle denaturation, annealing, and extension were repeated 35 times as described in previous studies\(^\text{17,18}\). Smart Cycler software (Cepheid, Sunnyvale, CA, USA) was used to analyze the results and the specificity of the PCR products was verified by melting curve analysis. The expression levels of DC-
Figure 2. Effects of propofol on DC-STAMP mRNA expression in RAW264.7 cells. The cells were stimulated with 0 (control), 10, 20, or 30 µM propofol in the presence of RANKL for 5 h (A), 1.5 days (B) and 4 days (C), and DC-STAMP mRNA levels were determined on day 4 of culture by real-time PCR. Bars indicate the mean ± standard deviation of three independent experiments. ***P<0.001 (vs. control).

Figure 3. Effects of propofol on OC-STAMP mRNA expression in RAW264.7 cells. The cells were stimulated with 0 (control), 10, 20, or 30 µM propofol in the presence of RANKL for 5 h (A), 1.5 days (B) and 4 days (C), and OC-STAMP mRNA levels were determined on day 4 of culture by real-time PCR. Bars indicate the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 (vs. control).
STAMP, OC-STAMP, LGR4, and RANK genes were normalized to that of GAPDH and then evaluated as ratios relative to the control.

**Statistical analysis**

Mean differences were tested by ANOVA and Tukey’s multiple comparison tests. Results with P < 0.05 were considered statistically significant.

**Results**

**Effects of propofol on DC-STAMP and OC-STAMP expression in RAW264.7 cells**

Gene expression of osteoclast fusion factors DC-STAMP and OC-STAMP in RAW264.7 cells stimulated with propofol for 5 h, 1.5 days, or 4 days in the presence of RANKL was examined by real-time PCR on day 4 of culture. Stimulation of cells with propofol for 5 h slightly increased the expression of DC-STAMP compared to that of the control (Fig. 2A). However, the changes were not statistically significant at any of the propofol concentrations (p > 0.05). In contrast, propofol stimulation for 1.5 or 4 days significantly reduced DC-STAMP expression compared to that of the controls at all propofol concentrations (p < 0.001) (Fig. 2B, C). Similar to that of DC-STAMP, stimulation of RAW264.7 cells with propofol for 5 h did not significantly change the expression levels of OC-STAMP compared to that of the control at any of the propofol concentrations evaluated (p > 0.05) (Fig. 3A). However, once again consistent with the findings for DC-STAMP, the expression of OC-STAMP was significantly reduced by stimulation with 10, 20, or 30 µM propofol for 1.5 or 4 days compared to that of the controls (p < 0.05) (Fig. 3B, C).

**Effects of propofol on the formation of TRAP-positive multinuclear cells**

Representative TRAP staining at day 4 of culture of RAW264.7 cells stimulated with propofol for 5 h, 1.5 days, or 4 days is shown in Fig. 4A. TRAP staining was slightly reduced by stimulation with 30 µM propofol for 5 h. The staining of the cells stimulated for 5 h with the lower propofol concentrations (10 and 20 µM) was comparable to that of the control (0 µM). In comparison, propofol stimulation for 1.5 days suppressed the formation of TRAP-positive osteoclast-like cells in a concentration-dependent manner and to the same extent as 4 days of continuous propofol stimulation (Fig. 4A, B). The TRAP-positive cells were divided into three groups according to their number of nuclei (Fig. 4C). Cells with 3-5 nuclei were classified as small, those with 6-9 nuclei were classified as medium, and cells with 10 or more nuclei were classified as large. As with the TRAP-staining analysis, no significant change was observed in the cells stimulated with 10 or 20 µM propofol for 5 h (p > 0.05), but a slight decrease in the number of osteoclast-like cells was seen in the cells stimulated with 30 µM propofol for 5 h (p < 0.05) (Fig. 4C). In addition, 1.5 days of propofol stimulation caused a decrease in the number of osteoclast-like cells in a concentration-dependent manner, regardless of the size of cells. This was also observed for 4 days of continuous propofol stimulation and to a similar extent (p < 0.05) (Fig. 4C).

**Effects of propofol on LGR4 and RANK expression in RAW264.7 cells**

The gene expression of LGR4 and RANK at day 4 of culture in RAW264.7 cells stimulated with propofol for 5 h, 1.5 days, or 4 days in the presence of RANKL was examined using real-time PCR. As shown in Fig. 5A, LGR4 expression was significantly increased in cells stimulated with 20 or 30 µM propofol for 5 h compared to that in the control cells (P < 0.01) (Fig. 5C). However, no significant change was observed compared to that of the control at 1.5 days of propofol stimulation at any concentration significantly increased LGR4 expression compared to that of the control (P < 0.01) (Fig. 5C). In contrast, RANK expression was significantly decreased by 5 h of stimulation with 20 µM propofol compared to that of the control (p < 0.05), but was
Figure 5. Effects of propofol on LGR4 mRNA expression in RAW264.7 cells. The cells were stimulated with 0 (control), 10, 20, or 30 µM propofol in the presence of RANKL for 5 h (A), 1.5 days (B) and 4 days (C), and LGR4 mRNA levels were determined on day 4 of culture by real-time PCR. Bars indicate the mean ± standard deviation of three independent experiments. *P< 0.05, **P< 0.01, ***P< 0.001 (vs. control).

Figure 6. Effects of propofol on RANK mRNA expression in RAW264.7 cells. The cells were stimulated with 0 (control), 10, 20, or 30 µM propofol in the presence of RANKL for 5 h (A), 1.5 days (B) and 4 days (C), and RANK mRNA levels were determined on day 4 of culture by real-time PCR. Bars indicate the mean ± standard deviation of three independent experiments. *P< 0.05, **P< 0.01, ***P< 0.001 (vs. control).
significantly increased by 5 h of stimulation with 30 µM propofol (P < 0.01) (Fig. 6A). Furthermore, stimulation with 20 or 30 µM propofol for 1.5 days significantly decreased RANK expression compared to that in the control cells (p < 0.05) (Fig. 6B). Expression of RANK was significantly decreased compared to that of the control by stimulation with propofol for 4 days at any concentration (p < 0.001) (Fig. 6C).

Discussion

When propofol is administered clinically only for a short-time sedation, such as local treatment, the findings of the current study suggest that transient administration has little effect on osteoclast differentiation. However, continuous administration of propofol for a day or longer, even if it is interrupted after that point, can affect bone remodeling to a similar extent as longer continuous propofol administration.

Direct propofol stimulation of osteoclast progenitor cells affects osteoclast differentiation differently from that of indirect propofol stimulation via osteoblasts. Kim et al. reported that continuous propofol stimulation induced osteoclast differentiation of mouse bone marrow cells of the macrophage lineage. On the other hand, Lee et al. demonstrated that the RANKL/OPG expression ratio was significantly decreased by the stimulation of propofol in osteoblasts isolated from mouse calvaria. Moreover, differentiation of osteoclast progenitor cells into osteoclasts was suppressed when they were cultured in medium containing the culture supernatant of osteoblasts stimulated with propofol for 7 days.

Unlike the study by Kim et al., the current study showed that direct propofol stimulation of osteoclast precursor cells suppressed osteoclast differentiation. Despite the difference between the effects of direct propofol stimulation and indirect effects via osteoblasts, our findings are consistent with those of Lee et al., which demonstrated the inhibitory effect of propofol on osteoclast differentiation.

The differences in experimental conditions between the study by Kim et al. and ours must be considered. First, the two studies used different cells as osteoclast progenitor cells. Kim et al. stimulated monocye lineage cells isolated from mouse bone marrow with propofol in the presence of M-CSF and RANKL. Meanwhile, we used RAW264.7 cells, which are often used as a model for RANKL-induced osteoclast differentiation, without the medium being supplemented with M-CSF. Second, different concentrations of propofol were used in the two studies. In the study by Kim et al., osteoclast differentiation was promoted by 50 µM propofol, but DC-STAMP expression and the number of TRAP-positive cells did not significantly change by stimulating with 5-20 µM propofol, remaining within the range of clinically assumed blood concentrations. In our study, 50 µM propofol markedly suppressed the proliferation of RAW264.7 cells (data not shown); however, propofol at a concentration within the clinical range (from 10-30 µM) exhibited suppressive effects on osteoclastogenesis without suppressing proliferation.

Upon binding of RANK to RANK, nuclear factor of activated T cells (NFAT) c1, a transcription factor involved in osteoclast differentiation, is translocated to the nucleus and binds to the promoter of the DC-STAMP gene. This initiates the transcription of DC-STAMP mRNA, which is essential for osteoclast fusion and multinucleation. Osteoclast formation is also promoted by OC-STAMP in the presence of RANKL. In the current study, decreased expression of both DC-STAMP and OC-STAMP was observed, not only in RAW264.7 cells continuously stimulated with propofol for 4 days, but also in cells that continued to be cultured in the presence of RANKL after transient propofol stimulation for 1.5 days. In addition, cells stimulated with propofol for 4 or 1.5 days exhibited decreased RANK expression. Unlike our findings regarding RANK expression, 1.5 days of propofol stimulation had no effect on the expression of LGR4, which is a RANKL receptor that suppresses osteoclast differentiation. However, 4 days of continuous propofol stimulation led to an increase in LGR4 expression. Based on these results, it was considered that the stimulation with propofol beyond a single day suppressed osteoclast differentiation by decreasing RANK expression. Furthermore, 4 days of continuous propofol stimulation may not only induce decreased RANK expression, but may also induce increased LGR4 expression, suppressing RANKL-induced osteoclast differentiation.

Kalkman et al. reported that exposure to general anesthetics in childhood is more likely to cause behavioral abnormalities than exposure to general anesthetics during subsequent surgery in later stages of life. In addition, Flick et al. reported that multiple anesthetic exposures in childhood, up to the age of 2 years, cause cognitive dysfunction. Long-term or repeated use of propofol (as investigated in our study) may cause the onset of propofol infusion syndrome (PRIS) in children. In the current study, inhibition of osteoclast differentiation was induced by continuous propofol stimulation, as well as by stimulation that was interrupted after 1 day. This suggests that propofol-induced suppression of osteoclastic bone resorption not only disrupts the balance of bone remodeling, but also affects bone wound healing in treatments involving bone invasion. Therefore, long-term or frequent use of intravenous anesthetics should be performed with caution, especially in patients at risk of bone disease, in treatments with invasion of the bone, and in children. Although changes in bone metabolism have not been reported in patients with PRIS, adverse effects of propofol on bone metabolism may be revealed, alongside extended applications of propofol. The effects of propofol on patients with background factors that affect bone metabolism, such as smoking, inflammatory diseases, and bisphosphonate use, require further investigation.

An increase in blood lipids due to the lipid emulsion contained in propofol infusion, and the involvement of macrophages in the phagocytosis of lipid droplets, have been implicated as the mechanism of PRIS development. The current study used a propofol preparation containing a lipid emulsion, consistent with its clinical use. The effects of the lipid emulsion itself on osteoclast differentiation have not been reported, but the findings of the present study suggest this needs to be examined in the future. In addition, the study by Kim et al. suggested that continuous, direct stimulation with propofol acts on p38 mitogen-activated protein kinase to increase OC-STAMP expression, thereby promoting the formation of osteoclasts. However, Merle et al. reported that the activity of the N-methyl-D-aspartate receptor, which is also found in osteoclasts, is involved during the final stage of osteoclast formation. Therefore, the mechanism of intracellular signal transduction by propofol stimulation, which was not examined in the current study, needs to be investigated in detail.

In conclusion, the findings of our study suggest that several hours of propofol administration does not affect RANKL-induced osteoclast differentiation. However, several days of propofol exposure may suppress the differentiation and fusion of osteoclasts due to decreased gene expression of DC-STAMP, OC-STAMP, and RANK, as well as increased expression of LGR4. Continuous administration of propofol may disrupt the balance of bone remodeling and also affect bone wound healing.

Acknowledgement

This study was supported by Sato Fund and Dental Research Center, Nihon University School of Dentistry.
Conflicts of Interest
All the authors of this paper declare that there are no conflicts of interest to be disclosed.

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