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Lipopolysaccharide and High Concentrations of Glucose Enhances Zoledronate-induced Increase in RANKL/OPG Ratio by Upregulating PGE2 Production in Osteoblasts

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Abstract: Preexisting diseases, such as diabetes and chronic inflammation in periodontal tissue, are risk factors associated with bisphosphonate-related osteonecrosis of the jaw. Osteoblasts produce prostaglandin (PG)E2 via cyclooxygenases (COX), and the autocrine action of PGE2 impacts the function of osteoblasts, including receptor activator of NF-kappa B ligand (RANKL) and osteoprotegerin (OPG) production. This study assessed the effects of the stimulation of zoledronate in the presence of lipopolysaccharide (LPS) and high concentrations of glucose on the expression of COX-2, RANKL, and OPG, in addition to PGE2 production in osteoblasts. MG-63 cells were cultured in medium containing 1 μg/ml LPS, 25 mM glucose (high glucose), and/or zoledronate (1×10^4, 1×10^5, 1×10^6, 5×10^6, or 1×10^8 M). The mRNA expression of COX-2, RANKL, and OPG genes was determined by real-time polymerase chain reaction. The concentrations of RANKL and OPG protein and PGE2 in the culture supernatant were examined by enzyme-linked immunosorbent assay. Zoledronate at a concentration of 5×10^6 M overwhelmingly increased COX-2 mRNA expression. The expression levels of RANKL and OPG as well as PGE2 production were significantly increased in cells stimulated with 5×10^8 M zoledronate in the presence of LPS and high glucose than in the unstimulated cells (control). NS398, a specific inhibitor of COX-2, blocked the stimulatory effects of zoledronate (in the presence of LPS and high glucose) on PGE2 production and the protein expression levels of RANKL and OPG. The ratio of RANKL/OPG was also increased following zoledronate stimulation. In addition, a significant difference was observed not in the stimulation with zoledronate alone, but by the stimulation of zoledronate in the presence of LPS and high glucose as compared that in controls. These results suggest that LPS and high concentrations of glucose enhance zoledronate-induced increase in RANKL/OPG ratio via the autocrine action of NS398-blocked PGE2 in osteoblasts.

Key words: PGE2, RANKL, Osteoprotegerin, Zoledronate

Introduction

Many clinical studies have demonstrated the effectiveness of bisphosphonates for the treatment of bone disorders, such as Paget disease, osteoporosis, and malignant disease of bone.1-2. Cell biological studies and animal experiments have also revealed that bisphosphonate inhibits osteoclastic bone resorption3-4. In contrast, bisphosphonate-related osteonecrosis of the jaw (BRONJ) has been recognized as an adverse effect, alongside the spread of bisphosphonate therapy.5. Prolonged treatment with bisphosphonates, smoking habits, tooth extraction, and preexisting diseases such as diabetes and chronic inflammation in periodontal tissue, are considered risk factors for BRONJ.6-7. Diabetes and periodontal disease are highly prevalent in adults.6. In periodontal disease, bacterial pathogenesis in subgingival plaque induces chronic inflammation in periodontal tissue. Lipopolysaccharide (LPS) derived from periodontal pathogens such as Porphyromonas gingivalis (P. gingivalis) is considered a trigger of the production of inflammatory cytokines, prostaglandins (PG), or active oxygen species, in macrophages, gingival fibroblasts, osteoblasts, and osteoclasts.8-11. Diabetic patients are predisposed to developing periodontal disease.12. The symptoms of periodontal disease, including destruction of periodontal tissue, are more severe in diabetic patients than in non-diabetic patients.13-14. These adverse effects of diabetes on periodontal disease are associated with a vulnerability to infection and delay in the healing process due to hyperglycemia; therefore, periodontal disease is a diabetic complication (diabetic periodontitis).15. Gum tissue, periodontal ligament, and alveolar bone surrounding the teeth contain many blood vessels; as such, these tissues are also exposed to high concentrations of glucose as well as periodontal pathogenesis in diabetic periodontitis.16,17.

The receptor activator of NF-kappa B (RANK) ligand (RANKL) and osteoprotegerin (OPG) control osteoclastogenesis.18. RANKL and osteoprotegerin (OPG) control osteoclastogenesis.18. RANKL and OPG are considered risk factors for BRONJ...
strongly induces the differentiation of osteoclasts via RANK expression on the cell surface of osteoclast precursor cells, whereas OPG blocks the interaction between RANKL and RANK\textsuperscript{10,19}. Osteoblasts produce both of these factors; therefore, osteoblasts play a pivotal role in osteoclastogenesis\textsuperscript{20}. Osteoblasts also produce prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) via cyclooxygenases (COX), and the autocrine action of PGE\textsubscript{2} has an impact on the function of osteoblasts, including RANKL and OPG production\textsuperscript{21,22}.

Several studies previously reported the effects of bisphosphonate on osteoblasts; the calcified nodule formation and the expression of angiogenic factor or RANKL in osteoblasts were suppressed by bisphosphonate\textsuperscript{23-25}. Treatment with bisphosphonate under diabetic periodontitis conditions might influence not only osteoclasts but also osteoblasts; however, there has been little basic research conducted on this issue. In the present study, the effects of bisphosphonate in the presence of LPS derived from \textit{P. gingivalis} and high concentrations of glucose on the expression levels of COX-2, RANKL, OPG, and PGE\textsubscript{2} production in osteoblasts were examined using zoledronate which is a nitrogen-containing bisphosphonate and MG-63 cells (a human osteoblastic cell line). In addition, we investigated the possibility of autocrine action of PGE\textsubscript{2} as a cause of the alteration in the RANKL/OPG ratio using NS398, which acts as a specific inhibitor of COX-2.

### Materials and Methods

#### Reagents

Alpha-minimal essential medium (α-MEM), fetal bovine serum (FBS), and penicillin-streptomycin solution were purchased from Gibco (BRL, Rockville, MD, USA), HyClone Laboratories (Logan, UT, USA), and Sigma Chemical (St. Louis, MO, USA), respectively. Zoledronate, 1,10,10,10-tetramethyl-1,1,1,1-tetraazacyclotetradecane-1,4,7,10,13-pentaaqua(1H)-dibenzo[d,f][1,10]phenanthroline-3,6-dionate, and NS398 were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). \textit{P. gingivalis} LPS was purchased from InvivoGen (Carlsbad, CA, USA). The SYBR Premix Ex Taq II\textsuperscript{TM} solution was purchased from Takara Bio (Otsu, Japan).

#### Cell culture and stimulation

MG-63 (human osteosarcoma cell line) cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). This cell line is widely used as a human osteoblast model because it shares the same typical characteristics of osteoblasts\textsuperscript{20}. Cells were maintained in α-MEM containing 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin-streptomycin solution at 37 °C in a 5% CO\textsubscript{2} humidified chamber. For experimental stimuli, the cells were seeded in 6-well plates at a density of 6×10\textsuperscript{3} cells/cm\textsuperscript{2} and left overnight to settle. After settling, cells were cultured in α-MEM containing 1 µg/ml LPS, 25 mM d-glucose, and/or zoledronate (1×10\textsuperscript{-8}, 1×10\textsuperscript{-7}, 1×10\textsuperscript{-8}, 5×10\textsuperscript{-9}, or 1×10\textsuperscript{-5} M) in addition to FBS and antibiotics.

#### Enzyme-linked immunosorbent assay (ELISA)

Cells were cultured in the presence or absence of 5×10\textsuperscript{-8} M zoledronate, 1 µg/ml LPS, 25 mM glucose, and/or 10 µg/ml NS398. After 3 days, the medium was replaced with serum-free α-MEM and the cells were cultured without stimulation for 24 h. Culture supernatants were collected and the concentrations of PGE\textsubscript{2}, RANKL, and OPG in the culture supernatant were measured using an ELISA kit (PGE\textsubscript{2} and OPG; RayBiotech, Norcross, GA, USA) according to the manufacturer’s instructions. Triplicate assays were performed on each specimen, and the data were converted to pg/ml using a standard curve.

#### Statistical analysis

Values are reported as mean ± standard deviation. Significant differences were determined using a one-way analysis of variance followed by a Tukey’s multiple comparison test. Differences with a p-value <0.05 were considered statistically significant. GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analyses.

### Table 1. PCR primers used in the experiments

| Target | Forward Primer | Reverse Primer | GenBank accession no. |
|--------|----------------|----------------|-----------------------|
| COX-2  | 5'-GGCTTGATCTCAATGCACCTA-3' | 5'-GAAGTACCAAGCTGGTCTGAAT-3' | NM_000963.4 |
| RANKL  | 5'-AATTGCGCCACTTGTGGAA-3' | 5'-TGGATGGCTTGAATATAAGCAACG-3' | NM_003701.4 |
| OPG    | 5'-TTTGAGGAAACAGCAACCTGAA-3' | 5'-CCGAAACAGTGAATCAACTCAA-3' | NM_002546.4 |
| GAPDH  | 5'-TCCACACCCCTGTGGTGT-3' | 5'-TGAACGGAAGCTCAGTGG-3' | NM_001357943.2 |

PCR; polymerase chain reaction
Results

The effects of zoledronate on COX-2 mRNA expression

The expression level of COX-2 mRNA was significantly increased in cells stimulated with $5 \times 10^{-6}$ and $1 \times 10^{-5}$ M zoledronate as compared with that in unstimulated cells (control) on days 1, 3, and/or 5 of culture (Fig. 1). Above all, $5 \times 10^{-6}$ M zoledronate overwhelmingly increased COX-2 mRNA expression levels on day 3, by 8.43-fold compared with the control. Based on these results, the concentration of zoledronate that stimulates MG-63 cells was $5 \times 10^{-6}$ M and the stimulation lasted 3 days in the following experiments.

The effects of LPS and high glucose on zoledronate-induced COX-2 mRNA expression

COX-2 mRNA expression significantly increased in both cells stimulated with $5 \times 10^{-6}$ M zoledronate alone and in cells stimulated with $5 \times 10^{-6}$ M zoledronate in the presence of 1 µg/ml LPS and 25 mM glucose (high glucose; HG) for 3 days, and COX-2 mRNA expression was assessed by real-time PCR. **P<0.01, ***P<0.001 (vs. control)

The effects of zoledronate, LPS and high glucose on PGE_2 production

PGE_2 production significantly increased in both cells stimulated with $5 \times 10^{-6}$ M zoledronate alone and in cells stimulated with $5 \times 10^{-6}$ M zoledronate in the presence of 1 µg/ml LPS and 25 mM glucose (high glucose) compared with unstimulated cells (control) by 2.01- and 4.16-fold, respectively (Fig. 3). The upregulation of PGE_2 production was significantly higher following stimulation with zoledronate in the presence of LPS and high glucose than that with the stimulation of zoledronate alone. Thus, LPS and high glucose enhanced the zoledronate-induced COX-2 mRNA expression levels in MG-63 cells. LPS and high glucose significantly increased COX-2 mRNA expression levels, independent of the stimulation of zoledronate; its level was the same as in the case of zoledronate stimulation alone.

The effects of zoledronate, LPS and high glucose on mRNA expression levels of RANKL and OPG

RANKL and OPG mRNA expression was significantly increased in cells stimulated with $5 \times 10^{-6}$ M zoledronate in the presence of 1 µg/ml LPS and 25 mM glucose (high glucose) as compared with unstimulated cells (control), by 2.94-fold and 7.60-fold, respectively (Fig. 4). The upregulation of RANKL and OPG expression levels was significantly higher following stimulation with zoledronate in the presence of LPS and high glucose compared with that following stimulation with zoledronate alone. Moreover, NS398, a specific inhibitor of COX-2, completely blocked PGE_2 production in cells stimulated with zoledronate in the presence of LPS and high glucose. Thus, LPS and high glucose enhanced zoledronate-induced PGE_2 production via COX-2. LPS and high glucose without zoledronate significantly increased PGE_2 production as compared with the control levels, by 2.26-fold; this was similar to the case of zoledronate stimulation alone.

The effects of zoledronate, LPS and high glucose on PGE_2 production

PGE_2 production significantly increased in both cells stimulated with $5 \times 10^{-6}$ M zoledronate alone and in cells stimulated with $5 \times 10^{-6}$ M zoledronate in the presence of 1 µg/ml LPS and 25 mM glucose (high glucose) compared with unstimulated cells (control) by 2.01- and 4.16-fold, respectively (Fig. 3). The upregulation of PGE_2 production was significantly higher following stimulation with zoledronate in the presence of LPS and high glucose than that with the stimulation of zoledronate alone. Thus, LPS and high glucose enhanced the zoledronate-induced COX-2 mRNA expression levels in MG-63 cells. LPS and high glucose significantly increased COX-2 mRNA expression levels, independent of the stimulation of zoledronate; its level was the same as in the case of zoledronate stimulation alone.

The effects of zoledronate, LPS and high glucose on mRNA expression levels of RANKL and OPG

RANKL and OPG mRNA expression was significantly increased in cells stimulated with $5 \times 10^{-6}$ M zoledronate in the presence of 1 µg/ml LPS and 25 mM glucose (high glucose) as compared with unstimulated cells (control), by 2.94-fold and 7.60-fold, respectively, whereas stimulation with $5 \times 10^{-6}$ M zoledronate alone did not affect their expression (Fig. 4). LPS and high glucose significantly increased the mRNA expression levels of RANKL, independent of zoledronate stimulation, as compared with the control levels; its levels were obviously lower compared with the stimulation of zoledronate case in the presence of LPS and high glucose.
The effects of zoledronate, LPS and high glucose on the protein expression levels of RANKL and OPG and the ratio of RANKL/OPG

RANKL and OPG protein expression levels increased in cells stimulated with $5 \times 10^{-6} \text{ M}$ zoledronate (Zol), 1 µg/ml LPS, and 25 mM glucose (high glucose) as compared with unstimulated cells (control), by 3.14-fold and 1.67-fold, respectively, whereas stimulation with $5 \times 10^{-6} \text{ M}$ zoledronate alone did not affect their protein expression (Fig. 5). LPS and high glucose also increased RANKL protein expression, independent of zoledronate stimulation as compared with controls, but its increased levels were markedly lower compared with the stimulation of zoledronate case in the presence of LPS and high glucose. Moreover, NS398 significantly decreased RANKL and OPG protein expression in cells stimulated with zoledronate in the presence of LPS and high glucose.

Based on the results of the protein expression of RANKL and OPG, the RANKL/OPG ratio was determined. The RANKL/OPG ratio was significantly increased by the stimulation of $5 \times 10^{-6} \text{ M}$ zoledronate in the presence of LPS and high glucose as compared with controls and the stimulation of zoledronate alone case (Table 2).

### Discussion

In this study, zoledronate at a concentration of $5 \times 10^{-4} \text{ M}$ and $1 \times 10^{-5} \text{ M}$ markedly increased COX-2 expression in the human osteoblastic cell line MG-63 cells. Previous studies reported that these concentrations of zoledronate had inhibitory effects on alkaline phosphatase (ALPase) activity, extracellular matrix protein expression, or calcified nodule formation in MG-63 cells23, mouse osteoblastic cell line MC3T3-E1 cells26,27, or primary human osteoblasts28. Manzano-Moreno et. al.25 previously

### Table 2. RANKL/OPG ratio in culture supernatant of MG-63 cells

|                | RANKL/OPG ratio a) |
|----------------|--------------------|
| Control        | 1.00 ± 0.18        |
| Zol            | 1.10 ± 0.62        |
| Zol + LPS + HG | 1.86 ± 0.07        |

a) Relative to average of control, *P<0.05 (vs. control), †P<0.05 (vs. Zol) Zol; zoledronate, HG; high glucose
assessed the effects of three types of bisphosphonate, zoledronate, alendronate, and clodronate, on mRNA expression involved in the osteoblast differentiation and function in MG-63 cells and human primary osteoblasts. In their experiments, treatment with zoledronate at concentrations ranging from 1×10^{-5} to 1×10^{-6} M decreased the expression of osteoblast differentiation-related transcription factors, collagenous, and non-collagenous protein, bone morphogenic protein, and ALPase, whereas the expression levels of transforming growth factor β and vascular endothelial growth factor were increased. They also revealed that the effects of zoledronate on these mRNA expression levels was found to be generally dose-dependent. In the present study, the amount of total RNA eluted from cells was less obvious in the stimulation of 1×10^{-6} M zoledronate, and COX-2 expression levels were lower in 1×10^{-6} M than 5×10^{-5} M cases. Although we did not assess the other type of osteoblastic cell line, except for MG-63 cells, it is possible that zoledronate, at a concentration exceeding 5×10^{-5} M, might have cytotoxic effects in osteoblasts.

There are two isoforms in COX. COX-1 normally appears as a housekeeping gene. COX-2 expression is low in normal cells, and many inflammatory stimuli induce COX-2 expression\(^{59}\). Previous studies using osteoblasts reported that LPS\(^{21,30}\), butyric acid\(^{15}\), interleukin-1β\(^{12,33}\), interleukin-17\(^{54}\), and mechanical stress\(^{35,36}\) induced PGE\(_2\) production by increasing COX-2 expression in osteoblasts. This study also confirmed that COX-2 expression in MG-63 cells was increased by 1 μg/ml of \(P.\) gingivalis LPS and 25 mM glucose (high glucose) in a diabetic periodontitis model\(^{37,38}\). In addition, bisphosphonates also have inflammatory effects, which is potentially considered one of the factors responsible for the onset of BRONJ\(^{59}\). Our results also indicated that zoledronate exerted inflammatory effects in osteoblasts; the stimulation of zoledronate alone induced COX-2 expression. Moreover, this upregulatory effect of zoledronate on COX-2 expression was enhanced by \(P.\) gingivalis LPS and high concentrations of glucose (high glucose). As expected, PGE\(_2\) production was also increased by about two-fold by the stimulation of 5×10^{-5} M zoledronate in the presence of \(P.\) gingivalis LPS and high glucose compared with the stimulation of zoledronate alone. Moreover, NS398, a specific inhibitor of COX-2, attenuated the elevated PGE\(_2\) production. These results suggested that \(P.\) gingivalis LPS and high glucose augmented zoledronate-induced PGE\(_2\) production by upregulating COX-2 expression in osteoblasts.

Next, we hypothesized that the zoledronate-induced PGE\(_2\) might have an effect on the expression levels of RANKL and OPG, the former being an essential factor, and the latter being a natural inhibitor of osteoclastogenesis in osteoblasts. We examined the expression of these factors in zoledronate-stimulated MG-63 cells in a diabetic periodontitis model (via the addition of LPS and high glucose) or a normal model. As a result, the stimulation of zoledronate in the presence of LPS and high glucose increased RANKL and OPG expression. Moreover, NS398 blocked the upregulation of the stimulatory effect of zoledronate on the protein expression of RANKL and OPG in the presence of LPS and high glucose. These results suggested that zoledronate stimulation in the diabetic periodontitis model induced RANKL and OPG expression via the autocrine action of increased PGE\(_2\) in osteoblasts.

In this study, the stimulation of 5×10^{-5} M zoledronate alone had less of an effect on the expression of RANKL and OPG in MG-63 cells; however, these results were different from those reported in a previous study, in which RANKL expression was decreased, whereas OPG expression was increased by the stimulation of zoledronate at concentrations ranging from 1×10^{-5} M to 1×10^{-9} M\(^{59}\). The duration of stimulation might explain this difference in the effects of zoledronate on RANKL and OPG expression. In this study, MG-63 cells were stimulated with zoledronate for 3 days and then assessed for RANKL and OPG expression levels. In contrast, the previous study\(^{59}\) examined the expression of RANKL and OPG in cells stimulated with zoledronate for 24 h. As such, zoledronate might directly downregulate RANKL expression and upregulate OPG expression in osteoblasts, but this direct effect might diminish over time.

The excessive inhibition of osteoclastic bone resorption is considered a mechanism underlying the onset of osteonecrosis in jawbone in which turnover is more frequent than other bone tissue\(^{40,41}\); however, the exact pathogenic mechanisms behind BRONJ remain unclear. Despite the anti-bone resorptive effects of bisphosphonate, a large number of osteoclasts were observed in BRONJ\(^{36,42}\). Previous studies\(^{43,44}\) that histologically assessed clinical specimens of several types of osteonecrosis in the jaw, including BRONJ, revealed that not only osteoclasts, but also cells that expressed RANKL and OPG, were more frequently observed in specimens of BRONJ subjects than in healthy subjects. Although the background factors such as preexisting diseases in BRONJ patients have not been elucidated in these previous studies, the results of this study might explain the increase in osteoclasts and RANKL- and OPG-positive cells in some BRONJ patients. In the case of preexisting periodontitis and diabetes (diabetic periodontitis), LPS and high glucose enhance zoledronate-induced PGE\(_2\) production by increasing COX-2 expression. Moreover, the autocrine action of PGE\(_2\) acts as a trigger for the upregulation of RANKL and OPG protein production in osteoblasts. This up-regulation was larger for RANKL than OPG. Thus, the RANKL/OPG ratio was also increased by zoledronate stimulation in the presence of LPS and high glucose, suggesting that the inhibitory effects of OPG on osteoclastogenesis were relatively attenuated. These alterations in the jawbone might occur in the case of the administration of zoledronate to patients with diabetic periodontitis.

In conclusion, zoledronate increased PGE\(_2\) production via the upregulation of COX-2 expression in osteoblasts, and LPS and high glucose enhanced the zoledronate-induced increase in the RANKL/OPG ratio via the autocrine action of NS398-blocked PGE\(_2\) in osteoblasts.

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Conflict of Interest
The authors have declared that no conflict of interest exist.

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