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# Zoledronic acid increases the circulating soluble RANKL level in mice, with a further increase in lymphocyte-derived soluble RANKL in zoledronic acid- and glucocorticoid-treated mice stimulated with bacterial lipopolysaccharide

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ABSTRACT

The nitrogen-containing bisphosphonate (BP) zoledronic acid (ZA) is a potent antiresorptive drug used in conjunction with standard cancer therapy to treat osteolysis or hypercalcemia due to malignancy. However, it is unclear how ZA influences the circulating levels of bone remodeling factors. The aim of this study was to evaluate the effects of ZA on the serum levels of soluble receptor activator of NF-kB ligand (sRANKL) and osteoprotegerin (OPG). The following four groups of C57BL/6 mice were used (five mice per group): (1) the placebo + phosphate-buffered saline (PBS) group, in which placebo-treated mice were injected once weekly with PBS for 4 weeks; (2) the placebo + ZA group, in which placebo-treated mice were injected once weekly with ZA for 4 weeks; (3) the prednisolone (PSL) + PBS group, in which PSL-treated mice were injected once weekly with PBS for 4 weeks; and (4) the PSL + ZA group, in which PSL-treated mice were injected once weekly with ZA for 4 weeks. At the 3-week time point, all mice were subjected to oral inflammatory stimulation with bacterial lipopolysaccharide (LPS). The sera of these mice were obtained every week and the levels of sRANKL and OPG were measured using enzyme-linked immunosorbent assay. At the time of sacrifice, femurs were prepared for micro-computed tomography (micro-CT), histological, and histomorphometric analyses. Our data indicated that ZA administration remarkably reduced bone turnover and significantly increased the basal level of sRANKL. Interestingly, the PSL + ZA group showed a dramatically elevated sRANKL level after LPS stimulation. In contrast, the PSL + ZA group in nonobese diabetic mice with severe combined immunodeficiency disease (NOD-SCID mice), which are characterized by the absence of functional T- and B-lymphocytes, showed no increase in the sRANKL level. Our data suggest that, particularly with combination treatment of ZA and glucocorticoids, surviving lymphocytes might be the source of inflammation-induced sRANKL. Thus, circulating sRANKL levels might be modulated by ZA.



## 1. Introduction

Bisphosphonates (BPs) are potent antiresorptive drugs widely used for the treatment of metabolic bone diseases involving excessive bone resorption, such as multiple myeloma, osteoporosis, Paget's disease, and osteolysis due to bone metastasis from solid tumors. One such BP, zoledronic acid (ZA), is used in conjunction with standard cancer therapy to treat osteolysis or hypercalcemia due to malignancy by negatively regulating bone turnover [1]. Bone remodeling is regulated by a number of growth factors, cytokines, systemic peptides, and glucocorticoids and is a closely

*Abbreviations:* ZA, zoledronic acid; BPs, bisphosphonates; sRANKL, soluble receptor activator of NF-kB ligand; OPG, osteoprotegerin; PBS, phosphate-buffered saline; PSL, prednisolone; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay; μCT, microfocus X-ray computed tomography; NOD-SCID mice, nonobese diabetic mice with severe combined immunodeficiency disease; BV/TV, bone volume (%); Tb.Th, trabecular thickness (μm); Tb.N, trabecular number (per mm); Ob.S/BS, osteoblast surface (%); OS/BS, osteoid surface (%); OV/BV, osteoid volume (%); Oc.S/BS, trabecular osteoclast surface (%); N.Oc/B.Pm, number of osteoclasts per bone perimeter (per 100 mm); ES/BS, eroded surface (%); TGF-β, transforming growth factor-β; TACE, tumor necrosis factor-α (TNF-α)–converting enzyme.

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coordinated metabolic process whereby old bone is removed by osteoclasts and replaced by osteoblasts.

The discovery of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) provided many insights into the regulation of bone remodeling. RANKL is an endogenous osteoclast-activating factor secreted mostly by osteoblasts and activated T cells. In contrast, OPG, which is secreted by osteoblasts or cells in the heart, kidney, liver, and spleen, acts as a decoy receptor for RANKL and prevents its function [2–4]. It was previously indicated that the expression of RANKL and OPG is modulated by various osteotropic factors, including commonly used drugs such as estrogen, glucocorticoids, and immunosuppressants [5,6]. Recently, several clinical studies have shown that soluble RANKL (sRANKL) or serum OPG concentrations are altered by BP treatment in patients with multiple myeloma [7], Paget's disease of bone [8], osteoporosis following allogeneic stem cell transplant [9], and postmenopausal osteoporosis [10]. Therefore, treatment with BPs may affect the regulation of circulating OPG and sRANKL [11]. However, it remains unclear how the balance between circulating sRANKL and OPG is influenced by ZA.

The objective of this study was to use mouse models to determine the effect of ZA administered as a weekly subcutaneous injection on both tissue-level bone remodeling and the concentration of circulating sRANKL and OPG. Specifically, our focus was on the change in the serum levels of sRANKL, although the influence of bone remodeling was also examined. As a comparison with ZA alone, additional animals were treated with glucocorticoid using a slow-release pellet of prednisolone (PSL). Before their sacrifice, all animals were stimulated with bacterial lipopolysaccharide (LPS) to induce a pathological inflammatory reaction. Our hypothesis was that ZA would significantly suppress bone turnover in all situations and change the balance of the circulating sRANKL level compared with vehicle-treated animals. Furthermore, we believed that the interaction between ZA and glucocorticoid would result in other effects on sRANKL and OPG, with these effects indicating the presence of an alternate mechanism.

## 2. Materials and methods

### 2.1. Experimental protocol

The protocol used here was approved by the Committee for Ethical Use of Experimental Animals at Saitama Medical University. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to use alternatives to *in vivo* techniques. Male C57BL/6JJc1 mice (CLEA Japan Inc., Tokyo, Japan) and male NOD.CB17-Prkdc$^{scid}$/J, so-called NOD-SCID, mice (Charles River Japan, Yokohama, Japan) obtained at 8 weeks of age were maintained for 1 week under controlled temperature and humidity with a 12-h light/12-h dark cycle and fed standard laboratory food and water *ad libitum*. All experiments were performed using C57BL/6JJc1 mice, and NOD-SCID mice were used as a control for the measurement of sRANKL and OPG levels. At the 3-week time point, 7 days before sacrifice, LPS (10 μg per mouse) (*Escherichia coli*. O55; Wako Pure Chemical Industries, Tokyo, Japan) was injected under the right buccal gingiva of the mandible. Peripheral blood samples were obtained from the tail vein every week. At the time of sacrifice, the femur and mandible bones were obtained, and the right distal femur was prepared for histomorphometric analysis.

### 2.2. BP and glucocorticoid administrations

Slow-release pellets (Innovative Research of America, Sarasota, FL) of placebo or 3.5 mg/kg/day of PSL were implanted into the lateral side of the neck of the mice according to the manufacturer's protocol. ZA was kindly provided as a hydrated disodium salt by Novartis Pharma AG (Basel, Switzerland). To examine the influence of an antiresorptive agent on the loss of bone density, we treated the mice with once-weekly subcutaneous injections of 100 μg/kg ZA dissolved in sterile water or phosphate-buffered saline (PBS) alone. Immediately after the first administration, the mice were implanted with 21-day release PSL or placebo pellets, and the weekly injections were continued over the 21-day release period.

### 2.3. Microfocus X-ray computed tomography

Cross-sectional views of the distal femoral metaphysis at 0.5 mm distal to the growth plate were obtained by microfocus X-ray computed tomography (μCT) (Comscan Tecno, Yokohama, Japan) and analyzed using TRI/3DBon analytical software (RATOC, Tokyo, Japan). For tomographic imaging, the following conditions were used according to the manufacturer's protocol: energy, 27 keV; current, 0.1 mA; and slice thickness, 9.12 μm (n = 4 animals per group).

### 2.4. Histological procedures

The extracted specimens, including soft tissues, were fixed in 4% neutral buffered formaldehyde, followed by decalcification in 10% EDTA solution for hard tissues. The specimens were dehydrated in increasing concentrations of ethanol and embedded in paraffin. Tissue sections (4-μm-thick) obtained from the paraffin-embedded tissue blocks were mounted on positively charged glass slides. Sections were stained with hematoxylin and eosin (H&E) according to standard procedures.

Immunohistochemistry was performed using the avidin–biotin peroxidase complex technique. In brief, slides were deparaffinized through a series of xylene baths and rehydrated with graded concentrations of ethanol/distilled water. The slides were then immersed in methanol containing 3% hydrogen peroxide for 10 min, incubated in 10% horse serum for 30 min at room temperature, and then incubated with a hamster anti-mouse T cell receptor (TCR) αβ antibody (Beckman Coulter Inc., Fullerton, CA) at 1:100 dilution and a hamster anti-mouse TCRγδ antibody (clone UC7-13D5; Beckman Coulter Inc.) at 1:100 dilution for 24 h at 4 °C. Immunodetection was performed using a Histofine® MouseStain Kit with diaminobenzidine (DAB) as the substrate chromogen (Nichirei Corp., Tokyo, Japan). Negative controls were achieved by using rabbit nonimmune serum of the same dilution instead of the primary antibody. Sections were counterstained with 1:15 diluted Mayer's hemalum solution. The numbers of TCR-positive cells were counted at the 4-week time point in the mice of each group. Quantification was performed in four non-overlapping fields at a magnification of ×200 by measuring the binary pixel area using ImageJ software (1.46r).

### 2.5. Bone histomorphometry

Bone histomorphometry can express, through a numerical value, information such as the histologic structure of bone, the functions of osteoblasts and osteoclasts at the bone surface, and the morphological changes in those cells. The information obtained from this measurement is extremely helpful to understand the pathophysiology of patients with various bone diseases and the pharmacological mechanisms of drugs, as well as bone growth and changes due to aging, and is considered essential for bone metabolism studies.

The bony specimens were fixed in 70% ethanol, and the undecalcified bone was embedded in glycolmethacrylate. Sections of 3-μm thickness were cut longitudinally in the distal region of the femur and stained with toluidine blue. Histomorphometry was