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Zoledronate inhibits receptor activator of nuclear factor kappa-B ligand-induced osteoclast differentiation *via* suppression of expression of nuclear factor of activated T-cell c1 and carbonic anhydrase 2

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ABSTRACT

Bisphosphonates (BPs) are widely used in the prevention of skeletal-related events (SRE), including osteoporosis, skeletal metastases of malignant tumours, and multiple myeloma. Osteonecrosis of the jaw (ONJ) is frequently reported as a major adverse effect induced by BP treatment. The receptor activator of the nuclear factor kappa-B ligand (RANKL) inhibitor, denosumab, has recently been used to prevent SRE, but the frequency of ONJ induced by denosumab is similar to that by BPs. This finding suggests that the inhibition of RANKL-mediated osteoclastogenesis may have a close relationship with the occurrence of ONJ. We therefore investigated the expression status of RANKL-inducible genes in zoledronate-treated mouse osteoclast precursor cells. The molecular targets of zoledronate in the RANKL signal pathway and additional factors associated with osteoclastogenesis were analysed by genome-wide screening. Microarray analysis identified that among 31 genes on 44 entities of RANKL-inducible genes, the mRNA expression level of two genes, *i.e.*, *nuclear factor of activated T-cells c1* (*NFATc1*) and *carbonic anhydrase 2* (*CAII*), was decreased in zoledronate-treated cells. Subsequent analyses verified that these two genes were significantly silenced by zoledronate treatment and that their expression was restored following inhibition of zoledronate action by geranylgeraniol. Zoledronate inhibited RANKL-induced osteoclast differentiation by suppression of *NFATc1* and *CAII* gene expression. Our results suggest that these genes might be common targets for zoledronate and denosumab in the mechanism underlying RANKL-induced osteoclast differentiation. A clear understanding of the common molecular mechanisms of bone-remodelling agents is thus essential for prevention of ONJ.

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1. Introduction

Bisphosphonates (BPs), widely used in the treatment of osteoporosis, skeletal metastases of malignant tumours, and multiple myeloma to prevent hypercalcemia, pain, and pathological fractures, are accumulated in the mineralised bone matrix and released during bone resorption [1]. Nitrogen-containing BPs (N-BPs) such as alendronate, pamidronate, risedronate, and zoledronate affect osteoclast activity and survive inhibition by mevalonate metabolism. These compounds inhibit a key enzyme, farnesyl pyrophosphonate (FPP) synthase, in the pathway for mevalonate biosynthesis [2,3]. Therefore, N-BPs interfere with a variety of cellular functions essential for bone resorption and osteoclast survival that lead to apoptosis, thus suppressing aberrant bone resorption [4].

The receptor activator of nuclear factor (NF)-κBligand (RANKL) is an essential mediator of osteoclast formation, activation, and survival. RANKL binds to its receptor, RANK, on the surface of osteoclasts, leading to their activation and differentiation and the subsequent induction of bone resorption [5,6]. Anti-RANKL agents such as denosumab are used for treating multiple myeloma and malignant tumours with skeletal metastases, preventing RANKL from binding to RANK and thereby inhibiting the development, activation, and differentiation of osteoclasts [7]. This is different from the mechanism of action of BPs, which bind to bone minerals and likely inhibit osteoclast differentiation mainly after being taken up by osteoclasts at sites of bone resorption.

Clinical reports describe surgical dental treatments in patients receiving BP treatment as being associated with osteonecrosis of the jaw (ONJ) [8–10]. ONJ occasionally causes worsening of the quality of life and general status because of unbearable pain and eating dysfunction, but the role of BPs in this mechanism is unknown [11,12]. However, with regard to the correlation of ONJ incidence with BP potency, it appears that inhibition of osteoclast function and differentiation may be a key factor in the underlying pathomechanism [13]. On the other hand, reports have been published of ONJ cases where invasive dental treatment, including treatment with anti-RANKL agents, denosumab, was administered, suggesting that these agents appear to have a mode of action similar to that of BPs [14,15]. Such evidence from previous reports suggests that both BPs and anti-RANKL agents can regulate RANKL-mediated osteoclast differentiation, and the identification of novel BP targets in RANKL-mediated osteoclastogenesis may nullify the mechanism of bone-remodelling agent-induced ONJ.

In this study, we hypothesized that BPs suppresses osteoclast differentiation by regulation of RANKL-mediated genes. To prove the hypothesis, we performed a genome-wide screening of RANKL-induced osteoclast differentiation among cells treated with or without zoledronate and identified two genes, *nuclear factor of activated T-cells c1* (*NFATc1*) and *carbonic anhydrase 2* (*CAII*), markedly inhibited by zoledronate.

2. Materials and methods

2.1. Cell culture

Osteoclast precursor cells (OCPCs) derived from ICR mice were purchased from the Primary Cell Co., Ltd. (Sapporo, Japan). The cells were maintained in alpha-minimum essential medium (a-MEM; Sigma, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS; Biowest, Nuaille, France) containing 100-ng/ml macrophage colony-stimulating factor (M-CSF; Peprotech, Rocky Hill, NJ, USA) according to the manufacturers' instructions. All the cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2. Tartrate-resistant acid phosphatase (TRAP) assay

OCPCs were seeded at 8.0×10^4 cells per well in 24-well EZ View glass bottom LB culture plates (Iwaki, Tokyo, Japan) and cultured for 5 days as described above. Next, the cells were cultured in the presence or absence of 100-ng/ml RANKL (Peprotech) with 0, 1, 3, 5, 7, 10-μM zoledronate (trade name Zometa, marketed by Novartis, Basel, Switzerland) for 4 days. To evaluate osteoclast formation in both groups, the cells were stained for TRAP and TRAP-positive multinuclear (>3 nuclei) cells were counted in triplicate and observed under a BZ-9000 microscope (Keyence, Osaka, Japan).

2.3. Cell viability assay

The number of viable cells was determined using a Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instructions (Dojindo). After the incubation of OCPCs for 24 or 48 h in 96-well plates with the indicated various concentrations of zoledronate, kit reagent WST-8 was added to the medium and incubated for a further 2 h. The absorbance of samples (450 nm) was determined using a scanning multiwell spectrophotometer that serves as an ELISA reader.

2.4. Microarray analysis

Total RNA was isolated from OCPCs incubated in the presence of 100-ng/ml M-CSF and/or 100-ng/ml RANKL with or without 10-μM zoledronate for 24 h using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol and quantified using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). A Low Input Quick Amp Labelling Kit (Agilent Technologies) was used to reverse transcribe 100 ng of total RNA. cDNAs were then transcribed *in vitro* for 2 h at 37 °C to produce biotinylated cRNA. Labelled cRNA was isolated using the RNeasy Mini Kit column. Quality of total RNA, cDNA synthesis, cRNA amplification, and cRNA fragmentation were monitored by capillary electrophoresis using Bioanalyzer 2100. Six hundred nanograms of fragmented cRNA was hybridised for 17 h at 65 °C with 10 rpm constant rotation, using a Sureprint G3 Mouse GE Microarray Kit 8 × 60k <G4851 A> (Agilent Technologies). Hybridized arrays were scanned using <G2505C> (Agilent Technologies) and analysed by Scan Control <version A.8.5.1> (Agilent Technologies).