Bisphosphonates Modulate RANKL and OPG Expression in Human Osteoblasts

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INTRODUCTION

Bisphosphonates are important inhibitors of bone resorption and widely used clinically to treat osteoporosis, metabolic bone diseases and other orthopaedic disorders [1, 2]. Inhibiting osteoclasts via the mevalonate pathway is recognized as the primary mechanism of its inhibitory action [3]. Recent evidence suggests that bisphosphonates also regulate essential signaling molecules involved in osteoclastogenesis such as RANKL (receptor activator of NF-kB ligand) which are synthesized by osteoblasts [4], and bisphosphonates also modulate OPG (Osteoprotegerin), a decoy receptor absorbing RANKL and preventing RANK activation [5]. We previously demonstrated that bisphosphonstes increase osteoblast proliferation and up-regulate expression of genes involved in new bone formation [6]. In this report we continue our investigations into the diverse bisphosphonate-osteoblast interactions in modulating essential signaling molecules such as RANKL and OPG.

MATERIALS AND METHODS

Bisphosphonates and Controls: Alendronate (ALN) (Merck, Rahway, NY), Risedronate (RIS) (Proctor & Gamble, Cincinnati, OH) and Zoledronate (ZOL) (Novartis, Basel, Switzerland) were dissolved in sterile PBS and stored frozen in aliquots of [10^{-3}M] to [10^{-8}M]. Frozen aliquots of 1, 25-(OH)-$VitD_3$ ($VitD_3$) and Dexamethasone (DEX) in sterile PBS were used as positive controls. Cells treated with media alone served as negative controls (CTR).

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CELL CULTURE AND TREATMENT

Dose Dependent Study 0.25×10^6 osteoblasts were plated in each well of 6-well plates with 2ml of α-MEM supplemented with 10% FBS, 1% penicillin / streptomycin and 1% L-glutamine, incubated at 37ºC and equilibrated in 5% CO_2 in air. After 24h incubation, fresh media was supplemented with 10mM β-glycerophosphate, 0.1mM ascorbic acid, and various concentrations of bisphosphonates ([10^{-10}M] to [10^{-6}M] final concentration ALN, RIS or ZOL) or controls ([10^{-8}M] $VitD_3$ or DEX). Cells were cultured for an additional 72 h.

Time Course Study: After an initiating 24 h culture identical to the dose dependent study, osteoblasts were treated with bisphosphonates and controls at [10^{-8}M]. Cultures were continued for 6, 12 and 24 h and cells harvested for RNA extraction.

Total RNA Extraction and Reverse Transcription: Total cellular RNA was extracted in TRIzol (Invitrogen, Carlsbad, CA) and 1µg of total RNA was reverse-transcribed into cDNA using SuperScript First-Strand Synthesis System (Invitrogen) according to manufacture's protocol.

TABLE I. Primer sequences used in Real-Time RT-PCR

RANKL	Left	5'-TCCCATCTGGTTCCCATAAA-3'
	Right	5'-GGTGCTTCCTCCTTTCATCA-3'
OPG	Left	5'-TTCCGGAAACAGTGAATCAA-3'
	Right	5'-CGCTGTTTTCACAGAGGTCA-3'
β-2-microglobulin	Left	5'-TTTCATCCATCCGACATTGA-3'
	Right	5'-ATCTTCAAACCTCCATGATG-3'

Cycle Parameter

Initial Denaturation	95	10m
Denaturation	95	10s
Annealing	60	5s
Extention	72	20s

Real-Time RT-PCR: RANKL, OPG and β-2-microglobulin primers were designed to span at least two intron/exon boundaries, referring to primer design software "USCS Genome Browser" (**http://genome.ucsc.edu/**) [7] and "Primer 3" (**http://frodo.wi.mit.edu/**) (TABLE I). As internal controls, GAPDH and β-2-microglobulin were selected to minimize errors in normalization of RANKL and OPG mRNA expression [8]. The cDNA of control samples were diluted in DNAse-free water to 20%, 10% and 5%, and then amplified by PCR to prepare a standard curve. The cDNA of other samples were diluted to 10%. Each PCR reaction contained 5 µl of diluted cDNA, 0.5

μM of forward and reverse primers, 4 μl of LightCycler FastStart DNA Master^{PLUS} SYBR Green I Master Mix (Roche Diagnostics, Indianapolis, IN), 1 μl of DMSO and PCR grade water (Roche Diagnostics, Indianapolis, IN) up to a final volume of 20 μl. Amplification was demonstrated in the LightCycler system (Roche Diagnostics, Indianapolis, IN) and detected by measuring the fluorescence signal of SYBR Green. The fluorescence response data were acquired once at the end of each extension segment. During PCR, these signals increased in proportion to the amount of double-stranded DNA PCR products. To rule out that the amplified data reflected primer dimers or other non-specific products, melting curve analysis was performed following PCR amplification. Briefly, adequate amplified PCR product was indicated by the presence of a peak at the proper melting temperature for each gene sequence. Moreover, Real-Time PCR products were analyzed using 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light.

RESULTS

Dose Dependent Study: RANKL and OPG mRNA expression were measured using Real-Time RT-PCR assay 72 h after treatment with a range of bisphosphonate concentrations. In our pilot experiments, expression levels of RANKL and OPG mRNA remained unchanged irrespective of normalization to either β-2-microglobulin or GAPDH (data not shown). β-2-microglobulin mRNA expression was more reproducible, so amplified products derived from RANKL and OPG mRNA were thus normalized to β-2-microglobulin RT-PCR products.

The individual bisphosphonates modulated RANKL mRNA expression in a unique dose-dependent manner. At the most effective concentration [10^{-8}M], ALN decreased RANKL mRNA expression by 38%, while ZOL dramatically increased RANKL expression by 62% (Insert Fig. 1a). RIS had no effect on RANKL mRNA expression at any concentration tested. OPG mRNA was relatively down-regulated by all bisphosphonates, and no significant dose-dependent effect was observed (Fig. 1b).

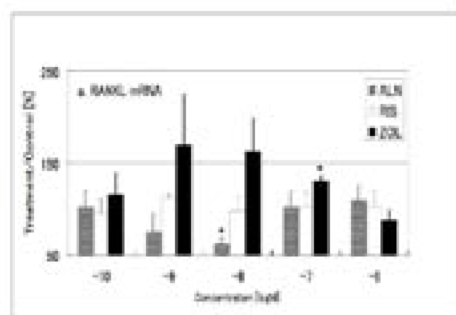

Fig. 1a

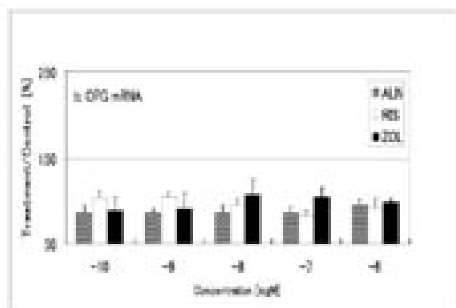

Fig. 1b

Fig. 1 (a) RANKL and (b) OPG mRNA expression in osteoblasts treated with [10^{-10}M] to [10^{-6}M] ALN, RIS and ZOL for 72h, amplified by Real-Time RT-PCR. Each value represents mean + SE of triplicate determination. Analysis was performed by one-way ANOVA and Student's two-tailed t-test. * $p < 0.05$, compared with control.

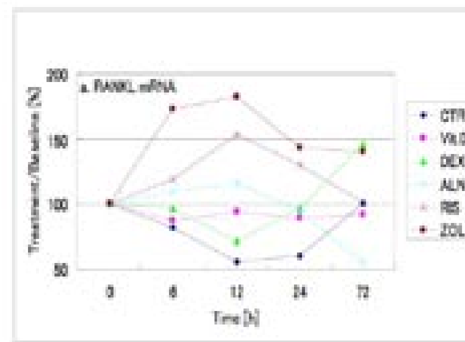

Fig. 2a

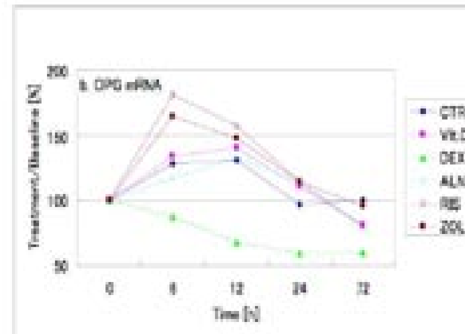

Fig. 2b

Fig. 2 (a) RANKL and (b) OPG mRNA expression in osteoblasts treated with [10^{-8}M] ALN, RIS, ZOL, Vit.D, DEX or media alone at various time period (6, 12 and 24h). Each value represents mean (n=5).

Time Course Study: The most effective dose of the bisphosphonates was observed at [10^{-8}M], consistent with our previous study [6], and was thus employed for time course studies. In control osteoblasts, RANKL mRNA level decreased and OPG mRNA levels increased gradually in a time-dependent manner, a phenomenon is attributed to osteoblast differentiation [9]. At all time periods, all bisphosphonates up-regulated RANKL mRNA expression as compared to control cells, but expression decreased after 12h. RANKL expression peaked 12h after treatment with ZOL (330%) and RIS (270%) as compared to controls (Fig. 2a). While neither of the bisphosphonates nor VitD$_3$ affected OPG mRNA expression, DEX strongly inhibited OPG mRNA expression by 50% at 12h (Fig. 2b).

Discussion: Significant strides have been made in understanding the mechanism of action of bisphosphonates. Recent evidence suggests that bisphosphonates may not only reduce osteoclast maturation and function directly but also act indirectly by activating osteoblast-stromal cells to synthesize mediators that interfere with osteoclastogenesis [10-12]. Bisphosphonates also regulate essential signaling molecules involved in osteoclastogenesis such as RANKL [13]. RANKL is synthesized by osteoblast-stromal lineage cells and combine with RANK, a cytoplasmic membrane receptor on osteoclast precursors, resulting in osteoclast proliferation and maturation [14]. Bisphosphonates also modulate OPG expression in osteo-

blasts [5]. OPG, a member of the tumor necrosis factor receptor family, is also secreted by osteoblast-stromal lineage cells and acts as a decoy receptor, absorbing RANKL and preventing RANK activation [14]. However, these effects of bisphosphonates on RANKL and OPG gene expressions in osteoblasts are still not well understood.

In our present study, we utilized Real-Time RT-PCR to obtain accurate and validated quantification of RANKL and OPG mRNA expression, and to unravel the complicated interaction of bisphosphonate effects on osteoblasts. Interestingly, our data suggests that the bisphosphonates alendronate, risedronate, and zoledronate modulate gene expression of RANKL and OPG in a unique dose- and time-dependent manner. Whereas ALN is likely to decrease RANKL/OPG ratio, which consequently suppresses osteoclast formation, ZOL appears to be the most potent activator of RANKL and OPG in osteoblasts at lower concentrations. Recent reports by Pan et al. [15] and Kim et al. [16] have presented data that do not agree with our findings. These groups reported that bisphosphonates do not impact RANKL and OPG gene expression [15, 16], but ZOL increases expression of the metalloprotease-disintegrin TNF-α converting enzyme (TACE) which cleaves the transmembrane RANKL, aborting its role in osteoclastgenesis [15]. The discrepancy in their data may be due to different cell; Kim et al. used mouse osteoblast-like cells [16] and Pan et al. used human trabecular bone derived cells [15]. Furthermore, Pan et al. used ZOL at concentrations nearly two orders of magnitude higher [$5x10^{-6}$M] as compared to our study [15]. We are continuing our investigations into bisphosphonate biology using a novel multiplexed protein profiling system.

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