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Revival of nitrogen-containing bisphosphonate-induced inhibition of osteoclastogenesis and osteoclast function by water-soluble microfibrous borate glass

He Yuan [a,1], Li-na Niu [b,1], Kai Jiao [b], Dan-dan Pei [c], Chandrani Pramanik [d], Ji-yao Li [a,2], Regina Messer [d], Satish Kumar [d,2], David H. Pashley [e], Franklin R. Tay [e,*]

[a] State Key Laboratory of Oral Disease, West China Hospital of Stomatology, Sichuan University, Chengdu, Sichuan, China
[b] State Key Laboratory of Military Stomatology, School of Stomatology, The Fourth Military Medical University, Xi'an, Shaanxi, China
[c] Department of Prosthodontics, Stomatology Hospital of Xi'an Jiaotong University College of Medicine, Xi'an, Shaanxi, China
[d] School of Materials Science and Engineering, Georgia Institute of Technology, Atlanta, GA, USA
[e] College of Graduate Studies, Augusta University, Augusta, GA, USA

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ABSTRACT

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a serious skeletal complication associated with the long-term oral or intravenous use of nitrogen-containing bisphosphonates (N-BPs). Here, we investigated the effects of an ionic cocktail prepared from water-soluble microfibrous borate glass on neutralizing the inhibitory effects of two heterocyclic N-BPs, risedronate or zoledronic acid, on osteoclastogenesis, apoptosis of differentiated osteoclasts and osteoclast function. Cell growth and proliferation assays were first performed on RAW 264.7 cells to optimize the concentrations of the ionic cocktail and N-BPs to be used for static cell culture. The pre-osteoclasts were then stimulated with RANKL to differentiate into osteoclasts. The effects of the ionic cocktail and N-BPs on osteoclast differentiation, apoptosis and function were subsequently examined using 3 series of experiments conducted at the gene, protein, morphological and functional levels. After concentration optimization, the ionic cocktail was found to partially reverse N-BP-induced inhibition of osteoclastogenesis, stimulation of osteoclasts apoptosis and reduction of osteoclast resorptive activity. Ultrastructural examination of osteoclasts that had been exposed to either N-BP identified classical features of late apoptosis and secondary necrosis, while osteoclasts exposed simultaneously to the concentration-optimized ionic cocktail and N-BPs exhibited only signs of early apoptosis that were possibly reversible. Taken together, the results of the 4 series of experiments indicate that the ionic cocktail produced from dissolution of borate glass dressings has the potential to rescue the adverse effects of heterocyclic N-BPs on osteoclast differentiation and function. These results warrant further confirmation using dynamic cell culture and small animal BRONJ models.

Statement of significance

Long-term oral and intravenous use of nitrogen-containing bisphosphonates (N-BPs) may result in bisphosphonate-related osteonecrosis of the jaw (BRONJ) due to the suppression of normal bone turnover. There is no effective treatment for such a complication to date. This work reported the use of an ionic cocktail derived from water-soluble microfibrous borate glass to revert heterocyclic N-BP-induced inhibition of osteoclastogenesis, stimulation of osteoclasts apoptosis and reduction of osteoclasts resorption in static cell culture condition. This ionic cocktail may have the potential to be further developed into a new adjunctive treatment for BRONJ.

1. Introduction

Osteoclasts are differentiated multinucleated macrophages that originate from the fusion of hematopoietic monocyte/macrophage precursors at or near the bone surface [1]. These cells play a

* Corresponding author at: College of Graduate Studies, Augusta University, Augusta, GA 30912-1129, USA.
E-mail addresses: jiyaoliscu@163.com (J.-y. Li), satish.kumar@gatech.edu (S. Kumar), ftay@gru.edu (F.R. Tay).
[1] These authors contributed equally to this work.
[2] Co-corresponding authors.

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significant role in bone remodeling and homeostasis because of their bone resorption activity. Bone disorders are caused by too little (e.g. osteopetrosis) or too much (e.g. osteoporosis) osteoclast activity. Bisphosphonates (BPs) are used for treating bone diseases relating to excessive osteoclasts resorption activity, including osteoporosis, Paget's disease, hypercalcemia and osteolysis associated with multiple myeloma and metastatic cancers [2].

There are two classes of BPs with different cellular mechanisms in inhibiting bone resorption. Non-nitrogen-containing BPs induce osteoclast apoptosis through inhibition of adenosine triphosphate-dependent enzymes [3]. Conversely, nitrogen-containing bisphosphonates (N-BPs) mainly inhibit the action of farnesyl diphosphate synthase (FPPS), a key regulating enzyme in the mevalonate pathway [4] that is required for osteoclast differentiation, survival and function. Hence, N-BPs are more potent drugs for the treatment of bone wasting diseases. Orally-administered risedronate and intravenously-administered zoledronate acid are the two frequently used N-BPs [5].

Despite their therapeutic potential, a serious skeletal complication associated with the long-term oral and intravenous use of N-BPs was reported as bisphosphonate-related osteonecrosis of the jaw (BRONJ) [6]. Case reports of BRONJ described that this complication as being triggered by prior surgical assault such as tooth extraction [7]. Approximately 0.028–4.3% of those who received N-BPs suffer from this condition [8]. In BRONJ, normal bone turnover is severely suppressed by N-BP-induced inhibition of osteoclast differentiation and function, to the extent that local micro-damages that arise from normal mechanical loading or injury cannot be repaired [9]. Management strategies of BRONJ range from conservative treatment to early surgical intervention in combination with adjunctive treatment. Because there is no effective treatment available to date, development of new strategies for treating this serious adverse effect resides on identification of novel methods of reversing the inhibition effect of N-BPs on osteoclast differentiation and function.

Immortal cell lines are frequently used as homogeneous cohorts for examining bone cell biology and function *in vitro*. However, there is no defined cell line available for mature osteoclasts. Osteoclastogenesis induced by cytokines from pre-osteoclast cell lines rarely undergo 100% differentiation. Macrophage colony-stimulating factor and receptor activator of nuclear factor kappa-B ligand (RANKL) are essential cytokines for differentiation and activation of human osteoclasts [1]. Because the murine pre-osteoclastic RAW 264.7 cell line requires only RANKL to differentiate into multinucleated osteoclasts, it is frequently employed as a model to study osteoclast formation, cell cycle and function *in vitro* [10].

Water-soluble glasses have been used in tissue engineering for repairing hard and soft tissue damages [11]. Biologically active ions released from some of these soluble glasses have been reported to be beneficial for cell proliferation and differentiation [12]. Water-soluble borate glass with faster dissolution rate appears to be more desirable for tissue engineering via rapid dilution and dissipation of the released ions to achieve an optimal concentration for cell function [13]. Ions released from a microfibrous borate glass (MBG) dressing were found to be capable of sequestering bone-bound N-BPs [14]. However, information is lacking on whether the ions released from MBG can modify the inhibitory effects of heterocyclic N-BPs on osteoclast differentiation and function.

Thus, we hypothesized that an ionic cocktail prepared from MBG dressing has no adverse side effects on osteoclasts, and that interaction of the ionic cocktail with heterocyclic N-BPs revives the inhibited osteoclast differentiation and function. To test these hypotheses, the effects of the MBG-derived ionic cocktail on neutralizing the inhibitory effects of risedronate or zoledronic acid on osteoclastogenesis, apoptosis of differentiated osteoclasts and osteoclasts function were investigated in a static cell culture model.

2. Materials and methods

2.1. Cell culture

A murine macrophage cell line (RAW 264.7, ATCC TIB-71, Manassas, VA, USA, referred thereafter as RAW cells) that has the capacity to differentiate into osteoclasts in the presence of RANKL [10] was used for investigation (Supplementary Information SI-1).

2.2. Reagents

Microfibrous borate glass (MBG) with the composition of 53.8 B_2O_3, 20.0 CaO, 12.1 K_2O, 4.6 Na_2O, 4.6 MgO, 3.8 P_2O_5 (in wt%; GL1550, Mo-Sci Corp., Rolla, MO, USA) were immersed in Dulbecco's Modified Eagle Medium (DMEM) at a ratio of 10 mg of MBG to 10 mL of DMEM for 72 h. A concentrated ionic cocktail (IC) prepared from MBG was collected by passing the infused-DMEM through 0.22 μm filters (Merck KGaA, Darmstadt, Germany). The elemental composition of the IC, as determined by inductively coupled plasma-mass spectrometry, was (in μg/mL): B (103.1), Ca (32.1), Mg (21.2), K (90.0), Na (41.8). The concentrated IC was stored at -20 °C for subsequent experiments. Stock solutions of risedronate sodium (RIS) and zoledronic acid (ZOL) (both from Sigma–Aldrich, St. Louis, MO, USA) were prepared by dissolving the respective powder in sterilized deionized water to achieve a final concentration of 1 mM and stored at −20 °C until use.

2.3. Experimental design

Four series of experiments were performed in the present study to: (a) determine the optimal IC and N-BP concentrations for RAW cell growth under static cell culture conditions; (b) evaluate the effects of concentration-optimized IC and N-BPs on osteoclastogenesis; (c) understand how apoptosis in differentiated osteoclasts is affected by concentration-optimized IC and N-BPs; and (d) examine how osteoclast resorption activity is affected by the presence of IC and/or N-BP.

2.4. Optimal IC and N-BP concentrations for growth of RAW cells

Paracelsus' observation that "all chemicals are potentially poisonous depending upon dosage" provides pedagogical insights on contemporary toxicologic testing [15]. Accordingly, the optimal IC and N-BP concentrations that were non-toxic to RAW cells under static cell culture conditions were first determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), LDH (lactate dehydrogenase) and BrdU (5-bromo-2′-deoxyuridine) assays. Each assay was conducted in sextuplicate (Supplementary Information SI-1).

Data obtained from each assay were analyzed using two-factor analysis of variance (ANOVA) to examine the effects of N-BP concentration and *IC, and the interaction of these two factors on the respective cell viability/proliferation parameter. For each assay, post hoc pairwise comparisons were conducted using the Holm–Sidak procedure. Parametric analyses were performed after validating the normality (Shapiro–Wilk test) and homoscedasticity assumptions (modified Levene test) of the respective data sets. When those assumptions were violated, nonlinear transformation of the data was performed to satisfy those assumptions prior to the use of parametric statistical methods. Statistical significance for all tests was set at $\alpha = 0.05$. The highest RIS or ZOL concentration that produced no significant difference in cytotoxicity