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Jaw bone marrow-derived osteoclast precursors internalize more bisphosphonate than long-bone marrow precursors

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ABSTRACT

Bisphosphonates (BPs) are widely used in the treatment of several bone diseases, such as osteoporosis and cancers that have metastasized to bone, by virtue of their ability to inhibit osteoclastic bone resorption. Previously, it was shown that osteoclasts present at different bone sites have different characteristics. We hypothesized that BPs could have distinct effects on different populations of osteoclasts and their precursors, for example as a result of a different capacity to endocytose the drugs. To investigate this, bone marrow cells were isolated from jaw and long bone from mice and the cells were primed to differentiate into osteoclasts with the cytokines M-CSF and RANKL. Before fusion occurred, cells were incubated with fluorescein-risedronate (FAM-RIS) for 4 or 24 h and uptake was determined by flow cytometry. We found that cultures obtained from the jaw internalized 1.7 to 2.5 times more FAM-RIS than long-bone cultures, both after 4 and 24 h, and accordingly jaw osteoclasts were more susceptible to inhibition of prenylation of Rap1a after treatment with BPs for 24 h. Surprisingly, differences in BP uptake did not differentially affect osteoclastogenesis. This suggests that jaw osteoclast precursors are less sensitive to bisphosphonates after internalization. This was supported by the finding that gene expression of the anti-apoptotic genes Bcl-2 and Bcl-xL was higher in jaw cells than long bone cells, suggesting that the jaw cells might be more resistant to BP-induced apoptosis. Our findings suggest that bisphosphonates have distinct effects on both populations of osteoclast precursors and support previous findings that osteoclasts and precursors are bone-site specific. This study may help to provide more insights into bone-site-specific responses to bisphosphonates.

Introduction

During the process of bone remodeling, bone is resorbed by osteoclasts. These multinucleated, tartrate-resistant acid phosphatase (TRACP)-expressing cells are formed by the fusion of mononuclear precursors present in the bone marrow. In diseases associated with excessive bone resorption, such as osteoporosis and cancers that have metastasized to the bone, bisphosphonates (BPs) are widely used as a treatment to reduce bone resorption by osteoclasts, thereby improving bone quality and reducing fracture risk.

Two different classes of BPs can be distinguished by the presence or absence of an amino group. Each class acts by a distinct mechanism to inhibit osteoclast function. After uptake, non-N-BPs, such as clodronate are transformed into non-hydrolysable forms of ATP, resulting in apoptosis [1,2]. Nitrogen-containing BPs (N-BPs) such as zoledronic acid, pamidronate, and risedronate, inhibit farnesyl pyrophosphate synthase (FPPS), thereby preventing prenylation of small GTPases [3,4]. Prenylation is a post-translational lipid modification important for cellular localization and function of small GTPases. Disruption of this process and inhibition of small GTPase function are therefore thought to be the mechanisms by which N-BPs lead to dysfunctional osteoclasts [5]. Besides their effect on prenylation, N-BPs can induce apoptosis directly through accumulation of the substrate of FPPS, IPP, which can be converted to the toxic ATP analogue triphosphoric acid 1-adenosin-5-yl ester 3-(3-methylbut-3-enyl) ester (ApppI) [6].

Abbreviations: ALP, alkaline phosphatase; BP, bisphosphonate; FPPS, farnesyl pyrophosphate synthase; Irf8, interferon regulatory factor 8; MafB, V-maf musculoaponeurotic fibrosarcoma oncogene homolog B; MFI, median fluorescence intensity; ONJ, osteonecrosis of the jaw; PAM, pamidronate; PBGD, porphobilinogen deaminase; RIS, risedronate; TRACP, tartrate-resistant acid phosphatase.

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In vitro, BPs exert effects on numerous different cell types [7–9]. *In vivo*, however, BPs bind rapidly and avidly to bone mineral, and as a result are thought to selectively affect osteoclasts, which release and take up the BP during resorption. Nonetheless, uptake of BPs by monocytes *in vivo* has also been reported [10]. A side-effect associated with bisphosphonates is osteonecrosis of the jaw (ONJ), which is defined as persistence of exposed bone in the maxilla or mandible for at least 8 weeks [11]. The prevalence is very low in patients receiving oral BPs for the treatment of osteoporosis; however it is estimated to range between 1 and 15% in cancer patients who receive high intravenous doses of the N-BPs pamidronate or zoledronic acid [12]. Several hypotheses regarding the etiology of BP-related ONJ have been proposed [12]. First, suppression of bone turnover and subsequent increase in bone mass could lead to avascularization followed by necrosis. Since bone turnover in the jaw is taken to be higher than in other bones [13], this effect might be more pronounced in the jaw. Next, high bone turnover and thus high local osteoclast activity could also lead to excessive release of BP from the bone and BP exposure and subsequent uptake by neighboring cells [8]. This might lead to death of cells other than osteoclasts such as other bone cells, endothelial cells, and bone marrow cells. Since osteoclasts are more active at a low pH [14], this effect could be even more pronounced in an acidic environment created by an infection or by the BP itself [12,15].

A potential explanation for ONJ could lie in the sensitivity of jaw osteoclasts and their precursors to bisphosphonates. Previously, it was shown that osteoclasts derived from various bone sites have different characteristics (reviewed in [16,17]). We hypothesize that precursors and osteoclasts from the jaw are more sensitive to bisphosphonates than those from long bone, resulting in excessive suppression of bone turnover in the jaw compared to other skeletal sites.

To investigate the effect of BPs on different osteoclast populations, we isolated jaw and long-bone marrow from mice and studied the effect of pamidronate on osteoclastogenesis *in vitro*. We also quantitatively and qualitatively assessed internalization of the fluorescently labeled N-BP risedronate (FAM-RIS) by jaw and long-bone marrow cells. This study will provide more insight into the differences between bone-site-specific osteoclasts and precursors, and may lead to a better understanding of the association between certain BPs and osteonecrosis of the jaw.

Materials and methods

Synthesis of FAM-RIS

FAM-RIS was synthesized from a commercially available mixture of 5- and 6-carboxyfluorescein (5(6)-carboxyfluorescein) according to the published procedure [18]. The sample was purified by TLC on silica gel then by preparative gradient HPLC (RP, A: 10% MeOH, 0.1 M TEAC, pH 7.0; B: 75% MeOH in 0.1 M TEAC, pH 7.5; A to 40% of B in 12 min then to 70% B in 100 min). After rotary evaporation to remove the solvent, the residue was dissolved in water and the concentration determined by UV–VIS ($\lambda_{max} = 492$ nm, $\varepsilon = 72{,}000\ M^{-1}\ cm^{-1}$). The sample was then divided into 0.5 mL aliquots (glass vials) which were then lyophilized and stored at −20 °C. The compound was characterized by ^{1}H and ^{31}P NMR (single peak); high resolution MS (M^{+}), calcd 715.1089 m/z; found 715.1055; and UV–VIS and fluorescent emission spectroscopy ($\lambda_{max} = 518$ nm).

Bone marrow isolation

Bone marrow cells from long bones and mandibles of six week old male C57BL/6J mice (Harlan, Horst, The Netherlands) were isolated and used for osteoclastogenesis experiments. Mice were injected intraperitoneally with a lethal dose of sodium pentobarbital (0.1 mL Euthestate, Ceva Sante Animale, Naaldwijk, The Netherlands). Tibiae, femurs, and mandibles were removed and were kept in α-minimal essential medium (α-MEM; Gibco, Paisley, UK) supplemented with 5% fetal calf serum (FCS; Hyclone, Logan, UT, USA), 1% antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B) (antibiotic antimycotic solution; Sigma, St. Louis, MO, USA), and heparin (170 IE/mL; Leo Pharmaceutical Products B.V., Weesp, The Netherlands). Soft tissue, condyles and the incisors were removed from the mandibles, leaving the jaw bone containing the molars and adjacent bone marrow space [19]. Bones were crushed in mortars and flushed with medium without heparin through a 21-gauge needle and filtered with a 70-μm pore-size cell strainer (Falcon/Becton Dickinson, Franklin Lakes, NJ, USA). Animal experiments were approved by the Animal Welfare Committee of the VU University (Amsterdam, The Netherlands).

Osteoclastogenesis

Freshly isolated bone marrow cells were seeded in 96-well plates (Cellstar, Greiner Bio-One, Monroe, NC, USA) at a density of 1×10^5 cells per well. Cells were seeded on plastic or on bovine cortical-long-bone slices. Osteoclastogenesis was induced with 150 μL medium containing 30 ng/mL recombinant mouse macrophage-colony stimulating factor (rmM-CSF; R&D Systems, Minneapolis, MN, USA) and 20 ng/mL recombinant mouse receptor activator of nuclear factor κB ligand (rmRANKL; R&D Systems). Stock solutions of 100 mM pamidronate (Sigma) in deionized water, 100 mM risedronate (LKT Laboratories, Inc., St. Paul, MN, USA) in PBS, 10 mM risedronate conjugated with 5(6)-carboxyfluorescein (FAM-RIS) in PBS (as described in Synthesis of FAM-RIS) [18], and 10 mg/mL FITC-dextran (Mw: 10,000; Life Technologies, Molecular Probes, Carlsbad, CA, USA) in PBS were prepared, filter sterilized and stored at −20 °C until use. Pamidronate (1–100 μM) was added to the culture medium during an early (days 0–3) or a later (days 3–6) stage of differentiation. For some experiments, bone slices were incubated with pamidronate (10–100 μM) for 20 h and PAM was washed away before seeding of the bone marrow. Culture medium with M-CSF and RANKL, but without BPs was used as a control. Cultures were maintained in a humidified atmosphere at 37 °C and 5% CO_2. The culture medium was replaced every three days. At the end of the culture period, cells were rinsed with PBS and fixed in 4% PBS-buffered formaldehyde (used for TRACP staining and confocal microscopy). Bone slices were stored in distilled water at 4 °C (used for resorption assay).

TRACP staining

Fixed cells on plastic or on bone slices were stained for TRACP using the leukocyte acid phosphatase kit (Sigma). Nuclei were visualized with 4′6-diamidino-2-phenylindole dihydrochloride (DAPI). The number of TRACP-positive cells with three or more nuclei was assessed and expressed per cm^2.

RNA isolation and real time quantitative PCR

RNA was isolated from cells cultured on bone slices using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. cDNA was synthesized using the MBI Fermentas cDNA synthesis kit (Fermentas, Vilnius, Lithuania). Real time qPCR was performed on an ABI Prism 7000 using SYBR Green mastermix (Applied Biosystems, Foster City, CA, USA) and primers are listed in Table 1. Gene expression was divided by expression of the housekeeping gene porphobilinogen deaminase (PBGD) for normalization.

Caspase 3/7-Glo assay

Caspase-3/7 activity was measured, 5 h after addition of 50 μM PAM at day 3 of culture in the presence of M-CSF and RANKL. The caspase 3/7-Glo assay (Promega, Madison, WI, USA) was performed according to