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Removal of matrix-bound zoledronate prevents post-extraction osteonecrosis of the jaw by rescuing osteoclast function

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ABSTRACT

Unlike other antiresorptive medications, bisphosphonate molecules accumulate in the bone matrix. Previous studies of side-effects of anti-resorptive treatment focused mainly on systemic effects. We hypothesize that matrix-bound bisphosphonate molecules contribute to the pathogenesis of bisphosphonate-related osteonecrosis of the jaw (BRONJ). In this study, we examined the effect of matrix-bound bisphosphonates on osteoclast differentiation in vitro using TRAP staining and resorption assay, with and without pretreatment with EDTA. We also tested the effect of zoledronate chelation on the healing of post-extraction defect in rats. Our results confirmed that bisphosphonates bind to, and can be chelated from, mineralized matrix in vitro in a dose-dependent manner. Matrix-bound bisphosphonates impaired the differentiation of osteoclasts, evidenced by TRAP activity and resorption assay. Zoledronate-treated rats that underwent bilateral dental extraction with unilateral EDTA treatment showed significant improvement in mucosal healing and micro-CT analysis on the chelated sides. The results suggest that matrix-bound bisphosphonates are accessible to osteoclasts and chelating agents and contribute to the pathogenesis of BRONJ. The use of topical chelating agents is a promising strategy for the prevention of BRONJ following dental procedures in bisphosphonate-treated patients.

1. Introduction

Bisphosphonates are effective drugs for the management of bone-wasting conditions [1]. They reduce the complications of bone metastatic lesions and minimize skeletal-related events (SRE) in several malignancies, such as breast and prostate carcinomas, and multiple myeloma. Bisphosphonates inhibit osteoclastic bone resorption, thus decreasing the risk of bone fractures in elderly cancer patients, improving the quality of life, and increasing the survival rates in lung cancer with bone metastasis [2], breast cancer [3,4], and prostate cancer [5]. Their direct anti-tumor effect is being investigated in clinical trials [6], after it has been proven in several preclinical trials [7–9].

However, bisphosphonate use has declined by >50% from 2008 to 2012, due to concerns over uncommon side effects, such as atypical femur fractures and osteonecrosis of the jaw [10,11]. The highest incidence of bisphosphonate-related osteonecrosis of the jaw (BRONJ) was reported in 5–10% of cancer patients on high doses of zoledronic acid (Zol) treatment [12,13]. Patient incompliance [14], as well as reduction of drug prescription, have led to increase in osteoporosis-related complications in recent years [10].

Newer anti-resorptive medications were also associated with ONJ [12]. On the other hand, since the majority of cases followed an invasive dental procedure, prevention strategies have so far depended on stopping the treatment before such procedures (drug holiday). However, unlike other anti-resorptive medications, bisphosphonates deposit in the bone matrix, due to their two phosphonate groups that form the bone hook [15,16]. The hydroxyl group on the R1 side-chain and even the R2 side-chain may also increase binding affinity, as well as therapeutic potency [17–20]. These properties cause bisphosphonates to accumulate in the bone matrix for years after cessation of treatment, with an average half- life of approximately ten years [21]. Therefore, while a drug holiday can reverse the systemic effect of bisphosphonates, as well as other anti-resorptive medications, it is unlikely to impact the activity of matrix-bound bisphosphonates. Previous studies have confirmed the accumulation of high levels of bisphosphonates in alveolar bone and periodontal ligament surrounding teeth [15,22]. The systemic effects of bisphosphonates have been extensively studied, including abnormal bone remodeling, systemic inflammation, and

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immune modulation [12]. Yet, the role of matrix-bound bisphosphonate molecules in the development of BRONJ has not been elucidated. The development of an effective preventive strategy for BRONJ, a largely untreatable and devastating condition, depends on addressing both the systemic and any potential local effect of the drug.

Ethylenediaminetetraacetic acid (EDTA) is widely-used for dental as well as medical applications as a chelating agent that can bind to metals via four carboxylate and two amine group [23]. It has been FDA approved and is used regularly to treat heavy metal poisoning in patients worldwide [24–26]. In dentistry, it is routinely used for removal of smear layer, root canal treatment, and root surface conditioning during periodontal treatments [23,27,28], where EDTA 17% is the most commonly used concentration in clinical dentistry as well as dental research [29–34]. A previous study has shown a significant decrease in bisphosphonate content from bone using EDTA decalcification ex-vivo [35].

We have previously shown that bisphosphonates accumulate in alveolar bone and that they continue to be susceptible to both systemic and local chelating agents [15]. In the current study, we hypothesized that matrix-bound bisphosphonates are both biologically accessible and active, and that they play a significant role in the pathogenesis of bisphosphonate-related osteonecrosis. Unlike other anti-resorptive medications, this property extends the availability of the drug beyond stopping the systemic treatment. We tested this overall hypothesis through the following specific aims: 1) we tested the hypothesis that matrix-bound zoledronate inhibited osteoclast differentiation and function in-vitro, using dentin discs, synthetic bone-mimicking matrix, and human bone, and 2) we tested the hypothesis that localized chelation of zoledronate from alveolar bone dental extraction sites could remove sufficient zoledronate to improve post-extraction socket healing and prevent the occurrence of osteonecrosis.

2. Methods

2.1. Preparation of dental discs

Extracted non-pathological human 3rd molars were collected (under human assurance committee exemption #94-02-194, 2009, Augusta University) and stored in sodium azide at 4°. Teeth were cut to generate 1 mm-thick dentin slices using an IsoMet® Low Speed Saw (Buehler, Inc. Lake Bluff, Illinois, USA). The dentin surfaces were ground and polished using grinding discs (CarbiMet Abrasive paper plain 320 grit, Buehler Inc., Lake Bluff, Illinois, USA). The resulting smear layer was removed by acid etching the surface using 37% phosphoric acid for 15 s to the discs before application of the bisphosphonate treatment.

2.2. Fluorescent imaging of AF647-Zol bound to dentin discs before and after chelation with EDTA

Dentin discs were treated with varying doses (0, 0.1 μM, 1 μM) of AF647 Zol (Biovinc, LLC, Los Angeles, CA, USA) in a 24 well plate for 24 h. The discs were then washed thoroughly with sterile phosphate – buffered saline (PBS) three times. Then discs were imaged using an Ami-X optical fluorescence imaging system (Spectral Instruments Imaging, LLC, Tucson, AZ, USA) with an excitation and emission Indocyanine green (ICG) filters. The fluorescent images were taken at 640 nm and 670 nm excitation and emission wavelength respectively. Regions of interest were standardized before the application of the fluorescent signals to prevent observational bias. Mean fluorescent intensity of the selected region of interest was plotted. Equal volumes of 0.5 ml EDTA 17 wt% (ethylenediaminetetraacetic acid and disodium salt dihydrate, Fisher Scientific Co, Aiken Rd., Asheville, NC, USA) was then applied to the discs for 30 min. The chelated solution was collected and its fluorescence was assessed by a plate reader (BioTek micro plate reader, BioTek instruments Inc., Winooski, VT, USA), and the discs were re-imaged to compare fluorescent intensity before and after EDTA application.

2.3. Mass spectrometry of dentin discs

Dentin discs were treated with vehicle (PBS) or increasing doses of Zol (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} mol) for 24 h, then EDTA was applied for 30 min after which, the supernatant was collected, additionally the dentin discs were centrifuged in spin columns and the fluid from the dentinal tubules was collected and added to the supernatant. Zoledronic acid standards and samples were first derivatized before the liquid chromatography-mass spectrometry (LC-MS) analysis as follows: First, the sample (100 μl) was diluted in isopropanol (400 μl). Then, trimethylsilyl diazomethane (TMS-DAM; 2.0 mol/L solution in hexane, 50 μl, Sigma-Aldrich, Inc., St. Louis, MO, USA) was added. After 1 h, the samples were evaporated to dryness under vacuum at 40 °C and subsequently re-dissolved in methanol:water (1:9, 100 μl) prior to injection.

Separation of the molecules was performed on a Shimadzu Nexera UHPLC system equipped with a Phenomenex Kinetex C18 column (100 × 2.1 mm, 1.7 μm) at a flowrate of 0.2 ml/min and column oven temperature of 40 °C. A gradient elution between 5% acetonitrile in 10 mM ammonium acetate (buffer A) and 90% acetonitrile in 10 mM ammonium acetate (buffer B) was used for separation with the following steps: 2% buffer B for 2 min; 2%–80% buffer B in 4 min; 80% buffer B for 1 min; 80% to 2% buffer B in 1 min and 2% buffer B for 7 min. The effluent was ionized using positive ion electrospray on an AB-SCIEX 4000 QTRAP mass spectrometry (Sciex, Ltd., Gurugram, Haryana 122015, India) with the following instrument settings: ion spray voltage 5500 V, curtain gas 20, temperature 450, gas 1 30, gas 2 20, and low/unit resolution for Q1/Q3. The optimal collision energy, de-clustering potential, entrance potential and exit potential were determined using standards. The MS was running in MRM mode monitoring two transitions of 329.1/203.1 and 329.1/135.0 for derivatized zoledronic acid to improve the selectivity of the assay. After the samples were analyzed, the integrated peak areas for the two transitions were calculated using Multiquant software (version 2.0; Sciex. Ltd., Gurugram, Haryana 122015, India).

2.4. Cell culture, osteoclast differentiation and resorption pit formation

Murine monocyte/macrophage lineage cell line (RAW264.7; American Type Culture Corporation (ATCC; Manassas, VA, USA) were cultured in Dulbeco's modified eagle medium (DMEM; ATCC) with 10%FBS and 1% Penicillin/Streptomycin, and incubated in 37 °C and 5% CO^2 and passaged at a low number to be used for the differentiation assay. Osteoassay plates (Corning Inc., New York, NY, USA) were pretreated with increasing doses (1 μM,10 μM) of zoledronic acid (Zol; Selleck Chemicals, Inc.; Houston, TX, USA), for 24 h, after which they were thoroughly washed to remove any unbound Zol. RAW 264.7 cells were seeded onto the OsteoAssay plates, and grown until 80% confluency. Osteoclast differentiation and resorption pit formation were assessed according to the manufacturer protocol. Briefly, differentiation medium was prepared using Minimum Essential Medium (MEM-Alpha), 10% FBS, 1% Pen/Strep and 50 ng/ml receptor activator of nuclear factor kappa-B ligand (Recombinant mouse RANKL; Abcam, Inc., Cambridge, MA, USA). Differentiation medium was changed every other day for 7 days, then the cells were TRAP stained with (Acid Phosphatase, Leukocyte (TRAP) Sigma Aldrich, Inc. St. Louis, MO, USA) according to manufacturer's instructions. The percentage of TRAP-positive cells was calculated using Image-J software (https://imagej.nih.gov/ij/), by counting the number of TRAP-positive cells and the total number of cells. The results were reported as a percentage: $\frac{\text{TRAP}-\text{positive cell}}{\text{Total number ofcells}} \times 100$. Resorption pits were assessed after 21 days of incubation, by bleaching plates to remove the cells followed by staining with toluidine blue to stain the resorption pits. For counting the TRAP-positive cells or the resorption pits, each well was divided into 4 quadrants and 8 random pictures were taken, 2 within each quadrant, using a light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). An