Rapid Communication

Risedronate and alendronate suppress osteocyte apoptosis following cyclic fatigue loading

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Abstract

Purpose: The purpose of this study was to determine whether bisphosphonate treatment can prevent or delay osteocyte apoptosis in a cyclic fatigue animal model and if there are differences between two different bisphosphonates in their effects on osteocyte apoptosis.

Introduction: Fatigue loading induces microdamage in long bones in rats and causes osteocyte apoptosis. In vitro data suggest that the bisphosphonates can prevent osteocyte apoptosis.

Materials and methods: Six month old female Sprague-Dawley rats (n=72) were given a daily subcutaneous (sc) injection of saline vehicle, risedronate (RIS: 0.05 μg/kg per day) or alendronate (ALN: 0.1 μg/kg per day). On the 8th day of drug treatment, an axial compressive load was applied to the right ulna using a load-controlled electromagnetic device (17N, 6000 cycles, 2 Hz, 10% loss of stiffness ∼1 h). Three, seven or ten days after loading, the animals were sacrificed. Immunohistochemistry for caspase-3 was performed to assess the extent of osteocyte apoptosis in loaded and non-loaded ulnas.

Results: Microdamage (Mdx) created by cyclic loading of the ulna induced a significant increase (p=0.03) in the number of apoptotic osteocytes compared to non-damaged regions of the same ulna, and compared to the contralateral non-loaded ulna. Risedronate and alendronate had an early effect (3 days after loading) on reducing load-induced osteocyte apoptosis. Risedronate significantly reduced the density of apoptotic osteocytes compared to vehicle-treated controls by ∼50% in the Mdx area, whereas alendronate reduced it by ∼40%. There were no differences among groups by seven days following loading.

Conclusions: (1) Low doses of risedronate or alendronate suppressed osteocyte apoptosis induced by fatigue loading of the ulna in rats. (2) There was no difference between the effects of risedronate or alendronate on osteocyte apoptosis at these doses.

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Introduction

Fatigue loading induces microdamage in long bones in rats [1]. The microdamage causes osteocyte apoptosis [22], which then signals to initiate a targeted remodeling response [11,13,14,22,23]. When microdamage-induced osteocyte apoptosis was prevented by giving the fatigue-loaded rats a caspase inhibitor, there was a subsequent reduction in remodeling [2]. This suggests that osteocyte apoptosis is a precondition for the remodeling response [9,22]. If this is true, then another mechanism for the inhibition of bone remodeling by bisphosphonates, beyond their well known effect of inhibiting the mevalonate pathway in osteoclasts, could be that they disrupt osteocyte signaling for osteoclast recruitment [19]. In cell culture studies, bisphosphonates inhibited apoptosis of MLO-Y4 osteocytic cells following treatment with glucocorticoids, a known inducer of apoptosis [18]. In mice treated with prednisolone [18], alendronate reduced osteocyte apoptosis,
demonstrating the effect in vivo. One limitation of the in vivo study, however, was that although prednisolone induced apoptosis in osteoblasts, it did not significantly increase the density of apoptotic osteocytes, so the capacity of the bisphosphonate to reduce osteocyte apoptosis could not be convincingly demonstrated. Recently, Gu et al. showed that osteocytes produce osteoclast inhibitors that have not yet been characterized. This inhibitory effect disappeared when osteocyte apoptosis was induced, and resulted in increased osteoclastic bone resorption. This suggests that local apoptosis of osteocytes could play a major role in triggering local bone remodeling [6].

The current study used a controlled loading model employing the rat ulna that is known to induce osteocyte apoptosis, and to stimulate intracortical remodeling in an animal in which it is normally absent [1,22]. To address the issue of prevention and time dependence-related bisphosphonate effects on osteocyte apoptosis, we tested two hypotheses:

1) Risedronate or alendronate treatment prevents or delays osteocyte apoptosis in a cyclic fatigue animal model;
2) There is no difference between the effects of risedronate or alendronate on osteocyte apoptosis.

Materials and methods

Experimental design

Six month old female Sprague–Dawley rats (n=72) (Harlan, Indianapolis, IN, USA) were housed two per cage at Indiana University’s Laboratory Animal Resource Center. Animal rooms were environmentally controlled at a temperature of 67–77 °F, and relative humidity of 30–70%. A light cycle of 12 h light and 12 h dark was maintained. Water and standard rat feed were provided ad libitum during the acclimation and experimental periods. All procedures were approved by Indiana University’s Institutional Animal Care and Use Committee.

Rats were divided by weight into 9 groups, 8 animals per group. Three groups of control rats (CTL; n=24) were given a daily subcutaneous (sc, 0.3 ml) injection of saline vehicle. Six groups of rats were injected sc daily with Risedronate (RIS, n=24) in saline vehicle at a dose of 0.05 μg/kg per day; or Alendronate (ALN, n=24) in saline vehicle at a dose of 0.1 μg/kg per day. Drug dosages were designed for low-dose equivalence based on the relative potency of risedronate and alendronate, and based on previous data in rats in which 0.5 to 5.0 μg/kg per day of risedronate produced optimal bone anti-resorptive activity [8]. This dose was chosen to determine whether a dose of bisphosphonate that does not suppress the initiation of intracortical remodeling in response to microdamage could prevent osteocyte apoptosis.

Ulnar axial loading

On the 8th day of drug treatment, rats were anesthetized with an intramuscular injection of ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg), and their body temperature maintained using a recirculating heating pad. One bout of axial compressive loading was applied to the right ulna using a load-controlled electromagnetic device. Loading had a peak magnitude of 17N (∼3000 microstrain at mid-ulna) at a frequency of 2 Hz for 6000 cycles (50 min generally equivalent to ∼10% loss of stiffness), as monitored using a CDD Laser Displacement Sensor (LK Serie, Keyence Corp. Osaka, Japan). Bisphosphonate treatments were continued after loading, and animals were sacrificed 3, 7 or 10 days after loading by cardiac injection with 4% phosphate-buffered formalin (PBF) (Fig. 1). The loaded ulnas were dissected free, decalcified in 10% EDTA at 4 °C for 4 weeks and then paraffin embedded.

Immunohistochemistry

Five micrometer thick cross-sections of the ulnas were prepared for immunostaining analysis. Apoptotic chondrocytes in the growth plates and a mixed population of apoptotic and healthy cells from Oncogene Research Products (ref QIA33 TdT-FragEL) were used as positive controls for the immunostain (Fig. 2). The protocol we used was adapted from Chrysis et al. and Verborgt et al. [3,23]. Sections were dewaxed in xylene and rehydrated in graded alcohol baths. For caspase-3 antibody, antigen retrieval was performed for 20 min (Epitope Retrieval: BioGenex Decal Retrieval Solution, San Ramon, CA, USA). Slides were then washed in 100% methanol CH₃OH (twice), 70% methanol, PBS and PBS + 0.3% Triton X-100. Endogenous peroxidase activity was quenched by incubating in 3% H₂O₂ in methanol for 5 min for caspase-3. Nonspecific binding was blocked by incubating with 1.5% goat serum blocking solution (VectorStain ABC Kit) diluted in PBS for 30 min. Primary antibody (Caspase-3 (H-277), sc-7148, Santa Cruz Biotechnology, USA) was diluted in PBS + 0.3% Triton X-100 + 0.1% bovine serum albumin (BSA). Caspase-3 was used at a 1:25 dilution. Sections were incubated overnight at +4 °C, washed several times in PBS containing 0.1% Tween-20 (PBS-Tw). The secondary antibody was biotinylated goat anti-rabbit IgG (Vector) diluted 1:200 in PBS containing 1.5% normal goat serum; sections were incubated in this solution for 45 min at room temperature. Finally, sections were washed several times in PBS-Tw, incubated with avidin-conjugated peroxidase (VectorStain ABC Kit, Vector) for 30 min. Peroxidase activity was detected using a solution of 0.05%DAB + 0.01%H₂O₂ that generates a brown color (D5905, Sigma, Saint Louis, MO, USA). Slices were counterstained with methyl green, dehydrated, cleared and mounted with Eukitt.

Detection of apoptosis

Apoptotic osteocytes were counted at ×850 magnification on blinded sections from two slides per ulna on a Nikon Optiphot fluorescence microscope (Nikon, Inc., Garden City, NY, USA) using the Bioquant digitizing system (R&M Biometrics, Nashville, TN, USA). Two measurement areas, each 340 × 455 μm, were chosen: one was located in the medial cortex in the microdamaged (Mdx) area and the other was located on the opposite cranio-lateral cortex (Non-Mdx area), an area where damage was not sustained (Fig. 3). In the non-loaded ulna, an area in the medial cortex comparable to the Mdx area of the loaded ulna was used as the sampling region. All osteocytes (positive and negative) were counted in each area. The density of positively stained osteocytes (apoptotic), the density of empty lacunae and the density of all osteocytes (positive, negative and empty lacunae) in each area (Mdx or non-Mdx) was calculated (Table 1).

Statistics

After verification of Gaussian distribution (Shapiro–Wilk normality test), a one-way ANOVA was used to compare the density of apoptotic osteocytes over time among the three different groups. Two outliers (one control and one ALN treated) were eliminated because apoptotic density was more than 10 standard deviations above the mean.
deviations beyond the group mean. The significance values are from Fisher’s following a significant Analysis of variance (ANOVA). When a significant ($p < 0.05$) $F$-value was detected, Fisher PLSD was used to compare groups. In addition, paired $t$-tests were used to compare the density of apoptotic osteocytes between sampling regions [Mdx area vs. non-Mdx area on the loaded ulna; Mdx area on the loaded ulna vs. the analogous region on the non-loaded ulna] and Student’s $t$-tests were used to compare the density of apoptotic osteocytes between timeline. Significance was assumed at $p < 0.05$.

Results

Cyclic loading created microdamage and induced a significant increase ($p < 0.03$) in apoptotic osteocytes in the medial cortex compared to the non-loaded forearm, and compared to the non-damaged cranio-lateral cortex of the loaded ulna, within 3 days following loading of vehicle-treated control animals (Fig. 4, Table 1). In groups treated with risedronate or alendronate, there were no significant differences between the density of apoptotic osteocytes in the Mdx area compared to either the nondamaged region of the loaded ulna, or to the nonloaded contralateral ulna, suggesting that bisphosphonate treatment prevented the load-induced increase in osteocyte apoptosis.

Treatment with risedronate significantly reduced the density of apoptotic osteocytes compared to vehicle-treated controls by about 50% in the Mdx area ($p=0.019$) by 3 days after the loading, and alendronate reduced it by about 40% ($p=0.047$)

Table 1

<table>
<thead>
<tr>
<th>N</th>
<th>Days after fatigue loading</th>
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<tbody>
<tr>
<td></td>
<td>10 days</td>
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<tr>
<td></td>
<td>RIS</td>
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<tr>
<td>Load non-Mdx</td>
<td>All osteocytes</td>
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<td></td>
<td>Positive osteocytes</td>
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<td></td>
<td>Empty lacunae</td>
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<tr>
<td>Load Mdx</td>
<td>All osteocytes</td>
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<td></td>
<td>Empty lacunae</td>
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<tr>
<td>Nonload Mdx</td>
<td>All osteocytes</td>
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<tr>
<td></td>
<td>Positive osteocytes</td>
</tr>
<tr>
<td></td>
<td>Empty lacunae</td>
</tr>
</tbody>
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Note that the area in the nonloaded ulna corresponded to the Mdx area in the loaded ulna.

$N$: Numbers of animals for each group shown in the table except for two groups: RIS 10 days, $n=7$; CTL 10 days, $n=6$.

* $p<0.05$ vs. corresponding CTL group value.
(Figs. 4 and 5, Table 1). By 7 and 10 days after loading, there were no significant differences among any of the groups (Fig. 5). By 7 days after loading, there were significantly more apoptotic osteocytes in the microdamaged region of the loaded ulna, than in the non-microdamaged region (Table 1). However, the apoptotic osteocyte density never reached the levels found in controls at 3 days, suggesting that osteocyte apoptosis was indeed suppressed. By 10 days, there were no differences in apoptotic density between different areas within groups.

Discussion

Using an in vivo model in which osteocyte apoptosis was significantly increased by load-induced fatigue damage to the bone, we have shown that bisphosphonates provide significant protection against osteocyte apoptosis. Plotkin et al. [18] previously showed that bisphosphonates reduced osteocyte apoptosis in mice treated with glucocorticoids. However, in that study, they were unable to demonstrate that the glucocorticoids increased osteocyte apoptosis in vivo, and so the demonstration of the bisphosphonate effects was not convincing. The in vivo data presented here validate Plotkin’s conclusions that the bisphosphonates can suppress osteocyte apoptosis at doses below those equivalent to doses used for the clinical treatment of osteoporosis.

Bisphosphonates attenuate osteocyte apoptosis by activating the extracellular signal regulated kinases (ERKs) by opening hemichannels formed by connexin Cx43 [15,16]. Romanello et al. found using osteoblast-like cell lines which express P2Y receptors that risedronate may affect osteoblasts by activating ERK through the upregulation of a heat shock protein, Hsp90 [20]. ERK activation in osteoblasts may be mediated by the L-type voltage-sensitive calcium channel (VSCC) which requires influx of Ca2+ from the extracellular space [10]. Calcium entry through the L-type voltage-sensitive Ca2+ channel (L-VSCC) is activated by shear stress [4], which would induce ERK activation and prevent apoptosis.

Levels of mechanical loading that do not create damage, i.e. lower loads than used in our experiments, may prevent osteocyte apoptosis through a similar pathway involving integrin-mediated activation of Src kinase. However, unlike the bisphosphonates that do not require nuclear translocation of ERKs to inhibit apoptosis, mechanical loading promotes nuclear translocation and new gene transcription [17]. Inhibition of apoptosis by estrogen also requires the nuclear accumulation of ERKs and activation of downstream transcription factors. Gu et al. suggested that the mechanism of glucocorticoid-induced osteoporosis may be due to the apoptosis of osteocytes, which can be opposed by estrogen [5]. Because both bisphosphonates and estrogen work through the Src kinase pathway but differ in their requirement for nuclear translocation, it is possible that the two in combination may be more effective in protecting the skeleton than either treatment alone [15].

Alternatively, it is possible that osteoblast apoptosis occurs through an extrinsic pathway that involves the binding of the ligand FasL to Fas, its cell-surface receptor. Interruption of this pathway can also prevent cell death. Kogianni et al. [7] observed that glucocorticoids increased cell membrane localization of Fas and induced osteocyte apoptosis; treatment with
bisphosphonates both reduced apoptosis and decreased localization of Fas in the cell membrane.

Because osteocyte apoptosis is thought to signal the initiation of bone remodeling [12, 22, 23], the early reduction of osteocyte apoptosis by bisphosphonates could disrupt the signaling mechanism in bone and may be one indirect pathway for their anti-remodeling effects. This could complement the anti-resorptive effects that bisphosphonates have through the inhibition of farnesyl diphosphate synthase in the melavonate pathway and subsequent osteoclast apoptosis. Using 16 different bisphosphonates with variable effects on bone resorption, Plotkin et al. very recently showed that the pro-apoptotic effects of bisphosphonates on osteoclasts are distinct from their anti-apoptotic effects on osteoblasts and osteocytes [16]. They were able to demonstrate that all 16 bisphosphonates prevented apoptosis in MLO-Y4 cells, but only 10 of the 16 bisphosphonates caused apoptosis in RAW-264.7 cell-derived osteoclasts. Kogianni et al. [7] had also demonstrated this dissociation when they showed an anti-apoptotic effect on MLO-Y4 cells even using variants of bisphosphonates that had reduced anti-resorptive effects. This dissociation is because the effects of the bisphosphonates act through two very different mechanisms in cells from the osteoblastic and osteoclastic lineages. As Plotkin et al. point out, this may explain why bisphosphonates are effective in some osteoporoses characterized by normal bone resorption but reduced bone formation.

Moreover, both Plotkin [16] and Kogianni [7] have shown in culture that the anti-apoptotic effects of the bisphosphonates on osteocytes and osteoblasts can occur at concentrations of drug three orders of magnitude lower than those that induce osteoclast apoptosis. Our results show that this anti-apoptotic effect in osteocytes occurs in vivo as well at doses that are 1–2 orders of magnitude below those that induce maximal anti-resorptive activity. The osteocyte apoptosis in response to microdamage occurs within 3 days after loading, and by 7 days the apoptotic phase has largely passed as shown by fewer apoptotic osteocytes in control animals. Both bisphosphonates prevented the osteocyte apoptosis at this early, 3 day, time point suggesting that, even below clinical treatment doses, bisphosphonates can have rapid pharmacologic effects. By 7 days, there were no significant differences between vehicle-treated controls and bisphosphonate treated animals.

There are limitations to this study. The immunostaining technique involves some subjectivity in evaluating sections for apoptosis and may introduce variability. The loading model creates a lot of damage and may not be indicative of the effect of bisphosphonates on osteocyte apoptosis with smaller amounts of damage. The bisphosphonate doses used were below the clinical doses used to induce maximal anti-resorptive activity reduce osteocyte apoptosis in a cyclic fatigue animal model.

Acknowledgments

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References


However, later-term induction of cellular caspase-3, as shown here 3–10 days after the stimulus was provided, is more likely an indicator of apoptosis [21].

Despite these limitations, the data clearly demonstrate that these two bisphosphonates given at a dose below the clinical treatment dose for osteoporosis significantly reduce osteocyte apoptosis in an animal model of fatigue loading induced microdamage.

We conclude that

1. Low doses of risedronate and alendronate 1–2 orders of magnitude below those that induce maximal anti-resorptive activity reduce osteocyte apoptosis in a cyclic fatigue animal model.
2. At these low doses, effects of risedronate or alendronate on osteocyte apoptosis were similar.


