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Bisphosphonates suppress periosteal osteoblast activity independently of resorption in rat femur and tibia

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Abstract

Recent studies demonstrate that bisphosphonates suppress bone resorption by leading to apoptosis of the osteoclast and inhibiting the differentiation to mature osteoclasts. The influence of bisphosphonates on bone formation is unknown, although it has been hypothesized that bisphosphonates inhibit osteoblast apoptosis and stimulate osteoblast proliferation and differentiation in vitro, leading to increased bone formation. The purpose of this study was to investigate the effect of bisphosphonates on bone formation. We administered risedronate at 0.05, 0.5 or 5.0 μg/kg/day or alendronate at 0.1, 1.0 or 10 μg/kg/day subcutaneously for 17 days to 6-month-old female Sprague–Dawley rats. Control rats were given a daily subcutaneous injection of saline. Following sacrifice, the femoral and tibial mid-diaphyses were harvested and mineralizing surface (MS/BS), mineral apposition rate (MAR) and bone formation rate (BFR/BS) were measured on periosteal and endocortical surfaces. In the femur, periosteal MAR was significantly lower in all treatment groups (22–29% for risedronate, 26–36% for alendronate) than in control. In the tibia, periosteal MAR and BFR of all treatment groups were significantly lower (41–50% for risedronate, 43–52% for alendronate) than in the control group. Because the periosteal surfaces of these bones are only undergoing bone formation in modeling mode, our results show that bisphosphonates suppress bone formation independently of bone resorption. Because this effect is seen on periosteal MAR rather than on periosteal MS/BS, we hypothesize that bisphosphonates affect the activity of individual osteoblasts at the cell level. This may help to explain the reason that the anabolic effects of teriparatide are blunted when administered concurrently with or following a course of bisphosphonates in humans.

Keywords: Rodent; Modeling and remodeling; Osteoblast; Bone histomorphometry

Introduction

The nitrogen-containing bisphosphonates, alendronate and risedronate, suppress bone turnover, increase bone mass (primarily through increases in mineralization) and reduce fracture risk in osteoporotic women [5,6,8,17,31,39]. They act by promoting osteoclast apoptosis and inhibiting differentiation to mature osteoclasts [16,25,30,32,40,41] or by reducing osteoclast activity [2,10,11,15,31,38,40,44], either through an inhibition of farnesyl diphosphonate synthase (FPP) in the mevalonate pathway, or by otherwise inhibiting prenylation of small GTPases. The influence of bisphosphonates on bone formation is unknown, although it has been hypothesized, based primarily on studies in culture, that bisphosphonates inhibit osteoblast apoptosis [1,35,38] and may stimulate osteoblast proliferation and differentiation [18,19,23,26,41,42], leading to increased bone formation.

Studies in animal models have produced equivocal results. In ovariectomized rats, risedronate had no effect on tibial periosteal surface apposition, and suppressed bone formation on the tibial endosteal surface [2,43]. In intact growing rats, on the other hand, alendronate significantly suppressed mineral apposition rate and bone formation rate on the periosteal surface of the tibia [3]. Furthermore in OPG knock-out mice, risedronate markedly suppressed both periosteal and endocortical mineral apposition rate [33]. These data suggest that bisphosphonates may suppress bone formation independently of bone resorption.

Specific effects of bisphosphonates on osteoblast activity and bone formation are difficult to test on remodeling surfaces where resorption and formation are coupled because suppression of bone turnover naturally reduces both bone resorption and bone formation. However, the periosteal

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surface of bone in mature rats does not undergo appreciable resorption but rather is known to undergo direct bone apposition in modeling mode. Based on data from the literature that suggested that bisphosphonates inhibit osteoblast apoptosis, we hypothesized that bisphosphonates would enhance periosteal bone formation. We tested this hypothesis by evaluating the effects of risedronate and alendronate on bone formation on periosteal surfaces known to be undergoing modeling formation in adult rats.

Materials and methods

Six-month-old female Sprague–Dawley rats ($n = 62$) (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 a relative humidity of 30–70%. A light cycle of 12 h light and 12 h dark 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 7 groups. Control rats (CNT; $n = 9$) were given a daily subcutaneous (sc) injection of saline vehicle. Six groups of rats were injected subcutaneously daily for 17 days with risedronate (RIS) in a saline carrier at a dose of 0.05 μg/kg per day (RIS-low; $n = 9$), 0.5 μg/kg per day (RIS-mid; $n = 9$) or 5 μg/kg per day (RIS-high; $n = 9$); or alendronate (ALN) at a dose of 0.1 μg/kg per day (ALN-low; $n = 9$), 1.0 μg/kg per day (ALN-mid; $n = 8$) or 10 μg/kg per day (ALN-high; $n = 9$). The middle doses are equivalent to the therapeutic dose used clinically. Drug dosages were designed for dose equivalence based on the relative potency of risedronate and alendronate.

All rats were given an intraperitoneal (ip) injection of calcein (10 mg/kg body mass; Sigma Chemical Co., St. Louis, MO, USA) on days 8 and 3 prior to killing. The right femurs and tibias were removed and cleaned of soft tissue. The diaphyses were dehydrated in graded alcohols, cleared in xylene and embedded in methylmethacrylate. Using a diamond-embedded wire saw (Histo-saw; Delaware Diamond Knives, Wilmington, DE, USA), transverse thick sections (70 μm) were removed from the middle of the diaphyses and mounted unstained on standard microscope slides. Histomorphometric parameters were measured at 100× magnification from two slides per limb on a Nikon Optiphot fluorescence microscope (Nikon, Inc., Garden City, NY, USA) using the Bioquant digitizing system (R&M Biometrics, Nashville, TN, USA). Total bone perimeter (B.Pm), single-label perimeter (sL.Pm) and double-label perimeter (dL.Pm) were measured on periosteal and endocortical surfaces at 100× magnification. Double-label area (dL.Ar) was collected at 200× magnification. Primary data were averaged for calculation of the derived parameters: mineralizing surface (MS/bone surface [BS] = [1/2 sL.Pm + dL.Pm]/B.Pm; %); mineral apposition rate (MAR = dL.Ar/dL.Pm per 5 days; μm/day); and bone formation rate (BFR/BS = MAR × MS/BS × 3.65; $\mu m^3/\mu m^2$/year). Terminology is in accordance with the American Society for Bone and Mineral Research (ASBMR) committee on histomorphometric nomenclature [34]. Statistical tests were performed using SAS software (SAS Institute, Inc.). Differences among treatment groups were evaluated using a one-way analysis of variance (ANOVA). When a significant overall F value ($P < 0.05$) was present, differences between individual group means were tested using Fisher's protected least-significant difference (PLSD) post hoc test. For all tests, $P < 0.05$ was considered statistically significant. Data are presented as mean ± standard deviation.

Results

There was a significant reduction in mineral apposition rate on the periosteal surfaces of both the femur and tibia at all doses of both bisphosphonates. On the femoral periosteal surface, this reduction ranged from 21% to 29% for risedronate and between

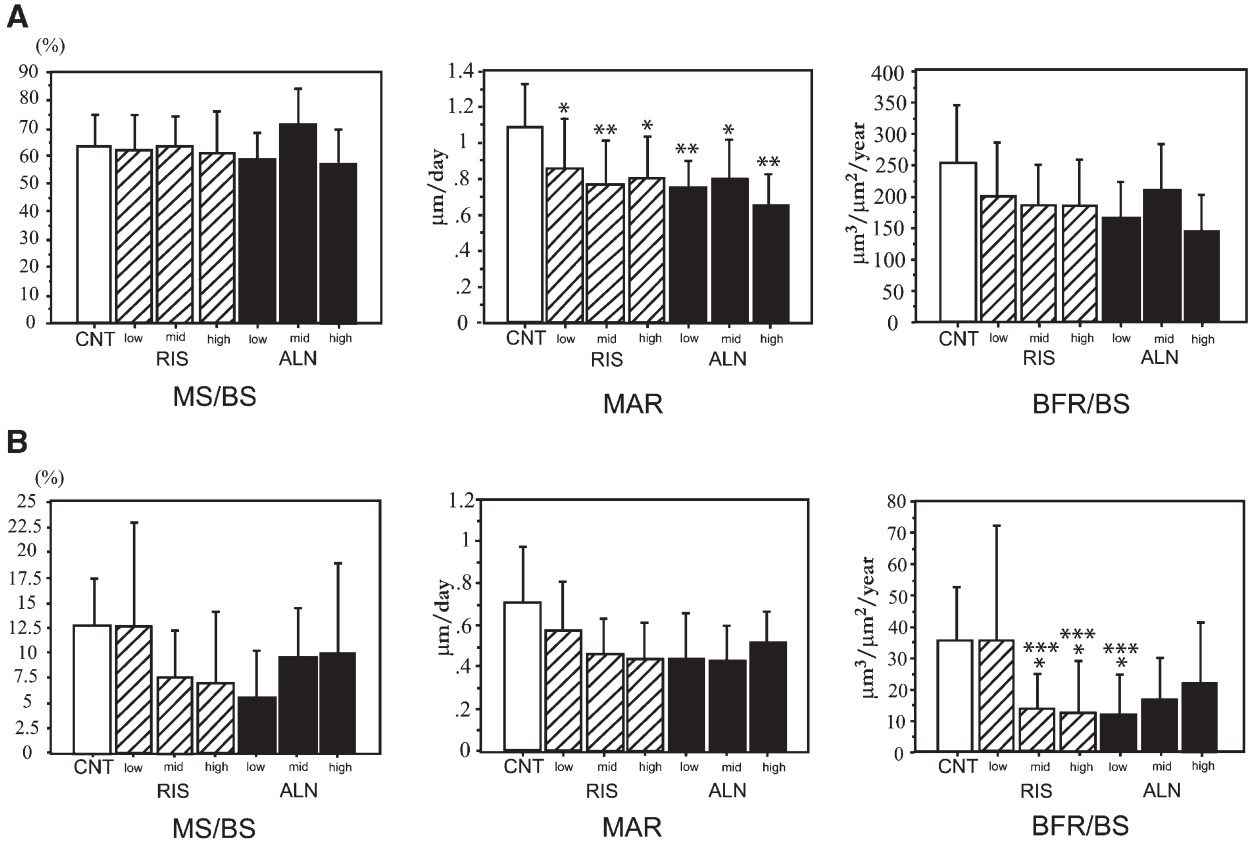

Fig. 1. Effects of bisphosphonate treatment on femur diaphyseal bone surface. (A) Periosteal surface, (B) endosteal surface. *$P < 0.05$ vs. CNT, **$P < 0.01$ vs. CNT, ***$P < 0.05$ vs. RIS-low.

26 and 36% for alendronate (Figs. 1 and 3A). On the tibial periosteal surface, the reduction was slightly greater than in the femur, ranging from 41% to 50% for risedronate and 43% to 52% for alendronate (Figs. 2 and 3B). For the tibial periosteal surface, but not for the femoral periosteal surface, this translated into a significant depression of bone formation rate. There were no significant differences between bisphosphonate-treated groups.

On endocortical surfaces, significant suppression compared to control animals was found only in bone formation rate in the femur (Fig. 1). This suppression ranged between 61% and 67%. No significant differences among groups were found for femoral MS/BS or MAR on the endocortical surface, nor were any significant differences found on the endocortical surface of the tibia (Fig. 2). Again, no significant differences were found between risedronate and alendronate-treated groups.

Discussion

Our data show that the bisphosphonates at clinical dose levels as well as lower levels significantly suppress bone formation independent of that found from coupled bone remodeling. This suppression was primarily found for mineral apposition rate, which is generally considered as an indicator of individual cell-level activity. No significant suppression was found for mineralizing surface, suggesting that bisphosphonates may not have a suppressive effect on osteoblast proliferation or differentiation but rather only on the individual activity of osteoblasts.

It is not certain whether the bisphosphonates suppress osteoblast activity through direct action on the osteoblast itself, or only indirectly through osteoclast regulation of osteoblast activity. Several studies show in vitro a direct effect of bisphosphonates on osteoblast proliferation and differentiation, independent of any effects that may occur through suppression of bone resorption. However, these studies differ on exactly how bisphosphonates may affect these cell-level processes, showing either an inhibitory effect on both proliferation and differentiation [27], a stimulatory effect [18,19,23,26,42], a stimulatory effect on differentiation, but a suppressive effect on proliferation [37], or no effect on either [20]. It is well known that osteoclasts ingest large amounts of bisphosphonates released from resorbed bone matrix, and that this prevents protein prenylation necessary for their proper function [10,11]. Direct effects of bisphosphonates on non-resorbing cells, including osteoblasts in culture, have been reported [12], although this apparently conflicts with data from the same group that suggests that risedronate, at least, is not internalized by osteoblasts [13]. Even if osteoblasts do internalize bisphosphonate, they do not internalize much [12] and because bisphosphonates cannot be metabolized by the osteoblast their intracellular concentration could increase over time. It is still possible that even a small amount of drug could interfere with cell function. Indeed, Gasser et al. [21] observed that bisphosphonates are able to prevent protein prenylation in osteoblasts.

Osteoprotegerin (OPG) production in osteoblasts, which is stimulated by bisphosphonates [41], also has a function in auto-

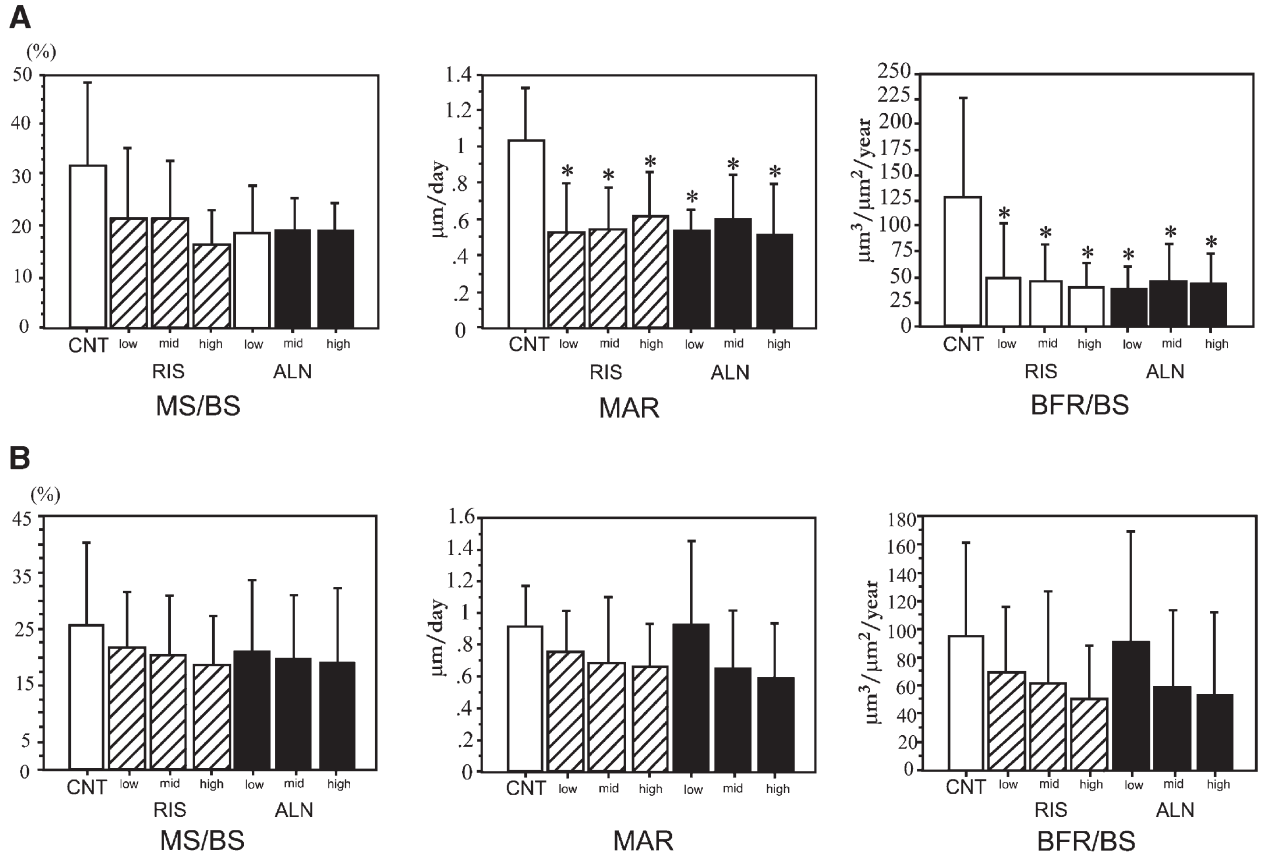

Fig. 2. Effects of bisphosphonates treatment on tibia diaphyseal bone surface. (A) Periosteal surface, (B) endosteal surface. *$P < 0.01$ vs. CNT.

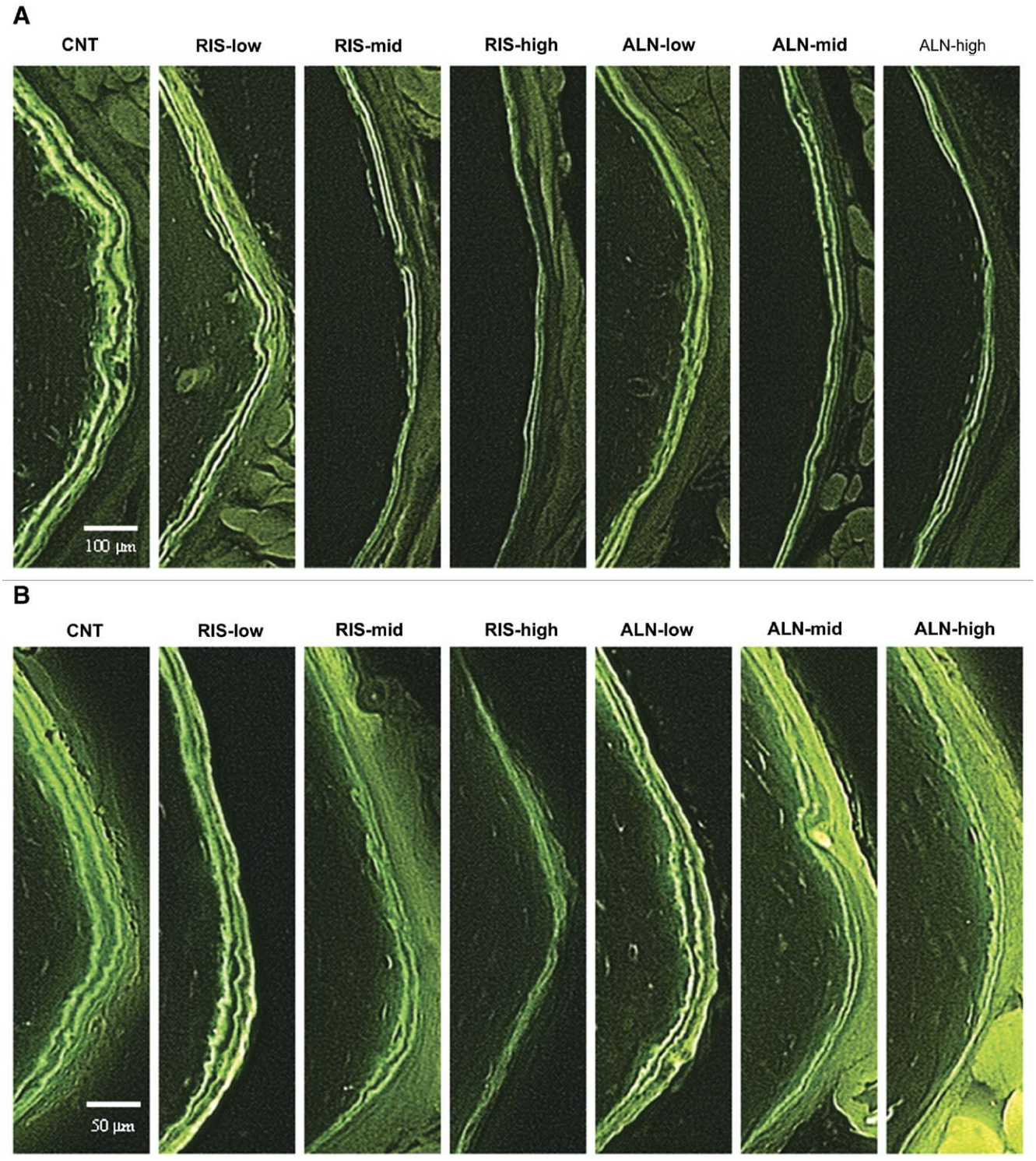

Fig. 3. Periosteal surface from cross section of diaphysis. (A) Femur, original magnification: ×100. (B) Tibia, original magnification: ×200.

regulation of osteoblastic bone formation. Nakamura et al. [33] showed that mineral apposition rate was significantly increased in OPG knock-out mice, but that this elevation of MAR was decreased in OPG−/− when risedronate was administered at 0.01 mg/kg/day for 30 days. This suppression of osteoblast function occurred without any change in the RANKL–RANK interaction. They also observed that risedronate treatment altered osteoblast shape from more cuboidal, typical of an active osteoblast, to flatter, more typical of a lining cell or a quiescent osteoblast. They suggest that osteoclasts regulate osteoblast function directly. This regulation could occur even at a remote site not undergoing coupled remodeling.

These results may in part explain why the anabolic effects of teriparatide are blunted by concurrent [4,15] or prior [14]

administration of bisphosphonates in humans. One explanation for the attenuation of teriparatide effects by bisphosphonates is that as bone formation is reduced as a consequence of lower activation frequency, there are fewer mature osteoblasts available for teriparatide to stimulate [28]. We offer the alternative explanation that bisphosphonates impair the activity of individual cells and make them less receptive to stimulation by teriparatide. We found no evidence of a suppressive effect of alendronate on bone's mineralizing surface (MS/BS), which would be indicative of fewer available fully differentiated osteoblasts, but only an effect to suppress mineral apposition rate. This is consistent with the observation of Gasser et al. [22] who showed reduced MAR in rats pre-treated with alendronate and then subsequently treated with PTH. Gasser et al. suggested that this blunting effect could occur through a direct effect on the lining cells, which do not convert to active osteoblasts because of the inhibitory effect of bisphosphonates on cytoskeletal function. This explanation coincides nicely with the observation of Nakamura et al. [33] that risedronate caused de-differentiation of active osteoblasts to flatter cells more typical of lining cells. Gasser et al, however, also showed that a two-month alendronate withdrawal period to allow washout of the drug prior to the initiation of PTH treatment did not restore the normal anabolic response to PTH. This suggests that there is also a recurring suppression of bone formation caused by continuing interaction of matrix embedded drug on the lining cells or active osteoblasts. This matrix interaction effect could still occur through direct influence on the osteoblast, independent of the effect on the portion of bone formation that occurs as the result of coupled remodeling.

It is known that there is an "anabolic window" following the initiation of teriparatide therapy. This is caused by an uncoupling of resorption and formation that allows formation to occur through direct apposition of bone to trabecular and periosteal surfaces [24,29] 3–4 months before the rise in coupled remodeling [7]. This may be one reason that teriparatide treatment following pretreatment with alendronate has its greatest attenuating effects within the first 6 months of teriparatide treatment [14].

This effect on bone formation also may help to explain the failure to find consistent increases in bone volume following treatment with bisphosphonates [9,36]. Indeed, Boivin et al. [5] have proposed that the prolonged period of secondary mineralization and increased mean tissue age caused by the bisphosphonates can account for almost all of the increased BMD observed over the first 3 years of treatment. One might expect that suppression of bone resorption by alendronate would nevertheless allow the refilling of the remodeling space by osteoblastic bone formation, resulting in a 3–5% increase in bone volume. However, this has been difficult to detect from human biopsy studies [9,36]. An independent suppressive effect on osteoblasts at the BMU level could account for the absence of these changes in bone volume.

In this study, we showed that bisphosphonates suppress bone formation on periosteal surfaces that are modeling in formation mode. This may indicate a direct effect on osteoblast activity and suppression of bone formation independently of bone resorption, but an indirect effect dependent on suppression of resorption at remote sites and perhaps mediated through OPG cannot be excluded. Regardless of which of these mechanisms is correct, the simple observation that bisphosphonates can have a suppressive effect on bone formation provides an explanation for the observation that the anabolic effects of teriparatide are blunted when administered in conjunction with bisphosphonate treatment in human patients.

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