

Original Full Length Article

Prolonged alendronate treatment prevents the decline in serum TGF- β 1 levels and reduces cortical bone strength in long-term estrogen deficiency rat model[☆]

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ABSTRACT

Introduction: While the anti-resorptive effects of the bisphosphonates (BPs) are well documented, many questions remain about their mechanisms of action, particularly following long-term use. This study evaluated the effects of alendronate (Ale) treatment on TGF- β 1 signaling in mesenchymal stem cells (MSCs) and osteocytes, and the relationship between prolonged alendronate treatment on systemic TGF- β 1 levels and bone strength.

Methods: TGF- β 1 expression and signaling were evaluated in MSCs and osteocytic MLO-Y4 cells following Ale treatment. Serum total TGF- β 1 levels, a bone resorption marker (DPD/Cr), three-dimensional microCT scans and biomechanical tests from both the trabecular and cortical bone were measured in ovariectomized rats that either received continuous Ale treatment for 360 days or Ale treatment for 120 days followed by 240 days of vehicle. Linear regression tests were performed to determine the association of serum total TGF- β 1 levels and both the trabecular (vertebrae) and cortical (tibiae) bone strength.

Results: Ale increased TGF- β 1 signaling in the MSCs but not in the MLO-Y4 cells. Ale treatment increased serum TGF- β 1 levels and the numbers of TGF- β 1-positive osteocytes and periosteal cells in cortical bone. Serum TGF- β 1 levels were not associated with vertebral maximum load and strength but was negatively associated with cortical bone maximum load and ultimate strength.

Conclusions: The increase of serum TGF- β 1 levels during acute phase of estrogen deficiency is likely due to increased osteoclast-mediated release of matrix-derived latent TGF- β 1. Long-term estrogen-deficiency generally results in a decline in serum TGF- β 1 levels that are maintained by Ale treatment. Measuring serum total TGF- β 1 levels may help to determine cortical bone quality following alendronate treatment.

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Introduction

Osteoporosis is a disease characterized by reduced bone mass and strength such that the risk of bone fracture is greatly enhanced. Currently, anti-resorptive agents are the first line of treatment for

osteoporosis. Randomized controlled clinical trials demonstrate that 3–6 years of treatment with anti-resorptive agents, especially bisphosphonates (BPs), reduce bone loss as well as the risk of vertebral and non-vertebral fractures through the inhibition of osteoclast lifespan and activity. During the early phase of treatments with BPs, there is a rapid reduction in osteoclastic bone resorption, followed by a delayed but continual reduction in osteoblast activity and new bone formation [1–4]. Over time, these changes result in reduced overall bone turnover, a modestly prolonged secondary mineralization phase, increased degree of bone mineralization and density, and improved bone strength [5–12]. However, despite over 15 years of clinical investigation of bisphosphonates, their mechanisms of action on osteoclasts and osteoblasts are still unclear.

Recently, prolonged bisphosphonate use has been associated with atypical or subtrochanteric femoral fractures, which has motivated further study of the cellular mechanisms of bisphosphonate action.

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In addition to the pro-apoptotic effect of bisphosphonates on osteoclasts, they also have anti-apoptotic effects on osteocytes [13–17]. Normally, the activity of osteoblasts and osteoclasts is coupled to maintain a constant bone mass, such that osteoclastic bone resorption is followed by migration of osteoblasts into the resorption site and the formation of osteoid that later is fully mineralized to provide bone strength. This coupling is attributed, in part, to the release of matrix derived osteogenic growth factors including TGF- β and IGFs [18–23]. However, in acute estrogen deficiency, this coupling is disrupted such that bone turnover is elevated, as is the release of matrix derived growth factors [24]. While the increased growth factor release may temporarily increase bone formation, eventually, prolonged estrogen deficiency may be associated with low bone turnover. The effect of prolonged bisphosphonate treatment on release of these growth factors into the bone marrow is not known.

While a number of studies have reported that the transcriptional regulation of osteoclast apoptosis is responsible for the anti-resorptive action of bisphosphonates, the TGF- β 1 pathway also has direct effects in regulating osteoblast differentiation [19,25,26]. In response to bone resorption, active TGF- β 1 recruits bone marrow stromal cells to bone remodeling sites [19,23,27–29]. Reduction in TGF- β signaling in osteoblasts has been shown to improve the elastic modulus of bone matrix, its mineral concentration, fracture toughness and bone mass in mice [30–32]. Based on these data, we hypothesize that prolonged bisphosphonate treatment might alter TGF- β 1 production and activity including its influence on bone turnover, bone material and mechanical properties. To address this hypothesis, we evaluated a possible role for TGF- β 1 signaling *in vitro* and *in vivo* following alendronate treatment. We report that exposure to alendronate treatment resulted in higher serum TGF- β 1 levels, likely by increasing the level of TGF- β 1 activity in osteoprogenitor cells. Additionally, serum total TGF- β 1 levels were found to negatively associate with cortical bone maximum load and ultimate strength with alendronate treatments in rats.

Materials and methods

Animal studies

Six-month old female Sprague Dawley rats were either sham operated or ovariectomized (OVX). Sixty days after OVX, the OVX rats received continuous alendronate treatment (0.5 μ g/kg/3 \times /week, by subcutaneous injection, SC) for 120 days (Ale-120d) or 360 days (Ale-Ale-Ale), or alendronate treatment for 120 days (0.5 μ g/kg/3 \times /week) followed by vehicle treatment for 120 days (Ale-120d/Veh-120d) or 240 days (Ale-120d/Veh-240d). Groups of animals ($n=12$) were sacrificed on days 60, 180 (Ale-120d), 300 (Ale-120d/Veh-120d) and 420 (Ale-120d/Veh-240d). University of California Davis Institutional Animal Care and Use Committee approved all the study protocols.

Urine samples were collected from all experimental groups, stored at -80°C until analyzed for deoxypyridinoline/creatinine, a marker of bone resorption (DPD/Cr (mmol/nmol), Quidel, San Diego, CA). Serum samples were used to measure total levels of TGF- β 1 (pg/mL) in its latent form using ELISA (R&D Systems, Minneapolis, MN). Bone mechanical properties were examined by compression tests on the 6th lumbar vertebra (LVB6) and three-point bending tests on the left tibia (MTS Model 831, Eden Prairie, MN) [5,11,33–40].

MicroCT measurements of bone architecture and mineral density of bone tissue

The 5th lumbar vertebral bodies and tibial shafts from all the studied animals were obtained for micro-computed tomography (microCT) (viva CT 40, Scanco Medical, Bassersdorf, Switzerland). A total of 424 slices covering the entire body region of each vertebra were scanned at the energy level of 70 kVp and intensity of 85 μ A with an isotropic resolution of 10.5 μ m in all three spatial dimensions.

In addition, the tibial shafts were scanned at the tibial–fibular level (95 slices with 10.5 μ m resolution and 325 ms integration time) to evaluate changes in the bone volume/tissue volume (BV/TV) and the degree of bone mineral density (DBM). The scan region was selected using reference lines positioned at the top of femoral head and at the base of medial condyle [11,41].

Cell culture and treatment protocols

Mouse bone marrow derived-MSCs were obtained under a material transfer agreement between UC Davis and Texas A&M Institute for Regenerative Medicine. These cells are a relatively pure population of stromal cells negative for CD11b, CD45 and CD34 and positive for CD29, CD31, and CD106. Passage 6 mouse MSCs were used [42]. The P-6 MSCs were seeded either treated with alendronate (Ale, Sigma, St. Louis, MO) at 10^{-10} – 10^{-6} M for 6 hours or cultured in osteogenic medium that contained alendronate. RNA was collected from the cultures at 6 hours, day 3, 7 and 14 to perform real-time reverse transcript PCR (RT-PCR) for genes encoding proteins in TGF- β 1 signaling pathway (TGF- β 1, *Smad2* and *Smad3*) and markers of osteoblast differentiation (*Runx2*), maturation (osteocalcin, *Bglap*) or mineralization (osteopontin, *Opn*) [42–44].

MLO-Y4 cells were cultured on collagen-coated (rat tail collagen type I, 0.15 mg/mL) surfaces and were grown in phenol red-free α -modified essential medium (α -MEM) supplemented with 2.5% FBS and 2.5% bovine calf serum, and incubated in a 5% CO₂ incubator at 37 $^{\circ}\text{C}$, as described previously. The cells were treated with Ale at 10^{-10} – 10^{-4} M for 6 hours [45–47].

A separate set of mouse MSCs or MLO-Y4 cells were treated with Ale at 10^{-10} – 10^{-6} M for 6 hours, from which conditioned media was harvested for a TGF- β 1 reporter bioassay (TGF- β 1 Luciferase/GFP Assay, SABiosciences, a Qiagen Company Frederick, MD).

RNA preparation and RT-PCR

Total RNA from bone marrow or the cultured cells were isolated using a modified two-step purification protocol employing homogenization (PRO250 Homogenizer, 10 mm \times 105 mm generator, PRO Scientific IN, Oxford CT) in Trizol (Invitrogen, Carlsbad, CA) followed by purification over a Qiagen RNeasy column (Qiagen). Real-time PCR was carried out on ABI Prism 7300 (Applied Bioscience) in a 25 μ L reaction that consisted of 12.5 μ L 2 \times SYBR Green Mix (SABioscience Inc.), 0.2 μ L cDNA, 1 μ L primer pair mix and 11.3 μ L H₂O. Primer sets for real-time PCR were purchased from SABioscience. All the test genes were expressed relative to a control gene, GAPDH using the delta-delta Ct method. The results were expressed as fold changes from WT group, where fold change is $2^{-\Delta\Delta\text{Ct}}$ [43,44,48].

Immunohistochemistry

The right distal tibiae were decalcified in 10% EDTA for 2 weeks and embedded in paraffin. Four micrometer-thick sections were prepared for immunohistochemistry (IHC) using primary antibodies against TGF- β 1 (Cell Signaling, Danvers, MA). TGF- β 1 detection was performed using Vectastain ABC system (Vector Laboratories, Burlingame, CA). Sections were counterstained with methyl green. Results were presented as the percentage of the positive stained osteocytes/total osteocytes counted at the tibial cortical bone region of $\sim 3\text{ mm}^2$. A Bioquant imaging analyzing system (Nashville, TN) was used for the measurements [43,47–49].

Biomechanical testing

For the vertebrae, the endplates of the lumbar vertebral body were polished using an 800-grit silicon carbide paper to create two parallel planar surfaces. The vertebral body's height and diameter were

measured using digital calipers; the diameter represents an average of six caudal and cranial diameter measurements. Samples were stored in Hanks' Balanced Salt Solution (HBSS) 12 hours prior to testing. Each lumbar vertebra was then loaded to failure under far-field compression along its long axis using an electro-servo-hydraulic MTS 831 testing system (MTS Systems Corp., Eden Prairie, MN) at a displacement rate of 0.01 mm/s while continuously recording their corresponding loads and displacements. The load data were used to calculate the maximum strength of the vertebrae by normalizing the maximum load by both the vertebral height and bone volume/trabecular volume (BV/TV).

To analyze the biomechanical properties of the tibiae, the ends of each tibia were removed with a low speed saw to decrease the possibility of buckling during testing. The tibia samples were soaked in HBSS prior to three-point bending tests at a span of 14.5 mm with the bone loaded such that the posterior surface was under tension and the anterior surface was under compression using the EnduraTEC electro force 3200 testing system (Bose Corp., Eden Prairie, MN). Each tibia was loaded to failure in 37 °C HBSS at a displacement rate of 0.01 mm/s while its corresponding load and displacement were measured using a calibrated 225 N load cell. Following testing, a two-point average of the diameter and a six-point average of the cortical shell thickness were measured at the fracture site of each tibia using digital calipers. The ultimate strength, σ , of the tibiae was calculated from the standard equation for a beam in three-point bending:

$$\sigma = \frac{PLy}{4I}$$

where P is the maximum load reached during the bending test, L is the span between the lower support pins, y is the distance from the center of mass, and I is the moment of inertia of the cross-section [5,11,36,39].

Statistical analysis

The group means and standard deviations (SDs) were calculated for all outcome variables. We performed Pearson correlations and

linear regression models to examine the effects of Ale treatments on serum total TGF- β 1 levels with DPD/Cr, and on the associations of serum total TGF- β 1 levels with vertebral and cortical bone architectural and biomechanical parameters. In addition, one-way analysis of variance (ANOVA) with the Tamhane's post-hoc test was used to control for unequal variances using SPSS Statistics 20 (Chicago, IL). A two-tailed p -value <0.05 was considered statistically significant.

Results

TGF- β 1 signaling in osteoblasts or in osteocytes following BP treatment

To determine if there was a direct effect of Ale in inducing TGF- β 1, we evaluated the effect of Ale on the expression of components of the TGF- β 1 pathway and on TGF- β 1 signaling activity *in vitro*. We cultured mouse mesenchymal stem cells (MSCs) or osteocyte-like MLO-Y4 cells with alendronate. We then evaluated TGF- β 1 signaling using Cignal SMAD Reporter Kit, and gene expression of specific components of the TGF- β 1/Smad pathway (TGF- β 1, *Smad2*, *Smad3*) and of osteoblast differentiation (*Runx2*), maturation (osteocalcin, *Bglap*) and mineralization (osteopontin, *Opn*).

When the MSCs were incubated with Ale for 6 hours, the activity of the TGF- β 1 pathway was increased, as detected by increased activation of the Smad3-luciferase reporter ($p < 0.05$ vs. control-treated cells; Fig. 1a). *Smad2* and *3* gene expression was also increased by low dose (10^{-10} M) Ale (Fig. 1b), along with the expression of osteocalcin and osteopontin (Fig. 1c). Higher doses of Ale ($>10^{-10}$ M) did not change TGF- β 1/*Smad2*, or *Runx2* and *Bglap* gene expression in the MSCs. Ale suppressed *Opn* expression at a higher dose, 10^{-6} M (Fig. 1c). Ale at all doses examined did not change Smad luciferase activity (Fig. 1d), gene expression in TGF- β 1 signaling pathway (Fig. 1e) or osteogenic gene expressions (Fig. 1f) in the MLO-Y4 cells.

When the MSCs differentiated in osteogenic medium for 3, 7 and 14 days in the presence or absence of Ale, lower doses of Ale treatment (10^{-10} M and 10^{-8} M) increased *Smad3* and osteoblast differentiation as indicated by *Runx2* expression but decreased osteoblast maturation (*Bglap*) and mineralization (*Opn*) at day 3 (Fig. 2). Ale

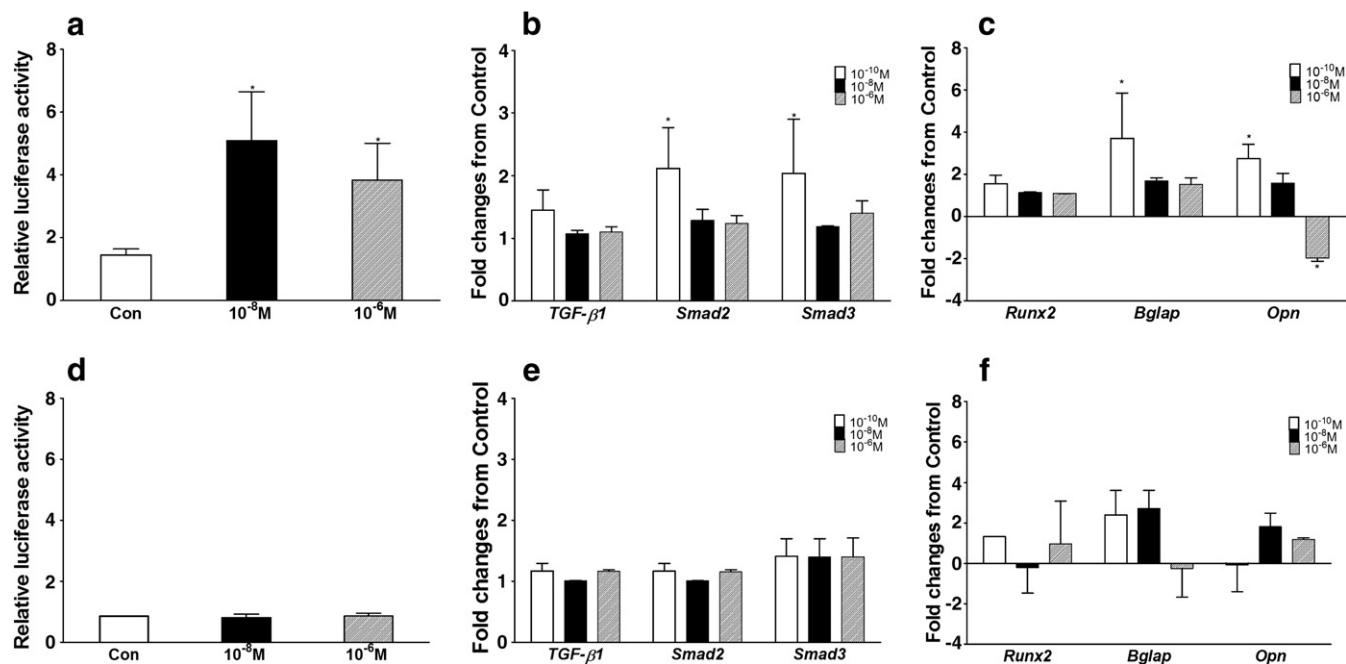


Fig. 1. Effects of alendronate on TGF- β 1 signaling *in vitro*. Mouse mesenchymal stem cells (a–c) or MLO-Y4 cells (d–f) were cultured with alendronate (10^{-10} – 10^{-6} M) for 6 hours. TGF- β 1 signaling was present in MSCs and MLY-O4 cells; TGF- β 1 gene expressions are shown in b (MSCs) and e (MLO-Y4 cells) and mineralization gene expressions are shown in c (MSCs) and f (MLO-Y4 cells). *, $p < 0.05$ vs. control. Data represented are the average from three sets of cultures.

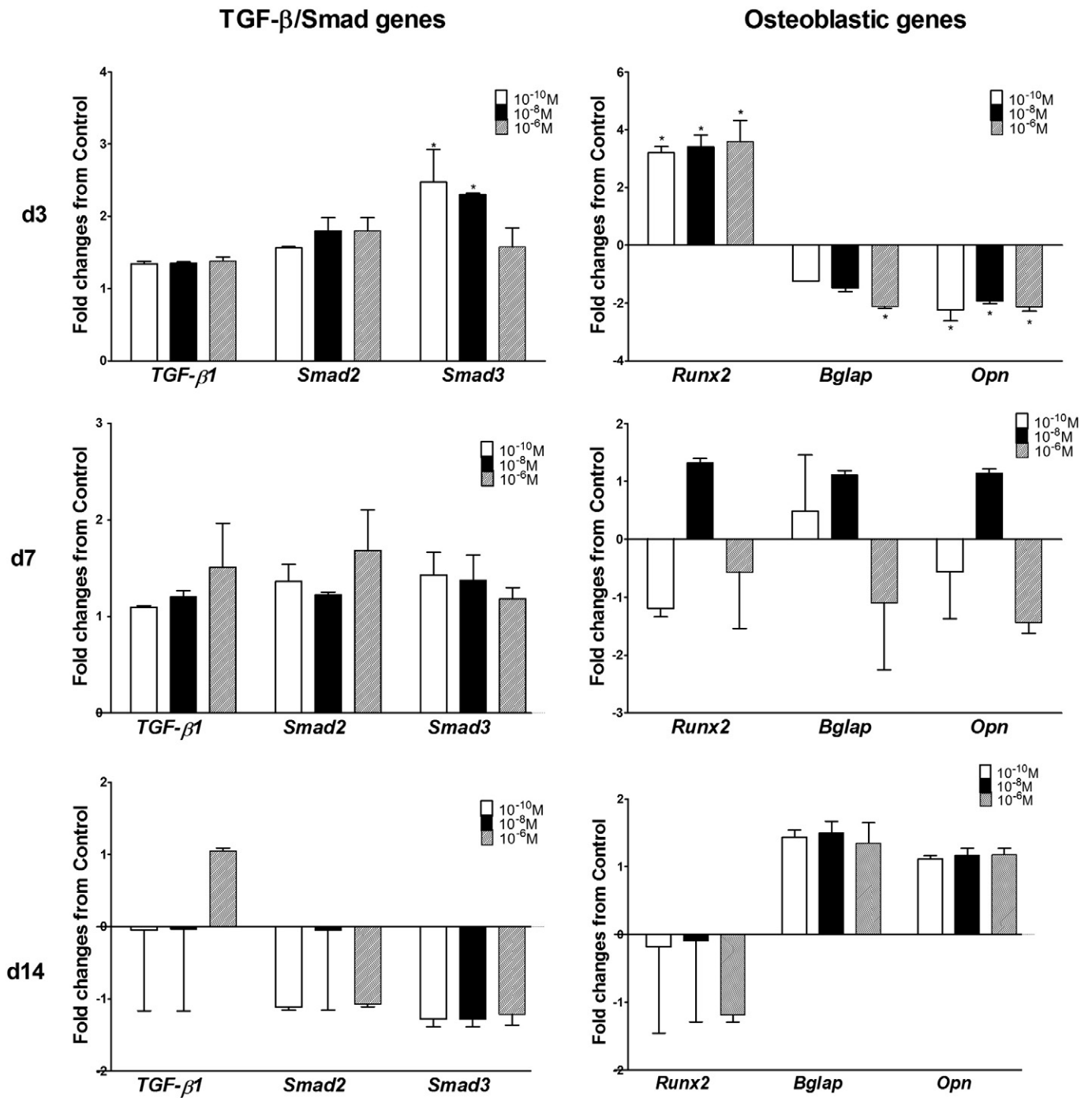


Fig. 2. TGF-β1 gene and mineralization gene expressions were measured in mouse mesenchymal stem cells cultured with alendronate (10^{-10} – 10^{-6} M) in osteogenic medium for 3, 7 or 14 days. Data represented average from three sets of cultures.

did not alter either TGF-β1 or osteogenic gene expression in MSCs differentiated for osteoblasts at 7 or 14 days (Fig. 2).

Bone turnover, degree of bone mineral density and bone strength with continuous Ale treatment or withdrawal

Ovariectomized rats had a significant increase in DPD/Cr and losses in both the trabecular and cortical bone DBM and bone stress at day 180 as compared to the sham groups. Ale treatment for 120 days decreased DPD/Cr and cortical bone ultimate stress and did not prevent the losses in trabecular and cortical bone DBM. Continuous Ale treatment for 360 days suppressed DPD/Cr, restored trabecular and cortical DBM but decreased cortical bone ultimate stress

as compared to the OVX group at the same time period. Ale treatment for 120 days followed by 240 days of vehicle resulted in continued suppression of DPD/Cr, partially restored trabecular DBM and maximum stress but cortical bone ultimate stress remained low as compared to the sham or OVX controls at days 420 (Table 1).

Dynamic changes in serum TGF-β1 levels with prolonged Ale treatment

To determine the effect of Ale on the level of serum TGF-β1, total TGF-β1 levels were assessed in serum. This analysis revealed an increase of nearly 150% in serum TGF-β1 levels by within 60 days of OVX relative to the sham controls (Fig. 3A, $p < 0.05$). At a later stage of estrogen deficiency (> 180 days post-OVX), we observed a

Table 1

Bone turnover marker (DPD/Cr), degree of bone mineral density, and bone strength measured in the trabecular (compressive stress) and cortical bone (maximum stress).

Parameters experimental groups	DPD/Cr (mmol/nmol)	Trabecular bone DBM (mgHA/mm ³)	Trabecular bone maximum strength (MPa)	Cortical bone DBM (mgHA/mm ³)	Cortical bone ultimate strength (MPa)
Sham-180d	46.8 ± 3.0	1119.0 ± 9.8	66.4 ± 9.8	1235.9 ± 20.7	197.5 ± 40.2
Sham-300d	30.8 ± 3.6	1115.6 ± 14.6	62.2 ± 11.6	1231.0 ± 14.7	149.2 ± 30.9
Sham-420d	30.8 ± 3.1	1135.5 ± 11.3	64.2 ± 12.4	1228.0 ± 15.0	171.0 ± 30.7
OVX-180d	50.1 ± 3.8 ^Δ	1100.1 ± 7.8 ^Δ	86.2 ± 22.4 ^Δ	1213.3 ± 12.5 ^Δ	172.7 ± 18.6 ^Δ
OVX-300d	35.7 ± 2.8	1105.4 ± 6.4	71.4 ± 8.5	1229.2 ± 20.5	168.8 ± 10.9
OVX-420d	33.9 ± 7.6	1118.3 ± 13.3 [#]	77.7 ± 23.1	1224.2 ± 18.4	169.4 ± 10.8
Ale-120d	18.6 ± 4.3 ^{Δ*}	1092.6 ± 18.1 ^Δ	67.3 ± 19.7	1209.6 ± 14.8 ^Δ	180.2 ± 21.4 ^Δ
Ale-360d	17.9 ± 2.4 [*]	1106.8 ± 13.3	57.8 ± 9.3	1229.9 ± 13.7 [#]	156.2 ± 18.3 [#]
Ale-120d/Veh-240d	15.3 ± 5.8 [*]	1124.0 ± 10.4 [#]	66.4 ± 9.2	1216.1 ± 12.6	156.9 ± 15.6 [#]

DBM: degree of bone mineral density; ^Δ*p*<0.05 vs. sham at the same time period; ^{*}*p*<0.05 vs. OVX at the same time period, vs. [#]*p*<0.05 vs. Ale-120d.

decrease in serum total TGF-β1 levels in OVX rats relative to the sham-operated animals (Fig. 3a). Compared to the OVX, animals treated with Ale–Ale–Ale or Ale–Veh–Veh did not have the decline in serum total TGF-β1 levels evaluated at 180, 300 and 420 days (Fig. 3a). Ale treatment for 120 days prevented this decline in serum total TGF-β1 levels for up to 240 days (Fig. 3a). These studies reveal a biphasic effect of ovariectomy on serum total TGF-β1 levels, such that serum total TGF-β1 levels are increased by OVX during the early stage (60 days) of estrogen deficiency and the serum total TGF-β1 levels decreased thereafter but remained elevated in Ale-treated groups at later stages (>180 days).

Immunohistochemistry (IHC) was then used to examine the effect of OVX and BP treatment on TGF-β1 expression and localization in both the trabecular and cortical bone from the tibiae [37,38]. Following 120 days of Ale-treatment, OVX animals had 100% more TGF-β1-positive osteocytes in cortical bone relative to vehicle treated controls (Figs. 3b and d; *p*<0.05 vs. OVX). Withdrawal of the alendronate

treatment for 120 and 240 days yielded similar numbers of TGF-β1-positive osteocytes and periosteal cells as the 120 days Ale-treated group, and were significantly higher than the OVX + Vehicle treated group (Figs. 3c and d). Bone marrow cells and osteocytes in the trabecular bone also stained positive for TGF-β1 but there were no significant differences detected among the groups. Therefore, Ale treatment prevented the long-term OVX-sensitive reduction in TGF-β1 protein in the serum as well as in the osteocytes in the cortical bone.

To determine if the alterations in serum TGF-β1 levels were related to bone remodeling or functional changes in bone quality, we performed linear regression analyses. The predictor was serum TGF-β1 levels and outcome variables were DPD/Cr, a bone resorption marker, and the vertebral compressive strength measured from the 5th lumbar vertebral bodies or tibial cortical bone biomechanical parameters. In the period of rapid bone remodeling within 60 days of OVX, total serum TGF-β1 levels were significantly associated with

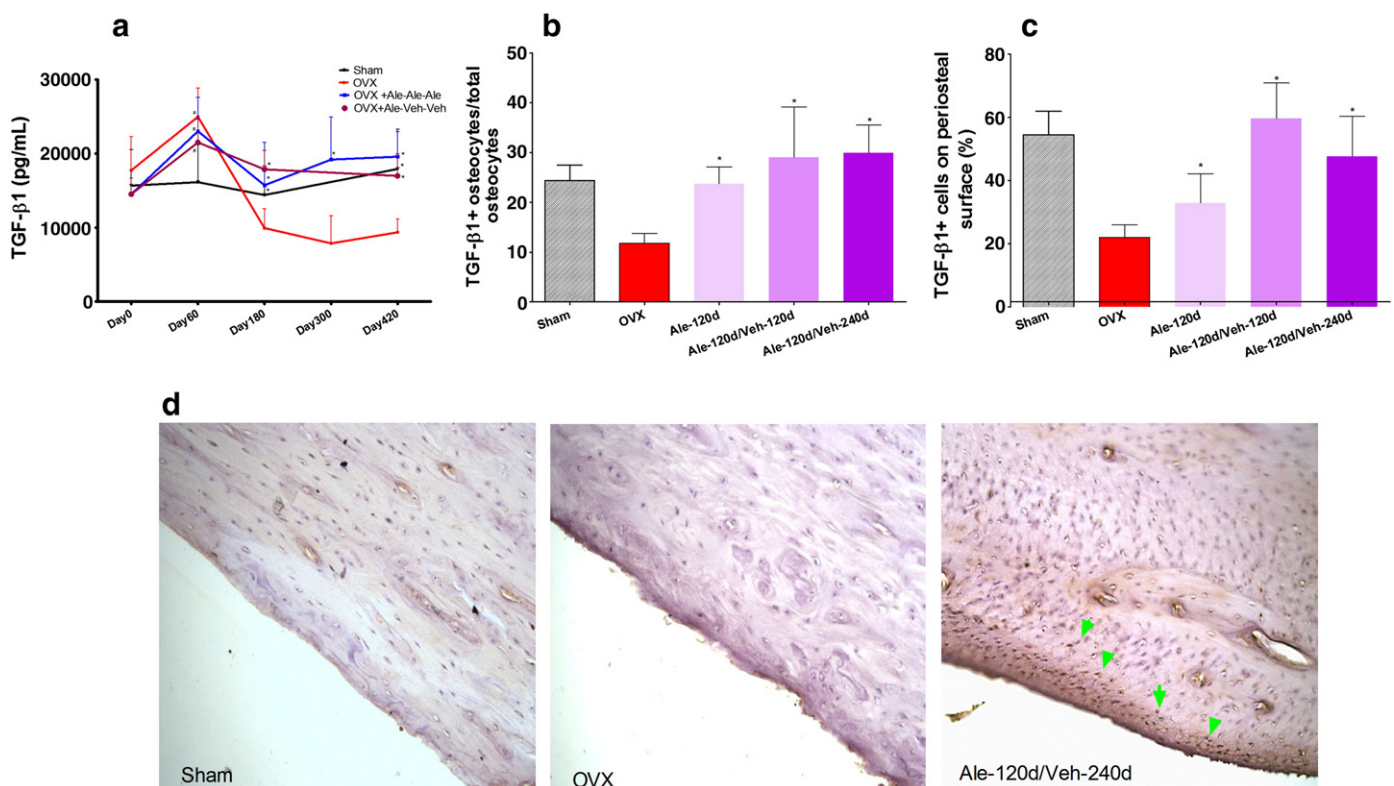


Fig. 3. Systemic TGF-β1 levels and distributions in rats treated with alendronate: a, total serum TGF-β1 levels in latent form following continuous alendronate treatment or withdrawal. Immunohistochemical staining of TGF-β1 in the tibial cortical bone (*n*=6/group). b, TGF-β1 + osteocytes/total osteocytes at the tibial cortical bone from continuous alendronate treatment or withdrawal; c, TGF-β1 + cells at periosteal surface of the tibial cortical bone from continuous alendronate treatment or withdrawal; d, representative cortical bone images from Sham, OVX or treated with alendronate for 120 days followed by 240 days of withdrawal. #, *p*<0.05 vs. sham; *, *p*<0.05 vs. OVX.

DPD/Cr (Fig. 4a). There was no correlation between DPD/Cr and total serum TGF- β 1 levels 120 days post-OVX (Fig. 4b). DPD/Cr positively correlated with serum total TGF- β 1 levels in Ale-treated groups (Fig. 4c). There were no associations between serum total TGF- β 1 levels and biomechanical parameters measured at the vertebral bodies (Fig. 5). A small but statistically significant negative association between serum TGF- β 1 levels and cortical bone maximum load and ultimate strength was observed (Figs. 6a and b). The maximum load of the cortical bone was decreased by 0.147 N when TGF- β 1 was increased by 1 pg/mL (Fig. 6a). The ultimate strength of the cortical bone was decreased by 0.171 MPa when TGF- β 1 was increased by 1 pg/mL (Fig. 6b).

Discussion

These studies suggest that long-term estrogen-deficiency causes a decline in serum TGF- β 1 levels that are prevented by Alendronate treatment through mechanisms that are indirect. Also, serum

TGF- β 1 levels were negatively associated with the cortical, but not the trabecular bone strength.

TGF- β 1 is a secreted factor that plays an important role in bone remodeling. It promotes bone formation by augmenting progenitor recruitment, proliferation and differentiation into matrix-producing osteoblasts [21,50,51]. TGF- β 1 is associated with peak bone mass acquisition [20] and TGF- β 1 polymorphisms correlate with bone mineral density (BMD) in humans [19,29,41,52]. While the elevated TGF- β 1 levels are associated with osteosclerosis [53], reduced TGF- β 1 levels are associated with osteopenia [23,28,54,55]. Although *in vitro* experiments have reported on the effects of exogenous TGF- β 1 on osteoclast activities that were both time and dose dependent, the results were dependent on the cell types used in these studies [56,57]. TGF- β 1, at low to moderately elevated levels, stimulated early osteoblast proliferation but inhibited terminal differentiation and mineralization [30,58–60]. Mice that are null for TGF- β 1 develop skeletal defects with shortened long bones and decreased tibial BMD [61], while inhibition of TGF- β 1 signaling has been shown to lead to higher bone mass and improved bone quality [30,32,62].

Our results suggest a biphasic effect of ovariectomy on serum TGF- β 1 levels, such that serum TGF- β 1 levels are increased by OVX during the early stage (60 days) of estrogen deficiency. This finding is consistent with the previous report by Dallas et al. that matrix bound TGF- β 1 was released by osteoclasts [63] during the early stage of ovariectomy where the osteoclasts were activated. We found that expression of TGF- β 1 and Smad genes was reduced after long-term estrogen deficiency in aged rats, consistent with prior reports that estrogen induces TGF- β 1 expression [44,49]. The release of TGF- β 1 from matrix stores in the cortical bone upon the increased remodeling in cortical bone following ovariectomy and the consequent rapid rise in serum TGF- β 1 levels following OVX suggest that endogenous TGF- β 1 stores may become “depleted” after the rapid period of bone resorption has ceased. At later stages of estrogen deficiency (>120 days post-OVX), serum TGF- β 1 levels are reduced relative to sham-operated animals, and alendronate treatment appeared to mitigate this decline in serum TGF- β 1 levels.

The ability of BPs to prolong the remodeling cycle may impact the deposition, storage and activation of latent TGF- β 1 in bone matrix. TGF- β 1 represses the master osteogenic transcription factor Runx2 through Smad3, to control extracellular matrix elastic modulus, a key component that determines bone material properties [30,52,62,64]. Smad3, which is expressed during the osteoblast maturation phase, may be one of the most critical mechanisms in coupling bone formation to bone resorption [60,62,64,65]. Our gene expression observations may be secondary to the alteration of bone turnover induced by the anti-resorptive treatments. Like estrogen [66,67], the bisphosphonates increase TGF- β 1/Smad3 as shown in our current and previous reports [5]. Low dose alendronate treatment *in vitro* increased TGF- β 1/Smad3 early on and may contribute to the bone sparing effects of the bisphosphonates through maintaining osteoblast proliferation [1,3,68]. Although TGF- β 1-positive osteocytes increased with long-term alendronate treatment, this may not be due to rapid direct effects of BP on TGF- β 1 signaling in osteocytes. In contrast, alendronate activated bone marrow MSC/stromal cells or osteoprogenitor cells at the periosteal surface to sustain TGF- β 1/Smad signaling and bone formation. Similar to the report by Bismar et al., we did not find an association between TGF- β 1 levels with trabecular bone density [54]. However, prolonged alendronate treatment sustained the elevation in serum total TGF- β 1 levels and was associated with reduced cortical bone strength.

The mechanism by which bisphosphonates augment bone strength in relationship to the changes in BMD is still being defined [41,47,53,55]. Bisphosphonates increase BMD by reducing osteoclast-mediated bone remodeling. This prolongs the secondary mineralization phase, resulting in more highly mineralized and uniform bone matrix, which is, associated with, improved trabecular bone strength [7–10,12,69–71]. Our research group and others have reported that the degree of

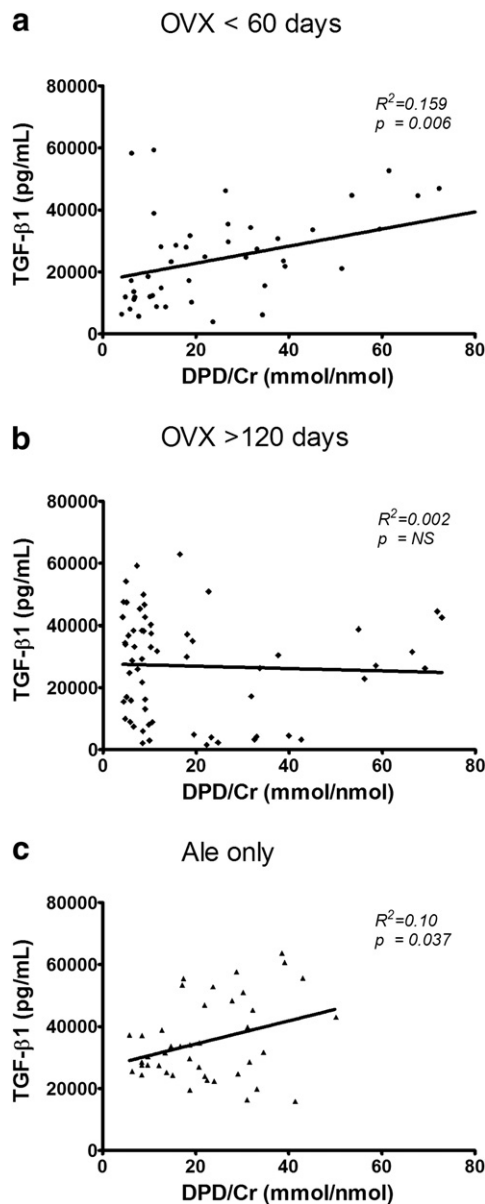


Fig. 4. Correlations between a, DPD/Cr and serum total TGF- β 1 in animals ovariectomized for 60 days; b, DPD/Cr and serum total TGF- β 1 in animals ovariectomized (OVX) for 120 days–420 days; c, DPD/Cr and serum total TGF- β 1 in animals treated with Ale.

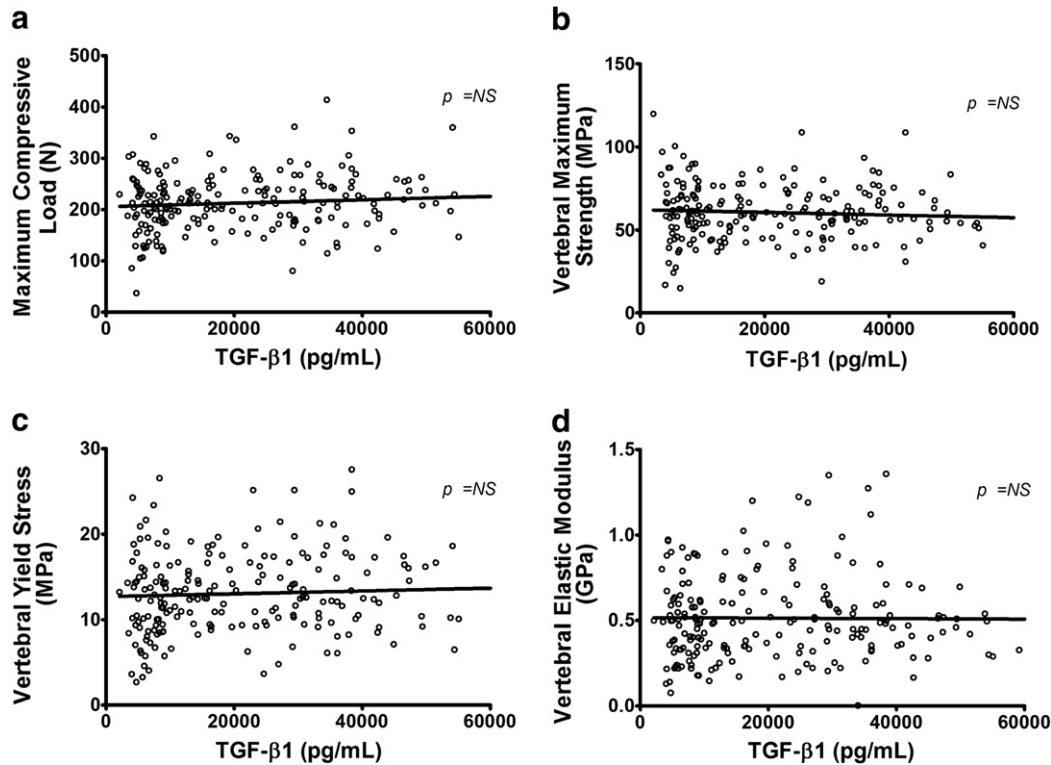


Fig. 5. Associations between serum total TGF- β 1 and a, maximum compressive load, b, maximum strength, c, elastic modulus and d, yield stress mineral density of the trabecular bone measured at the 6th lumbar vertebral body.

mineralization in trabecular bone is a strong predictor of bone strength [5,11,33,40,72]. A majority of studies of bisphosphonates on bone mineralization are short-term intervention studies of 3–6 months, which report an increased degree of bone mineralization that might benefit trabecular bone strength [6,7,10,70]. However, these medications are

used clinically for many years. Long-term use of oral bisphosphonates appears to maintain vertebral bone strength over 10 years, and this benefit may derive from changes in bone mass or the degree of bone mineralization [73]. Interestingly, we have previously reported that femoral fracture toughness tended to decrease with higher doses of ibandronate and

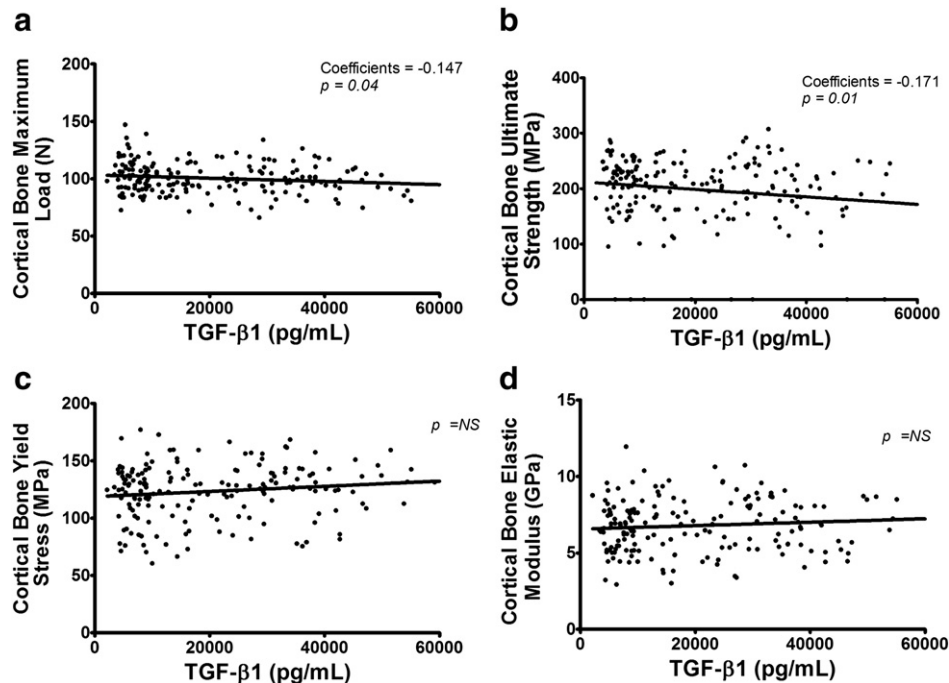


Fig. 6. Associations between serum total TGF- β 1 and a, maximum load, b, ultimate strength, c, elastic modulus and d, yield stress mineral density of the cortical bone measured at the tibial shafts.

risedronate treatments [41], despite the ability of these BPs to restore vertebral bone mass and the biomechanical property (vertebral maximum load) and the biomaterial property, maximum stress, a parameter that is independent of bone size and shape [74]. Fracture toughness is a parameter that describes the material properties of the cortical bone which affect the whole bone fracture resistance and is especially relevant if crack-like defects are present, as has been found with higher doses of BP or prolonged treatment periods [41,75]. Our observations in rats are similar to the report by Donnelly et al., who studied bone tissue properties near fragility fracture sites in the proximal femur in postmenopausal women treated with bisphosphonates. They found that bisphosphonate treatments reduced mineral and matrix heterogeneity and they hypothesized that this may diminish tissue-level toughening and permit fracture propagation [47]. A recent report by Bala et al. also suggested long-term alendronate therapy in postmenopausal women was associated with higher collagen maturity and lower mineral crystallinity that was independent of bone mineralization in the iliac cortical bone [76]. Therefore, long term bisphosphonate treatments may have negative effects on cortical bone mechanical and material property despite their well-known beneficial effects on the trabecular bone sites.

In summary, we found that prolonged reduction in bone turnover following alendronate treatment in female rats increased TGF- β 1 production by bone marrow stromal cells and periosteal osteoprogenitors. Elevated serum total TGF- β 1 levels had a modest negative association with cortical bone biomechanical and material properties. These data motivate further investigations to better understand the relationship between bisphosphonates, TGF- β 1, and cortical bone quality. Additional studies may lead to the use of serum TGF- β 1 as a biomarker to assess cortical bone strength with prolonged bisphosphonate treatment and may help to guide clinical decisions regarding duration of bisphosphonate treatment in osteoporotic patients.

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