High dose $1,25(OH)_2D_3$ inhibits osteoblast mineralization \textit{in vitro}

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Abstract. Vitamin D is essential for optimal calcium absorption needed for maintaining normal bone mineral density (BMD). Consequently, vitamin D-deficiency leads to poorly mineralized bone with diminished strength and load bearing capacity. Surprisingly, several animal and clinical studies have identified suppressive effects of high dose vitamin D supplementation on bone formation. These data suggest that while vitamin D is necessary for basal bone homeostasis, excessive concentrations may be detrimental to the skeleton. To further examine the direct effects of high dose vitamin D on the function of osteoblasts we differentiated primary osteoblast precursors and MC3T3 preosteoblastic cells, in the presence of supraphysiological doses of the active metabolite, $1,25$-dihydroxyvitamin D$_3$ [$1,25(OH)_2D_3$]. \textit{In vitro} osteoblast mineralization was potently suppressed by high dose $1,25(OH)_2D_3$. To investigate the mechanism we used a bioassay to examine nuclear factor-κB (NF-κB) activation in MC3T3 cells. Although NF-κB agonists are generally potent inhibitors of osteoblast differentiation, surprisingly, $1,25(OH)_2D_3$ dose-dependently suppressed, rather than stimulated, NF-κB activation. Interestingly, $1,25(OH)_2D_3$ also suppressed Smad activation induced by the osteoblast commitment and differentiation factors transforming growth factor-β (TGF-β) and bone morphogenetic protein 2 (BMP2), which may account for the inhibitory activities of $1,25(OH)_2D_3$ on mineralization. Our data suggest that vitamin D has complex pleiotropic effects on osteoblast signal transduction. As the net balance of high dose $1,25(OH)_2D_3$ appears to be an inhibitory action on osteoblasts, our data suggest that the therapeutic value of vitamin D to maximize bone mass through indirect actions on calcium absorption may need to be carefully balanced with potential inhibitory direct effects on mineralizing cells. Our data suggest that indiscriminate over-dosing may be detrimental to bone formation and optimal concentrations need to be established for humans \textit{in vivo}.

Introduction

Vitamin D is a hormone recognized to play a critical function in bone metabolism. This is evidenced by formation of poorly mineralized bone during vitamin D deficiency leading to rickets in children and osteomalacia in adults. This is largely a consequence of the necessity for vitamin D to promote efficient calcium absorption in the small intestine. Any decline in serum calcium concentrations due to inadequate calcium absorption leads to a secondary hyperparathyroidism that catabolizes the skeleton to maintain a physiological level of calcium necessary for normal cellular metabolism (1). While a minimum of 10 ng/ml of 25(OH)D is sufficient to prevent rickets and osteomalacia (2) recent studies have demonstrated that a minimum threshold 25(OH)D level of 29.7 ng/ml is necessary for protection from fracture (3). However, there is a paucity of data as to the optimal vitamin D concentration for fracture prevention and to complicate matters it is now appreciated that vitamin D plays a number of extraskeletal roles including promotion of innate and adaptive immune function, prevention of cancers, and prevention of hypertension (2,3). The doses of vitamin D needed to achieve these extraskeletal actions may be considerably higher than that needed to affect its positive actions on the skeleton (2).

In this study we investigated the impact of high dose $1,25(OH)_2D_3$ on osteoblast activity \textit{in vitro} using the MC3T3 osteoblastic cell line and primary bone marrow stromal cells (osteoblast precursors). Our data suggest that high dose $1,25(OH)_2D_3$ suppresses osteoblast mineralization despite reducing nuclear factor-κB (NF-κB) activation, an action expected to promote osteoblast activity. This net suppressive effect was likely a consequence of an additional inhibitory effect of $1,25(OH)_2D_3$ on other key osteoblastic pathways, including activation of Smad signaling.

Materials and methods

Materials. α-minimal essential medium (α-MEM) and antibiotics (penicillin and streptomycin) were purchased from
Invitrogen Corp. (Carlsbad, CA). Fetal bovine serum (FBS) was from HyClone. Tumor necrosis factor-α (TNF-α), TGF-β and BMP2 were from R&D Systems (Minneapolis, MN). Antibodies for western blot analysis were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). 1,25(OH)₂D₃ and all other reagents were purchased from the Sigma-Aldrich Chemical Corp., (St. Louis, MO) unless otherwise specified.

Cell culture. The preosteoblastic cell line MC3T3-E1, clone 14 (MC3T3) was purchased from the American Type Culture Collection (Manassas, VA) and cultured as previously described (4,5). Primary mouse bone marrow stromal cells (early osteoblast precursors) were isolated and purified from mouse long bones as previously described (5).

Osteoblast differentiation assays and Alizarin red-S staining. MC3T3 cells were plated and cultured for 72 h in α-MEM (1.0 ml/well) containing 10% FBS in 12-well dishes at a density of (1.0x10⁵ cells/well). Medium was aspirated and changed to mineralization medium (α-MEM supplemented with 10% FBS, 100 µg/ml L-ascorbic acid and 4 mM β-glycerophosphate) as previously described (5-7). 1,25(OH)₂D₃ was added at the indicated dose and cells were replenished with fresh medium every 3 days. At 18 days cells were rinsed with PBS and mineralization nodules visualized by fixing the cells in 75% ethanol for 30 min at 4°C followed by staining with Alizarin red-S (40 mM, pH 6.2) for 30 min at room temperature. Excess stain was removed by copious washing with distilled water. Plates were imaged using a flatbed scanner (Epson Perfection V600 Photo).

NF-κB constructs and luciferase assays. The NF-κB responsive reporter pNF-κB-Luc (BD Biosciences) or pGL3-Smad, responsive to all R-Smads, was used as previously described by us (5,7). Briefly, reporter plasmid was transfected into MC3T3 cells (1x10⁵ cells/well) using Lipofectamine 2000 reagent (Invitrogen Corp.) in α-MEM without FBS and antibiotics. Five hours later the medium was changed to α-MEM containing 10% FBS plus antibiotics and cells were treated with TNF-α (1 ng/ml) to increase NF-κB activity. Parallel groups received 1,25(OH)₂D₃ at the indicated dose. Cells were extracted with passive lysis buffer (Promega Corporation, Madison, WI) 24 h later, and luciferase activity was measured using the luciferase assay system of Promega, on a microplate luminometer (Turner Designs, Sunnyvale, CA, USA).

Western blotting. Cells were plated in 35-mm wells at a density of 1x10⁶ cells/well in 2 ml of medium (α-MEM + 10% FCS and antibiotics) and cultured for 24 h prior to addition of 1,25(OH)₂D₃ (10 µM) for an additional 24 h, followed by addition of TNF-α (10 ng/ml) for 60 min. Cultures were subsequently lysed for preparation of cytosolic and nuclear extracts as previously described (8) for western blotting using antibodies against Smad4. Loading controls consisted of β-actin for cytosolic proteins, or proliferating cell nuclear antigen (PCNA) for nuclear proteins.

Statistical analysis. Statistical significance was determined using GraphPad InStat version 3 for Windows XP (GraphPad Software, Inc., La Jolla, CA). Multiple comparisons were
performed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons post-test for parametric data. Gaussian distribution was assessed using the Kolmogorov and Smirnov test. A P-value <0.05 was considered statistically significant.

Results

1,25(OH)\(_2\)D\(_3\) suppresses mineralization of MC3T3 cells and primary osteoblasts in vitro. The direct effects of 1,25(OH)\(_2\)D\(_3\) on bone formation and mineralization are unclear. To address this issue we investigated the action of 1,25(OH)\(_2\)D\(_3\) on osteoblast differentiation and mineralization in vitro. MC3T3 preosteoblastic cells and primary bone marrow stromal cells (early osteoclast precursors) were differentiated into mineralizing osteoblasts in vitro in the presence or absence of 1,25(OH)\(_2\)D\(_3\) (10 or 100 nM) in mineralizing medium for 18 days and stained for calcium deposition with Alizarin red-S. Vitamin D was found to potently suppress mineralization in both MC3T3 cells (Fig. 1A) and in primary bone marrow stromal cell cultures (Fig. 1B). Vitamin D did not appear to mediate direct toxic effects on the cultures as cells proliferated robustly over the culture period, and were still alive and visibly attached to the plate at the end of the experiment.

1,25(OH)\(_2\)D\(_3\) is a direct inhibitor of TGF-\(\beta\) and BMP2-induced Smad activation. TGF-\(\beta\) and BMPs such as BMP2 are anabolic agents that signal through the Smad signaling pathway. We thus examined the effect of 1,25(OH)\(_2\)D\(_3\) on basal, BMP2-induced, and TGF-\(\beta\)-induced, Smad activation using a Smad-luciferase reporter responsive to all Smad species (5). Interestingly, 1,25(OH)\(_2\)D\(_3\) had no direct effect on basal Smad activation (Fig. 3A); however, it potently and significantly diminished Smad-activation induced by TGF-\(\beta\) (Fig. 3B) and BMP2 (Fig. 3C).

1,25(OH)\(_2\)D\(_3\) downregulates TGF-\(\beta\)-induced translocation of the Smad complex to the nucleus. Because TGF-\(\beta\) upregulates

Figure 3. Vitamin D suppresses TGF-\(\beta\) and BMP2-induced Smad activation in MC3T3 osteoblast precursors. (A) MC3T3 cells were transfected with pG5.3-Smad luciferase reporter plasmid and luciferase activity quantitated in (A) unstimulated (basal), (B) TGF-\(\beta\)-stimulated (10 ng/ml), or (C) BMP2-stimulated (0.5 ng/ml) cells, in the presence or absence of a dose range of vitamin D (0.1, 1, 10, 50, 100 or 250 nM). ***P<0.001 vs. control (grey bar). Data are presented as mean ± SD of 5 replicate wells per data point and are representative of three independent experiments.

Figure 4. Vitamin D suppresses TGF-\(\beta\)-induced nuclear translocation of Smad4 in MC3T3 osteoblast precursors. (A) MC3T3 cells were treated for 24 h with a dose range (0, 1, 10 or 100 nM) of vitamin D or (B) with vitamin D (100 nM) for 0, 1, 3 or 24 h. (C) MC3T3 cells were treated for 24 h with vitamin D (100 µM) and then stimulated for 1 h with TGF-\(\beta\) (10 ng/ml). In all experiments cytosolic and nuclear protein extracts were prepared for Smad4 detection by western blotting using \(\beta\)-actin as a cytosolic loading control and PCNA as a nuclear loading control. Data are representative of at least two independent experiments.
Interestingly, we found that TGF-β inducers of angiogenesis and osteoblast differentiation (22) by bone morphogenetic proteins (BMPs) (5,14,19), potent to sites of bone remodeling (21), is potently antagonized by factor (20) and inducer of osteoblast-precursor migration Smad induction by TGF-β impacts multiple stages and pathways involved in osteoblast responsible. While TNF-α pathways necessary for osteoblast differentiation may be mineralization. While the reason for the latter effect is unclear, fact, 1,25(OH)2D3 mineralization typically observed with NF-κB activation similarly antagonizes Smad activation in osteoblast precursors, high dose 1,25(OH)2D3 actually had a potent inhibitory effect on mineralization. In fact the NF-κB actions in osteoblast precursors, high dose 1,25(OH)2D3 failed to promote osteoblast differentiation and mineralization typically observed with NF-κB antagonism. In osteoblast biology however, the NF-κB signal transduction system is a pathway that we (5,7,9-12) and others (13-17) have reported to potently suppress osteoblastic bone formation. Indeed, TNF-α, a potent inducer of NF-κB activation, significantly reduces the basal bone formation rate leading to diminished peak bone mineral density (BMD) in mice (5). A surprising finding was that despite exhibiting anti-NF-κB actions in osteoblast precursors, high dose 1,25(OH)2D3 failed to promote osteoblast differentiation and mineralization typically observed with NF-κB antagonism. In fact, 1,25(OH)2D3 actually had a potent inhibitory effect on mineralization. While the reason for the latter effect is unclear, our data suggest that modulation of other signal transduction pathways necessary for osteoblast differentiation may be responsible. While TNF-α-induced NF-κB activation likely impacts multiple stages and pathways involved in osteoblast differentiation (18), we (5) and others (13,19) have shown that Smad induction by TGF-β, an early osteoblast commitment factor (20) and inducer of osteoblast-precursor migration to sites of bone remodeling (21), is potently antagonized by NF-κB signaling.

NF-κB activation similarly antagonizes Smad activation by bone morphogenetic proteins (BMPs) (5,14,19), potent inducers of angiogenesis and osteoblast differentiation (22). Interestingly, we found that TGF-β- and BMP2-induced Smad activation was potently suppressed by 1,25(OH)2D3. Inhibition of this pathway may account for some or all of the suppression observed in our in vitro study.

The relevance of these results for in vivo bone formation remains to be studied in detail; however the implications of our findings may be of great importance, given the clinical use of vitamin D supplements for fracture prevention. Although vitamin D supplementation is commonly used to combat osteoporosis, currently the optimal dose of vitamin D required for fracture prevention is contentious. Recent meta-analysis have suggested that supplementation of greater than 400 IU of vitamin D may reduce fractures (23), however the mechanism is unclear and may be associated in part with decreased risk of falling as a consequence of improved neuromuscular function (2).

Our data suggest that doses beyond that required for optimal intestinal calcium absorption, may actually be detrimental to bone formation though direct inhibitory actions on osteoblast differentiation and/or function. While at first glance these findings would seem to be in conflict with historical precepts that vitamin D is beneficial for the skeleton, in fact there are already a number of clinical and animal studies that suggest the potential for vitamin D to lower in vivo bone formation.

In a clinical study of bedridden older patients with chronic secondary hyperparathyroidism, low dose (400 IU/day) vitamin D supplementation led to a significant increase in the amino-terminal propeptide of type I procollagen (PINP), a marker of in vivo bone formation. These gains were completely negated by a high dose (1200 IU/day) vitamin D supplementation, while indices of bone resorption did not significantly change with either regimen (24). In another study wintertime vitamin D supplementation of healthy men led to a significant dose-dependent decline in bone specific alkaline phosphatase, a marker of in vivo mineralization (25).

As the vast majority of studies involve vitamin D supplementation in the context of antiresorptive therapy, typically a bisphosphonate, it becomes extremely difficult to assess the effects of vitamin D alone on bone turnover given that antiresorative agents themselves potently suppress bone formation as a consequence of coupling.

Furthermore, the amelioration of secondary hyperparathyroidism by vitamin D supplementation is often associated with a decline in bone turnover (26). This may be a consequence of reduced parathyroid hormone (PTH)-driven bone resorption leading to reduced bone formation as a consequence of coupling. However, our studies suggest that reduced bone formation may be exacerbated by a direct inhibitory effect of 1,25(OH)2D3 on bone formation. Further evidence in support of this notion comes from animal studies in which the confounding effects of vitamin D on PTH were masked by administration of 1,25(OH)2D3 in the context of thyroparathyroidectomized rats infused with PTH. Under stable PTH conditions, vitamin D led to ~2-fold decline in mineral apposition rate and bone formation rate (27).

In conclusion, our data suggest that high dose vitamin D may have significant inhibitory effects on mineralization. Achieving optimal 1,25(OH)2D3 concentrations for intestinal calcium absorption and for non-skeletal benefits may need to be balanced against potential inhibitory effects on bone forming cells.
References


