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ORIGINAL ARTICLE

The effect of the alendronate on *OPG/RANKL* system in differentiated primary human osteoblasts

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Abstract Alendronate is a well-established treatment for osteoporosis and suppresses bone resorption by a direct effect on osteoclasts and their precursors. The effect of alendronate on osteoclasts is produced, at least in part, by the receptor activator of nuclear factor kappaB ligand (RANKL) and the osteoprotegerin (OPG) synthesized by the osteoblasts. This study analyzes the effect of alendronate in cell viability, alkaline phosphatase (ALP) activity and *RANKL* and *OPG* expression in primary human osteoblasts (hOB). Alendronate at concentrations lower than 10^{-5} M did not have a toxic effect on hOB in vitro and did not modify the ALP activity at least for 72 h. Alendronate did not change *OPG* expression in basal, 10% fetal bovine serum (FBS), and vitamin D-treated cultures. Similar results were observed at the protein level. Unexpectedly, alendronate at 10^{-7} and 10^{-5} M concentrations increased the *RANKL* expression with the presence of vitamin D in differentiated hOB, and this induction of *RANKL* mRNA levels by alendronate was dose-dependent. However, this effect was not observed in basal and 10% FBS culture conditions. Thus, we conclude that alendronate does not affect the ALP activity and *OPG* gene expression in differentiated hOB, but may increase *RANKL* gene expression induced by vitamin D.

Keywords Primary human osteoblasts · Alendronate · RANKL · Osteoprotegerin

Introduction

Bisphosphonates (BPs) are used in the treatment of osteoporosis and diseases with high bone turnover, as well as in skeletal related events secondary to malignant disease. BPs are stable analogs of pyrophosphate and they are traditionally divided into non-nitrogencontaining (non-N-) and nitrogen-containing (N-) (reviewed in [1, 2]). Non-N-BPs such as etidronate and clodronate suppress bone resorption by incorporating within non-hydrolyzable ATP analogs that have no releasable energy content and lead to osteoclast death. N-BPs such as alendronate, ibandronate, risedronate, and zoledronate inhibit the enzyme farnesyl diphosphate synthase (FPPS) and block prenylation of small GTPases such as Ras, Rac, Rho, and cdc42 [3–6]. This results in the accumulation of constitutively active unprenylated GTPases in the cytoplasm of the osteoclast, which causes inappropriate activation of downstream signaling pathways, leading to the disruption of normal osteoclast function and survival [7]. Thus, N-BPs suppress bone resorption by a direct effect on osteoclasts and their precursors [1, 2, 7].

BPs bind avidly to hydroxyapatite crystals, and although all BPs share the same phosphorus–carbon–phosphorus core, the different BPs differ markedly in their affinity for

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hydroxyapatite binding [8, 9]. These differences in bone binding affinity and the different ability to inhibit FPP synthase may explain clinical differences among the BPs, in terms of speed of onset anti-fracture efficacy at different skeletal sites and the degree and duration of suppression of bone turnover. At present, alendronate is a well-established treatment for osteoporosis, and clinical trials show that this BP decreases the risk of osteoporotic fractures [10, 11 reviewed in 12].

Several findings suggest that the effects of BPs on osteoclasts could also be produced, at least in part, by modulation of the synthesis of resorption-promoting or resorption-inhibiting factors by osteoblasts [13]. Previous studies have proposed that receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) could be related to some of these factors [14–16]. Bone resorption is dependent on RANKL, a TNF family member that is essential for osteoclast formation, activity, and survival in normal and pathological states of bone remodeling (reviewed in [17, 18]). The catabolic effects of RANKL are prevented by OPG, a TNF receptor family member that binds RANKL, and thereby prevents activation of its single cognate receptor called RANK (reviewed in [17, 18]). Thus, osteoclast activity depends, at least in part, on the relative balance of RANKL and OPG, mainly produced by osteoblasts. Several hormones and cytokines regulate the production of RANKL and OPG by osteoblasts, including parathyroid hormone (PTH), PTH-related protein (PTH-rP), 1,25-dihydroxyvitamin D3 [1,25(OH)2D3 or vitamin D], estrogens, transforming growth factor (TGF), interleukin-1 (IL-1), and tumor-necrosis factor (TNF) (reviewed in [19]). Several studies have analyzed the effects of alendronate and other BPs in OB (stromal cells, primary cells and OB cell lines) and have shown that these drugs influence the differentiation, proliferation and maturation [9, 20–28], apoptosis [9, 20, 29–33] and gene expression [13–16, 24, 26, 30, 34, 35] of these cells. Despite this, the knowledge of the effect of alendronate on the mature osteoblast cells, especially in the *OPG/RANKL* system, is still incomplete and it has not been totally clarified.

The aim of the present study was to explore the alendronate effect in the regulation of the *OPG/RANKL* system on in vitro primary human osteoblasts (hOB) and its influence on the vitamin D action.

Results

Alendronate effects on the viability and alkaline phosphatase (ALP) activity of hOB cells

Non-passage primary hOB, after the synchronization procedure, displayed a characteristic pattern of gene expression and protein production of various osteoblastic differentiation markers (osteocalcin gene expression, type I collagen synthesis and ALP activity) (data not shown). To assess the cytotoxic effect of alendronate in hOB, cells were treated with different concentrations of alendronate in several culture conditions (non-stimulated, supplemented with 10% fetal bovine serum (FBS) and with vitamin D at 100 nM) for 48 and 72 h. We observed that alendronate showed a cytotoxic effect in all culture conditions at concentrations higher than 10^{-5} M (Fig. 1). We also observed that cells cultured in supplemented medium were more sensitive to high concentrations of alendronate. Thus, after treatment at 10^{-3} M, only 64 and 27% of cells remained viable at 48 and 72 h, respectively (Fig. 1). Remarkably, although alendronate at 10^{-5} M decreases osteoblast viability in all culture conditions, the number of viable cells at this concentration remains higher than 80% for 72 h (Fig. 1). Thus, we considered that alendronate at a

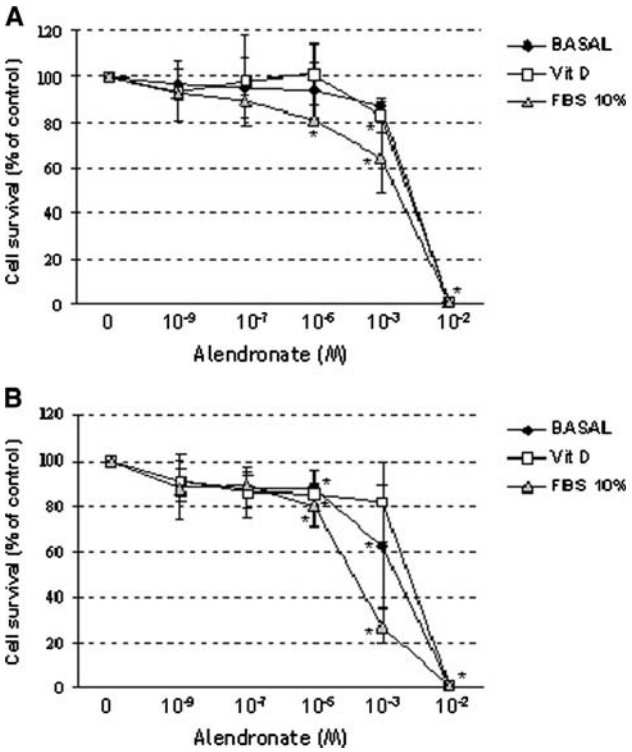

Fig. 1 Alendronate effect in the survival of hOB. Survival of primary human osteoblast cells with increasing concentrations of alendronate was measured at 48 h (**a**) and 72 h (**b**) in several culture conditions [non-stimulated (basal), supplemented with 10% FBS and with vitamin D at 100 nM]. Cell viability was determined using an MTT colorimetric assay, and the absorbance was read at 450 nm. The cell survival is expressed as the percentage of absorbance versus nontreated cells (control). Results are expressed as mean ± SD of the individual experiments performed with hOB taken from three patients. Culture experiments were performed in duplicate for each sample, treatment and culture conditions. * indicates significant differences ($P < 0.05$) in the absence or presence of alendronate

concentration of 10^{-5} M or lower had no toxic effect for hOB after 72 h of treatment.

On the other hand, we analyzed the effect of alendronate in the osteoblast phenotype and differentiation stage quantifying the ALP activity of hOB cells. These cells were treated with different concentrations of this BP in all culture conditions for 24, 48, and 72 h. Vitamin D significantly increased the ALP activity of synchronized hOB, and this induction was 1.3-, 1.4-, and 1.3-fold higher at 24, 48, and 72 h, respectively (Fig. 2a). At 72 h, the highest degree of stimulation in ALP activity was achieved with vitamin D, followed by non-stimulated and supplemented medium (10% FBS) conditions. This result shows that the increment of ALP activity at 48/72 h is lower in proliferative (10% FBS) than in nonproliferative conditions (basal and vitamin D). Alendronate did not have a significant effect on the ALP activity at 10^{-9}, 10^{-7} (data not shown) and 10^{-5} M in any of the culture conditions and did not interfere with the vitamin D effect (Fig. 2a).

Thus, we considered that alendronate at 10^{-5} M has no toxic effect on primary hOB in vitro and does not modify the ALP activity in these cells, at least for 72 h.

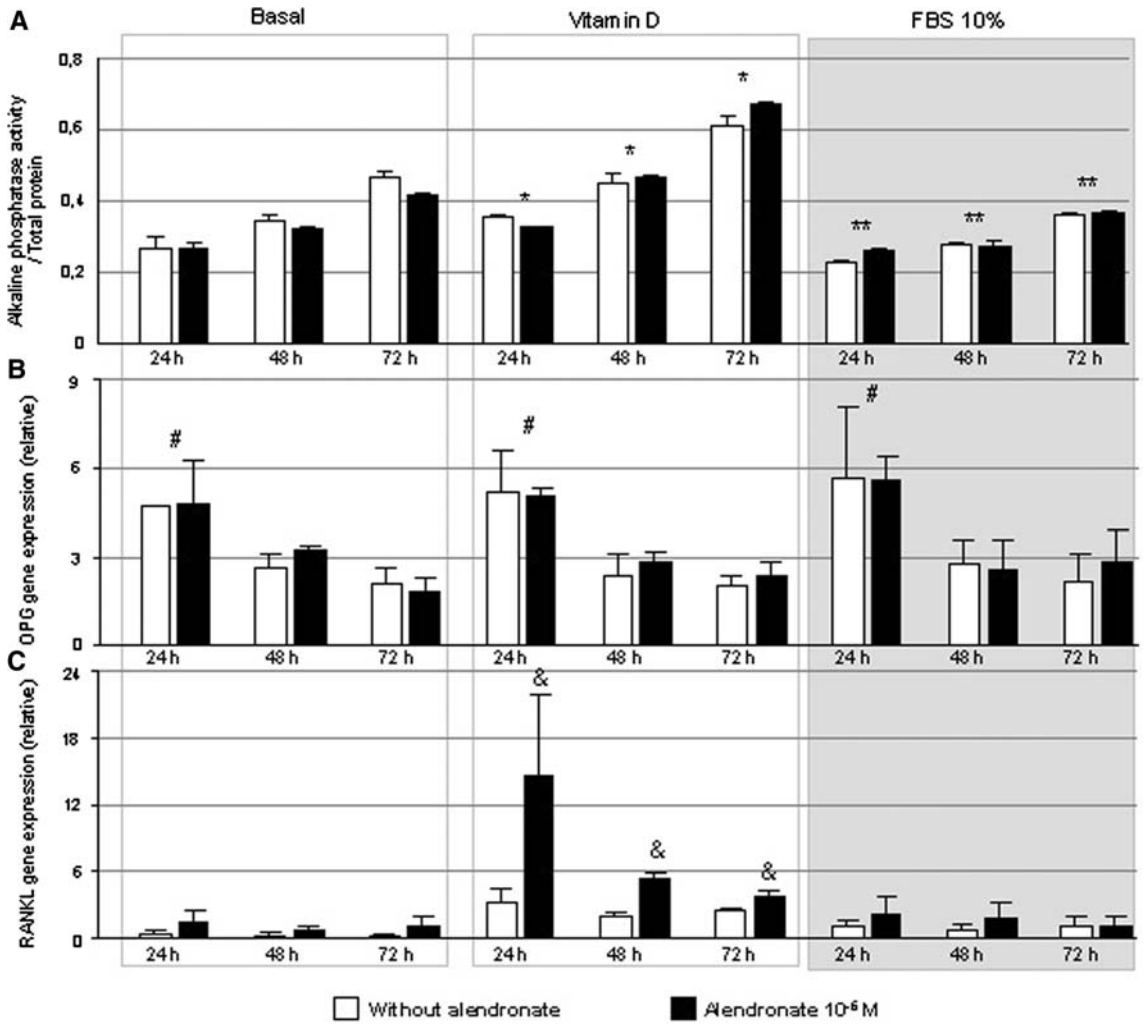

Fig. 2 Alendronate effect in ALP activity and *RANKL* and *OPG* gene expression of hOB. The experiments were performed with alendronate at 10^{-5} M in several culture conditions [nonstimulated (basal), supplemented with 10% FBS and with vitamin D at 100 nM] at 24, 48, and 72 h. **a** ALP activity was measured using the *p*-nitrophenylphosphate (pNPP) metabolization and the results were expressed as relative activity (versus total protein) using arbitrary units. **b–c** *OPG* and *RANKL* transcripts were quantified by real-time PCR analysis, and results were expressed as relative gene expression (versus *β-actin*) using arbitrary units. All the experiments were performed in duplicate. Treatments are indicated below the graphs and culture conditions are indicated above. Results are expressed as mean ± SD of the individual experiments performed with hOB taken from three patients (ALP activity) and six patients (*OPG* and *RANKL* gene expression). Alendronate at 10^{-5} M do not affect the ALP activity and *OPG* gene expression (**a**, **b**). * $P < 0.05$ versus basal and 10% FBS experiments performed with or without alendronate. ** $P < 0.05$ versus basal experiments performed with or without alendronate. # $P < 0.05$ versus 48 and 72 h with or without alendronate and $P < 0.05$ versus cultures without alendronate

Alendronate effect in *OPG* and *RANKL* gene expression

To further characterize the stimulatory effects of alendronate on *OPG* and *RANKL* gene expression, time course experiments were performed. hOB were treated with alendronate at 10^{-5} M for 24, 48, and 72 h in basal, 10% FBS, and 1,25-dihydroxyvitamin D3 (vitamin D) culture conditions.

Our results show that 10% FBS and vitamin D at 100 nM did not modify *OPG* mRNA levels on differentiated hOB, and these mRNA levels were significantly higher at 24 h in all culture conditions (basal, vitamin D, and 10% FBS, $P < 0.05$ with respect to 48 and 72 h) (Fig. 2b). Regarding alendronate effect, we observed that this BP at 10^{-9}, 10^{-7}, and 10^{-5} M did not change the *OPG* gene expression in basal, 10% FBS, and vitamin D-treated cultures of hOB (Fig. 2b) (10^{9} and 10^{-7} M data not shown). A similar result was observed at the protein level. OPG protein levels (corrected by the number of viable cells) were not significantly different at 24 h in cultures with (3.64 pmol/l ± 1.42) and without alendronate (3.32 pmol/l ± 1.23) ($P = 0.8$). Therefore, we conclude that alendronate at nontoxic concentration did not affect the *OPG* expression by differentiated hOB.

On the other hand, our results show that vitamin D stimulates *RANKL* mRNA levels compared with basal conditions (3.25 ± 1.34 vs. 0.43 ± 0.19, $P < 0.05$) in hOB cells. Remarkably, we found that alendronate significantly increases the *RANKL* gene expression in the presence of vitamin D at 24 h (14.70 ± 7.12 vs. 3.20 ± 1.31, $P < 0.05$), 48 h (5.35 ± 0.54 vs. 2.09 ± 0.24, $P < 0.05$), and 72 h (3.69 ± 0.72 vs. 2.58 ± 0.21, $P < 0.05$) (Fig. 2c). In order to examine the dose–effect of alendronate on the *RANKL* mRNA levels, we added different quantities of alendronate (10^{-9}, 10^{-7}, and 10^{-5} M) to vitamin D-treated cultures for 24 h. We selected this time exposure because the highest *RANKL* expression levels were obtained at 24 h (Fig. 2c). We observed that the induction of *RANKL* mRNA levels by alendronate was dose-dependent and at the most effective dose of alendronate (10^{-5} M) this induction was 4.4-fold (at a concentration of 10^{-9} M of alendronate this stimulation was not significant) (Fig. 3). However, this effect was not observed in basal and 10% FBS culture conditions (Fig. 3). Therefore, we conclude that alendronate at 10^{-7} and 10^{-5} M in presence of vitamin D increases *RANKL* gene expression on differentiated hOB in vitro.

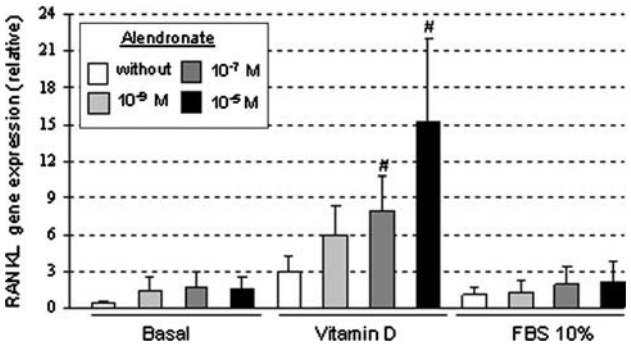

Fig. 3 Dose-dependent effect of alendronate in *RANKL* mRNA expression in hOB. The experiments were performed at several concentrations of alendronate (10^{-9}, 10^{-7}, and 10^{-5} M) for 24 h in several culture conditions [non-stimulated (basal), supplemented with 10% FBS and with vitamin D at 100 nM] (indicated below). *RANKL* transcripts were quantified by real-time PCR analysis, and results were expressed as relative gene expression (versus *β-actin*) using arbitrary units. All the experiments were performed in duplicate. Results are expressed as mean (±SD). Significant differences of a given culture condition in the absence or presence of alendronate are indicated by the # symbol ($P \leq 0.05$)

Discussion

The mechanism of the action of BPs has been well-described in previous studies, indicating a direct effect on osteoclasts (reviewed in [17]); however, some authors suggest that the effect of BPs on these cells could also be produced, at least in part, by the modulation of the synthesis of RANKL and OPG in the osteoblasts [14–16]. In this study we described the effects of alendronate on the viability, ALP activity and *RANKL* and *OPG* expression in differentiated primary hOB.

In our study we observed that alendronate, at concentrations lower than 10^{-5} M (10^{-5}, 10^{-7} and 10^{-9} M), has no toxic effect on primary hOB in vitro and does not modify the ALP activity in these cells, at least for 72 h. Several studies have evaluated the effect of alendronate and other BPs in the differentiation, proliferation, and maturation of osteoblasts, with controversial results [20–28]. Among these, we remark on the Im et al. study [24], showing that alendronate and risedronate (from 10^{-5} to 10^{-8} M) are promoters of proliferation and maturation of both primary hOB and MG-63 osteoblast-like cells. However, in this study we have not observed an increment of the maturation process (reflected by the ALP activity) induced by alendronate at 10^{-5} M, despite performing the experiments with concentrations of alendronate similar to those previously reported. Another study, performed by Reinholz et al. [26], describes that pamidronate and zoledronate directly regulate cell proliferation, differentiation, and gene expression in human fetal osteoblasts (hFOB). On the other hand, Garcia-Moreno et al. [22] reported that high concentrations of alendronate inhibited osteoblast proliferation of primary hOB and they found that at lower concentrations ($\leq 10^{-5}$ M) this drug had no significant proliferative effects compared to controls. Our results are in agreement with this study, and suggest that

alendronate at this concentration ($\leq 10^{-5}$ M) does not affect proliferation and viability of primary hOB.

The second step of this study evaluated the effect of alendronate in the gene expression of RANKL/OPG pathway. We observed that alendronate at 10^{-9}, 10^{-7}, and 10^{-5} M does not change *OPG* gene expression in basal, 10% FBS, and vitamin D-treated cultures of mature hOB. A similar result was observed at the protein level. These results are in concordance with the results described by Kim et al. [14], but not with those obtained by Viereck et al. [15]. Kim et al. tested the effect of alendronate and pamidronate in mouse osteoblastic cells, and concluded that both BPs inhibited osteoclast formation and bone resorption, but failed to alter the *RANKL* and *OPG* mRNA expression. Although the experimental model of Viereck et al. is very similar to our study, their results are totally inconsistent with ours. Thus, these authors showed that pamidronate and zoledronate increased *OPG* mRNA levels and induced OPG production in a dose- and time-dependence on primary hOB. Although the reasons for these differences are not completely elucidated, the distinct effect among BPs (pamidronate, zoledronate, and alendronate) and the differences in the hOB cultures (for example, the age of the bone trabecular samples specimens) could play a role [9]. Despite this, our results are very consistent and replicable. Therefore, we conclude that alendronate does not affect the *OPG* gene expression and protein synthesis in differentiated primary hOB.

Finally, we evaluated the effect of alendronate in the *RANKL* gene expression in basal, 10% FBS, and vitamin D-treated cultures of mature hOB. Surprisingly, we found that alendronate increased *RANKL* gene expression in the presence of vitamin D, and we observed that this induction of *RANKL* mRNA levels by alendronate was dose-dependent. Actually, the opposite effect of alendronate in *RANKL* expression would be expected. Although our results confirm the described stimulation of *RANKL* gene expression by vitamin D in hOB, the enhanced effect of alendronate in this stimulation is not consistent with the role of the RANKL in osteoclastogenesis and with the antiresorptive effect of alendronate. We would like to emphasize that this effect was observed with alendronate concentrations of 10^{-7} and 10^{-5} M, and that the finding of the effect of the RANKL stimulation in bone remodeling in this particular case is unknown. Nevertheless, it should be pointed out that these results should be interpreted with caution because of uncertainty about the concentration of alendronate that hOB are exposed in vivo. The alendronate concentrations in the culture media used in this study were within plasma concentrations observed in a pharmacokinetic study of 70 mg of alendronate sodium tablets [36]. Concerning the effect of alendronate in vitamin D action, Gomez-Garcia et al. [34] showed that alendronate interacts with the inhibitory effect of vitamin D on parathyroid hormone-related protein expression in human osteoblastic cells. In this study the authors showed that alendronate did not affect the vitamin D receptor (VDR) mRNA expression or osteocalcin secretion in hOB, with or without vitamin D, and suggest that an increase in calcium influx appears to be involved in the mechanism mediating this effect of alendronate. We do not exclude that a similar mechanism may be involved in the effect of alendronate on *RANKL* gene expression by vitamin D.

In summary, in this study we observed that alendronate does not affect the ALP activity and *OPG* gene expression in differentiated hOB, but can increase *RANKL* gene expression induced by vitamin D.

Materials and methods

Human primary osteoblast cultures

Bone cell cultures were established from trabecular bone specimens using a modification of Robey and Termine's procedure [37]. Bone pieces were obtained from six patients undergoing a hip replacement for osteoarthritis, (age range 58–77 years old) without features of metabolic bone disease or previous treatment that could affect bone metabolism, such as BPs or glucocorticoids. Samples were obtained according to procedures approved by the Hospital Clinic Ethics Committee. Trabecular bone pieces were processed according to previously described protocol [38] and only cells in the first passage were used for the experiments. Cells were grown in DMEM/HAM F-12 (1:1) medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS (Invitrogen). Cells were characterized as osteoblasts by determination of ALP activity and osteocalcin mRNA expression, measured by a histochemical technique and reverse transcription PCR, respectively.

Non-passaged primary hOB were seeded at a density of 10^4 cells/cm^2 in 6-well culture plates (for gene expression studies) and in 12-well culture plates (for OPG protein quantification) and were grown with DMEM/HAM F-12 medium supplemented with 10% of FBS. When osteoblasts reached confluence, in order to synchronize, culture medium was replaced with medium containing 100 μg/ml of ascorbic acid and cells were incubated for 48 h. Subsequently, osteoblast cells from each patient were cultured with or without FBS or/and 1,25-dihydroxyvitamin D3 (Vitamin D) 100 nM for 24, 48, and 72 h with several alendronate concentrations. Alendronate (Merck, Darmstadt, Germany) was dissolved in NaCl 0.9% at pH 7.4 to prepare the stock solution at a concentration of 0.1 M. Results are expressed as mean ± SD of the individual

experiments performed with hOB taken from the six patients.

Cytotoxicity assays

Primary osteoblast cells from each subject were plated in 24-well tissue plates and were incubated in DMEM/HAM F-12 (1:1) medium supplemented with 10% of FBS. After synchronization, cells were incubated for 48 and 72 h with different concentrations of alendronate (10^{-9}–10^{-2} M). Cell survival was determined by MTT-dye reduction during 4 h, using the MTT colorimetric assay Kit (Roche, Basel, Switzerland). The absorbance was read at 450 nm with an ELISA reader, and the survival was expressed as the percentage of absorbance versus nontreated cells (control). Cell cytotoxicity tests for each cell sample and treatment were performed in duplicate. Results are expressed as mean ± SD of the individual experiments performed with hOB taken from the three patients.

ALP activity assay

Primary osteoblasts from three subjects were plated in 12-well tissue plates and were incubated in supplemented medium. After synchronization, cells were incubated for 24, 48 and 72 h with different concentrations of alendronate in medium with 10 μg/ml of ascorbic acid. Following this, cells were washed with phosphate-buffered saline (PBS) and lysated with a lysis buffer (CelLytic M; Sigma-Aldrich). Cell extracts were incubated with 2 mg/ml of *p*-nitrophenylphosphate (pNPP) in a 0.05 M glycine buffer containing 0.5 mM $MgCl_2$ (pH 10.5) at 37°C for 30 min. The reaction was stopped by the addition of 0.4 N NaOH of reaction mixture and ALP activity was quantified by absorbance at 405 nm. Total protein content was determined with the BCA method in aliquots of the same samples with the Quick Start Bradford Protein Assay (Bio-Rad Laboratories; Madrid; Spain), read at 562 nm, and calculated according to a series of albumin (BSA) standards. ALP levels were normalized by the total protein content. ALP activity measurements were conducted in triplicate. Human fibroblast cell line was used as a negative control of ALP activity.

Extraction of total RNA and reverse transcription

Total RNA was prepared from osteoblast cells using Trizol reagent (Invitrogen) according to the manufacturer's protocols. Reverse transcription of RNA, for first-strand complementary DNA (cDNA) synthesis, was performed using 600 ng of total RNA and 25 ng/ml of oligo (dT) 12–18 primer (Invitrogen) in a final volume of 6.5 μl. The reaction was incubated at 70°C for 10 min and immediately chilled on ice. Primer extension was then performed at 42°C for 3 h after the addition of reaction buffer containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl and 3 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM of each dNTPs and 100 U of SuperScript™ III Reverse Transcriptase (Invitrogen) in a final volume of 10 μl. The reaction was inactivated by heating at 70°C for 10 min. cDNA was stored at −20°C.

Study of gene expression by real-time PCR

Designed human Taqman assays (Applied Biosystems, CA, USA) were used to quantify gene expression of *TNFRSF11B* (OPG) and *TNFSF11* (RANKL) (Hs00900358_m1 and Hs01092186_m1, respectively). Real-time PCR was performed in a volume of 25 μl containing 5 μl of cDNA (1/10 dilution of reverse transcriptase mixture), 900 nM of each primer, 50 nM TaqMan-MGB probe, and 10 μl TaqMan Universal PCR Master Mix 2X in 96-well plates with the ABI PRISM 7900 HT Detection System. Results were analyzed using the SDS TM Applied Biosystems Software Ver.2.1. Results were expressed as relative gene expression (versus *β-actin* gene expression) using arbitrary units. All real-time PCR reactions for the individual samples from the six patients were performed in duplicate.

OPG protein measurement

OPG protein from supernatant of 12-well culture plates was measured using a highly sensitive commercially available polyclonal antibody-based sandwich enzyme immunoassay (Biomedica GmbH, Vienna, Austria) with intra- and interassay variabilities of 9 and 10%, respectively. The absorbance was read at 450 nm with an ELISA reader and the OPG levels were expressed as the percentage of absorbance versus quantity of viable cells measured using an MTT assay. All samples were performed in duplicate, and the results were then averaged.

Statistics

All data analyses were performed using the SPSS 14.0 statistical package. Significant differences between any two groups were determined by Student's *t*-test. When multiple groups were compared, one- or two-way ANOVA was utilized, followed by a Student–Newman–Keuls test, when applicable. A value of $P \leq 0.05$ was considered significant.

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