ACTA ODONTOLOGICA SCANDINAVICA, 2016
VOL. 74, NO. 7, 550–557
http://dx.doi.org/10.1080/00016357.2016.1217041

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ORIGINAL ARTICLE

Alendronate alters osteoblast activities

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ABSTRACT

Objective: Due to accumulation in the bone matrix and a half-life of at least 10 years, it is important to understand the cellular impact of bisphosphonates (BPs). This study assessed the effects of alendronate (ALN) on human primary osteoblasts.

Material and methods: Osteoblasts were incubated with ALN (5, 20 and 100 μM), and both cells and cell culture media were harvested after d 1, 3, 7 or 14. Proliferation was evaluated by ^{3}H-thymidine incorporation and tetrazolium dye (MTT) colorimetric assay, and viability by the lactate dehydrogenase (LDH) activity in the medium. Differentiation was evaluated using protein Luminex multiplex assays and RT-PCR.

Results: ALN had no significant effects on cell viability. The lower concentrations enhanced the proliferation, whereas 100 μM diminished the proliferation. mRNA expression of osteocalcin (OC), alkaline phosphatase (ALP) and α-1 type 1 collagen were reduced, whereas ALN enhanced the expression of leptin mRNA and the secretion of interleukin-8 (IL-8) and regulated on activation normal T cell expressed and secreted (RANTES).

Conclusions: ALN enhanced the secretion of immune factors from human osteoblasts. Combined with a lower rate of proliferation and a decline in differentiation, this indicates that higher dosages or accumulation may cause undesirable local changes in bone.

ARTICLE HISTORY
Received 15 April 2016
Revised 17 June 2016
Accepted 20 July 2016
Published online 16 August 2016

KEYWORDS
Bisphosphonates; osteonecrosis; cytotoxic; bone

Introduction

Alendronate (ALN) is a drug classified as a nitrogen-containing bisphosphonate (BP), a group of antiresorptive drugs which are widely used to prevent, cure or palliate diseases causing bone deficiency, such as osteoporosis and Paget's disease.[1,2] BPs are synthetic analogues of inorganic pyrophosphate, an endogenous regulator of bone turnover that inhibits bone resorption and mineralization *in vitro*.[3] BPs have a high affinity for hydroxyapatite and are resistant to metabolism by endogenous phosphatases.[3,4] ALN administration has shown significant, progressive increase in bone mineral density, and is known to reduce the incidence of hip, vertebral and non-vertebral fractures.[5] The structure of bone in the human skeleton varies, depending on site and developmental lineage. After seeing local variations in bone marrow stromal cells from one donor, Stefanik et al. concluded in a study from 2008 that there was a possibility of dysregulation of mandibular bone homeostasis after exposure to BPs.[6] An important factor in the ALNs potent effects on the bone metabolism is its pooling in the bone matrix.[7] The paradox is that this might also play a part in causing one of the more severe adverse drug reactions (ADR), this drug has been known to facilitate, BP-related osteonecrosis of the jaw (BRONJ). Furthermore, the usage of oral BPs, such as ALN, is associated with an increased risk of subtrochanteric femoral fractures.[8,9]

There is limited detailed insight into the effects of BPs on primary normal human osteoblasts, and unveiling more of the osteoblasts' response might hold some of the answers to the pathogenesis of BRONJ. Since the first documented cases of BRONJ were reported in 2003,[10] the condition has been the subject to numerous studies and case reports. On a cellular level, the established effect of BPs on osteoclasts, with the promotion of apoptosis, has been known for several years.[11] More recently, this has been accompanied by a suggestion of an inhibition of the migration of osteoclast precursors during osteoclastogenesis.[12] What are less mapped out are the effects of BPs on primary human osteoblasts. With yet no specific regime for treatment or prevention,[13] it is essential to look for other factors that may be involved in the pathogenesis of BRONJ. Analysing the response from primary human osteoblasts after treatment with high concentrations of ALN, might disclose parts of the answer.

We aim to verify the effects of various concentrations of ALN, a commonly used BP, on proliferation, viability, the expression and secretion of bone markers and cytokines/chemokines from primary osteoblasts originated from more than one donor.

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Materials and methods

Study design

Commercially available primary human osteoblasts (NHO; Cambrex BioScience, Walkersville, MD) from femur of two donors (both male, aged 10 (D1) and 22 (D2)), were grown in Osteoblast Basal Media (OBM) (Cambrex BioScience), containing ascorbic acid, foetal calf serum and gentamicin. Cells were subcultured at 37 °C in a humidified atmosphere of 5% CO_2 prior to confluence according to manufacturer's instructions.

Cells, at passage 5 (D1) and 6 (D2), were seeded in 6-well plates and incubated with ALN (Sigma-Aldrich Biotechnology, Saint Louis, MO) dissolved in OBM to final concentrations of 5, 20 and 100 μM. The 5 μM was thought to be of clinical relevance for human osteogenic cells in general,[14] whereas 20 and 100 μM ALN was chosen to simulate potentially higher concentrations due to local accumulation. Incubation was done at 37 °C and in 5% CO_2 air. Cells and cell culture media were harvested after 1, 3, 7 or 14 d of incubation, with the last change of medium with or without ALN 24 h prior to harvest. Untreated cells at each time point were used as control.

Cell viability and proliferation

Cell viability was confirmed by monitoring the activity of lactate dehydrogenase (LDH) in the cell culture medium. The LDH was measured using the microplate-based Cytotoxicity Detection Kit (LDH; Boehringer, Mannheim, Germany). In accordance with the manufacturers' protocol, 50 μl aliquots of cell culture medium were used and the absorbance was read using a microplate reader (Elx800, BioTek, Bad Friedrichshall, Germany) at 450 nm.

The proliferation rate of the cells was measured using ^{3}H-thymidine incorporation (1.7×10^4 cells/well in 24 well plates) and a MTT colorimetric assay (4×10^3 cells/well in 96 well plates). For the thymidine incorporation, sub confluent cells were incubated with cell culture medium containing 5, 20 or 100 μM ALN for 1 and 3 days prior to harvest. Untreated cells were used as control at each time point. The cells were pulsed with 1 μCi ^{3}H-thymidine/well 12 h prior to harvest. The medium was removed and the cells were washed twice with PBS and twice with 5% trichloroacetic acid (TCA) to remove unincorporated ^{3}H-thymidine. The cells were solubilized in 125 μl of 1 M sodium hydroxide (NaOH), and 100 μl of the solubilized cell solution was transferred to 2 ml scintillation fluid (Lumagel; Lumac LSC BV; Packard, Groningen, The Netherlands) and counted for 3 min in a liquid scintillation counter (Packard 1900 TR, Packard Instruments, Meriden, CT).

A MTT colorimetric Cell Growth Assay Kit CT02 from Chemicon (Merck KGaA, Darmstadt, Germany) was used as a second measure of proliferation. Cells were cultured as in the thymidine incorporation, and the assay was performed as described by the supplier. The absorbance was measured using an ELISA plate reader (BioTek Instruments, Winooski, VT) with a test wavelength of 570 nm and a reference wavelength of 630 nm.

Protein quantification in cell culture medium

Multianalyte profiling was performed using the Luminex 200TM system (Luminex Corporation, Austin, TX) and the XY-platform and acquired fluorescence data were analysed by the 3.1xPONENT software (Luminex). Prior to analysis, the cell culture medium was concentrated five times using Microsep Centrifugal tubes with 3 kDa cut-off (Pall Life Science, Ann Armor, MI).

The concentrations of cytokines in cell culture media were determined using the 25-Milliplex Human Cytokine Immunoassay kit (Millipore, Billerica, MA). Further, the level of bone markers osteoprotegerin (OPG), osteocalcin (OC), leptin (LEP), osteopontin (OPN), parathyroid hormone (PTH), adiponectin, insulin and sclerostin were determined using Milliplex Human Bone Panel 1B Immunoassay kit (Millipore). All analyses were performed according to the manufacturers' protocols.

mRNA isolation prior to RT-PCR quantification

Treated and untreated cells were washed twice with PBS, and lysed in 100 μl lysis/binding buffer (100 mM Tris–HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 0.5 mM dithiothreitol (DTT), and 1% sodium dodecyl sulfate (SDS)). Total RNA was extracted using the RNeasy mini kit (Sigma, St. Louis, MO). RNA concentration was measured at 260 nm in a Nanodrop ND 2000 spectrophotometer (Nanodrop, Thermo Scientific, Wilmington, DE). RNA fractions with the ratio of absorbance 260 and 280 nm around 2.0 were used for analysis of gene expression using RT-PCR. One microgram of RNA was used to synthesize first-strand complementary DNA (cDNA) with oligo dT primers using RevertAid™ First Strand cDNA Synthesis kit #K1622 (Thermo Scientific, Waltham, MA).

RT-PCR quantification of target genes

RT-PCR were done on biological triplicates of the samples using SsoAdvanced™ SYBR® Green supermix (Bio-Rad, Hercules, CA). The total reaction volume was 10 μl. The thermal profile consisted of three steps: enzyme activation/initial DNA denaturation 95 °C 30 s, denaturation 95 °C 5 s and annealing/extension 60 °C 30 s. A melt curve (65–95 °C) with 0.5 °C increments was used to access the appropriate melting temperature of the PCR products. RT-PCR was performed in 384-well plates using a ViiA™ 7 instrument (Applied Biosystems Inc., Carlsbad, CA).

The primer sequences for the target genes are listed in Table 1. The housekeeping genes β-actin and GAPH were used as reference. The efficiency of each set of primers was between 95–100%. The relative changes in mRNA were measured by comparative quantification (ΔΔCt method), with efficiency (E) correction for the individual transcripts. Relative expression $= (E_{target})^{Ct\ target\ (control-sample)} / (E_{ref})^{\Delta Ct\ ref\ (control-sample)}$.

Table 1. Sequences of primers used for real-time PCR.

Gene	Sequence of left primer	Sequence of right primer
LEP	5′- ACA GAA AGT CAC CGG TTT GG -3′	5′- GGA AGG CAT ACT GGT GAG GA - 3′
COL1A1	5′- CAT CTC CCC TTC GTT TTT GA -3′	5′- CCA AAT CCG ATG TTT CTG CT -3′
OC	5′- GCA AGT AGC GCC AAT CTA GG -3′	5′- GCT TCA CCC TCG AAA TGG TA -3′
ALP	5′- GAC AAG AAG CCC TTC ACT GC -3′	5′-GCC ATT TGT GAA AGG AGA GC-3′
CD44	5′- AAG GTG GAG CAA ACA CAA CC -3′	5′- ACT GCA ATG CAA ACT GCA AG -3′
Gapdh	5′- ACC CAG AGA AGA CTG TGG ATG G-3′	5′-CAC ATT GGG GGT AGG AAC C-3′
B-actin	5′-CCA ACT GGG AGG ACA TGG AG-3′	5′-CGG TTG GCC TTA GGG TTC AG-3′

LEP: leptin; COL1A1: alpha-1 type 1 collagen; OC: osteocalcin; ALP: alkaline phosphatase; CD44: CD44 antigen; Gapdh: Glyceraldehyde 3-phosphate dehydrogenase; B-actin: Beta-actin.

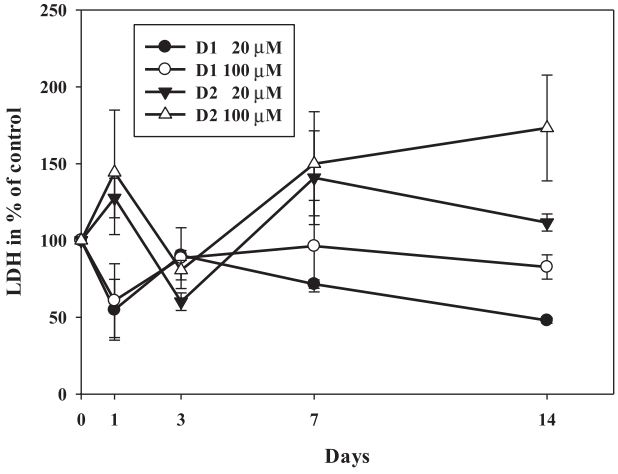

Figure 1. Lactate dehydrogenase (LDH) activity in cell culture medium presented in percentage of control. The effect of ALN present at concentrations of 20 or 100 µM for 1, 3, 7 or 14 d. ALN induced no significant differences in LDH activity compared to untreated osteoblasts at any concentration or time point tested. The notations D1 and D2 are abbreviations used for donor 1 and donor 2, respectively.

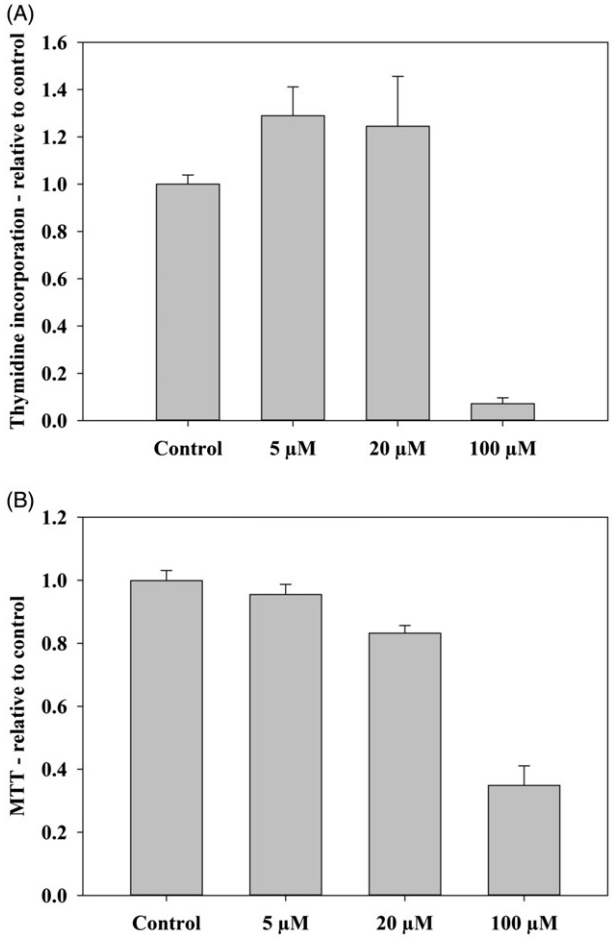

Figure 2. Effect of ALN on cell proliferation. The proliferation rate of the human osteoblasts was measured using ^{3}H-thymidine incorporation (A) and an MTT colorimetric assay (B) on sub confluent cells incubated for 3 d with 5, 20 or 100 µM alendronate ($n = 4$). Data presented relative to untreated control at each time point =1.

Statistical analysis

Statistical evaluation was performed using the software SigmaPlot 11.0 and 13.0 (Systat Software, San Jose, CA). Untreated cells were used as control at each individual time point, and statistical significance was assessed by student's t-test and p value set to 0.05. For the RT-PCR data, statistical evaluation of the significance of differences between measured Ct-values was carried out using the GenEx standard package (http://www.biomcc.com).

Results

High dosage of ALN abolish osteoblast proliferation

ALN induced no significant differences in LDH activity compared to untreated osteoblasts at any concentration or time point tested (Figure 1). Thymidine incorporation indicated a 20% increase ($p = 0.012$) in cell proliferation when treated with 5 µM ALN compared to control, while it was reduced to less than 10% ($p < 0.001$) of control after treatment with the 100 µM solution (Figure 2(A)). The 20 µM ALN, however, failed to induce significant changes in proliferation compared to control. This resembled the results obtained from the MTT assay (Figure 2(B)), with a 20% decline in proliferation rate after treatment with 20 µM ALN ($p = 0.002$) and a reduction to 35% of control ($p < 0.001$) with 100 µM concentration. Compared to untreated cells, 5 µM ALN resulted in an insignificant 5% reduction.

Reduction in markers of osteoblast differentiation

The treatments reduced the expression of OC (Figure 3(A)), alkaline phosphatase (ALP) (Figure 3(B)) and alpha-1 type 1 collagen (COL1A1) (Figure 3(C)) mRNAs during the study period. After administration of ALN for fourteen days, both 20 and 100 µM ALN induced a decrease in expression of

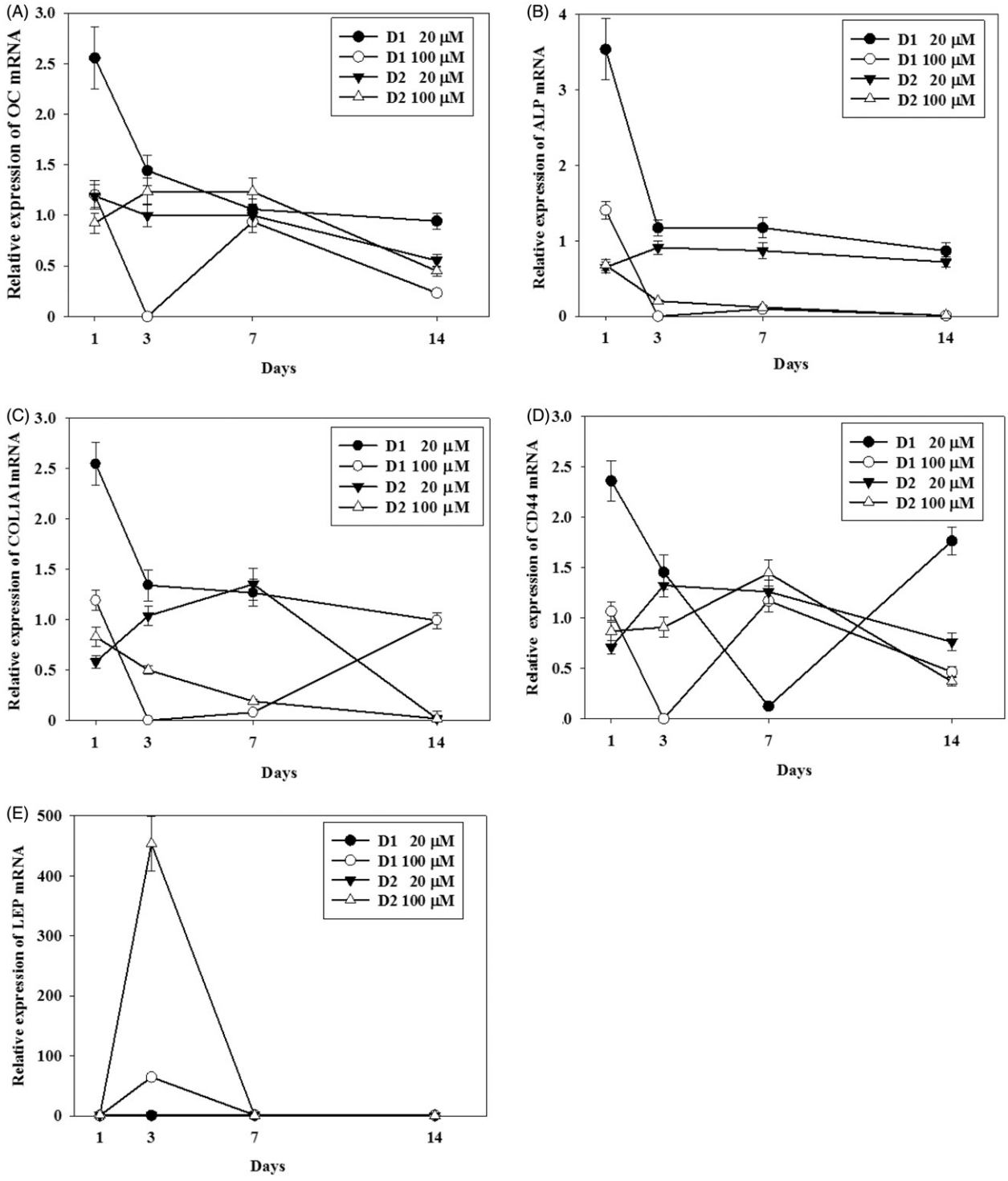

Figure 3. Relative mRNA expression of osteocalcin (OC) (A), alkaline phosphatase (ALP) (B), alpha-1 type 1 collagen (Col1A1) (C), CD44 (D) and leptin (LEP) (E). Measured in cell lysate from cultures of human osteoblasts treated with ALN at 20 or 100 µM for 1, 3, 7 or 14 d. The notations D1 and D2 are abbreviations used for donor 1 and donor 2, respectively.

CD44 (Figure 3(D)). Treatment with both 20 and 100 µM ALN enhanced the expression of LEP mRNA at all time points tested (Figure 3(E)), with a 450-fold increase at day three with the higher dosage of ALN.

There was an acute reduction in the secretion of OPN at day one ($p < 0.001$) (Figure 4(A)). The levels of sclerostin in the cell culture medium were reduced upon treatment with 100 µM ALN ($p = 0.003$), whereas 20 µM (Figure 4(B)) induced no significant change ($p = 0.414$).

Increased levels of pro-inflammatory cytokines

The secretion of the pro-inflammatory cytokines, interleukin-8 (IL-8) ($p < 0.001$) and RANTES ($p < 0.001$) were increased after treatment with the highest dosage of ALN after 14 d (Figure 5(A) and Figure 5(B), respectively). Interferon-α2 (IFN-α2) secretion was non- significantly reduced after incubation with 20 µM for 14 d ($p = 0.106$), whereas 100 µM ALN almost abolished the secretion ($p < 0.001$) at day 14 (Figure 5(C)).

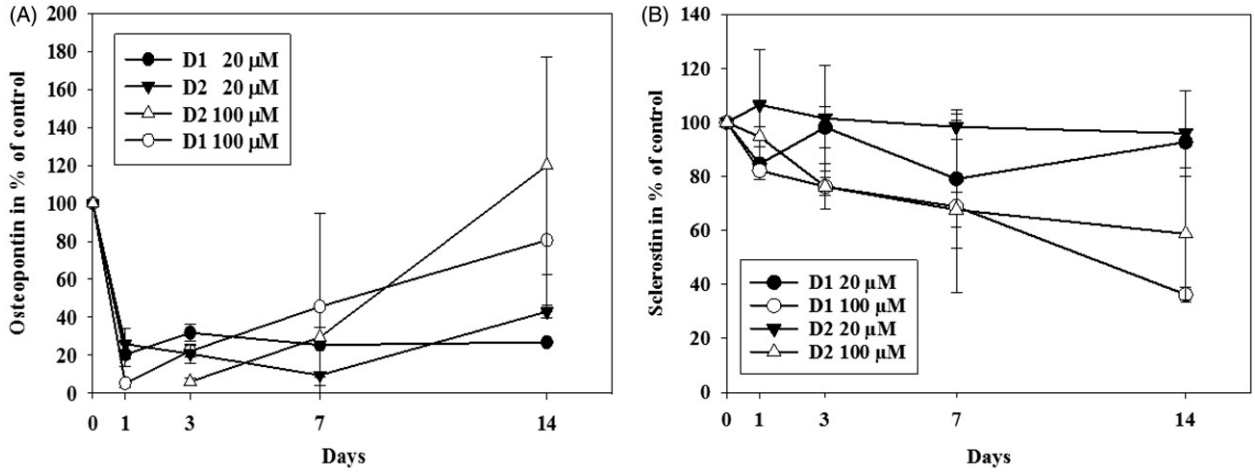

Figure 4. Concentration of osteopontin (OPN) (A) and sclerostin (B) measured in medium from cultures of human osteoblasts treated with ALN (20 and 100 µM) for 1, 3, 7 or 14 d. Data are presented in percentage of control. The notations D1 and D2 are abbreviations used for donor 1 and donor 2, respectively.

There was also a reduction in the secretion of IL-7 ($p < 0.001$) (Figure 5(D)) and IL-15 ($p < 0.001$) (Figure 5(E)).

The secretion of OPG, OC, adiponectin, granulocyte colony-stimulating factor (G-CSF), IL-1β, IL-4, IL-5, monocyte chemoattractant protein-1 (MCP-1), soluble IL-2 receptor alpha (sIL-2Rα), tumour necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF) were not found to be affected by the ALN treatments. The concentrations of LEP, insulin, eotaxin, IL-1Rα, IFN-γ, IL-2, IL-6, IL-10, IL-12p40, IL-13, IL-17, IP-10, macrophage inflammatory protein-1α (MIP-1α) and MIP-1β in cell culture media were below the set levels of detection.

Discussion

High dosage of ALN diminished the proliferation of human osteoblasts after 14 d of treatment, whereas lower dosages induced a 20% increase compared to control. Differentiation of osteoblasts appeared to be unchanged after incubation with 20 µM of ALN, while 14 d of 100 µM ALN caused a marked reduction, concomitant to an increase in the secretion of pro-inflammatory cytokines.

Most previous studies have described the effects of BPs on osteoblast-like cells,[15–18] however, to the best of the authors' knowledge, this is the first study on primary human osteoblasts evaluating the effects of BPs on viability, proliferation, and the secretion of cytokines and bone markers to the cell culture medium. Previous studies have used sub 5 µM as clinically relevant concentration [14] *in vitro*; however, the aim of this study was to evaluate the response in primary human osteoblasts to higher dosages. ALN has a documented half-life in humans of more than 10 years,[19] and the turnover in alveolar bone is suggested to be higher than the turnover in the long bones.[20,21] Consequently a higher concentration of BP in the jaw might be expected, potentially resulting in a continuous release of drug, exposing the osteoblasts in the jaws to BPs for a longer period of time. Thus, relevant concentrations and time points for *in vitro* studies might be difficult to estimate. We observed donor variation in response to ALN e.g. proliferation, underlining the importance of using cells from more than one donor. Contrary to previous studies on the effects of BPs on osteoblasts *in vitro*,[22,23] this study found no significant effects on osteoblast viability, even when treated with the highest dosage of 100 µM ALN.

Von Knoch et al. [24] found little or no effects on the proliferation of bone marrow stromal cells (BMCS) treated with 10 nM ALN. In contrast, Manzano-Moreno et al. [25] reported reduction in proliferation, arrest in cell cycle and apoptosis in an osteosarcoma cell line (MG-63) incubated with various BPs, including ALN, at concentrations between 10 and 100 µM. Taken together with our data, this suggests that primary human osteoblasts and BMCS may respond differently to ALN in terms of proliferation than transformed cells like the osteosarcoma cell line MG-63. Stefanik et al. [6] found that human BMSC isolated from iliac crest responded differently than stem cells originating from mandible bone marrow to incubation with pamidronate. They tested various concentrations, equivalent to 8.5 and 106.4 µM of pamidronate, and found that the proliferation of cells from axial skeletal sites was slightly enhanced by the lowest concentration, followed by a dose-dependent reduction to ~50% of control by the higher dosages. In comparison, the proliferative response of cells from the orofacial sites dropped to ~22% of control by the lowest dosage and to below 10% of control by pamidronate concentrations of 10 µg/ml (equivalent to 42.6 µM) or higher. In addition to cell type, site of origin appears to be of importance for bone cell response.

Reduction in collagen secretion and no change in ALP activity in cell culture medium from human osteoblasts incubated with pamidronate, ibandronate or zoledronate have previously been reported.[26] In our study, LEP mRNA expression in the osteoblasts was enhanced after exposure to ALN, however, the concentration in cell culture medium was below detection and the observation could not be confirmed on the protein level. Several studies have evaluated the role of leptin on bone metabolism,[27,28] yet the effects of BPs on leptin production in bone cells have not been described. There are a few studies describing the effects of BPs on the circulating levels of leptin, but the results are conflicting. Sebastian-Ochoa et al. [29] found that treatment of

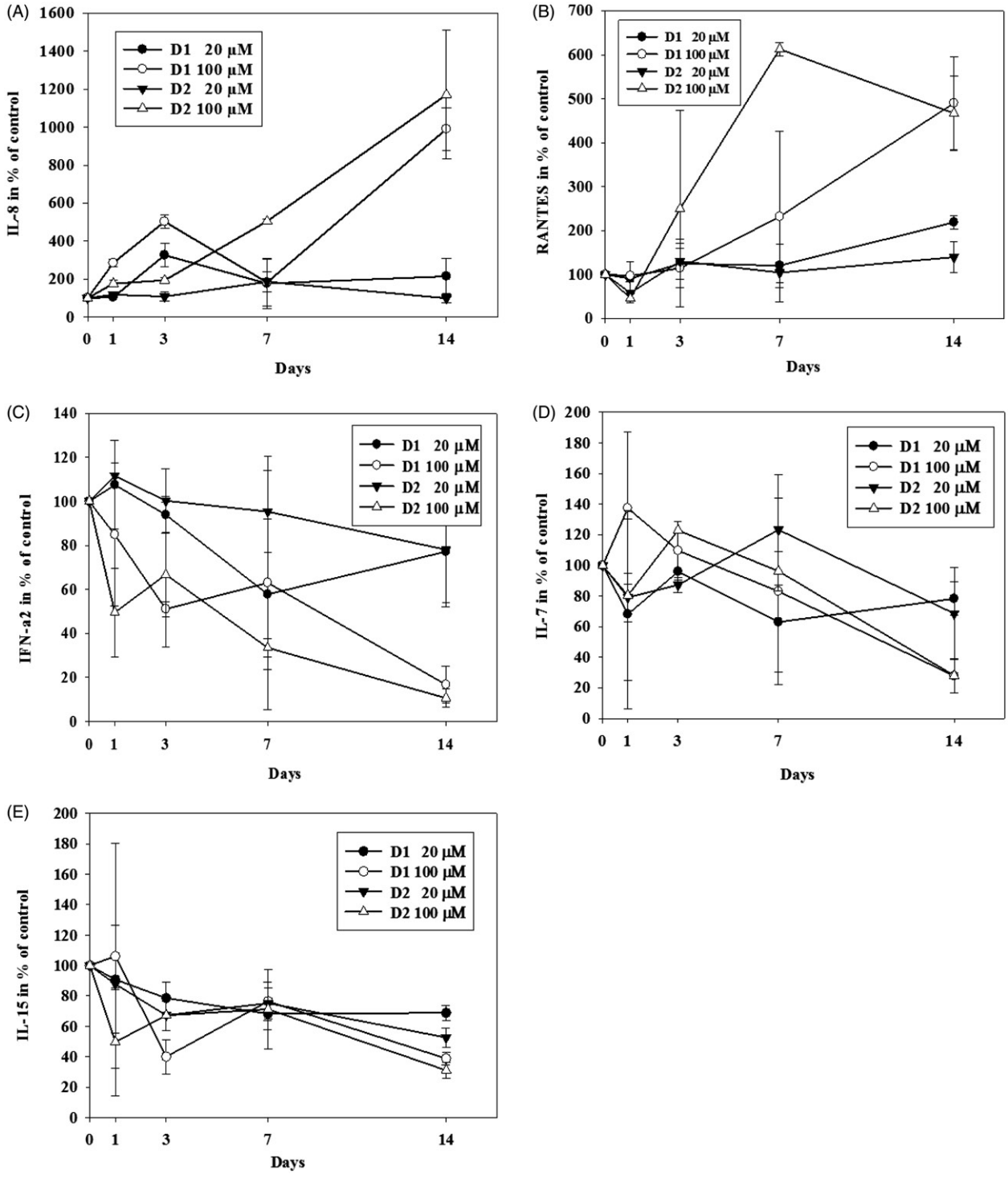

Figure 5. Concentration of IL-8 (A), Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) (B), interferon-alpha2 (IFN-A2) (C), IL-7 (D) and IL-15 (E) in medium from cultures of human osteoblasts treated with ALN (20 and 100 µM) for 1, 3, 7 or 14 d. Data are presented in percentage of control. The notations D1 and D2 are abbreviations used for donor 1 and donor 2, respectively.

menopausal women with ALN had no effects on circulating levels of leptin, whereas Tariq et al. [30] found that treatment with ibandronate induced a significant decrease in a similar population.

Our results also indicate that ALN might impair the differentiation of osteoblasts concomitant to an increase in proliferation, and consequently induce an altered bone quality.

Recent research suggests that a reduction of OPN might be linked to BRONJ-related suppression of bone turnover.[31] We observed an immediate reduction in the secretion of OPN from osteoblasts after exposure to the ALN. In addition, we found an increase in the secretion of the pro-inflammatory IL-8 and RANTES from the treated osteoblasts. With both cytokines suspected of playing important parts in the aetiology of osteolytic conditions,[32,33] there are indications that the cellular response from normal human osteoblasts treated with ALN could be involved in a local adverse development in bone. Kashii et al. [34] found by nanoindentation

analysis on femoral bone of rats that ALN (35 μg/kg/week) induced a significant decrease in Young's modulus, indicating that ALN causes the microstructure of bone matrix to become less anisotropic and thus resulting in a weakening of bone material properties.

Conclusion

High dosage of ALN increases the secretion of pro-inflammatory immune parameters from human osteoblasts. In combination with a reduction in proliferation and differentiation, high dosages of ALN may cause undesirable local effects in bone. Our findings are based on *in vitro* studies of a single cell type, and further studies are needed to determine the clinical impact.

Acknowledgements

The authors would like to thank Aina-Mari Lian for her invaluable skills and moral support (Clinical Oral Research Laboratory, Faculty of Dentistry, University of Oslo).

Disclosure statement

None to declare.

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