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Combined effect of strontium and zoledronate on hydroxyapatite structure and bone cell responses

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ABSTRACT

The influence of the simultaneous presence of the two inhibitors of bone degradation, strontium and zoledronate, on the direct synthesis of hydroxyapatite was explored in the range of Sr concentration up to 50 atom% at two different bisphosphonate concentrations (ZOL7 and ZOL14). The results of structural analysis indicated that HA can be obtained as a single crystalline phase up to a Sr concentration in solution of 20 and 10 atom% within the ZOL7 and ZOL14 series respectively. Both Sr substitution and ZOL incorporation affect the length of the coherently scattering crystalline domains and the dimensions of HA nanocrystals. At greater Sr content, XRD full profile fitting data indicate that zoledronate provokes the segregation of Sr in two crystalline apatitic phases, at different strontium content. Co-cultures of osteoblast-like MG63 cells and human osteoclast show that ZOL displays a greater inhibitory influence than Sr on osteoclast proliferation and activity. On the other hand, the results obtained on osteoblast surnatant and on gene expression indicate that Sr exerts a greater promotion on osteoblast proliferation and differentiation. The co-presence of Sr and ZOL has a combined effect on the differentiation markers, so that HA containing about 4 wt% ZOL and 4 Sr atom%, and even more HA containing about 4 wt% ZOL and 8 Sr atom%, result the best compromise for osteoblast promotion and osteoclast inhibition.

1. Introduction

Bisphosphonates (BPs) are considered as the most active inhibitors for bone degradation [1–3]. The chemical structure of non-hydrolysable BPs is analogous to that of pyrophosphate, where the oxygen atom connecting the two phosphates is replaced by a carbon atom. The different pharmacological activity and affinity for hydroxyapatite (HA) displayed by the different bisphosphonates depend on the different sidechains bound to the α-carbon [4,5]. BPs with nitrogen containing sidechains (N-BPs) inhibit the key enzyme in the mevalonate pathway, farnesyl pyrophosphate synthase, thereby preventing prenylation of small GTPases [6,7]. The results of inhibition are disruption of many osteoclast activities and cells apoptosis [4]. The direct antitumour effect of N-BPs is ascribed to the same molecular mechanism [8,9]. Moreover, interaction with macrophages and endothelial cells contributes to N-BPs indirect anticancer activities [10].

Clinical studies showed that long term uses of N-BPs resulted in excellent antifracture action, which persists after treatment suspension [11]. On the other hand, possible increased risk of atypical subtrochanteric fractures due to long term exposure to one of the most potent N-BPs, alendronate, has been reported [12]. A further side effect of systemic administration of N-BPs therapy is jaw osteonecrosis, which occurs especially in intravenous formulations as in the case of zoledronate (ZOL), and is more frequent in oncologic patients who receive high doses of the drugs [13-15].

Strontium administration as strontium ranelate has recently been shown to reduce fracture risk in osteoporotic patients [16,17], and it has been suggested as a therapeutic option following long-term BPs treatment [18]. *In vivo* and *in vitro* studies demonstrate that Sr reduces bone resorption through inhibition of osteoclast activity and differentiation and it enhances pre-osteoblastic cells replication and osteoblastic differentiation, promoting new bone formation [16,19]. We have previously shown that Sr can influence

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bone cells even if incorporated into hydroxyapatite structure: Sr substituted HA stimulates osteoblast activity and differentiation, whereas it hinders osteoclast proliferation in a dose dependent manner [20–22]. A similar inhibitory action on osteoclast proliferation and activity, has been demonstrated for HA nanocrystals containing about 7 wt% zoledronate [23].

The simultaneous presence of Sr and ZOL in HA composite nanocrystals should have an additive effect on bone cells. To this aim, we synthesized Sr substituted HA at different ZOL content and we investigated the HA structural modifications induced by their simultaneous presence, as well as the *in vitro* osteoblast and osteoclast response in co-culture system.

2. Materials and methods

2.1. Synthesis and general characterization

Strontium containing hydroxyapatites were synthesized in N_2 atmosphere as previously reported [20]. Briefly, 50 ml of solutions with different Sr/(Ca + Sr) ratios were prepared by dissolving the appropriate amounts of $Ca(NO_3)_2 \cdot 4\ H_2O$ and $Sr(NO_3)_2$ in CO_2-free deionized water and adjusting pH to 10 with NH_4OH. The total cation concentration was 1.08 M. 50 ml of 0.65 M $(NH_4)_2HPO_4$ solution, pH 10 adjusted with NH_4OH, was added drop-wise under stirring to the cationic solution heated at 90 °C. The precipitate was maintained in contact with the reaction solution for 5 h under stirring, then centrifuged at 10,000 rpm for 10 min and repeatedly washed with distilled water. The product was dried at 37 °C overnight. Samples were synthesized up to 50 Sr atom% and labelled according to Sr concentration as SrX, where *X* indicates Sr atom%.

Zoledronate containing hydroxyapatites were obtained by adding disodium zoledronate tetrahydrate (Chemos GmbH) to the phosphate solution. Samples were synthesized at two different zoledronate concentrations, 7 and 14 mM, calculated on final volume, and accordingly labelled ZOL7 and ZOL14.

Hydroxyapatites containing both strontium and zoledronate were prepared as reported above, by adding $Sr(NO_3)_2$ to calcium-containing solution and disodium zoledronate tetrahydrate to phosphate-containing solution, in the appropriate amounts. The two series of samples at different BP content were labelled SrXZOL7 and SrXZOL14, where *X* indicates Sr concentration in solution as specified above.

X-Ray diffraction analysis was carried out by means of a PANalytical X'Pert PRO powder diffractometer equipped with a fast X'Celerator detector. Ni-filtered CuKα radiation was used (40 mA, 40 kV). For phase identification the 2θ range was investigated from 10 to 60 2θ degrees with a step size of 0.1° and time/step of 100 s. To evaluate the coherence lengths of the crystals, further X-ray powder data were collected by means of step scans with a fixed counting time of 300 s for each 0.033°/step and 900 s for each 0.050°/step for *002* and *310* reflections respectively.

Data used in the full profile pattern refinement were collected counting for 1200 s at each 0.033° (2θ) and processed with the Rietveld routine of the HighScore Plus software package (PANalytical). The structural data of the apatitic phases [24] were used as starting data. During the refinement process the following parameters were refined in the order: zero, scale factor, background, *a*, *c*, peak width, peak shape, width dependence on 2θ, phase relative amount, strontium content.

Calcium and strontium contents in the solid products were determined by means of an ICP spectrometer (ICP Optima 4200DV, Perkin Elmer). Powders were previously dissolved in 0.1 M HCl. Results from this analysis represent the mean value of three different determinations.

Bisphosphonate content was determined spectrophotometrically via complex formation with Fe(III) ions using a Varian Cary50Bio instrument ($\lambda = 290$ nm) [25].

For TEM investigations, a drop of sonicated calcium phosphate suspension in ethanol was transferred onto holey carbon foils supported on conventional copper microgrids. A Philips CM100 transmission electron microscope, operating at 80 kV was used.

The surface area was measured using a Carlo Erba Sorpty 1750 BET analyser using constant volume N_2 absorption with desorption at 80 °C.

In vitro tests were performed on disk-shaped samples (∅ = 6.0 mm). Each disk was prepared by pressing 60 mg of powder into cylindrical moulds by using a standard evacuable pellet die (Hellma), and sterilized using gamma rays (Cobalt-60) at a dose of 25 kGy.

2.2. In vitro tests

2.2.1. Osteoblast and osteoclast co-culture

MG-63 human osteoblast-like cells (Istituto Zooprofilattico Sperimentale IZSBS, Brescia, Italy) were cultured in DMEM medium (Sigma, UK) supplemented with 10% FCS, and 1% antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Cells were detached from culture flasks by trypsinization, and centrifuged; cell number and viability were checked with trypan blue dye exclusion test. MG-63 were plated at a density of 1×10^4 cells/ml in 24-well plates containing sterile samples of Sr5, Sr10, ZOL7, ZOL14, Sr5ZOL7, Sr10ZOL7, Sr5ZOL14, Sr10ZOL14. The same concentration of cells was seeded in empty wells for control of experiment alone (CTR) or for co-culture (CTR/cc). Medium was changed with DMEM additioned with β-glycerophosphate (10^{-2} M) and ascorbic acid (50 μg/ml) to activate osteoblasts and plates were cultured in standard conditions, at 37 °C ±0.5 with 95% humidity and 5% CO_2 ±0.2.

Osteoclast precursor 2T-110 (OCP, Poietics™ Osteoclast Precursor Cell System, Lonza Walkersville, Inc., MD, USA) at a concentration of 1×10^5 cells/ml were differentiated in 24 well inserts 0.4-μm pore size (Millipore, Ireland) in DMEM additioned with macrophage colony-stimulating factor (MCSF, 25 ng/ml) and receptor activator for κB factor ligand (RANKL, 30 ng/ml). Osteoclasts were co-cultured with osteoblasts seeded on experimental biomaterials and CTR/cc wells up to 21 days. Both cell types were cultured in osteoblast:osteoclast differentiation medium.

2.2.2. Cells proliferation

Osteoblast and osteoclast proliferation and viability (14 and 21 days) was evaluated by WST1 colourimetric reagent test (WST1, Roche Diagnostics GmbH, Manheim, Germany). The assay is based on the reduction of tetrazolium salt to a soluble formazan salt by a reductase of the mitochondrial respiratory chain, active only in viable cells. 50 μl of WST1 solution and 450 μl of medium (final dilution: 1:10) were added to the osteoblasts and osteoclasts separately, and the multi-well plates were incubated at 37 °C for the next 4 h. Supernatants were quantified spectrophotometrically at 450 nm with a reference wavelength of 625 nm. Results of WST1 are reported as optical density (OD) and directly correlated with the cell number.

2.2.3. Osteoblast activity and differentiation

At the end of experimental times the supernatant was collected from all wells and centrifuged to remove particulates, if any. Aliquots were dispensed in Eppendorf tubes for storage at −70 °C and assayed for Alkaline Phosphatase (ALP, immunoenzymatic assay, Immunodiagnostics, UK), Type I Pro-Collagen (Coll1, immunoassay kit, USCN Life Science, Wuhan, China), Osteocalcin (OSTC immunoassay kit, USCN Life Science), Osteoprotegerin (OPG, enzyme Immunoassay kit, Bender MedSystems, Vienna, Austria), and Receptor Activator for Nuclear factor KB Ligand (RANKL, enzyme immunoassay kit, Bender MedSystems), Transforming Growth Factor-β1 (TGF-β1, Immunoassay, R&D Systems, MN, USA). Co-culture medium additioned with RANKL was evaluated and subtracted from RANKL samples values. All the measured concentration and activity were normalized by Total Protein (TP, Total Protein micro-Lowry kit, SIGMA, MO, USA) content.

2.2.4. Osteoblast morphology

Samples for each material, at the end of the experiment, were processed for Scanning Electron Microscopy (SEM): osteoblasts grown on the materials were fixed in 2.5% glutaraldehyde, in pH 7.4 phosphate buffer 0.01 M for 1 h and dehydrated in a graded ethanol series. After a passage in hexamethyldisilazane, the samples were air dried. The samples were sputter-coated with Pd prior to examination with a Philips CM100 Scanning Electron Microscope.

2.2.5. Quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from all samples at 24 h (baseline) and at 14 days. Phenol–chloroform extraction was performed using TRIzol Reagent (Invitrogen, Carlsbad, CA). Purified RNA was reverse transcribed with Superscript VILO cDNA Synthesis kit (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. The resulting cDNA was quantified with Quant-iT Pico-Green dsDNA assay kit (Invitrogen) and diluted to the final concentration of 5 ng/ml. Each sample (10 ng) was tested in duplicate. qPCR analysis was performed in a LightCycler Instrument (Roche Diagnostics GmbH, Mannheim, Germany) using the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany). The protocol included a denaturation at 94 °C for 15′, 35–40 cycles of amplification (94 °C 15″, appropriate annealing temperature for each target as detailed in Table S1 for 20″ and 72 °C for 20″) and a melting curve to check for amplicon specificity.

The crossing point values (i.e., the cycle number at which the detected fluorescence exceeded the threshold value) were determined for each sample, and these values were used for comparative gene expression analysis employing the $2^{-\Delta\Delta Ct}$ method.

2.2.6. Osteoclastogenesis

TRAP-staining was performed to assess osteoclast differentiation according to manufacturer's instructions (SIGMA, Buchs, Switzerland). The positive cells developed red colour of different intensity. The number of TRAP-positive multinucleated cells (three or more nuclei each cell) was counted under the microscope and results are given as percentage of CTR.

2.2.7. Statistical analysis

Statistical evaluation of data was performed using the software package SPSS/PC$^+$ Statistics™ 10.1 (SPSS Inc., Chicago, IL, USA). The results presented are the mean of six values. Data are reported as mean ± standard deviations (SD) at a significance level of $p < 0.05$. After having verified normal distribution and homogeneity of variance, a one-way ANOVA was done for comparison between groups. Finally, a post-hoc multiple comparison tests was performed to detect significant differences among experimental groups and control.