Journal of Dental Sciences (2015) 10, 8—15

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jds.com

ORIGINAL ARTICLE

Effects of zoledronic acid on osteoblasts in three-dimensional culture

Flora Thibaut [a,b†], Tanguy Watrin [b†], Fleur Meary [a,b], Sylvie Tricot [b], Virginie Legros [b], Pascal Pellen-Mussi [b], Dominique Chauvel-Lebret [a,b*]

[a] *Pôle d'odontologie et de chirurgie buccale, Centre Hospitalier Universitaire, Rennes, France*
[b] *Laboratoire de Biomatériaux en Site Osseux, UMR CNRS 6226 — Institut des Sciences Chimiques de Rennes, Faculté d'Odontologie, Université de Rennes 1, France*

Received 26 May 2014; Final revision received 11 July 2014
Available online 26 September 2014

KEYWORDS
bisphosphonates;
hFOB 1.19;
multicellular spheroids;
proliferation;
zoledronic acid

Abstract *Background/purpose:* Bisphosphonates (BPs) are synthetic drugs with antitumor and bone antiresorptive activities. The use of BPs is suspected to favor the emergence of osteonecrosis of the jaw (ONJ), a putative side effect whose pathogenesis remains unclear. Thus, we aimed to get insights about the impact of BPs on osteoblasts functions, using a three-dimensional (3D) culture model, which is suggested to be more similar to the *in vivo* tissues than monolayers.
Materials and methods: Effects of low (0.1 μM) and high (10 μM) concentrations of zoledronic acid were investigated on osteoblasts (hFOB 1.19), cultured as multicellular spheroids (MCS). Proliferation, apoptosis, spheroid growth kinetics, and morphology were studied using the 3-(4,5-dimethyl-thiazoyl-2yl) 2,5-diphenyl-tetrazolium bromide (MTT) and acid phosphatase (APH) assays, caspase 3 Western-blotting, phase contrast imaging and scanning electron microscopy (SEM).
Results: Proliferation, apoptosis, and spheroid morphology showed that 10 μM zoledronic acid (ZA) induced a significant reduction in the relative viable cell number, correlated with morphological alterations of spheroids, and induction of apoptosis. A lower ZA concentration (0.1 μM) promoted cell proliferation without affecting growth kinetics or spheroid morphology.
Conclusion: ZA sensitivity of osteoblasts depends on concentration and experimental models. The dual dose-dependent effects of ZA on osteoblasts cultured as spheroids, thereby promoting or inhibiting cell proliferation, may provide opportunities in tissue engineering. At last, the hFOB spheroid culture system represents a valuable model for the exploration of the molecular

* Corresponding author. Laboratoire de Biomatériaux en Site Osseux, Faculté d'Odontologie, Université de Rennes 1, 2 Avenue du Pr. Léon Bernard, 35043 Rennes Cedex, France.
E-mail address: dominique.lebret-chauvel@univ-rennes1.fr (D. Chauvel-Lebret).
† Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jds.2014.07.004

basis of BPs action on osteoblasts and for the development and evaluation of implantable biomaterials in bone sites.

Introduction

Bisphosphonates (BPs) are synthetic compounds, both metabolically stable and structurally similar to inorganic pyrophosphate. They bind strongly to hydroxyapatite crystals and inhibit bone resorption by reducing physicochemical bone mineral dissolution and decreasing osteoclast activity. Due to their antiresorptive properties, these drugs are widely used in the clinical treatment of bone diseases with increased bone resorption, such as postmenopausal osteoporosis, Paget's disease, or lytic bone metastasis.[1] BPs can be divided into two groups depending on their molecular mechanism of action at the cellular level.[2] Simple BPs, such as clodronate and etidronate, are metabolized intracellularly to nonhydrolyzable analogues of ATP. The accumulation of these metabolites in the cytosol of osteoclasts induces cell death, probably by inhibiting ATP-dependent intracellular enzymes.[3,4] The more potent amino-BPs [e.g., zoledronic acid (ZA), alendronate, and ibandronate], which are not metabolized, alter osteoclast function by inhibiting enzymes of the mevalonate biosynthetic pathway, notably the farnesyl diphosphate synthase (FPPS). Inhibition of FPPS leads to depletion of the metabolites FPP and geranylgeranyl diphosphate, necessary for the post-translational modification (prenylation) of small GTPases, thereby affecting subcellular localization and function of these signaling proteins essential for osteoclast survival and activity.[5]

The use of BPs has been linked to osteonecrosis of the jaw (ONJ), particularly in patients after intravenous therapy in the setting of malignancy. BPs-related ONJ (BRONJ) can be caused by oral trauma or dental extraction, but it can also occur spontaneously.[6,7] Although causality between BPs exposure and ONJ is still controversial, a clinical staging system of BRONJ has been developed to more appropriately diagnose and clinically manage patients, ranging from Stage 0 (no clinical evidence of necrotic bone, but nonspecific clinical findings and symptoms) to Stage 3 (exposed necrotic bone with pain, infection, pathologic fracture, extra oral fistula, oral antral/oral nasal communication, etc.).[8] Various hypotheses have been put forward to explain BRONJ pathophysiology, which is likely to be multifactorial. It is postulated that BRONJ localizes on the jaws because of the heavy bone turnover in this area, causing BPs to accumulate preferentially in jaws at cytotoxic concentrations. Pathophysiology may also involve suppression of bone turnover and angiogenesis, altered function of oral mucosal cells, microbial flora, antiinflammatory effects, and genetic predispositions.[9] Beyond well-described direct effects of BPs on osteoclastic activity, significant clinical and experimental evidence indicates that BPs also influence osteoblast functions.[10,11] *In vitro* and *in vivo* studies demonstrated dose-dependent effects of BPs on cell proliferation, cell differentiation, apoptosis, and matrix mineralization. High concentrations (>10 μM) generally produce adverse effects on these processes that may account for the occurrence of BRONJ.[12–17] Lower BP concentrations act positively on osteoblastic function by promoting survival, proliferation, and differentiation of bone-forming cells.[17–20] While this dual effect of BPs on osteoblasts is now well established, uncertainty remains concerning the threshold of cytotoxic and clinically relevant concentrations of BPs, probably because of multiple experimental models used for investigations.

The objective of this *in vitro* study was to evaluate the effects of ZA — a commonly prescribed amino-BP — on osteoblasts in a three-dimensional (3D) culture system, namely multicellular spheroids (MCS).[21] The MCS model is based on the cell's propensity to self-aggregate when cultured in nonadhesive conditions. By restoring cell-cell and cell-matrix interactions, this 3D system more closely mimics living tissue than monolayer cultures do,[22,23] making MCS-based assays more predictive of *in vivo* response to drugs. By examination of cell proliferation, cellular apoptosis, and spheroid morphology after ZA treatment, we demonstrated that the dual dose-dependent effect of ZA already observed in monolayer cultures is conserved in these osteoblastic microtissue-like cultures.

Materials and methods

Cell culture

The human fetal osteoblast cell line hFOB 1.19 (hFOB) was purchased from ATCC. hFOB cells were maintained in a 1:1 mixture of phenol red-free Dulbecco's Modified Eagle's medium/Ham's F-12 medium, supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 20 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid buffer, 2 mM L-glutamine, and 300 μg/mL G418. For cell expansion, hFOB cells were incubated at 33.5°C and 5% CO2 and fed twice a week. On reaching 80% confluency, they were passaged using 0.05% trypsin-0.02% Ethylenediaminetetraacetic acid.

Generation and culture of MCS

Formation of hFOB spheroids was achieved using agarose-coated 96-well tissue culture plates (50 μL of 1.5% agarose in phosphate-buffered saline (PBS). The seeding density was 2×10^3 cells/well. After 96 hours of incubation, spheroid cultures were treated by replacing 50% (= 100 μL) of supernatant with normal medium or medium