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Exposing human epithelial cells to zoledronic acid can mediate osteonecrosis of jaw: an *in vitro* model

Running title: Epithelial cells in ONJ by zoledronate

Key Words: epitellial cells; osteoblasts; zoledronic acid; ONJ; cytokines.

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ABSTRACT

Background: Osteonecrosis of the jaw (ONJ) is a chronic complication of bisphosphonate therapy, mainly when intravenous, in cancer patients with bone metastases and myeloma. Its pathophysiology is not yet fully elucidated; in particular, the molecular/cellular events triggering ONJ remain unclear. This complication could result from the effect of bisphosphonates released from bone into the soft-tissues, or from osteolysis induced by soft-tissues directly exposed to bisphosphonates.

This research investigated the possibility that ONJ may be evocated by changes induced in osteoblast activity by factors released by soft-tissue cells exposed to zoledronic acid.

Methods: An "in vitro" model was used, in which human osteoblast-like MG-63 cells were grown in medium conditioned by human keratinocytes NCTC 2544, exposed or not to zoledronic acid (5 or 50 µM). 5 µM zoledronic acid was also directly administered to MG-63 cells. Results: In NCTC 2544 cells, zoledronic acid decreased proliferation via decreased hydroxy-3-methyl-glutaryl-CoA reductase, suggesting that a decrease of healing capability can occur in case of injury. An increased pro-inflammatory potential was also observed.

Osteoblasts grown in medium conditioned in presence of zoledronic acid showed decreased proliferation and osteogenic properties, and increased ability to induce osteoclast differentiation and inflammatory process. Zoledronci acid direcly administered to MG-63 modulated only some parameters and in a lesser extent.

Conclusions: The research evidenced, for the first time, the direct involvement of epithelial cells in zoledronic-acid-triggered molecular mechanisms leading to osteonecrosis of the jaw, by modulating both osteoblast and osteoclast properties.

INTRODUCTION

Osteonecrosis of the jaw (ONJ) is a chronic complication of bisphosphonate (BP) therapy, mainly when intravenous, in cancer patients with bone metastases and myeloma. First described in 2003, the ONJ incidence ranges from 1.3% to 19%, the highest frequency being in mandible (1-3). The most commonly reported associated event is tooth extraction, but dental implants, mandibular exostoses, periodontal disease, and local trauma have also been described as preceding ONJ (4,5). Among BPs, zoledronic acid is a nitrogen-containing drug of the third generation, and is one of the most potent available (6,7). At present, the pathophysiological mechanisms underlying ONJ are not yet elucidated, but the fact that it occurs spontaneously suggests a multifactorial pathogenesis.

BPs appear to affect intracortical bone mandible remodeling, as evidenced in skeletally-mature beagle dogs after 3 years of daily oral treatment with alendronate. Suppression of remodeling could allow accumulation of nonviable osteocytes. Moreover, BPs also possess anti-angiogenic effect, which probably compromised post-extraction healing (8). The above reported effect of BPs could act in a synergic manner in the jaw where, alongside marked vascularity, bone turnover is high.

An important and as yet unsolved problem concerns the ONJ initiation: this complication could result from the effect on the soft-tissues of BPs released from the bone, or might be due to osteolysis triggered by soft-tissues exposed to BPs. As the name implies, ONJ is assumed to be primarily a bone condition. A recent study reported that zoledronic acid, released from the bone, affects oral mucosa, inducing early apoptosis and subsequently reducing cell growth (9).

Little research has as yet deeply examined the true role of soft-tissues in ONJ induction. The present research examined the possibility that ONJ is evocated by changes induced in osteoblast activity by factors released by epithelial cells exposed to zoledronic acid. For this purpose, an "in vitro" model was developed, in which human osteoblast-like MG-63 cells were grown in culture medium conditioned by human keratinocytes NCTC 2544, exposed or not to zoledronic acid. Parameters relating to proliferation, viability, inflammation, and mevalonate pathway were examined.

MATERIALS AND METHODS

Cell treatments

Human keratinocytes NCTC 2544 were seeded (20,000/cm^2) in modified Eagle's medium (MEM) supplemented with 2 mM L-glutamine, 1% (v/v) antibiotic/antimycotic solution, 1 mM sodium pyruvate and 10% (v/v) fetal bovine serum (FBS), and maintained at 37°C in a 5% CO2 atmosphere. Twenty-four hours after seeding, NCTC cells were exposed to zoledronic acid (5or 50 µM) dissolved in PBS. After 48 hours, medium was removed from NCTC cells, centrifuged at 3000 rpm per 10 min (centrifuge J-6B Beckman, Brea, CA, USA), and used to grow human osteoblast-like cells MG-63. These cells were chosen because, even if derived form human osteosarcoma, they are considered very similar to normal osteoblasts and usually used in biocompatibility studies.

MG-63 cells were seeded 20,000/cm^2 in modified Eagle's medium (MEM) supplemented with 2 mM L-glutamine, 1% (v/v) antibiotic/antimycotic solution, 1 mM sodium pyruvate and 10% (v/v) FBS. After 24 hours, culture medium was removed and replaced with medium

conditioned by NCTC 2544 cells for 48 hours. Analyses on MG-63 cells and culture media were carried out after a further 48 hours.

When zoledronic acid was directly added to MG-63 cells, cells were seeded 20,000/cm^2 in modified Eagle's medium (MEM) supplemented with 2 mM L-glutamine, 1% (v/v) antibiotic/antimycotic solution, 1 mM sodium pyruvate and 10% (v/v) FBS. After 24 hours, zoledronic acid was added at the final concentration 5 µM. Analyses were carried out after a further 48 hours.

Cell growth and viability

Cells present in monolayer and culture medium were counted using a Burker chamber. Viability was determined as lactate dehydrogenase (LDH) release in the medium. LDH activity was assessed spectrophotometrically after medium centrifugation at 600 x g for 10 min, and expressed as nmoles NADH oxidized//min/ml of medium (10).

Flow cytometry

The percentage of cells with hypodiploid content of DNA, typical of apoptotic cells (sub G0/G1 peak), was evaluated by determining the DNA content through propidium iodide staining, as described elsewhere (11).

Real-time polymerase chain reaction (real-time PCR)

Total RNA was extracted from NCTC 2544, MG-63 cells grown in the conditioned medium and MG-63 cells directly exposed to zoledronic acid with a RNEasy® Mini Kit (Qiagen, GmbH, Germany) following the manufacturer's protocol. One µg RNA was reverse transcripted using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City,

CA, USA). Real-time PCR was performed using IQ™ SYBRGreen Supermix (Bio-rad, Hercules, CA) in a iCycler system (Bio-Rad, Hercules, CA).

Each sample was tested three times. The changes were quantified as relative expression compared to that of corresponding control cells, and calculated as $2^{-\Delta\Delta Ct}$, where ΔCt = Ctsample - CtGAPDH and $\Delta\Delta Ct = \Delta Ctsample - \Delta CtT0$.

ELISA analysis

Cytokines (IL-6, IL-1β and TNF-α) were evaluated in the culture medium of NCTC 2544 or MG-63 cells using the Enzyme Linked Immuno Sorbent Assay (Bender Medsystems, Vienna, Austria).

Statistical analysis

Data are expressed as means ± S.D. The significance of differences between group means was assessed by variance analysis, followed by the Newman-Keuls test ($p < 0.05$).

RESULTS

The effect of zoledronic acid on growth and viability of NCTC cells is reported in Figure 1. Exposure to this BP decreased cell proliferation in a dose-dependent manner, inhibition being 12.1 % and 37.8%, respectively, in the presence of 5 and 50 μM zoledronic acid. The viability determination showed that zoledronic acid did not significantly induce necrosis, whereas a significant increase of apoptotic cell percentage (+35% and +182%, in presence of 5 and 50 μM zoledronic acid, respectively) was observed.

Figure 2 reports the mRNA content of factors involved in regulating inflammation and proliferation in NCTC cells exposed to zoledronic acid. With regard to pro-inflammatory cytokines, no change was observed in IL-1β, whereas a significant increase was observed in TNF-α (+90% and +130%, for 5 and 50 μM zoledronic acid, respectively). Similarly, ELISA determination of IL-1β and TNF-α release in the culture medium confirmed no change for the former and an increase for the latter (data not shown).

In Figure 2 is also reported the significant decrease found in the mRNA content of PPARα (peroxisome proliferation-activated receptor) and HMGR (hydroxy-3-methyl-glutaryl-CoA reductase) in the presence of both zoledronic acid concentrations.

MG-63 cells grown in medium conditioned by zoledronic acid-exposed NCTC 2544cells showed decreased cell numbers versus those grown in medium conditioned by unexposed NCTC 2544 cells (Figure 3). The decrease was dose-dependent: the percentage inhibition was 20.8 and 60.0%, in presence of 5 or 50 μM zoledronic acid, respectively. In MG-63 cells grown in medium conditioned by NCTC cells exposed to BP, no significant induction of necrosis, andapoptosis was observed. The number of MG-63 cells directly exposed to 5 μM zoledronic acid for 48 hours was significantly reduced in comparison with all other experimental groups. No change was observed in viability.

Figure 4 shows the mRNA content of inflammatory parameters and molecules involved in regulation of bone homeostasis in MG-63 cells. A significant increase of the pro-inflammatory cytokines IL-1β (+220%), IL-6 (+320%) and IL-8 (550%) was evidenced in MG-63 cells grown in the medium conditioned by NCTC 2544 cells exposed to 50 μM zoledronic acid; on the contrary, TGF-β2 decreased significantly (-90%). With regard to markers of bone homeostasis, the expression of BMP-4 was significantly reduced (-50%) in presence of both concentrations used; ALP was significantly reduced (-90%)only in presence

of the highest BP concentration. A significant increase of RANK ligand (RANKL) occurred, whereas osteoprotegerin (OPG) mRNA was increased, even if in a no significant way .

In MG-63 cells directly exposed to 5 μM zoledronic acid no significant change was observed in IL-1β, IL-8, ALP and OPG mRNA content in comparison with control cells and cells grown in medium conditioned by NCTC 2544 cells in presence of 5 μM zoledronic acid.

IL-6 mRNA content was similar to that of control cells and lower than that in MG-63 cells grown in medium conditioned by NCTC 2544 cells in presence of zoledronic acid.

With regard to TGF-β2, the mRNA content was increased in comparison with control cells (+30%) and much more in comparison with MG-63 cells grown in medium conditioned by NCTC 2544 cells in presence of zoledronic acid (about 330%). The expression of RANK-L was increased in a no significant way in comparison with control cells, and it was lower than in MG-63 cells grown in medium conditioned by NCTC 2544 cells in presence of zoledronic acid.

DISCUSSION

Despite the significant incidence of ONJ, and the increasing interest in elucidating its patho-physiology, the underlying molecular mechanisms are still only partially known. In particular, "in vivo" observations do not contribute to understanding the mutual influence between soft-tissues and bone, in triggering ONJ. At the present, experimental studies reported in the literature (12-14) mainly investigated the direct effect of BPs on epitellial cells, not the consequences that soft-tissue exposition to BPs can trigger on surrounding microenvironment. For this reason, our "in vitro" model aimed to investigate in depth the signal transduction pathways activated by zoledronic acid in the soft-tissues that may modify osteoblast/osteoclast activities. In particular, cell proliferation and viability, and the

production of inflammation mediators and molecules responsible for osteoclast activation, were evaluated.

The results regarding NCTC 2544 cells showed zoledronic acid to decrease cell proliferation, also inducing an increase in apoptosis in presence of highest zoledronic acid concentration used. The reduction of cell proliferation is probably mediated by the decreased expression of HMGR, which is the rate-controlling enzyme of the mevalonate pathway that produces both cholesterol and isoprenoids, molecules essential for cell proliferation (15). The reduced proliferation capacity of soft-tissue after exposure to BPs suggests that BP treatment significantly decreases its healing ability after injury, being this crucial in case of ONJ consequent to extraction or other invasive procedure.

Another important finding was that zoledronic acid increased the "pro-inflammatory potential" of NCTC 2544 cells: in treated cells, PPARα decreased, while TNFα increased. PPARα is a ligand-activated transcription factor, and a member of the nuclear-hormone receptor superfamily. Evidence showed that PPARα plays a beneficial role in reducing inflammation. Activation of this receptor appears to influence both acute and chronic inflammatory disorders involving neutrophils and macrophages (16). In our experimental protocol, the decrease of this well-known anti-inflammatory factor was coupled with an increase of pro-inflammatory TNFα, both contributing to generating a "pro-inflammatory microenvironment" that might be crucial in ONJ pathogenesis.

The results relating to osteoblasts, grown in medium conditioned by zoledronic-acid-exposed NCTC 2544 cells, showed that changes in the culture microenvironment, due to this BP, decrease osteoblast proliferation and osteogenic properties, while increasing the cells' ability to induce osteoclast differentiation and inflammatory processes. These observations demonstrate, for the first time, that exposing soft-tissues to zoledronic acid significantly increases production of pro-inflammatory factors, and decreases that of anti-inflammatory

ones. In turn, this affects osteoblast properties: osteoblasts grown in culture medium conditioned in presence of zoledronic acid showed reduced proliferation, which translated into a reduced regenerating ability during the healing process. This reduction could be determinant in the case of tooth extraction, when bone must be regenerated.

Osteoblasts grown in culture medium conditioned in presence of zoledronic acid also showed a reduced expression of BMP-4, TGFβ2 and ALP, indicating a decreased ability to produce bone, a fundamental event in tooth socket healing. The decrease in TGFβ2 could also be important in reducing angiogenesis process during healing process. In fact, other than to stimulate osteoblast proliferation and differentiation, and extracellular matrix production, this subtype can induce VEGF expression (17).

Moreover, in these osteoblasts, an increased expression of RANKL (a factor involved in stimulating osteoclast activation) occurred. This observation strongly suggests that altered bone remodeling in ONJ could be due partly to the increased soft-tissue-mediated osteoclastogenic properties of osteoblasts.

Results from experiments where MG-63 cells have been directly treated with zoledronic acid evidenced that this experimental condition only partially affect osteoblast properties, as regards osteogenic and osteoclastogenic properties. Moreover, zoledronic acid only partially increases IL-6 expression.

Since BP-induced osteonecrosis only occurs in the jaw bones, the results of this research appear to indicate that the effect of BPs on soft-tissues plays an important role, both in reducing the regenerative capability of both soft-tissues and bone, and in increasing bone remodeling via induction of osteoclast differentiation and activation. These changes are particularly negative in cases of tooth extraction and other events determining bone exposure. It may be hypothesized that ONJ occurs in consequence of the close contact between soft-tissues and bone, a condition not occurring in other body districts. In this anatomic condition,

it is possible that BPs accumulated in the bone and released in not-toxic amount affect soft-tissue properties, these in turn modulating osteoblast and osteoclast functions.

In conclusion, this research evidenced the possible direct involvement of the soft-tissues in zoledronic acid-triggered molecular mechanisms leading to ONJ. It should be noted that exposing epitellial cells to zoledronic acid can affect the properties of epithelial cells themselves, of osteoblasts and osteoclasts, all of which contribute to the onset of ONJ.

The experimental model used could also be useful to investigate other aspects of this complex oral disease.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

All the authors confirm that there are no conflicts of interest.

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Figure legends

Figure 1. Effect of zoledronic acid on NCTC 2544 cell proliferation and viability.

Cells were treated with zoledronic acid for 48 hours. Data are means ± S.D. from 4 experiments. For each panel, means with different letters are significantly different from one another ($p<0.05$) as determined by analysis of variance followed by *post-hoc* Newman-Keuls analysis.

C, control cells; 5, cells treated with 5 µM zoledronic acid; 50, cells treated with 50 µM zoledronic acid.

Figure 2. mRNA **c**ontent of IL-1β, TNF-α, PPARα and HMGR in NCTC 2544 cells treated or not with zoledronic acid for 48 hours.

Data are means ± S.D. from 4 experiments. For each parameter, means with different letters are significantly different from one another ($p<0.05$) as determined by analysis of variance followed by *post-hoc* Newman-Keuls analysis.

C, control cells; 5, cells treated with 5 µM zoledronic acid; 50, cells treated with 50 µM zoledronic acid.

Figure 3. Effect of medium conditioned by NCTC 2544 cells exposed or not to zoledronic acid, and of direct administration of zoledronic acid on proliferation and viability of MG-63 cells.

MG-63 cells were exposed to conditioned medium or to zoledronic acid for 48 hours. Data are means ± S.D. from 4 experiments. For each panel, means with different letters are

significantly different from one another ($p<0.05$) as determined by analysis of variance followed by *post-hoc* Newman-Keuls analysis.

C, MG-63 control cells; 5 CM, MG-63 cells grown in medium conditioned in presence of 5 µM zoledronic acid; 50, MG-63 cells grown in medium conditioned in presence of 50 µM zoledronic acid; 5 ZA, MG-63 cells directly exposed to 5 µM zoledronic acid.

Figure 4. mRNA content of inflammatory, osteogenic, and osteoclastogenic parameters in MG-63 cells grown in medium conditioned by NCTC 2544 cells exposed or not to zoledronic acid for 48 hours, and in MG-63 cells directly exposed to zoledronic acid.

Data are means ± S.D. from 4 experiments. For each parameter, means with different letters are significantly different from one another ($p<0.05$) as determined by analysis of variance followed by *post-hoc* Newman-Keuls analysis.

C, MG-63 control cells; 5 CM, MG-63 cells grown in medium conditioned in presence of 5 µM zoledronic acid; 50, MG-63 cells grown in medium conditioned in presence of 50 µM zoledronic acid; 5 ZA, MG-63 cells directly exposed to 5 µM zoledronic acid.

Figure 1

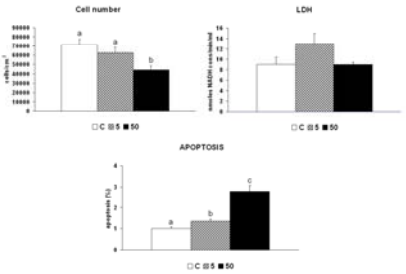

Figure 2

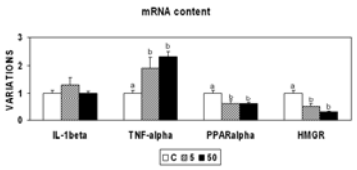

Figure 3

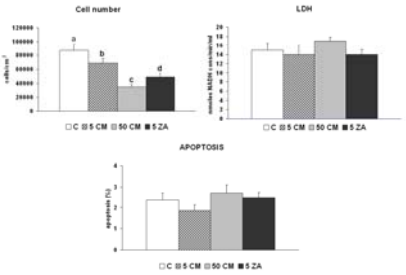
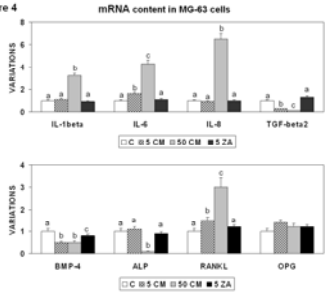
Figure 4