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Title Page

Article title: Effects of zoledronic acid and geranylgeraniol on angiogenic gene expression in primary human osteoclasts

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Abstract

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a serious complication associated with bisphosphonate treatment. Zoledronic acid (ZA) is a commonly used bisphosphonate due to its effectiveness in increasing bone density and reducing skeletal events, with evidence that it alters angiogenesis. **Objectives:** To determine the effects of ZA on angiogenic gene expression in primary human osteoclasts, and to investigate replacement of the mevalonate pathway using geranylgeraniol (GGOH). **Study design:** Osteoclast cultures were generated from peripheral blood mononuclear cells of three patients using the ACCUSPIN™ System-HISTOPAQUE and phenotyped by phase contrast microscopy, tartrate-resistant acid phosphatase staining, and pit assays. **Results:** Primary osteoclasts were found to express a number of key angiogenic molecules at very high levels. Gene expression levels for 84 human angiogenic factors were determined using PCR arrays. Three genes with significant fold regulation (FR) in response to ZA were: TNF (FR = +2.57, P = 0.050), CXCL9 (FR = +39.48, P = 0.028) and CXCL10 (FR = +18.52, P = 0.0009). The co-addition of geranylgeraniol with ZA resulted in the significant down-regulation of these three genes along with CCL2, TGFBR1, ENG, and CXCL1. **Conclusion:** GGOH reversed the gene changes induced by ZA and may offer a promising treatment for BRONJ.

Key words: zoledronic acid; geranylgeraniol; mevalonate pathway; osteoclasts; bisphosphonate-related osteonecrosis of the jaw

Introduction

Bisphosphonates are widely used to inhibit osteoclast activity in benign and malignant bone diseases such as osteoporosis, Paget's disease, multiple myeloma and some metastatic cancers (1, 2). The rationale for using bisphosphonates in these diseases is to increase bone mineral density, decrease the severity and incidence of skeletal complications, reduce metastatic bone pain and fracture risk, and improve the patient's quality of life (3). Bisphosphonate-related osteonecrosis of the jaw (BRONJ) has been described as a complicated side effect of long-term bisphosphonate therapy. There is currently a lack of specific evidence as to the pathogenesis of BRONJ, and that there are no definitively accepted treatments (4). The two most accepted hypotheses are the influence of bisphosphonates on angiogenesis and the inhibition of normal bone remodelling. Inhibition of bone remodelling is thought to lead to the accumulation of areas of micro-damage resulting in reduced mechanical strength and ultimately to areas of bone necrosis (5, 6). The most commonly accepted cellular target for bisphosphonates is osteoclasts and evidence suggests an important regulatory mechanism for nitrogen-containing bisphosphonates is to inhibit enzymes of the mevalonate pathway (MVP) (6, 7-9). Inhibition of specific enzymes of the MVP results in alterations to the process of protein prenylation, which is required for the post-translational maturation of a family of proteins including the small GTP-binding proteins (Ras, Rho, Rac, and Rab). These proteins play a critical role in cellular growth and differentiation, cytoskeletal reorganization, gene expression and membrane ruffling. The inhibition of these small GTPases may interfere with osteoclast functioning and thus contribute to BRONJ (5, 10). In order to develop an effective treatment strategy for BRONJ, a clearer understanding of the cellular and molecular responses of cells, when exposed to bisphosphonates, is necessary. The present study investigated the effects of the commonly used nitrogen containing bisphosphonate, zoledronic acid (ZA) on primary human osteoclasts.

The processes of osteoclastogenesis and angiogenesis are co-ordinated during physiological and pathological bone remodelling (11). The molecular signalling between these two processes is still largely unknown however several possible connections have been identified. Anatomically osteoclasts are located in close proximity to blood vessels within each bone-remodelling compartment, thus occupying an ideal position to signal blood vessels and promote angiogenesis (12). Tanaka et al, (2007) showed that osteoclasts could stimulate angiogenesis by direct secretion of pro-angiogenic factors such as VEGFA and osteopontin (11). Previous studies have also shown that osteoclasts secrete many different angiogenic factors, including VEGF, IL-8, osteopontin, bFGF, PDGF, angiopoietin-1 and angiopoietin-2, which could act directly on endothelial cells and stimulate angiogenesis (13). However, there are still only a limited number of studies demonstrating direct angiogenic signalling by osteoclasts. Yin et al (2011), presented the hypothesis that bisphosphonates may inhibit osteoclast generated stimulation of angiogenesis and thus play a role in BRONJ pathogenesis (14). At present there are no reports investigating this hypothesis.

Previously, Zafar et al (2014, 2016), have shown that ZA can alter the proliferation, migration and angiogenic gene expression of primary human gingival fibroblasts and primary human alveolar osteoblasts (8, 9). They have further shown that the replenishment of the MVP by geranylgeraniol (GGOH) was able to partially rescue angiogenic gene expression after ZA treatment. The aim of the present study was to examine the expression of angiogenic genes in primary human osteoclast and the effect of ZA alone and in combination with GGOH.

Materials and Methods

Establishment of human osteoclast cultures from peripheral blood mononuclear cells

Three human osteoclast cell cultures were generated from peripheral blood mononuclear cells (PBMC). Whole blood (50 mL) was collected from three healthy premenopausal females (aged 18-45 yrs). The study was approved by the Lower South Regional Ethics Committee (LRS/10/09/038) and all participants provided signed informed consent prior to blood collection. The osteoclasts were generated using the method described by Henriksen et al, (2012) and Agrawal et al, (2012) with minor modifications (15, 16). Briefly, whole blood was purified using the ACCUSPIN™ System-HISTOPAQUE®-1077 (Cat No. A0561, Sigma-Aldrich, St Louis, MO, USA). Twenty five mL of blood was layered over 15 mL of HISTOPAQUE®-1077 and centrifuged (RT, 800 g for 15 min). The lymphocyte and mononuclear cell layer in the HISTOPAQUE®-1077 was collected, washed with warm PBS (Cat No. SALB010, Invitrogen, Carlsbad, CA, USA; without $Ca2^+$ and $Mg2^+$), and centrifuged (RT, 150 g for 15 min). The cells were then plated in a 25 cm^2 cell culture flask (Cat No. GR690175, Greiner bio-one, Frickenhausen, Germany) with pre-warmed osteoclast expansion medium containing α-MEM with GlutaMAX, antibiotic-antimycotic reagent (100 unit penicillin, 100 µg/mL streptomycin, 250 ng/mL amphotericin B), 50 µg/mL gentamycin, 20 ng/mL M-CSF (R & D Systems, Minneapolis, MN, USA) and supplemented with 10% FBS (Gibco Invitrogen, Carlsbad, CA, USA). Once 80% confluence was achieved, the cells were transferred into a 75 cm^2 (T-75) cell culture flask (Cat No. GR658175, Greiner bio-one, Frickenhausen, Germany) and subsequently transferred into a 6 x 6 well plate (Cat No. 657160, Greiner bio-one, Frickenhausen, Germany) for the experiments. At this stage the medium was further supplemented with 25 ng/mL RANK-L as osteoclast growth medium (R & D Systems, Minneapolis, MN, USA). Cells were grown in 5% CO_2 /95% air.

Characterization of osteoclasts

The initial characterization of osteoclasts was carried out using phase-contrast microscopy, which allowed monitoring of the differentiation of the monocytes into mature osteoclasts over time. Tartrate-resistant acid phosphatase (TRAcP) activity, as an osteoclast specific marker, was determined using histochemical staining. The three patients osteoclasts were seeded into 8-well glass Lab-Tek II chamber slides (Cat No. 154534, Nunc, Thermofisher Scientific, Inc, USA) at a density of 1 x 10^5 cells/cm^2 (or 30,000 cells/well) in 0.4 mL/well of osteoclast growth medium containing both 20 ng/ml M-CSF and 25 ng/ml of RANK-L, and cultured for 14 days, with the medium changed twice weekly. Osteoclasts were allowed to attach for six hours and then the medium was refreshed and the cells cultured for three days. The culture medium was then removed and the cells were washed in PBS, and fixed in 10% neutral-buffered formalin (Cat No. BSPFS426.25, Thermofisher Scientific, Inc, USA) for 5 min at RT, followed by a final rinse with deionized water. The cells were stained according to the manufacturer's instructions using an Acid Phosphatase Leukocyte Kit (Cat No. 387A, Sigma-Aldrich, St Louis, MO, USA). Pit assays were conducted on 0.5 mm thick bovine cortical bone discs in 96 well plates. Osteoclasts were cultured for 14 days in the presence of 20 ng/ml M-CSF and 25 ng/ml of RANK-L. Pit enumeration was conducted with 5% sodium hypochlorite for 10 min followed by staining with 0.1% toluidine blue.

Gene expression assays

Gene expression assays were conducted using qRT^2-PCR technology. Osteoclasts (n=3) were seeded at a density of 1 x 10^5 cells/cm^2 in a 6 x 6 well plate and grown for 4 days in the presence of osteoclast expansion medium containing 20 ng/mL M-CSF to expand the osteoclast precursor population. The cell culture medium was replaced with osteoclast growth

medium containing both 20 ng/mL M-CSF and 25 ng/mL RANKL to generate osteoclasts which were then grown for 6 days with a medium change on the third day. Once the mature osteoclasts formed, fresh osteoclast growth medium/10% FBS containing either control (PBS) or treatment (30 µM ZA) was added. The effect of replacing the MVP was also examined with the addition of 30 µM ZA and 50 µM GGOH at the same time point. The timeline for the experimental assay is given in Fig. 1.

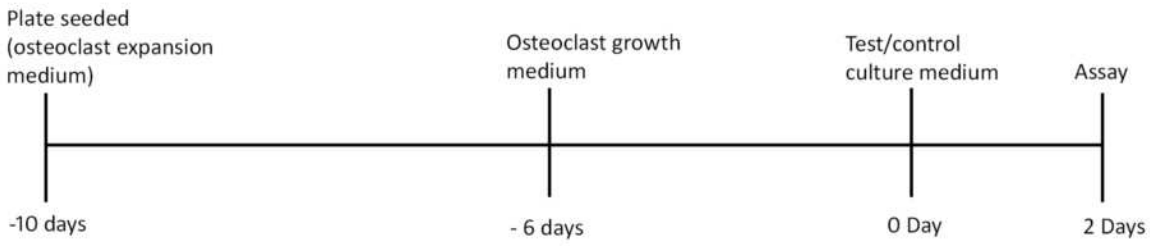

Fig. 1. Timeline of experimental assay.

Cellular morphological examination of osteoclasts

Osteoclasts cultured for gene expression assays were also examined for any morphological change in the presence of control (PBS), 30 µM ZA alone or in combination with 50 µM GGOH under an inverted microscope (Nikon Eclipse Ti-S microscope with TS-TCC5.0 ICE cooled camera, Coherent Scientific Ply. Ltd).

RNA extraction and quantitative real-time-PCR

RNA isolation was carried out using the phenol-chloroform extraction technique, and purified with a silica-based spin column using the Ambion Purelink^{TM} RNA Mini Kit (Cat No. 12183-018A, Applied Biosystems, CA, USA). TRIzol (500 µl) reagent (Cat No. 15596-026, Applied Biosystems, CA, USA) was added directly into the 6 x 6 well plates. The culture medium was

removed from the first well and TRIzol then added and incubated for 5 min while being pipetted repeatedly to assist in the cellular dissociation. The medium from the second well was removed and the TRIzol from the first well was transferred; this process was repeated until all the wells of each sample were processed. The RNA extraction was carried out using the manufacturer's protocol for the Ambion Purelink™ RNA Mini Kit**.** Genomic DNA contamination was removed using on-column PureLink DNase treatment (Ambion, Foster City, CA, USA). RNA concentration and purity were assessed using a NanoVue Spectrometer (GE Healthcare, Little Chalfont, UK). Total RNA (100-200 ng) was reverse transcribed using a RT^2 First Strand Kit (SABiosciences, Frederick, USA). Real-time assays were performed using the 96-well RT^2 Profiler™ PCR Array System (SA Biosciences Frederick, MD, USA) with the Human Angiogenesis Growth factor (PAHS-072) arrays. An ABI 7500 Real Fast PCR instrument (Applied Biosystems, Foster City, CA, USA) was utilized for thermal cycling.

Statistical analysis

Analysis for the gene assays was conducted using the raw quantification cycle (Cq) values of the tested genes normalized against the mean Cq of the reference genes. GeNorm software (Visual Basic Application applet for Microsoft Excel) was used to determine the most stable reference genes from a panel of five reference genes (ACTB, B2M, HPRT1, RPLPO and GAPDH). An M-value cut-off of 1.0 was used to select the most stable reference genes (B2M, HPRT1, RPLPO and GAPDH). Data were analyzed using the SABiosciences Microsoft Excel-based PCR Gene Data Analysis Template with Graphpad PRISM software (Version 6.00 for Windows, GraphPad Software, San Diego, CA, USA). Genes with mean Cq values of 34 or greater were excluded as being only minimally present. Fold regulation in gene expression was determined by comparison of the mean of the normalized expression

values, between test and control groups using the ΔΔCq method. Genes that showed statistically significant differences in expression between control and treatment (no treatment *vs* ZA; ZA *vs* ZA plus GGOH) were identified. The levels of statistical significance were determined using a T-test. Genes with a fold regulation of ± 2.0 and a $P \leq 0.05$ were considered to be significantly regulated.

Results

Phenotyping of primary human osteoclasts

Human peripheral blood mononuclear cells (PBMCs; n=3 patients) were incubated in the presence of RANK-L and M-CSF. Phase contrast microscopy revealed that initially the cells were small, spherical and mononuclear (Fig. 2A and 2Ai). After 7 days of culture the cells showed changes in morphology consistent with transformation into osteoclast-like cells. There were marked differences in their structure as compared to the PBMCs with an enlarged cytoplasm and the presence of multinucleate cells (Fig. 2B and 2Bi). These changes were more pronounced by day 14 (Fig. 2C and 2Ci) with very large multinucleate cells evident. All three primary human osteoclast cell cultures stained positively for the presence of TRAcP+ cells at 14-day of culture (Fig. 3) and pits were evident (data not shown).

Morphological examination of osteoclasts after treatment

RANK-L and M-CSF differentiated human osteoclasts were treated for 48 h with either 30 μM ZA alone or in conjunction with 50 μM GGOH and compared to control medium alone. The morphology of the osteoclasts cultured in control conditions showed very large multinucleate cells consistent with being osteoclasts (Fig. 4A and 4Ai). Osteoclasts cultured in the presence of 30 μm ZA for 48 h (Fig. 4B and 4Bi) were smaller and had a less defined

structure which were at times seen lifting from the plate. Osteoclasts that were cultured with 30 µM ZA and 50 µm GGOH had both large multinucleate cells as well as smaller less defined cells (Fig. 4C and 4Ci).

Relative qRT^2-PCR expression of angiogenic genes mRNA levels

The 84 genes tested in the angiogenic array where corrected for the levels of HGK and 70 genes (83%) had △Cq < 32.2 and were included in the future analysis. Higher viability was noted in expression in those genes with less RNA and thus higher △Cq values (Fig. 5). VEGFA, VEGFB and VEGFD were all expressed with VEGFB having the highest expression levels. The FLT1 and KDR receptors were both present however NRP1 and NPR2 receptors were very highly expressed. ENG also known as CD105 was present in high levels as were TGFB1, CXCL8, FN1 and HIF1A. The matrix metalloproteins all had △Cq < 25 indicative of osteoclast protease activity.

The treatment of the human osteoclasts with 30 µM ZA as compared to control conditions resulted in a trend towards up-regulation of genes (Fig. 6A). The significantly up-regulated genes include tumour necrosis factor (TNF; FR = +2.57, $P = 0.050$), C-X-C motif chemokine ligand 9 (CXCL9; FR = +39.48, $P = 0.028$) and CXCL10 (FR = +18.52, $P = 0.0009$). To assess if GGOH could reverse the effects of ZA, osteoclast cells were treated with 30 µM ZA and 50 µm GGOH and compared to those treated with 30 µM ZA alone. Genes were both up- and down-regulated in response to the GGOH however only seven genes were significantly regulated and these were all down-regulated (Fig. 6B). The three previously significantly up-regulate genes (ZA compared to control), were now down-regulated with GGOH. TNF was down-regulated FR = -3.68 with a $P = 0.081$ approaching significance, while CXCL9 (FR = -12.11, $P = 0.036$) and CXCL10 (FR = -13.06, $P = 0.003$) were significantly down-regulated.

Other down-regulated genes included CCL2, CXCL1, TNF, ENG, and TGFBR1. Two pro-angiogenic molecules VEGFA (FR = 3.76, P = 0.074) and ANGPTL4 (FR =14.04, P = 0.076) were up-regulated with *P*-values approaching significance.

When the gene response of ZA treated cells as compared to control were compared to the ZA+ GGOH cells, seven genes where identified as significant in primary human osteoclasts (Fig. 7).

Discussion

Osteoclasts are known to be an important cellular target for bisphosphonates when used clinically to increase bone density. Nitrogen containing bisphosphonates such as ZA act by inhibiting enzymes of the MVP in osteoclasts (6-8). The prenylation of proteins as a result of activation of the MVP plays a critical role in cellular growth and differentiation, cytoskeletal reorganization, gene expression and membrane ruffling (10). The inhibition of these small GTPases can then interfere with osteoclast cell functioning, which may play a role in BRONJ (5). The present study investigated the effects of 30 μM ZA alone, and with 50 μM GGOH as a potential reversal agent, on angiogenic gene expression in human osteoclasts derived from the monocyte fraction of peripheral blood. Currently, there are only a limited number of investigations into the effects of bisphosphonates on gene expression using cell culture models (8, 9, 17, 18). Nakagawa et al, (2015) carried out a microarray analysis in order to identify the molecular targets of ZA in the RANKL signalling pathway, and factors associated with osteoclastogenesis using osteoclast precursor cells (purchased from the Primary Cell Co., Ltd. Sapporo, Japan) (19). The results of their microarray analysis identified two genes, nuclear factor of activated T-cell c1 (NFATc1) and carbonic anhydrase 2 (CAII) that were significantly down-regulated in the presence of ZA and restored by GGOH. The present study

focused on the reported antiangiogenic effects of ZA and the potential of GGOH to restore angiogenic gene functioning (6). The use of primary human osteoclasts differentiated from blood monocytes collected from three participants, was in contrast with recent studies that used osteoclasts derived from mice to investigate the effect of ZA (19, 20, 21). To the authors knowledge, this is the first cell culture study that has utilized a primary human osteoclast cell culture model to determine the effects of ZA on angiogenic gene expression.

Osteoclasts were confirmed as large multinuclear TRAcP+ cells *in vitro*. CXCL10, CXCL9 and TNF were all up-regulated in response to ZA as compared to control and then down-regulated with GOOH. TNF is an inflammatory cytokine generally considered to be antiangiogenic *in vitro* with continuous administration but with potential angiogenic properties with pulsatile administration (22). In this study TNF mRNA was present in a moderate level, which was up-regulated in response to ZA and corrected with GGOH. It has also been identified that TNF up-regulation is a primary pathway contributing to CXCL10 production (23). Fold regulation changes were highest and most significant for CXCL10 and CXCL9 genes, which increased significantly from the low levels present in control osteoclasts. These CXC molecules have been shown to have pro-inflammatory and anti-angiogenic properties (24, 25). Furthermore, there are indications that CXCL9 also plays an important role in the repair of tissue damage (26). CXCL10 and CXCL9 share CXCR3 as a common receptor for their activities. When CXCL10 binds to the CXCR3 receptor, it mediates immune responses which initiate activation and recruitment of leukocytes including T cells, eosinophils, monocytes and NK cells (27). CXCL10 is also been identified along with RANKL as supporting osteoclast differentiation (28). These results suggest that the increased expression of CXCL10 and CXCL9 mRNA may result in further enhancement of the anti-angiogenic properties of ZA thus interfering with wound healing or repair of tissue. CXCL1,

another gene from the same family was significantly down-regulated (in the presence of ZA and GGOH compared with ZA alone). ZA alone did not cause any statistically significant difference in the regulation of this gene. In summary the regulation of TNF and chemokine (C-X-C motif) ligands may represent a pathway which is important during osteoclast ZA treatment and recovery.

Endoglin is a cell adhesion molecule which is highly expressed on vascular endothelial cells and is also found in bone marrow pro-erythroblasts, activated monocytes, mesenchymal stem cells, and leukemic cells of lymphoid and myeloid lineages. Higher expression of ENG has been reported in actively proliferating endothelial cells during angiogenesis and neo-vascularization. ENG mRNA is known to be present in high amounts in adherent monocytes *in vitro* as well as in endothelial cells (29). Decreased expression of ENG has been associated with abnormal angiogenesis *in vitro* and atypical vascular development and function *in vivo* (30-32). In the present study, ENG was highly expressed in the osteoclasts and the treatment with ZA did not significantly affect the expression of ENG, however the simultaneous addition of ZA and GGOH resulted in significant down-regulation of ENG mRNA levels.

CCL2 is a chemotactic factor for monocytes and its expression is important for the formation of osteoclasts (33). In osteoclasts CCL2 was significantly down-regulation in response to GGOH while previous research on osteoblasts found it was significantly up-regulated in response to GGOH (9). This differing regulation may be important in the control of bone formation and turnover.

An interesting finding of this research was the high production of some angiogenic associated genes in the primary osteoclasts. HIF1A was highly expressed even though the cells were

cultured in normoxic conditions. As a major regulator of angiogenesis HIF1A has also been identified as essential for osteoclast formation and bone resorption (34). VEGFB was present at higher levels than VEGFA suggesting a possible role in osteoclasts and their associated bone resorption. NRP1 and NRP2 are co-receptors and bind the VEGF family and their up-regulation in osteoclasts *in vitro* and *in vivo* has been described (35). These transcripts, present in abundant quantities, provide new information about potential angiogenic pathways regulated by primary osteoclasts.

This is the first paper to report on the effects of ZA and GGOH on angiogenic gene regulation in primary human osteoclasts, as one of the leading cell types associated with BRONJ. The results show that the potent anti-angiogenic ligands, CXCL9 and CXCL10 are up-regulated in response to ZA in osteoclasts. The present data supports the concept that long-term exposure to ZA causes angiogenic suppression through up-regulation of CXCL9 and CXCL10 mRNA, which may lead to BRONJ. However, the present study only investigated mRNA levels and further research to analyze CXCL9 and CXCL10 protein expression in response to ZA is warranted. Future studies on primary human osteoclasts examining changes in gene expression as a result of ZA and GGOH treatment with extended timepoints at lower doses would add to the data presented here. Also, further investigation of the prenylation levels of selected small GTP-binding proteins would provide insights into the MVP proteins affected in BRONJ. In summary the present study utilized an *in vitro* model with primary human osteoclasts and identified the regulation of angiogenic genes in response to ZA and the potential of GGOH to reverse these effects.

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