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. Replacement of the mevalonate pathway using geranylgeraniol (GGOH) was studied to determine the effects of ZA on angiogenic gene expression in primary human osteoclasts. Osteoclast cultures were generated from peripheral blood mononuclear cells of three patients using the peripheral blood mononuclear cell isolation. These cells were phenotyped by phase-contract microscopy, trarate-resistant acid phosphatase staining, and pit assays. 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 as follows: tumor necrosis factor (FR = +2.57, P = 0.005), CXCL9 (FR = +39.48, P = 0.028), and CXCL10 (FR = +18.52, P = 0.0099). The co-addition of geranylgeraniol with ZA resulted in the significant down-regulation of these three genes along with CCL2, TGFBR1, and in combination with GGOH. Subsequently, they showed that the replenishment of the MVP by geranylgeraniol during physiological and pathological bone remodeling [11]. The molecular signaling 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-remodeling 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 that have demonstrated direct angiogenic signaling 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) showed that ZA can alter the proliferation, migration and angiogenic gene expression of primary human gingival fibroblasts and primary human alveolar osteoblasts [8,9]. Subsequently, they showed 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 osteoclasts 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 (PBMCs). Whole blood (50 mL) was collected from three healthy premenopausal females (aged 18-45 years). 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 [Henriksen et al., Bone Research Protocols, Methods in Molecular Biology, 159-175, Humana Press; Helfrich, MH, 2012; Agrawal et al., Methods in Molecular Biology, 357-375, Humana Press, 2012]. Briefly, whole blood was purified using the ACCUSPIN System-HISTOPAQUE-1077 (Cat No. A0561, Sigma-Aldrich, St Louis, MO, USA). Twenty-five milli-
liliters of blood were layered over 15 mL of HISTOPAQUE-1077 and then 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 then centrifuged (RT, 150 g for 15 min). Next, the cells were plated in a 25 cm² 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). Once 80% confluence was achieved, the cells were transferred into a 75 cm² (T-75) culture flask (Cat No. GR658175, Greiner bio-one) and subsequently transferred into a 6 × 6 well plate (Cat No. 657160, Greiner bio-one) for the experiments. At this stage, the medium was further supplemented with 25 ng/mL RANK-L as the osteoclast growth medium (R & D Systems). Cells were grown in 5% CO₂, 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 osteoclasts from three patients were seeded into 8-well glass Lab-Tek II chamber slides (Cat No. 154553, Nunc, Thermo Fisher Scientific, Waltham, MA, USA) at a density of 1 × 10⁴ cells/cm² (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 were cultured for 14 days, with the medium changed twice weekly. Osteoclasts were allowed to attach for 6 h and then the medium was refreshed and the cells were 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. BSPIFS426.25, Thermo Fisher Scientific) 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). Pit assays were conducted on 0.5 mm thick bovine cortical bone disks 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 aRT²-PCR technology. Osteoclasts (n = 3) were seeded at a density of 1 × 10⁴ cells/cm² in a 6 × 6 well plate and grown for 4 days in the presence of an 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 using 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.

**Cellular morphological examination of osteoclasts**

Osteoclasts cultured for gene expression assays were also examined under an inverted microscope (Nikon Eclipse Ti-S microscope with TS-TC5.0 ICE cooled camera, Coherent Scientific Pty. Ltd) for any morphological changes in the presence of the control (PBS), 30 µM ZA alone or 30 µM ZA in combination with 50 µM GGOH.

**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 RNA Mini Kit (Cat No. 12183-018A, Applied Biosystems, Foster City, CA, USA). TRIzol (500 mL) reagent (Cat No. 15596-026, Applied Biosystems) was added directly into the 6 × 6 well plates. The culture medium was removed from the first well and TRIzol was 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. 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, Waltham, MA, USA). The 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 RT² First Strand Kit (SABiosciences, Frederick, MD, USA). Real-time assays were performed using the 96-well RT² Profiler PCR Array System (SABiosciences) with the Human Angiogenesis Growth factor (PAHS-072) arrays. An ABI 7500 Real Fast PCR instrument (Applied Biosystems) was utilized for thermal cycling.

**Statistical analysis**

Analysis of 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 and the ∆∆Cq 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 FR of ±2.0 and a P ≤ 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, Ai). 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, Bi). These changes were more pronounced by day 14 (Fig. 2C, Ci) with very large multinucleate cells evident. All three primary human osteoclast cell cultures stained positively for the presence of TRAcP⁺ cells at 14 days of culture (Fig. 3) and pits were evident (data not shown).
Morphological examination of osteoclasts after treatment
RANKL 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 were compared to the control medium alone. The morphology of the osteoclasts cultured in control conditions showed very large multinucleate cells that were consistent with being osteoclasts (Fig. 4A, Ai). Osteoclasts cultured in the presence of 30 μM ZA for 48 h (Fig. 4B, Bi) were smaller and had a less defined structure and 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 and smaller less defined cells (Fig. 4C, Ci).

Relative qRT²-PCR expression of angiogenic gene mRNA levels
The 84 genes tested in the angiogenic array were corrected for the levels of HGK and 70 genes (83%) had ΔCq < 32.2 and were included in the subsequent analysis. Higher viability was noted in the expression of those genes with less RNA and thus there were higher ΔCq 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 NRP2 receptors were expressed at very high levels. ENG, also known as CD105, was present in high levels as were TGFB1, CXCL8, FN1, and HIF1A. The matrix metalloproteins all had ΔCq < 25, which was indicative of osteoclast protease activity.

The treatment of the human osteoclasts with 30 μM ZA as compared to control conditions resulted in a trend toward up-regulation of the genes (Fig. 6A). The significantly up-regulated genes included tumor necrosis factor (TNF; FR = +2.57, P = 0.050), C-X-C motif chemokine

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Fig. 2 Phase-contrast microscopy showing the cellular morphology of primary human osteoclasts. (A and Ai) Day 0: Cells were small, spherical, and mononuclear. (B and Bi) Day 7: cells were more dispersed and the cytoplasm was enlarged, cells tended to cluster in small groups and were beginning to fuse. (C and Ci) Day 14: cells were larger, multinucleated, and displayed an osteoclast-like appearance. Magnification: (A, B, C) scale bar = 200 μm; (Ai, Bi, Ci) scale bar = 100 μm

Fig. 3 TRAcP-stained osteoclasts. Cells were cultured with M-CSF and RANKL for 14 days and were TRAcP stained: (A) scale bar = 200 μm; (Ai) scale bar = 100 μm

Fig. 4 Cellular morphology of primary osteoclasts (M-CSF and RANKL treated) under phase-contrast microscopy after 48 h of treatment/control conditions. (A & Ai) control cells: cells were large, multinucleated, and display an osteoclast-like appearance. (B & Bi) cells in the presence of 30 μM ZA: cells were smaller and with less evidence of an osteoclast-like morphology. (C & Ci) cells with 30 μM ZA and 50 μM GGOH: cells were larger, multinucleated, and displayed an osteoclast-like appearance. Magnification (A, B and C): scale bar = 200 μm; (Ai, Bi & Ci): scale bar = 100 μm

Fig. 5 qRT²-PCR gene expression levels. The ΔCq values for control (48 h of media) gene expression levels in primary osteoclast expression (M-CSF and RANKL treated) when corrected for the housekeeping gene. Highly expressed genes have a ΔCq < 20 and low expressing genes have a ΔCq >32.5. (A) Angiogenic genes and receptors. (B) Angiogenic related genes. Mean ± SD
ligand 9 (CXCL9; FR = +39.48, P = 0.028) and CXCL10 (FR = +18.52, P = 0.0009). To assess whether GGOH could reverse the effects of ZA, osteoclast cells were treated with 30 μM ZA and 50 μM GGOH and were compared with 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-regulated genes (ZA compared to the 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 responses of ZA treated cells as compared to the control were compared to the ZA+ GGOH cells, seven genes were identified as significantly different in primary human osteoclasts (Fig. 7).

**Discussion**

Osteoclasts are known to be an important cellular target for bisphosphonates when used to increase bone density clinically. Nitrogen-containing bisphosphonates, such as ZA, act by inhibiting enzymes of the MVP in osteoclasts [6-8]. The preymlation 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 [6]. 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 have been only a limited number of investigations into the effects of bisphosphonates on gene expression using cell culture models [8,9,15,16]. Nakagawa et al. (2015) carried out a microarray analysis to identify the molecular targets of ZA in the RANKL signaling pathway and factors associated with osteoclastogenesis using osteoclast precursor cells (purchased from the Primary Cell Co., Ltd. Sapporo, Japan) [17]. 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 [17-19]. To the authors’ best knowledge, this is the first cell culture study that 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 the control and then down-regulated with GGOH. TNF is an inflammatory cytokine generally considered to be antiangiogenic in vitro with continuous administration but has potential angiogenic properties with pulsatile administration [20]. In this study, TNF mRNA was present at 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 [21]. FR 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 antiangiogenic properties [22,23]. Furthermore, there are indications that CXCL9 also plays an important role in the repair of tissue damage [24]. 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 [25]. CXCL10 has also been identified, along with RANKL, as supporting osteoclast differentiation [26]. These results suggested that the increased expression of CXCL10 and CXCL9 mRNA may result in further enhancement of the antiangiogenic properties of ZA, thus interfering with wound healing or the 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 that is important during osteoclast ZA treatment and recovery.

Endoglin is a cell adhesion molecule that is highly expressed on vascular endothelial cells and it 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 to actively proliferate endothelial cells during angiogenesis and neo-

**Fig. 6** (A) Scatter plot of gene regulation in response to 48 h of 30 μM zoledronic acid (ZA) treatment as compared to the control medium. (B) Scatter plot of gene regulation in response to 48 h of 30 μM zoledronic acid (ZA) with 50 μM GGOH treatment as compared to 30 μM zoledronic acid (ZA) as the control. These genes with FR ± 2 and P < 0.05 are shown as statistically significantly up-regulated (green) and down-regulated (red) genes.

**Fig. 7** Significantly regulated genes are shown and their responses to 30 μM zoledronic acid (ZA) or 30 μM zoledronic acid (ZA) with 50 μM GGOH treatment for 48 h were compared.
vascularization. ENG mRNA is known to be present in high amounts in adherent monocytes in vitro as well as in endothelial cells [27]. Decreased expression of ENG has been associated with abnormal angiogenesis in vitro and atypical vascular development and function in vivo [28-30]. In the present study, ENG was highly expressed in the osteoclasts and 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 [31]. In osteoclasts, CCL2 is significantly down-regulated in response to GGOH, while previous research on osteoblasts showed 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 [32]. 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 [33]. These transcripts, present in abundant quantities, provide new information about potential angiogenic pathways regulated by primary osteoclasts.

This is the first 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 showed that the potent angiogenic ligands, CXCL9 and CXCL10, are up-regulated in response to ZA in osteoclasts. The present data supports the concept that the 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 time points 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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Conflict of interest

None declared.

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