1. Introduction

Bisphosphonates (BPs) such as zoledronic acid (ZA: Zometa) are widely used to treat complications of bony metastases in cancer patients. A serious adverse event occurs in 1–12% of patients on BP therapy, osteonecrosis of the jaw (BPONJ). BPONJ develops after oral trauma and is manifested by poor wound healing and soft-tissue breakdown followed by exposure and necrosis of intra-oral bone. Currently, there is no effective clinical treatment for BPONJ.

Abstract

Objective: Bisphosphonates (BPs) like Zometa (ZA) are widely used to treat complications of bony metastases in cancer patients. A serious adverse event occurs in 1–12% of patients on BP therapy, osteonecrosis of the jaw (BPONJ). BPONJ develops after oral trauma and is manifested by poor wound healing and soft-tissue breakdown followed by exposure and necrosis of intra-oral bone. Currently, there is no effective clinical treatment for BPONJ.

Design: We evaluated the effect of ZA on the proliferation, apoptosis and migratory capacity of the cell lines CRL-7408, an oral fibroblast culture and OKF6, an oral epithelial cell line.

Results: In both oral epithelium and fibroblasts, ZA exposure inhibited proliferation and elevated apoptosis; however oral fibroblasts were differentially influenced versus oral epithelial cells. In oral fibroblasts, ZA treatment significantly inhibited motility. Further, quantitative real-time PCR demonstrated that ZA treatment of oral fibroblasts inhibits expression of both the COL1A1 and COL1A2 chains of type-I collagen, consistent with a loss of collagen immunofluorescent staining.

Conclusions: These data support a model wherein ZA treatment impedes oral wound healing by blocking the growth and migratory capacity of oral fibroblasts as well as downregulating the transcription of type-I collagen, functions necessary to deposit the granulation tissue needed for re-epithelization. Therefore, BP released from bone following tooth extraction may delay wound healing of the oral mucosal barrier and contribute to BPONJ pathogenesis.
painful condition that affects 1–12% of the patients receiving IV BP therapy.5,6 BPONJ is manifested by poor oral wound healing (particularly following invasive dental procedures), oral soft-tissue breakdown, and exposure of the underlying intra-oral bone, culminating in necrosis of the exposed bone. A working definition of BPONJ has been standardized by the American Association of Oral and Maxillofacial Surgeons and includes prior or current treatment with a BPs followed by persistent (>8 weeks) exposed necrotic bone in the maxillomandibular region, in the absence of radiation therapy to the jaws.1 Early stages of BPONJ are difficult to note via conventional radiography, which will not detect alterations until 30–50% of the bone is demineralized.1,12 BPONJ patients present with exposed necrotic bone, sites ranging in size from mm to cm, often with surrounding inflammation and pain. Systemic antibiotic treatment and/or an oral antimicrobial rinse are used in patients with asymptomatic exposed bone.50 As debridement is usually unsuccessful and carries the potential for further exposure of healthy bone, a conservative palliative approach is often recommended.10 Currently there is no highly effective clinical treatment for advanced BPONJ, and management may be limited to analgesia and control of disease progression, as complete healing may never occur.10

Presently, as the underlying pathophysiology of BPONJ is uncharacterized, there is a significant gap in our knowledge of the aetiology of BPONJ disease. To evaluate the role of BP therapy on oral wound healing, the current study evaluated the effect of ZA on the proliferation, apoptosis and migratory capacity of CRL-7408, an oral fibroblast cell line and OKF/6, a cell line derived from the oral epithelium. These results suggest a mechanism wherein ZA may affect oral wound healing via the inhibition of interstitial collagen synthesis by oral fibroblasts. In addition to providing an integrative analysis of the effects of BPs on repair and remodelling of oral soft tissues, ultimately we aim to identify risk factors and novel treatment strategies related to the initiation and progression of BPONJ.

2. Materials and methods

2.1. Cell cultures and treatments

Human oral fibroblasts (CRL-7408) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% foetal bovine serum, 100 unit/ml penicillin and 100 μg/ml streptomycin. Human oral epithelial cells (tert-immortalized) were the generous gift of Dr. James Rheinwald of Harvard University (Boston, MA). These OKF/6 cells were cultured in keratinocyte-SFM (Gibco, Carlsbad, CA) supplemented with 25 μg/ml bovine pituitary extract, 0.2 ng/ml rEGF, 0.4 mM CaCl2, 100 unit/ml penicillin and 100 μg/ml streptomycin. All oral cells were maintained at 37 °C in a humidified, 5% CO2 atmosphere. Zoledronic acid (Zometa, ZA) was obtained from Novartis (Basel, Switzerland) and diluted in a 0.9% NaCl infusion solution. Then, this mixture was placed in culture media at final concentrations of 5, 10, 30, 50 75, 100 and 300 μM. Oral epithelial cells and fibroblasts were treated with ZA at concentrations ranging from 0–100 μM for the indicated times.6,15 Control cells were incubated with vehicle only (0.9% NaCl).

2.2. Cell viability

Cells were seeded in 96-well plates with 3000 cells/well. After growing to 80% confluency, cells were treated with ZA or 0.9% NaCl vehicle for 24 h. CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay MTS kit (Promega, Fitchburg, WI) was used according to the manufacturer’s protocol. All experiments were repeated in triplicate.

2.3. Cell apoptosis assay

Cells were treated with either ZA or 0.9% NaCl vehicle for 48–66 h. Apoptotic cells were identified by staining with the annexin V–FITC apoptosis detection kit (BioVision, Mountain View, CA). Briefly, the cells were washed three times with phosphate-buffered saline and incubated with annexin V–FITC and propidium iodide (PI) for 5 min in the dark at room temperature. Annexin V–FITC binding was analysed using a FACScan cytometer using FITC signal detector FL1 and PI staining by the phycoerythrin emission signal detector FL2. Histograms show FITC staining of propidium iodide-negative cells. All experiments were repeated in triplicate.

2.4. Scratch wound healing

Scratch wounds were generated in confluent monolayers of cells using a sterile 200 μl pipette tip.16,17 After washing away suspended cells with PBS, the cells were cultured in serum-free medium in the presence of ZA (10 μM) or 0.9% NaCl alone. Migration into the wound space was photographed using an inverted microscope equipped with a digital camera at the time of the initial wound and at time intervals up to 66 h post wounding. The relative distances between edges of the injured monolayer were obtained via pixel counts at a minimum of ten sites/wound using NIH Image J software, with time-controlled

<table>
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<tr>
<th>Target gene</th>
<th>Tm (°C)</th>
<th>Primer sequences</th>
<th>Amplicon size</th>
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| Type-I collagen pro alpha (COL1A1) | 57.9    | Forward: 5’-GAACCGGTGTCATCCCTTG-3’  
Reverse: 5’-GAACGGGTAGTCCTTCAGCACA-3’ | 94 bp         |
| Alpha-2 collagen Type-I (COL1A2) | 55.0    | Forward: 5’-AGTCTGCTGTTGCTTTGCT-3’  
Reverse: 5’-GCCCTGCTCACTCTTCCCTCGA-3’ | 114 bp        |
| GAPDH                     | 58.3    | Forward: 5’-GACAGGCACTGGTCTGGCATGAG-3’  
Reverse: 5’-GACAGGCACTGGTCTGGCATGAG-3’ | 87 bp         |
comparisons analysed via non-parametric ANOVA ($p < 0.05$). All experiments were repeated in triplicate.

2.5. Real-time PCR

Fibroblasts (CRL-7408) were treated with either ZA (10 $\mu$M) or 0.9% NaCl in serum-free medium for 48 h. Total RNA was extracted with the RNeasy Mini Kit (QIAGEN, Venlo, Netherlands). Following the isolation of total RNA, synthesis of cDNA from genomic RNA was carried out immediately using the RT$^2$ First Strand Kit (SABiosciences, Venlo, Netherlands). Two specific primer pairs (type-I collagen protalpha F/R-11089 and alpha-2 collagen type-I F/R-10268) and the housekeeping gene GAPDH primer set (F/R-227) sequences were retrieved from a quantitative PCR primer database.$^{18}$ The sequences of all primers and the real time PCR amplification parameters are available in Table 1.$^{18}$ Real-time PCR was carried out in a final volume of 25 $\mu$l, containing 200 nM of each primer set and 5 $\mu$l of cDNA of type-I collagen (1 $\mu$l of GAPDH cDNA) in a 2× RT$^2$ SYBR green mix (SABiosciences). Following an initial 10 min denaturization/activation step at 95 $^\circ$C, the mixture was subjected to 50 cycles of amplification (denaturation for 15 s at 95 $^\circ$C, annealing and extension for 40 s at 60 $^\circ$C), in an iCycler IQ real-time detection system (Bio-Rad, Hercules, CA). Relative quantification through $2^{-\Delta\Delta CT}$ was used$^{19}$ in this study to estimate the relative changes in mRNA levels of type-I collagen subunits in fibroblasts cultured with or without ZA. Data normalization was carried out versus the transcript of the housekeeping gene GAPDH. A T-test was used to determine if the gene expression was significantly different. All reactions were performed in triplicate and repeated on separate occasions using biologic triplicates.

2.6. Immunofluorescent staining

Fibroblasts (CRL-7408) were seeded on a glass coverslip in each well of a 6-well plate to 70–80% confluence prior to treatment with ZA (10 $\mu$M) or 0.9% NaCl in serum-free medium for 48 h.

![Fig. 1 – Effect of ZA on proliferation of oral cells. Cells were cultured for 48 h in the presence of increasing concentrations of ZA or vehicle control, as indicated. Proliferation was assayed using an MTS-based proliferation assay as described in Section 2. Results are presented as proliferation relative to vehicle-treated control cells. (A) OKF/6 oral epithelial cells; (B) CRL-7408 oral fibroblasts. Compared to controls, MTS assays indicated a dose-dependent inhibition of proliferation in epithelial and fibroblast cultured cells incubated with ZA. This response is more marked in oral fibroblasts. Data represent mean ± SEM. *$p < .05$; **$p < .005$.](image1.png)

![Fig. 2 – Effect of ZA on apoptosis of oral cells. Cells were cultured for 48 h in the presence of increasing concentrations of ZA or vehicle control, as indicated. Apoptosis was evaluated by flow cytometry using annexin V staining as described in Section 2. Histograms show FITC staining of propidium iodide-negative cells. Mean percentages of apoptotic cells are shown relative to untreated controls. (A) OKF/6 oral epithelial cells; (B) CRL-7408 oral fibroblasts. A dose-dependent increase in apoptosis of oral epithelial and fibroblast cultured cells incubated with ZA is observed. This response is much more pronounced in oral fibroblasts. Data represent mean ± SEM. *$p < .05$; **$p < .005$.](image2.png)
The conditioned medium was collected for a later gelatin zymography experiment, and the cells were fixed in 4% paraformaldehyde in PBS containing 0.12 M sucrose for 20 min, blocked with 10% BSA for 1 h, then incubated with anti-collagen type-I (sc-80565, Santa Cruz Biotechnology, Santa Cruz, CA) mouse monoclonal antibody 1:1 dilution in 1% BSA for 1 h. After washing, cells were incubated for 30 min with a secondary antibody (1:500 in 1% BSA) conjugated to a fluorescence probe (Alexa Fluor 488, Invitrogen, Carlsbad, CA). All such procedures were performed at room temperature. The coverslip was then mounted on a slide with mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) and stored at 4°C overnight. The cells were visualized using an Olympus IX-81 inverted spinning disc confocal microscope.

2.7. Gelatin zymography

Conditioned medium collected from the immunofluorescent staining experiment was electrophoresed under non-reducing, non-denaturing conditions on 8% SDS-polyacrylamide gels containing 0.12% gelatin at 100 V for 2 h at room temperature. The gels were removed and incubated in 2.5% Triton X-100 buffer for 30 min with two changes. Following washing three times with DI water, the gels were incubated with Zymo buffer (20 mM glycine, 10 μM CaCl₂, 1 μM ZnCl₂, pH 8.3) for 24–48 h at 37°C, and then were stained with Coomassie Blue. Following electrophoresis, gels were washed and incubated at 37°C for 24 h prior to Coomassie staining. Lane: (1, 2) conditioned medium from control oral fibroblasts; (3, 4) conditioned medium from ZA-treated fibroblasts; (5) MMP standard containing pro-MMP2 (arrow) and active MMP-2 (arrowhead). Results indicate no apparent differences in proMMP-2 expression and/or activation between control and ZA-treated fibroblasts.

Fig. 3 – Effect of ZA on in vitro wound healing. Scratch wounds were generated in confluent monolayers of (A, B) OKF/6 oral epithelial cells or (C, D) CRL-7408 oral fibroblasts. Cells were then cultured in the presence of 10 μM ZA or vehicle control. Cultures were photographed at time zero and at time intervals as indicated. Results are presented as the % of wound closure relative to the original wound width. Versus controls, scratch-wound analyses indicated that oral cells cultured with ZA differ significantly in the rate of wound closure. Oral epithelial cells exhibited a higher rate, whilst oral fibroblasts showed a slower rate of wound closure. Data represent mean ± SEM. *p < .05; **p < .005.

Fig. 4 – Gelatin zymography of conditioned medium from ZA-treated oral fibroblasts. CRL-7408 cells were cultured for 48 h in the presence or absence of 10 μM ZA as indicated prior to electrophoresis under non-reducing, non-denaturing conditions on 8% SDS-polyacrylamide gels containing 0.12% co-polymerized gelatin. Following electrophoresis, gels were washed and incubated at 37°C for 24 h prior to Coomassie staining. Lane: (1, 2) conditioned medium from control oral fibroblasts; (3, 4) conditioned medium from ZA-treated fibroblasts; (5) MMP standard containing pro-MMP2 (arrow) and active MMP-2 (arrowhead). Results indicate no apparent differences in proMMP-2 expression and/or activation between control and ZA-treated fibroblasts.
blue for 30 min and distained for about 1 h. Gelatinolytic MMP activity was observed as clear zones of lysis in the gel.

3. Results

3.1. Effect of ZA on proliferation and apoptosis of oral cells

To examine the potential consequences of bisphosphonate treatment on cell populations in the oral cavity, the effect of ZA treatment on oral epithelial cells (OKF/6) and oral fibroblasts (CRL-7408) was evaluated. Cells were cultured for 24 h in the presence of increasing concentrations of ZA (0–100 μM) followed by analysis of viability/proliferation using a MTS assay. Significant toxicity was observed with ZA concentrations as low as 10 μM (Fig. 1A and B). Oral fibroblasts were more susceptible than oral epithelial cells to ZA toxicity, exhibiting an IC₅₀ of approximately 35 μM relative to approximately 90 μM for epithelial cells. Using annexin V exposure as an indicator of apoptotic cells, ZA treatment at concentrations up to 100 μM induced minimal apoptosis in oral epithelial cells, i.e., <10% of cells (Fig. 2A). In contrast, significant apoptosis was induced in oral fibroblasts treated with 30–100 μM ZA (Fig. 2B).

3.2. Effect of ZA on in vitro oral wound healing

Based on these results, the effect of ZA on healing of oral wounds was investigated in vitro. Cells were cultured to confluence, monolayers were wounded with a pipette tip, and the rate of wound closure was determined in the presence and absence of ZA. Oral epithelial cells rapidly migrated to close the wound, with complete closure observed by 48 h. ZA treatment did not delay OKF/6 cell migration, but rather resulted in an enhanced rate of wound closure relative to untreated cells (Fig. 3A and B). In contrast, wounds created in oral fibroblast cultures closed more slowly (72 h) and wound closure was significantly delayed in CRL-7408 cells by ZA treatment (Fig. 3C and D).

Wound healing in vivo requires the deposition of collagen to stimulate re-epithelialization. To determine whether ZA treatment may induce the expression of collagen-degrading matrix metalloproteinases (MMPs), gelatin zymography was used to visualize proteolytic activity in conditioned medium. No evidence for expression of MMP-1 was obtained.
even after 48 h incubation of zymograms prior to staining (not shown). Both ZA-treated and control fibroblasts expressed pro-MMP-2. However, no differences in relative expression or zymogen activation (potentially indicative of MMP-14 activity) were evident (Fig. 4).

As these data suggest that rates of collagen breakdown were unaltered by ZA treatment, additional experiments evaluated collagen synthesis. Oral fibroblasts were exposed to ZA for 48 h followed by analysis of type-I collagen subunit mRNA expression by qPCR. A significant inhibition of collagen expression was observed (Fig. 5A), as evidenced by a 70% decrease in COL1A1 and a 60% decrease in COL1A2 expression levels, respectively. Loss of collagen deposition was confirmed by immunofluorescence microscopy. Whilst vehicle-treated cells clearly show deposition of collagen at the basal surface, collagen staining is not evident in ZA-treated cells (Fig. 5B).

4. Discussion and conclusions

In the current study, we evaluated the effect of ZA on the proliferation, apoptosis and migratory capacity of oral fibroblasts (CRL-7408) and oral epithelial cells (OKF/6). Our results show a differential effect of ZA on these distinct cell populations. Although proliferation of both cell types was inhibited in a ZA concentration-dependent manner, oral fibroblasts were significantly more susceptible to ZA-induced apoptosis relative to oral epithelial cells. This is distinct from a recent study by Scheper and co-workers, who reported enhanced susceptibility of HaCat keratinocytes to ZA-induced apoptosis relative to gingival fibroblasts when treated with ZA concentrations up to 3 μM.4 It is currently unclear whether the epidermal (HaCat) versus oral mucosal (OKF/6) origin of the epithelial cells used in the Scheper study versus the current report may influence the apoptotic response. In this regard, it is interesting to note that proliferation of primary murine oral mucosal keratinocytes was inhibited by the bisphosphonate Pamidronate, whilst apoptosis was unaffected.22 Furthermore, it is difficult to predict the concentration of ZA that would be available to oral epithelium and fibroblasts after drug infusion in vivo. ZA plasma concentrations in humans rapidly decline from a post-infusion $C_{\text{max}}$ of 0.9 μM for a 4 mg dose infused over 15 min to <1% of $C_{\text{max}}$ at 24 h post-dose.24 However, drug concentrations in the bone of rats and dogs are reported to be >100-fold higher than plasma $C_{\text{max}}$ and are maintained for at least 6 months post-dose.24 Thus the relative proximity of cells to ZA-rich bone as well as the relative rate of drug release from bony tissues likely influence pericellular ZA exposure in vivo.

ZA treatment enhanced the migratory capacity of oral epithelial cells and resulted in a significant reduction in the closure rate of in vitro scratch wounds generated in cultures of oral fibroblasts. Although a similar effect was not observed in experiments using NIH3T3 fibroblasts, mice injected with zoledronic acid for 7 days followed by a tooth extraction showed a delay in closure of the tooth extraction socket.23 Cell migration is a complex process involving actin polymerization to extend the leading edge of the cell, reversible cycles of integrin-mediated cell–substratum adhesion, actomyosin contraction to move the cell body forward, and release of adhesive interactions at the rear of the cell.25 The differential effect of ZA on migration of oral epithelial cells and fibroblasts suggests a need for future studies to evaluate the molecular mechanism(s) by which ZA alters cellular motility. In this regard, it is interesting to note that oral wound repair following a dental extraction is initiated by clotting and the formation of granulation tissue, followed by fibroblast migration into the wound, proliferation and deposition of collagen to increase wound strength.20,21 Next, fibroblast apoptosis leaves a collagen-rich environment that stimulates re-epithelialization. This provisional matrix is essential for the formation of a viable wound bed. Subsequently, epithelial cells migrate without proliferation, then proliferate to generate additional migratory cells for wound re-epithelialization and eventual contraction at the wound surface. Our data showed a loss of collagen immunofluorescent staining in the matrix that underlies ZA-treated oral fibroblasts. This was not due to an increase in collagen-degrading MMPs, as shown by gelatin zymography. Instead, quantitative real-time PCR results demonstrated that ZA treatment inhibited expression of both the COL1A1 and COL1A2 chains of type-1 collagen. Together these data support a model wherein ZA treatment impedes oral soft tissue wound healing by blocking collagen expression by oral fibroblasts, thereby resulting in a deficit in deposition of the collagen-rich granulation tissue needed for re-epithelialization. Via this mechanism, BP released from bone following tooth extraction may delay wound healing of the oral mucosal barrier and contribute to BPONJ pathogenesis.

This model is supported by clinical data showing that oral soft-tissue breakdown accompanies exposure of subjacent intra-oral bone, suggesting that soft-tissue toxicity may contribute to the onset of BPONJ in humans. Oral BPs are known to cause inflammation and/or ulceration of the gastrointestinal epithelium.5,11 Furthermore, the mucosal lining of the oral cavity is relatively thin and in close proximity to underlying osseous structures.5 BPONJ is usually associated with an invasive procedure that causes trauma to oral soft tissues. Toxicity to overlying soft tissues can result from BP accumulated in bone and released during osteoclast-mediated resorption or remodelling of mandibular elements undergoing repair.7 This may delay wound healing of the mucosal barrier, thus prolonging deleterious exposure of underlying bone to oral microbes. Indeed, wound healing is both a complex and fragile process susceptible to interruption or failure, and this can lead to the development of chronic non-healing wounds.20

Whilst our analyses focused on the influence of BP therapy on oral fibroblasts and epithelial cells, a series of studies have addressed the role of BPs in osteogenesis. In this regard, BPs are observed to positively affect osteoblast differentiation and proliferation as well as increase type-I collagen expression, tissue mineralization and wound healing.26–30 In contrast, a recent study demonstrated that BPs, particularly ZA, negatively influence osteoblast proliferation, mineralization, type-I collagen expression and alkaline phosphatase activity.31 There are several potential explanations for this disparity, including the specific BP used in the study.27,30,31 Further, some of these investigations used osteocytes obtained from femur samples,7–30 whilst others examined calvarial cells or tissues.26,31 It is well known that load-induced responses differ between cortical bone in the cranium versus post-cranium32,33 and such variability may mitigate the role of BPs
on osteogenesis amongst skeletal sites. Future comparative studies on the differential responses of cranial versus postcranial bony and soft tissues to BP exposure are warranted.

The sizable number of patients receiving BP therapy combined with the extremely long half-life of BP in bone (8–10 years) indicates that the prevalence of BPONJ is likely to increase significantly. There is no present means of detecting which subpopulation of patients on IV BP therapy are at risk for development of BPONJ. However, documented risk factors include use of nitrogen-containing BPs, particularly zoledronic acid,1 dento-alveolar surgery, and cumulative dose. Median exposure to zoledronic acid prior to BPONJ onset ranges from 9–30 months, but can be as brief as 3 months.1 The cumulative hazard increases from 1% in 1 year of zoledronic acid treatment to 15% at 4 years of treatment.1 Further, it is unclear why BP-related ONJ may be a critical factor in the initiation and progression of proper matrix deposition and remodelling of oral soft tissues, oral fibroblasts, the release of BPs from oral bone may inhibit cavity may demonstrate enhanced BP sequestration. Given that maxillomandibular elements relative to other skeletal sites.7–10 As BPs accumulate at sites of active bone turnover,2,3 mechanically active regions in the oral cavity may demonstrate enhanced BP sequestration. Given that ZA treatment impedes the growth and migratory capacity of oral fibroblasts, the release of BPs from oral bone may inhibit proper matrix deposition and remodelling of oral soft tissues, which is a critical mechanistic aspect of wound healing. This, in turn, may be a critical factor in the initiation and progression of BPONJ.

**References**


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**Competing of interest**

Not declared.

**Ethical approval**

Not required.

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