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Zoledronic acid induces S-phase arrest via a DNA damage response in normal human oral keratinocytes

Hisashi Ohnuki [a,b], *Kenji Izumi* [a,*], *Michiko Terada* [a], *Taro Saito* [a,b], *Hiroko Kato* [a], *Akiko Suzuki* [a], *Yoshiro Kawano* [a], *Kayoko Nozawa-Inoue* [a], *Ritsuo Takagi* [b], *Takeyasu Maeda* [a]

[a] *Division of Oral Anatomy, Niigata University Graduate School of Medical and Dental Sciences, 2-5274, Gakkocho-dori, Chuo-ku, Niigata City 951-8514, Japan*

[b] *Division of Oral and Maxillofacial Surgery, Niigata University Graduate School of Medical and Dental Sciences, 2-5274, Gakkocho-dori, Chuo-ku, Niigata City 951-8514, Japan*

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ABSTRACT

Objective: This study aimed to clarify the effects of zoledronic acid (ZOL) on human primary oral mucosal keratinocytes growing in a monolayer culture and on a tissue-engineered oral mucosal construct.

Design: Changes in the viability and proliferation of oral keratinocytes incubated with ZOL were measured. Following treatment with 10 μM ZOL, histological examinations and immunohistochemical analyses for Ki-67, Geminin, and phospho-histone (γ)-H2A.X were performed on tissue-engineered oral mucosa. Cell cycle distribution and the degree of apoptosis were also measured by flow cytometry. Additionally, we measured the expression of cell cycle regulatory proteins as well as phospho-Chk1 and -Chk2.

Results: ZOL treatment suppressed cell viability and proliferation in a dose-dependent manner. Compared with untreated tissue-engineered oral mucosa, ZOL treatment resulted in a thinner epithelium in which the basal cells appeared less-organised. This is consistent with the observed significant reduction in the Ki-67 labelling index. In contrast, the Geminin labelling index was significantly higher than that in the untreated sample. In spite of the presence of a few apoptotic cells, ZOL induced an arrest in S-phase, which was confirmed by our observed alterations in the expression levels of cyclin A, B1, $p27^{KIP1}$, Rb and phospho-Rb. When the proteasome inhibitor MG132 was added to the ZOL-treated cells, we observed a partial restoration of the expression of cyclin A, cyclin B1, and $p27^{KIP1}$. Expression of phospho-Chk1 was detected, and a significant increase in the labelling index of γ-H2A.X was also seen.

Conclusions: These results indicate that a 10-μM ZOL treatment induces a DNA damage response in oral keratinocytes that activates the ubiquitin-mediated proteolysis of cell cycle regulators, resulting in cell cycle arrest and repressive effects on cell viability, proliferation, and epithelial turnover.

* *Corresponding author*. Tel.: +81 25 227 2816; fax: +81 25 223 6499.
E-mail address: izumik@dent.niigata-u.ac.jp (K. Izumi).

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1. Introduction

Bisphosphonates (BPs), which are potent inhibitors of bone resorption, are commonly prescribed for the treatment and prevention of osteoporosis and Paget's disease as well as for the treatment of skeletal-related events in malignancy associated with multiple myeloma, breast, prostate, and lung cancers. The efficacy of BPs has greatly contributed to improving patients' quality of life.[1–4] The potency of BPs depends upon the side chain that is attached to the geminal carbon in the P-C-P backbone, which binds avidly to hydroxyapatite on the bone surface.[5,6] Nitrogen-containing BPs (N-BPs) are more efficacious in suppressing bone resorption. Zoledronic acid (ZOL) is the most widely used intravenous N-BP that reduces skeletal morbidities under both benign and malignant conditions. Despite the benefits observed in some clinical settings, bisphosphonate-related osteonecrosis of the jaw (BRONJ) has recently emerged as a severe complication in patients receiving treatment with intravenous N-BPs such as ZOL.[7–9] BRONJ is characterised by exposed necrotic bone in the maxillofacial region that persists for more than 8 weeks.[10]

The pathophysiology of BRONJ remains uncertain. The prevailing theory on developing BRONJ centres on the impact of BPs on bone, particularly on their ability to inhibit osteoclast function, which results in the suppression of normal bone turnover.[7,9,11,12] The anti-angiogenic potency of ZOL is also thought to influence the development of avascular necrotic bone.[13,14] In particular, it is unclear why BRONJ selectively localises to the jaw. Because of the unique environment of the oral structure, which is exposed to a variety of constant physiologic (mastication/occlusion), inflammatory (periodontal diseases) and/or iatrogenic (dental procedures, denture wearing) stresses, jaw bones have a high bone remodelling rate and a rich blood supply.[9,11,15,16] Thus, it has been postulated that higher levels of BP accumulate in the jaws than in other bones, as BPs are primarily deposited in bones with high turnover rates.[17] Consequently, the oversuppression of bone turnover may compromise the healing potential of the jaw in response to microdamage from physiological stress and injuries such as tooth extraction and local trauma.

Apart from the ''inside-out'' theory that considers the bone as a central factor in the initiation of BRONJ, an ''outside-in'' hypothesis that focuses on the loss of oral mucosa integrity as a cause of BRONJ has been proposed.[16,18] Reid et al.[19] also speculated that the high levels of BP in the jaw may induce soft tissue toxicity, resulting in compromised oral mucosa wound healing after invasive procedures, which may be followed by exposure of the underlying bone upon secondary infection. Recently, investigations have shown a proapoptotic effect of BPs on cellular components in the oral mucosa soft tissue, including endothelial cells, fibroblasts, and keratinocytes, which may serve as triggering factors for BRONJ.[13,20–25] Because retrospective studies have introduced BRONJ cases without a history of dento-alveolar interventions,[8,26] it is highly likely that a debilitation of the oral mucosal epithelium precipitates the onset of latent BRONJ through the loss of epithelial continuity, facilitating the progression into clinical ''stage 1'' from ''at risk''.[15]

The epidermis, including the oral mucosa and skin, maintains both its tissue integrity and a barrier function that protects the body from any external stimuli (physical, chemical, microbial) through constant renewal and repair.[27] Primarily for clinical use, we developed an *ex vivo*-produced oral mucosa equivalent (EVPOME) that mimics native oral mucosa.[28] This tissue-engineered construct, which lacks submucosal cellular components, can be utilised to assess the general effects of BPs on oral epithelium *in vitro*.[29] We hypothesise that treatment with ZOL may compromise oral keratinocyte cellular activities and the three-dimensional epithelial structure, leading to a spontaneous breach of the thin epithelium. The present study was undertaken to examine the anti-proliferative effects of ZOL on human primary oral mucosa keratinocytes grown in a monolayer culture and within the EVPOME model. We analysed cell viability, cell proliferation, cell cycle effects, and the protein levels of cell-cycle regulators. Histological and immunohistochemical examinations were also performed with the EVPOME model.

2. Materials and methods

2.1. *Procurement of keratinised oral mucosa samples and primary oral keratinocyte cultures*

The protocol for obtaining human oral mucosa samples was approved by the Niigata University Faculty of the Dentistry Internal Review Board (22-R18-10-07). Patients that had been subjected to third molar removal were given sufficient information regarding this study, and all individuals (total 39: 13 males, 26 females, with a mean age of 24.2 years) signed an informed consent form. At the Oral and Maxillofacial Surgery outpatient clinic, keratinised oral mucosa was harvested from the area adjacent to the area of tooth extraction without causing any morbidity. The mucosal sample was transported in a 15-mL conical tube containing 5 mL of a basic keratinocyte culture medium, EpiLife® (Invitrogen, Carlsbad, CA, USA). Primary oral keratinocyte cultures were established, and cells were serially passaged as previously described, with minor modifications.[30] Briefly, a tissue specimen kept in a 15-mL conical tube for 2 h was transferred into a 0.04% trypsin solution (Invitrogen) containing 1.5% of antibiotic–antimycotic (Invitrogen) and soaked overnight at room temperature (RT). After transfer into a 0.0125% defined trypsin-inhibitor solution (DTI; Invitrogen), oral keratinocytes were mechanically dissociated from the underlying connective tissue and then resuspended in a chemically defined, animal product-free culture medium, EpiLife® (Invitrogen), supplemented with EpiLife Defined Growth Supplements (EDGS; Invitrogen), 0.06 mM Ca^{++}, Gentamicin (5.0 μg/mL), and Amphotericin B (0.375 μg/mL). Cells were then seeded at a density of 4.0–5.0 × 10^4 cells/cm^2. Cells were fed every other day. Once they reached 70–80% confluence, they were detached with a 0.025% trypsin/ethylenediaminetetraacetic acid (EDTA) solution (Invitrogen), neutralised with DTI, centrifuged and re-plated at a density of 0.7–1.0 × 10^4 cells/cm^2. Keratinocytes were then placed into serial culture. The majority of cells used in this study were 4th