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Research Paper
Wound Healing

Zoledronic acid impairs re-epithelialization through down-regulation of integrin αvβ6 and transforming growth factor beta signalling in a three-dimensional in vitro wound healing model

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Abstract. This study examined the negative effects of zoledronic acid on the re-epithelialization of oral mucosa in a three-dimensional in vitro oral mucosa wound healing model. A living oral mucosa equivalent was constructed by seeding a mixture of primary human oral keratinocytes and fibroblasts, at a cell density of 1.5×10^5 cm^2 each, onto human cadaver dermis. This was cultured in a submerged condition in 1.2 mM Ca^{2+} EpiLife for 5 days, and then in an air–liquid interface for 14 days. The equivalent was wounded by excising a linear 2-mm-wide epithelial layer on day 8 and subsequently incubated with 10 μM zoledronic acid for an additional 11 days. Histological and immunohistochemical observations revealed zoledronic acid to significantly suppress the epithelial thickness and Ki-67-labelling index. Zoledronic acid also abolished integrin αvβ6 expression, implying impaired keratinocyte migration. Zoledronic acid did not attenuate the total transforming growth factor beta 1 (TGF-β1) production into the supernatant, but down-regulated TGF-β receptor types I and II expression and Smad3 phosphorylation, as was also confirmed by immunofluorescence microscopy. This study therefore showed zoledronic acid to abrogate integrin αvβ6 expression, cause the down-regulation of TGF-β/Smad signalling in oral keratinocytes, and impair re-epithelialization, suggesting compromised oral mucosa homeostasis in patients receiving zoledronic acid.

Keywords: bisphosphonate; re-epithelialization; oral mucosa; 3D in vitro model; TGF-β signalling.

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Introduction

The precise pathoetiology of osteonecrosis of the jaws (ONJ) in patients receiving bisphosphonates (BPs) remains elusive despite proposals of several possible factors of pathogenesis in the literature, including the suppression of bone turnover, toxicity to the bone, anti-angiogenic action, changes in oral microflora, and immune dysfunction.[1] Reid et al.[2] stated that BPs might have a cytotoxic effect on the oral mucosa. Landesberg et al.[3] also demonstrated mucosa cell damage by pamidronate, proposing an 'outside–in' hypothesis at the initiation of ONJ. This hypothesis is in line with clinical conditions observed as a spontaneous mucosa breach, leading to the development of ulceration in the oral mucosa, triggered by ill-fitted dentures.[4] Kyrgidis et al. proposed that researchers should provide the experimental design to monitor molecular changes associated with BPs, not only of bone cells but also of keratinocytes.[5] A three-dimensional (3D) oral mucosa tissue-engineered model, in which keratinocytes differentiate and are spatially organized to show the biomimetic structures seen in vivo, would be more appropriate to investigate wound healing in vitro compared with the use of a monolayer culture system to show that BPs impair the ability of keratinocyte migration.[6,7]

Wound healing requires re-epithelialization from the wound margin through keratinocyte proliferation and migration, which is a rate-limiting event. It is well-known that integrin αvβ6, not seen in the healthy oral epithelia and expressed in keratinocytes, promotes cell migration and activates transforming growth factor beta 1 (TGF-β1). This is supported by the fact that integrin αvβ6 expression in the basal and lower suprabasal cell layers increases remarkably during re-epithelialization of oral mucosa wound healing, accompanied by the maximum level of TGF-β1.[8,9] TGF-β1 appears to have a critical impact on keratinocytes and the epithelialization during wound healing, because keratinocytes are the major target cells of TGF-β1 through paracrine and autocrine signals.[10] Upon binding of TGF-β1 to the TGF-β receptor II followed by heterodimerization and phosphorylation of TGF-β receptor I, the complexes activate the downstream Smad proteins, leading to ligand-induced gene transcription.[11]

We hypothesized that zoledronic acid (ZA) could reduce integrin αvβ6 expression levels in oral keratinocytes, and further postulated that ZA would down-regulate TGF-β1 signal transduction, resulting in impaired keratinocyte migration. Thus, in this study we analyzed the negative effects of ZA on the re-epithelialization of the oral mucosa using a 3D in vitro oral mucosa wound healing model, co-cultured with oral fibroblasts.

Materials and methods

Procurement of oral mucosa samples

The protocol for obtaining human oral mucosa samples had internal review board approval. Patients who had been subjected to 3rd molar removal were given sufficient information regarding this study, and all individuals (total 16: 6 males and 10 females, with a mean age of 23.6 years) signed an informed consent form.

Primary oral keratinocyte and fibroblast cultures

Keratinized oral mucosa was harvested from the area adjacent to the site 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 (Life Technologies, Carlsbad, CA, USA). Primary oral keratinocyte cultures were established, and cells were serially passaged as described previously.[12] The majority of cells used in this study were 4th and 5th passage, with the range of passages used being 3rd to 7th.

Oral fibroblast cultures were established from the underlying connective tissue after the epithelial layer was scraped off. Primary fibroblasts were isolated from those mucosa samples by an explant culture technique. The connective tissue was minced into 2-mm^2 explants. These were placed in a 60-mm Petri dish (Corning, New York, NY, USA) and incubated in a moist atmosphere of 5% CO_2 at 37 °C in a Dulbecco's modified Eagle's medium (DMEM) culture medium (Wako Chemical, Osaka, Japan) supplemented with 10% foetal bovine serum (FBS) (Nichirei, Tokyo, Japan), gentamicin (5.0 μg/ml; Life Technologies), and amphotericin B (0.375 μg/ml; Life Technologies). Cells were fed every other day. Once the cell outgrowth was sufficient, cells were detached with a 0.025% trypsin/ethylenediaminetetraacetic acid (EDTA) solution (Life Technologies), neutralized with a 0.0125% defined trypsin-inhibitor solution (DTI) (Life Technologies), then replated in another culture vessel (0.8×10^4 cells/cm^2). The 3rd–5th passaged cells were used in this study.

Fabrication of a 3D, full-thickness living oral mucosa equivalent (LOME) ($n = 10$)

The LOME, consisting of a stratified squamous epithelium (oral keratinocytes) grown on human cadaver dermis in which oral fibroblasts were repopulated, was reconstructed as reported previously elsewhere.[13] Briefly, an equal number of oral keratinocytes and oral fibroblasts (1.5×10^5) was resuspended in EpiLife supplemented with EpiLife defined growth supplement and 1.2 mM Ca^{2+}. The cell suspension was seeded onto a papillary surface of human cadaver dermis, AlloDerm (LifeCell, Branchburg, NJ, USA), which had been cut into circular pieces that fit in a 48-well microplate (1 cm^2), and pre-soaked with human type IV collagen (5 μg/cm^2; Sigma-Aldrich, St. Louis, MO, USA) overnight at 4 °C. After the LOME had been cultured in a submerged condition for 5 days with the medium changed every 24 h, it was raised to an air–liquid interface and cultured for another 3 days to allow oral keratinocytes to differentiate and stratify with the same culture medium. The medium was changed every 3 days.

Ex vivo wound healing assay

After culturing the LOME for 8 days, the middle of the epithelial surface of the LOME was wounded using a 2-mm-wide linear excision tool.[14] The 2-mm-wide strip composed of an epithelial layer and the underlying AlloDerm was excised using sterile scissors. This resulted in a linear, partial thickness mucosa defect. ZA (Zometa) was purchased from Novartis Pharmaceuticals Corp. (East Hanover, NJ, USA), dissolved in sterile ddH_2O and used at a final concentration of 10 μM. To examine the effects of ZA on subsequent epithelial regeneration and re-epithelialization over the wounded area on the LOME, 10 μM of ZA was added to the culture medium and the LOME cultured for an additional 11 days. As a control, no ZA was added.

Histological and immunohistochemical examinations

ZA-treated as well as untreated LOMEs that had been cultured for a total of 19 days, as well as native oral mucosa tissue not used for cell culture, were fixed with 4% paraformaldehyde (PFA) in 0.1 M PBS and embedded in paraffin. Paraffin sections of 4.5 μm in thickness were stained with haematoxylin and eosin for histological examination. A part of the