Effect of sclerostin removal *in vivo* on experimental periodontitis in mice

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(Received December 5, 2015; Accepted February 8, 2016)

Abstract: We explored the effects of sclerostin removal *in vivo* on experimental periodontitis in mice. A ligature of *Porphyromonas gingivalis*-saturated collagen silk was applied to the cervical region of the first molar tooth in 10 wild-type (WT) mice and 10 sclerostin-knockout (SOST-KO) mice, and the animals were fed 10% sucrose for 2 months. Another 10 WT mice and 10 SOST-KO mice were similarly treated, but then fed a normal diet for 2 months. The maxillae were then harvested for morphological and molecular examinations. The mice with periodontitis showed significantly more severe alveolar bone loss than control mice, the most significant absorption being observed in WT mice. Immunohistochemical staining demonstrated upregulation of RANKL and ERK1/2-MAPK expression and downregulation of OPG expression in mice with periodontitis, especially WT mice. Therefore, removal of sclerostin appears to modestly protect the alveolar bone from resorption in this experimental setting.

(J Oral Sci 58, 271-276, 2016)

Keywords: experimental periodontitis; sclerostin; alveolar bone loss.

Introduction

Periodontitis is an oral inflammatory disease associated with many systemic conditions, including cardiovascular disease, diabetes, rheumatoid arthritis, preeclampsia and preterm birth (1). Periodontitis is clinically significant because it leads to progressive alveolar bone destruction and connective tissue attachment loss. Although periodontitis sometimes may be caused during orthodontic treatment due to various reasons (2), it is well known that certain pathogenic bacteria play a crucial role in periodontitis development (3,4). One such bacterium is *Porphyromonas gingivalis* (*P. g.*) (5), which causes periodontitis by inducing changes in the expression of bioactive molecules or cytokines, and activating or inhibiting the pathways in which they act. During this process, some bone-related molecules in the periodontal region may be altered (6), inducing protective or negative effects on inflammatory bone resorption (7). The most commonly studied biomarkers in the context of periodontitis research include osteogenic factors [e.g. osteoproteprin (OPG)], osteoclast-activating factors [e.g. receptor activator of NFκB ligand (RANKL)], and related pathways [e.g. mitogen-activated protein kinase (MAPK)] (8,9).

Sclerostin is a bone matrix protein produced by osteocytes that has an inhibitory effect on bone formation as it is a potent antagonist of WNT signaling. High sclerostin expression, resulting from inhibition of the WNT/betacatenin pathway, can induce bone resorption and reduce bone formation in patients with diseases such as osteoporosis (10). In contrast, sclerostin deficiency in patients with sclerosteosis and Van Buchem disease (11,12) can...
cause alteration of bone composition, reduction of matrix mineralization, and upregulation of proteoglycan in bone subcompartments (13). SOST, the gene for sclerostin, is located at position 11.2 on the long arm of chromosome 17. SOST knockout (KO) in mice may not cause abnormality of tooth structures such as dentin, but may alter the bone and cementum phenotypes (14). Furthermore, inflammation can induce sclerostin expression (15). Since inflammatory factors play a crucial role in the progressive bone destruction that characterizes periodontitis, and sclerostin has a pivotal role in bone metabolism, it is reasonable to assume that sclerostin deficiency would affect the progression of periodontitis. We hypothesize that during periodontitis progression, SOST KO might facilitate bone formation and thereby inhibit bone resorption via the MAPK signaling pathway. As no previous study has investigated differences in experimental periodontitis between sclerostin-deficient and normal animals to confirm the role of sclerostin in periodontitis progression in vivo, here we explored whether removal of sclerostin would prevent alveolar bone loss in mice with experimental periodontitis.

Materials and Methods

Animal experiments were conducted according to a protocol approved by the Animal Experimentation Committee of the State Key Laboratory of Oral Disease, Chengdu, China (WCCSIRB-D-2015-070). Twenty wild-type (WT) and 20 SOST-KO 8-week-old male C57BL/6 mice were used.

Periodontitis groups

The experimental periodontitis groups comprised 10 wild-type (WT) mice (Group A) and 10 SOST-KO mice (Group B). Both groups were fed 10% sucrose and a standard solid mouse chow for 2 months. A ligature of 6-0 collagen silk saturated with P. g. (wild-type strain 33277, ATCC) was applied to the cervical region of the first molar teeth. The P. g. had been grown and maintained in a Schaedler broth (Thermo Fisher Scientific, Beverly, CA, USA) in an anaerobic chamber containing 85% N₂, 10% H₂, and 5% CO₂ at 37°C. The silk was displaced apically into the gingival sulcus weekly to ensure it maintained a subgingival position, and was replaced as necessary. The ligating silk strands were processed as follows. First, two strands of thin steel wire were inserted through the space between the maxillary first and second molars. Second, 6.0 silk was passed between the steel wires. Third, the steel wires with the silk were pulled from the buccal side. Fourth, the silk was ligated around the maxillary first molar. Finally, the silk was tied and positioned around the neck of the molar. The mice were maintained in cages at a constant temperature of 25°C under a 12-h/12-h light/dark cycle.

Control groups

Ten WT mice (Group C) and 10 SOST-KO mice (Group D) were employed as controls. The mice were provided with sterile water and a standard solid mouse chow for 2 months and maintained in cages at a constant temperature of 25°C under a 12-h/12-h light/dark cycle.

Morphometric and staining analysis

Maxillae in each group were harvested, de-fleshed, washed, fixed in 10% formalin at 4°C overnight and demineralized in a 10% ethylenediaminetetraacetic acid (EDTA)-buffered solution (pH = 7.0) at 4°C. Samples were stained with methylene blue (0.003%, Sigma-Aldrich, St Louis, MO, USA) and observed by stereomicroscopy. For microarchitectural assessment, three-dimensional (3D) images of the maxillae were reconstructed using a Viva CT40 micro-computed tomograph (CT, Scanco Medical, Bruttisellen, Switzerland). After scanning, the resulting gray-scale images were imported into Mimics (version 15.0, Materialise, Leuven, Belgium) and reconstructed into 3D images. The areas between the cement-enamel junction (CEJ) and the alveolar bone crest (ABC) of the first molars were then calculated and presented as exposure volume and surface area. The specimens were then paraffin-embedded and made into 4-μm-thick serial sections. Hematoxylin and eosin (HE) staining and immunohistochemical (IHC) staining for OPG, RANKL and possible pathways of MAPK including JNK, p38MAPK, ERK1/2 were conducted, and then observations were conducted using a light microscope (E600, Nikon, Tokyo, Japan). Semi-quantitative analysis of optical density (OD) after IHC staining was performed using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

Data for each group were presented as means ± standard deviation (SD), and statistical calculations were conducted on SPSS 17.0. Differences between the groups were assessed by two-way analysis of variance (ANOVA) with Student’s t-test. A test for normal distribution was performed in advance. Differences at P < 0.05 were considered to be statistically significant.

Results

Morphometric observation

Stereomicroscopy and micro-CT both showed that
the WT mice in Group A had more severe alveolar bone destruction, as evidenced by root exposure, in comparison with Groups B, C and D. In addition, HE staining indicated good arrangement of the periodontal membrane fibers and alveolar crest as well as relatively complete morphology of the alveolar bone in Groups C and D. However, Group A shows looser periodontal fibers, as well as degeneration and disorder of the periodontal membranes, relative to Group B (Fig. 1).

### Quantitative analysis of bone resorption

Root exposure in the area between the ABC and the CEJ demonstrated in micro-CT images was calculated and compared in terms of volume and surface area. The volumes of root exposure in Groups A, B, C, and D were 0.2655 ± 0.0441, 0.2468 ± 0.2024, 0.2246 ± 0.0082, and 0.2263 ± 0.0099 mm³, respectively, and the corresponding surface areas were 1.2240 ± 0.0629, 1.1828 ± 0.0598, and 1.1577 ± 0.1026 mm², respectively. There was a significant difference in both surface and volume exposure between Groups A and C (P < 0.05), but not between Groups B and D (P > 0.05) (Table 1).

### Immunostaining results

OPG and RANKL were expressed mainly in osteocytes, osteoclasts, osteoblasts, and periodontal ligament fibroblasts around the first molars. The expression of OPG was higher in the control groups than in the experimental groups, and was lower in Group C than in Group D. These results were confirmed by semi-quantitative analyses of OD after IHC. By contrast, RANKL expression was higher in the periodontitis groups, especially for WT mice with periodontitis versus WT control mice, which was also consistent with the semi-quantitative results.

Possible inflammatory pathways of MAPK including JNK, ERK1/2 and p38MAPK were detected. However, JNK and p39MAPK IHC staining revealed no positive signals. Only ERK1/2 positivity was evident, being much higher in Group A than in Groups B and C (Fig. 2).

### Discussion

Our present results are based on an experimental animal model of periodontitis, which has been widely used for investigations of periodontal disease. Although various similar animal models of periodontitis have been reported, application of P.g.-impregnated silk ligature
is a common and essential feature (16). Despite some limitations, animal models are more accurate than in vitro experiments because they can mimic the complex cellular interactions occurring in vivo. Moreover, they can be more experimental than clinical trials because the latter often preclude the manipulation of functional factors (e.g. removal of sclerostin) due to ethical considerations. However, since the importance of sclerostin came to light, many studies have focused on the effects of upregulation or downregulation of sclerostin expression. This led to development of the SOST-KO mouse model, which was used for comparison with WT mice in terms of changes to dental and periodontal structures (14). Most studies have suggested that sclerostin deficiency mainly alters alveolar bone and cementum phenotypes. Consistent with the characteristics revealed in the present study, SOST-KO mice show a stronger and more compact bone composition. Furthermore, our results indicate that during the progression of experimental periodontitis, SOST-KO mice have less alveolar bone resorption and lower RANKL expression, but increased OPG expression, suggesting that sclerostin might have a protective role against periodontitis progression.

The mechanism underlying the protective role of sclerostin against periodontitis progression is still unclear. Recent studies suggest that it might be associated with a number of factors, such as higher levels of pro-inflammatory and osteoclastogenic cytokines (17). Inflammatory cytokines are considered to play a major role in disease progression as, together with WNT signaling, they can form a positive feedback loop for modulation of bone remodeling (18). WNT signaling is considered to be an important pathway in bone formation and loss (19). Sclerostin and dickkopf-related protein 1, as WNT-beta-catenin signaling antagonists, are known to be involved in chronic periodontitis (20). Moreover, inflammatory factors can stimulate sclerostin expression in estrogen-deficient conditions (21), and in obese mice (15). This may occur through activation of MAPK signaling, which is induced by tumor necrosis factor (TNF)-α and TNF-related weak inducer of apoptosis (TWEAK) (22). In addition, Ras/ERK1/2 MAPK signaling, as the probable pathway in inflammatory bone resorption, is activated by TNF-α via the enhanced transcription of Smad ubiquitination regulatory factor 1 and is dependent on Runx2, which activates bone formation-related cytokines (23,24). ERK1/2-Runx2 signaling is also related to mechanical stress (25), which may explain why periodontitis frequently occurs during orthodontic treatment. Our present data for the two MAPK signaling pathways suggest that JNK and p38MAPK are not activated during periodontitis progression, while the ERK1/2 pathway in SOST-KO and WT mice is activated to different extents. This implies that the inflammatory process and bone resorption are probably due to the ERK1/2-MAPK pathway, resulting in changes in osteogenic and osteoclastic cytokines such as OPG and RANKL. Therefore, on the basis of this experimental animal model of periodontitis and the possible mechanism involved, it is reasonable to conclude that anti-sclerostin antibody may be valuable for clinical treatment of periodontitis.

This study had some unavoidable limitations. First, the experimental mouse model of periodontitis we employed cannot accurately simulate human periodontitis.
Furthermore, HE and IHC staining only show possible manifestations, but cannot yield quantitative results. Thus, our findings need to be confirmed by more precise examinations and further studies.

It is has been confirmed that anti-sclerostin antibody can enhance bone formation, bone mass, bone strength, and implant fixation in a rat model (26,27). Thus, we assume that anti-sclerostin antibody is able to stimulate bone regeneration after experimental periodontitis, and even for periodontitis which sometimes may be caused by orthodontic treatment or obesity. (28,29). In addition, a single-dose placebo-controlled randomized clinical trial of anti-sclerostin antibody has suggested that further clinical investigations of sclerostin inhibition as a potential therapeutic strategy would be justified for patients in whom increased bone formation is necessary (30). Therefore, taken together, the available data suggest that local application of anti-sclerostin antibody might be feasible for periodontitis patients in the future, since removal of sclerostin can reduce bone destruction in mice with experimental periodontitis and modestly protect against alveolar bone resorption.

References
23. Kanno T, Takahashi T, Tsujiyawa T, Aiyoshi W, Nishihara T (2007) Mechanical stress-mediated Runx2 activation is...


