Archives of Oral Biology 98 (2019) 132–139

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Peptides from rice endosperm protein restrain periodontal bone loss in mouse model of periodontitis

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ARTICLE INFO

Keywords:
Food peptide
Periodontitis
Inflammation
Bone loss
Osteoclastogenesis

ABSTRACT

Objective: Food-derived peptides have been reported to exhibit antibacterial activity against periodontal pathogenic bacteria. However, no effect has been shown on inflammation and bone resorption in periodontal pathology. The overall objective of the current study was to investigate how rice peptides influence biological defense mechanisms against periodontitis-induced inflammatory bone loss, and identify their novel functions as a potential anti-inflammatory drug.

Design: The expression of inflammatory and osteoclast-related molecules was examined in mouse macrophage-derived RAW 264.7 cell cultures using qPCR. Subsequently, the effect of these peptides on inflammatory bone loss in mouse periodontitis was examined using a mouse model of tooth ligation. Briefly, periodontal bone loss was induced for 7 days in mice by ligating the maxillary second molar and leaving the contralateral tooth unligated (baseline control). The mice were microinjected daily with the peptide in the gingiva until the day before euthanization. One week after the ligation, TRAP-positive multinucleated cells (MNCs) were enumerated from five random coronal sections of the ligated sites in each mouse.

Results: Rice peptides REP9 and REP11 significantly inhibited transcription activity of inflammatory and osteoclast-related molecules. Local treatment with the rice peptides, in mice subjected to ligature-induced periodontitis, inhibited inflammatory bone loss, explaining the decreased numbers of osteoclasts in bone tissue sections.

Conclusion: Therefore, these data suggested that the rice peptides possess a protective effect against periodontitis.

1. Introduction

Various bioactive peptides have currently drawn attention due to their multidisciplinary application as pharmaceutical and medicinal agents, cosmetics, and food. In particular, a large number of peptides are applied to medicament. Increasing evidence suggest the diverse effects of bioactive peptides on the regulation of multiple functions, including antioxidant, antimicrobial, enzyme-inhibitory, and anticancer. These bioactive peptides are generated from the animal and vegetable proteins available in different foods. Previous studies had reported that peptides obtained from corn gluten show antioxidant property (Jiang, Zhang, Lin, & Cheng, 2018; Jin, Liu, Zheng, Wang, & He, 2016) and those derived from fermented goat milk could account for most of angiotensin-1-converting-enzyme inhibitory, antioxidant, and antimicrobial activity (Ibrahim, Ahmed, & Miyata, 2017; Moreno-Montoro et al., 2017; Quiros, Hernandez-Ledesma, Ramos, Amigo, & Recio, 2005). Thus, application of bioactive peptides may have an impact on overall health of all animals and humans.

Periodontitis is the most prevalent chronic inflammatory disease in humans, caused by dysbiotic oral microflora, which in turn affects

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https://doi.org/10.1016/j.archoralbio.2018.11.021
Received 13 July 2018; Received in revised form 16 November 2018; Accepted 19 November 2018

periodontal tissue, including alveolar bone and connective tissue, resulting in tooth loss (Hajishengallis, 2015; Hajishengallis & Lamont, 2016). In periodontitis treatment, numerous anti-plaque agents have been formulated into mouth rinses and toothpastes(Serrano, Escribano, Roldan, Martin, & Herrera, 2015; Steinberg & Friedman, 2017), and tested as an adjunct to mechanical plaque removal. Specifically, it has been reported that CL (14–25) dodecapeptide from rice inhibits the growth and pathogenic activity of *Porphyromonas gingivalis*. *P. gingivalis* is gram-negative, anaerobic bacteria and one of the major periodontal infectious pathogens (Takei et al., 2013; Taniguchi et al., 2014). *P. gingivalis* has a strong pathogenicity, involving various virulence factors, such as lipopolysaccharide (LPS), fimbriae, hemagglutinins, and the proteinase gingipain, which can induce inflammatory responses in periodontal tissue. Rice peptide CL has also been evaluated clinically for its antiplaque effect (Takayama et al., 2015). Moreover, green tea extract epigallocatechin-3-gallate has been shown to alleviate *P. gingivalis*-induced periodontitis (Cai et al., 2015). Rice and green tea, respectively, represent the most popular food and beverages worldwide. In addition, antimicrobial genes have been reported to be expressed less in patients with severe periodontitis, than in healthy individuals (Jourdain et al., 2018). In such a case, application of the rice peptide may increase the antimicrobial effect on human health. We had earlier demonstrated that selective elimination or dissociation of *P. gingivalis* from dysbiotic community is a more effective strategy to treat periodontitis than traditional antimicrobial approaches (Maekawa, Krauss, et al., 2014). Although antimicrobial ability was well explored in epidemiological and clinical approaches, the other important effects of bioactive reagents on inflammation or bone metabolism are yet to be fully understood. In case of periodontitis, LPS, derived from the outer membrane of gram-negative bacteria (i.e. *P. gingivalis*), induces activation of monocytes and macrophages, resulting in pro-inflammatory cytokine production, such as proinflammatory cytokines interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor (TNF), leading to severe inflammation and bone loss in periodontal tissue. These LPS responses are caused via the activation of Toll like receptors (TLR), especially in subtype 4 (TLR4) (Akira, 2003). Rice extract has been reported to possess anti-oxidant and anti-inflammatory characteristics, which get manifested by the inhibition of the NF-kB signaling pathway (Limtrakul, Yodkeeree, Pitchakarn, & Punfa, 2015; Lin et al., 2010). Recently, we identified and characterized a peptide from enzymatic hydrolysates of rice protein and demonstrated its LPS-neutralizing activity (Taniguchi, Saito, et al., 2017). In a chronic inflammatory condition, persistent inflammation leads to osteoclastogenesis, and is consistently accompanied by activation of nuclear factor of activated T-cells, cytoplasmic 1 (Nfatc1) and Tnfrsf11a (RANK). Osteoclast precursors expressing RANK differentiate into osteoclasts via activation of Nfatc1, a transcription factor responsible for important mediators of the osteoclastogenesis and inflammatory processes (Boyle, Simonet, & Lacey, 2003; Jimi et al., 2004; Kobayashi, Udagawa, & Takahashi, 2009; Simonet et al., 1997). Thus, targeting RANK and Nfatc1 expression has been a promising strategy for regulating bone loss.

In order to eliminate the bacteria, resolve the inflammation and osteoclastogenesis, and treat severe periodontitis, oral hygiene with applicable antibiotics is vital. However, the frequent usage of antibiotics is of concern due to the development of drug-resistant bacteria. Bioactive peptides, from natural products, are beneficial due to their minimal side effects on human immune system. In this regard, we focused on the effect of the bioactive rice peptides on periodontitis. Despite the discovery of several drugs and compounds, effective and safe bioactive reagents for periodontitis are yet to be found. Therefore, in the present study, we hypothesized the rice peptide to exert anti-inflammatory and anti-osteoclastogenesis effects on a mouse model of experimental periodontitis.

2. Materials & methods

2.1. Rice-peptides

Rice endosperm protein (REP) powder (3.0 *g*) was suspended in 60 mL of ultrapure water and homogenized using a POLYTRON homogenizer (KINEMATICA; Bohemia, NY). The pH of the resulting REP suspension was adjusted to 7.8 using 1 M NaOH. Trypsin and chymotrypsin (60 mg) were added to the REP suspensions and incubated at 37 °C for 5 h. These enzymes were inactivated by incubating at 90 °C for 10 min. The mixture was then centrifuged at 12,000 × *g* for 30 min at 4 °C. After dialysis against ultrapure water using Spectra/Por Dialysis Tubing (MWCO, 500–1000 Da; Spectrum Laboratories, Inc., Rancho Dominguez, CA), peptides with low molecular weights were removed and the resulting supernatants were freeze-dried and stored for subsequent separation stages. Freeze-dried REP hydrolysates (200 mg) were dissolved in 50 mL of ultrapure water and fractionated by ampholyte-free isoelectric focusing (autofocusing) using a Rotofor (Bio-Rad, Richmond, CA), as described previously (Taniguchi, Kameda, et al., 2017). Autofocusing is a preparative isoelectric focusing technique, based on the ampholytic properties of peptide mixtures containing tryptic hydrolysates of food proteins. The constant power and run times were adjusted to 12 W and 2 h, respectively; samples were collected into 20 fraction tubes, and pH values were recorded.

2.2. Cell preparation and culture

Raw264.7 cells, representing a mouse macrophage cell line, were grown in Dulbecco's modified Eagle's medium [DMEM] (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (Japan Bioserum Co. Ltd., Hiroshima, Japan), 100 U/mL penicillin, and 100 μg/mL streptomycin (Wako Pure Chemical Industries) at 37 °C and 5% CO_2 in humidified air . For the stimulation experiments, the cells (5.0×10^5 cells/mL) were seeded onto three 12-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ) and cultured in DMEM supplemented with 10% FBS at 37 °C and 5% CO_2 in humidified air. After 60 min of incubation, peritoneal macrophages were pretreated with 50 μg/mL rice-peptides fractions 9, 11 and 15 (REP9, REP11 and REP15) for 1 h and then stimulated with 100 ng/mL *P. gingivalis* LPS (Pg-LPS) (InvivoGen, San Diego, CA). This was added onto 96-well plate (Becton, Dickinson and Company) and cultured in DMEM with RANKL (85 ng/mL) and rice-peptides REP9, REP11 and REP15 (50 μg/mL). Furthermore, the cells were incubated for 6 days in the same medium containing the supernatant, which was collected later. The various compounds were used at concentrations indicated in the figure legends, and determined as described below.

2.3. Quantitative real-time PCR

Total RNA was extracted from Raw264.7 cells and mouse gingival tissue using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH), and quantified by spectrophotometry at 260 and 280 nm. The RNA was reverse transcribed using SuperScript VILO Master Mix (Thermo Fisher Scientific), and quantitative PCR with the cDNA was performed using the StepOnePlus real-time PCR system (Thermo Fisher Scientific), according to the manufacturer's protocol. Data were analyzed using the comparative CT (ΔΔCt) method. TaqMan probes, sense primers, and antisense primers for the expression of a housekeeping gene (GAPDH), along with IL-6, IL-1β, Nod-like receptor protein 3 (Nlrp3), TNF, IL-10, transforming growth factor-β (TGF-β), and RANK, Acid Phosphatase 5 (Acp5, encoded TRAP), Cathepsin K (Ctsk) and B-cell lymphoma 6 (Bcl6) were purchased from Thermo Fisher Scientific.

2.4. Cell viability

Raw264.7 cells were seeded in 96-well plates at a density of

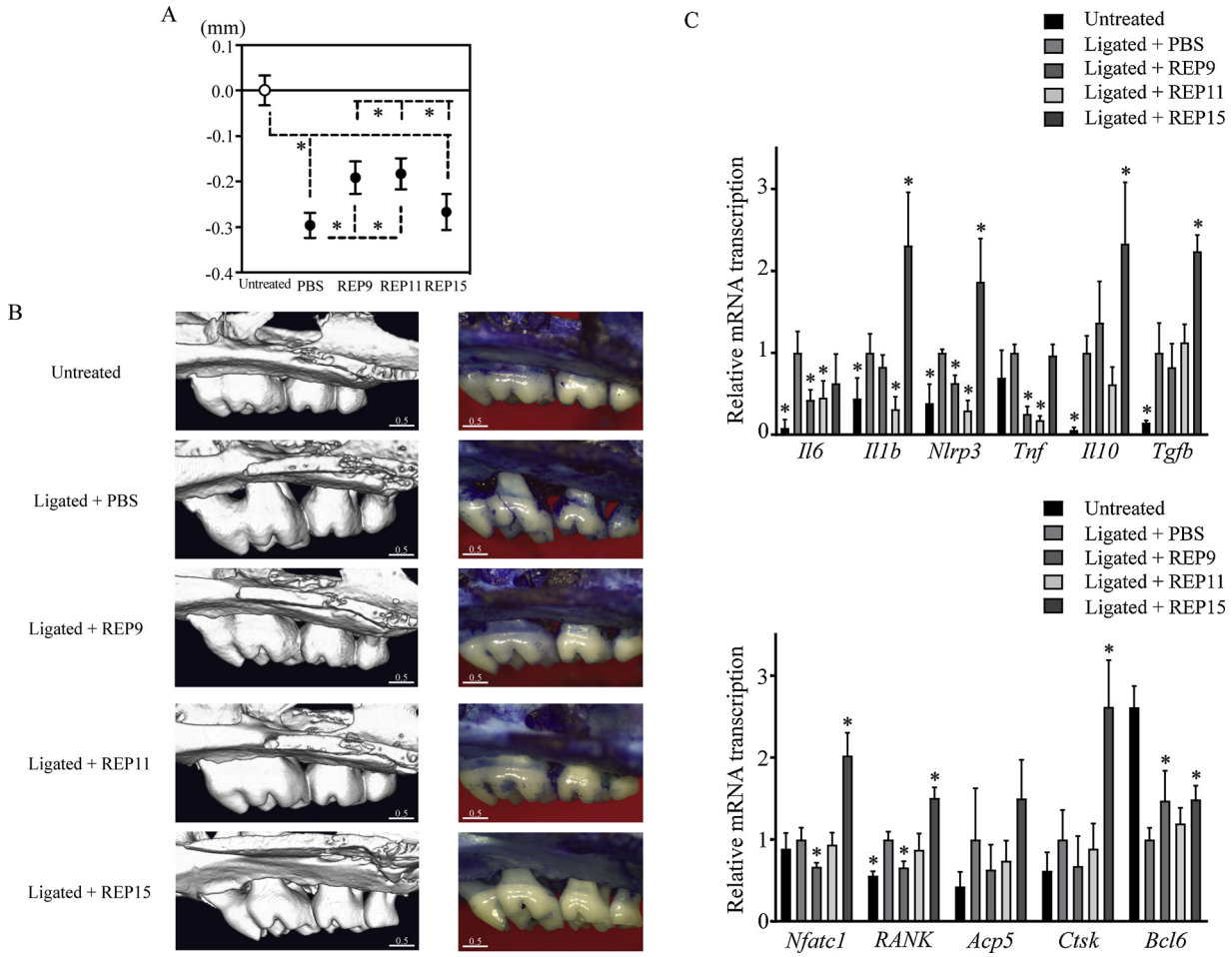

Fig. 1. Rice peptides REP9 and REP11 inhibit ligature-induced periodontitis. Periodontal bone loss was induced in mice for 7 days by ligating a maxillary second molar and leaving the contralateral tooth un-ligated (baseline control). Groups of mice were locally microinjected into the palatal gingiva with phosphate-buffered saline (PBS; control), REP9 (5 μL), REP11 (5 μL), and REP15 (5 μL), every day until the day before sacrifice (day 6). (A)The distance from CEJ to pinnacle of alveolar bone was measured at five predetermined points on the ligated second molar and the affected adjacent regions. Bone loss was calculated by subtracting the sum of values from the five-sites from the relevant distance in the un-ligated regions. Negative values (in mm) indicated bone loss relative to the baseline (un-ligated control). (B) Representative images of 3D reconstructed with micro CT scanning and stereoscopic microscope from indicated groups (scale bar = 0.5 mm). (C) In the same mice, ligature-induced periodontal inflammation and bone resorption was monitored in dissected gingiva and processed for quantitative polymerase chain reaction (qPCR), to determine messenger RNA (mRNA) expression of the indicated cytokines. *P < 0.05 with compared to the indicated group.

5.0×10^4 cells/well and incubated for 2 h. Cells were treated with REP9, REP11 and REP15 (50 μg/mL) for 5 h. Cell viability was assessed by MTT assay, in which MTT (400 μg/mL) was directly added to the cultures, followed by incubation at 37 °C and 5% CO2 in humidified air. Subsequently, the supernatant was aspirated and 200 μL of lysis solution (90% isopropanol, 0.5% SDS, 0.04 N HCl, DW) was added to dissolve the formazan dye. Optical density (OD) was measured at 570 nm using a microplate reader. Mean OD of the control group was set as 1, and the experimental groups were compared to control.

2.5. Mice and mice tooth ligating model

Balb/c mice, purchased from Nihon CLEA (Tokyo, Japan), were maintained in individually ventilated cages and provided sterile food and water ad libitum under specific pathogen-free conditions. They were used for experiments at the age of 12 weeks. All animal experiments were approved by the Institutional Animal Care and Use Committee of Niigata University (SA00181). First, to generate ligature-induced periodontitis, a 5-0 silk ligature was tied around the maxillary second molar (Abe & Hajishengallis, 2013). REP9, REP11, REP15, or PBS was microinjected into the palatal gingiva of the ligated second maxillary molar, once a day for six days, in intervention experiments. The mice were euthanized 7 days after placement of the ligatures. Gingiva were dissected and processed for qPCR determination of inflammatory cytokine mRNA expression levels. Periodontal bone loss was assessed morphometrically in defleshed maxillae using a stereoscopic microscope (35×). Specifically, the distance from cement-enamel junction to pinnacle of alveolar bone was measured at five predetermined points on the ligated second molar and adjacent affected regions. Bone loss was calculated by subtracting the sum of values from the five-sites from CEJ-to-pinnacle distance of alveolar bone in the un-ligated regions. Negative values (in mm) indicated bone loss relative to the baseline (un-ligated control).

2.6. Micro-computed tomography (micro CT) analysis

Samples from the maxillae of the mouse models of tooth ligation were scanned using the high-resolution micro scanner CosmoScan GX (Rigaku Corporation, Tokyo, Japan). Maxillae were first cleaned of the excess tissue, and micro CT was run with isometric resolution of 20-μm; the X-energy was set at 90 KV and 88 μA with an exposure time of 14 min. The CosmoScan GX software was used to reconstruct the three-dimensional image.

2.7. Histologic analysis

The maxillae from the models of tooth ligation were collected 7 days after ligation. For the standard histologic and subsequent quantitative histomorphometric analyses, the maxillae were fixed in 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries) for 24 h, followed by decalcification in Decalcifying Solution B (Wako Pure Chemical Industries) for 1 week at 4 °C. The specimens were then embedded in O.C.T. Compound (Sakura Finetek USA), which was frozen in liquid nitrogen, and coronal sections cut with cryostat. Sections were stained with tartrate-resistant acid phosphatase (TRAP) (Wako Pure Chemical Industries) and hematoxylin (Sakura Finetek Japan, Tokyo, Japan); TRAP-positive multinuclear giant cells (MNCs) were enumerated from five random coronal sections of the ligated sites from each mouse.

2.8. Statistical analysis

Data were evaluated by analysis of variance (ANOVA) and Dunnet multiple-comparison test using the InStat program (GraphPad Software). A p-value of $< 0.05$was considered statistically significant.

3. Results

In a preliminary experiment, REP was hydrolyzed with a mixture of chymotrypsin and trypsin for 5 h. The hydrolyzed REP was then fractionated into 20 fractions using autofocusing, and the osteoclast differentiation amongst the fractions was investigated. Based on the results, REP9 and 11 were selected as candidates for further consideration, and REP15 as the negative control (Sup.1)

Rice peptides REP9 and REP11 and not REP15 showed an effect of restraining ligature-induced periodontitis. To determine whether the rice peptides involved in osteoclast differentiation can regulate bone loss in vivo, we tested REP9 and REP11 for inhibiting ligature-induced periodontal bone loss in mice. To this end, we microinjected REP9, REP11, REP15 or PBS in the gingiva of mice subjected to ligature-induced periodontitis (Maekawa et al., 2015; Shin et al., 2015). We measured the distance from CEJ of the maxillary second molar of each group to pinnacle of alveolar bone with a stereoscopic microscope (35×). Mice that were subjected to ligature-induced periodontitis for 7 days spontaneously developed bone loss (Fig. 1A, B). Importantly, the induction of bone loss was significantly inhibited by REP9 and REP11 treatment. ($P < 0.05$; Fig. 1A, B). Moreover, gene expression profile of maxillary second molar and palatal gingiva was determined by qPCR. REP9 and REP11-mediated inhibition of bone loss was associated with significantly reduced levels of gingival mRNA expression of inflammatory (IL-6, Nlrp3, TNF) and osteoclast differentiation-related factors (Nfatc1, RANK). Furthermore, REP9 up-regulated expression of Bcl6, which related to suppress osteoclast differentiation and function (Fujie et al., 2015; Miyauchi et al., 2010). In the bone resorption activity related factor (TRAP, cathepsin K), REP9 and REP11 tended to reduce, but this reduction was not found to be significant. Some these effects were not showed by REP15 ($P < 0.05$; Fig. 1C, D).

Rice peptides REP9 and REP11, but not REP15 reduced TRAP-positive osteoclasts of the alveolar bone perimeter

Histomorphometric analysis was performed in TRAP-stained maxillae sections to determine the effect of REP9, REP11 and REP15 on osteoclast differentiation (Maekawa et al., 2017). In the ligated group, TRAP-positive osteoclasts, lining the alveolar bone perimeter of the maxillary second molar, were observed. In addition, induced periodontitis caused enlargement of the periodontal ligament space and bone resorption. Consistent with the bone loss measurements, local treatments with REP9 and REP11 in a replica experiment caused a significant reduction in the number of osteoclasts in bone tissue sections. ($P < 0.05$; Fig. 2).

3.1. Effect of rice peptides (REP9 and REP11 and REP15) on inflammatory cytokine in RAW 264.7 cells

The above-mentioned in-vivo data are consistent with the hypothesis that rice peptides REP9 and REP11 can mediate a protective role in periodontitis. To determine whether the rice peptides were involved in inflammation and osteoclast differentiation in vitro, their effect on mRNA expression of pre- and anti-inflammatory cytokines was examined. We used murine RAW 264.7 macrophages and exposed them to a bacterial proinflammatory stimulus, specifically LPS from *P. gingvalis*. Expression of *Il6*, *Il1b and Il10* mRNA, induced by *P.gingivalis*-LPS stimulation, was significantly suppressed by REP9 (REP11 significantly suppressed IL-6 expression only). Subsequently, we investigated whether REP9, REP11 and REP15 had cytotoxicity in RAW 264.7 cells. REP9, REP11 and REP15 had no effect on cell viability at the tested concentrations, as demonstrated by the MTT assay (Fig. 3 C).

3.2. Effect of rice peptides (REP9, REP11 and REP15) on the osteoclast differentiation in RAW 264.7 cells

We examined the effect of rice peptides on osteoclast differentiation in the RANKL-stimulated RAW 264.7 cells. We enumerated TRAP-positive MNCs that were incubated in culture medium with control or rice peptides (REP9, REP11 and REP15) and with RANKL for 7 days. The number of TRAP-positive MNCs in REP9- and REP11-added groups was significantly decreased. Subsequently, the effect of REP9, REP11 and REP15 on mRNA expression of osteoclast differentiation-related factors in RAW 264.7 cells was investigated. Expression of osteoclast differentiation and functional markers *Nfatc1* and *Tnfrsf11a* mRNAs was significantly suppressed by REP9. In addition, REP9 was significantly increased expression of *Bcl6* mRNA, which was related to the inhibition of osteoclast differentiation and function. ($P < 0.05$; Fig. 4 B, C).

4. Discussion

Periodontitis is a worldwide oral disease resulting in the destruction of tooth supporting tissues, including the destruction of alveolar bone and periodontal ligament. Emerging evidence indicates that periodontitis is caused by the dysbiotic microbial community within pathogenic species emerge (Hajishengallis, 2015; Hajishengallis & Lamont, 2016). However, innovative treatments and therapeutics are needed for periodontitis, which is often unresponsive to conventional treatment combined with antimicrobial therapy. In this regard, numerous novel antimicrobial-bioactive products have been tested. We previously reported that C3 inhibitor can selectively eliminate *P. gingivalis* from periodontitis to maintain the bacterial community symbiotic in mice and Non-human primates (Maekawa, Abe, et al., 2014; Maekawa et al., 2016). On the other hand, rice peptide REP used in this study contained large number of bioactive fractions or peptide without cytotoxic activity (Uraipong & Zhao, 2016). Intriguingly, *P. gingivalis* needs tissue breakdown products, including peptides and heme-containing compounds to survive (Hajishengallis, 2014). REP may selectively inhibit the growth of *P. gingivalis* owing to its binding affinity to heme-iron. Our previous study reported that CL, cationic peptide from soybean protein and rice bran, exhibited antimicrobial activity, especially against *P. gingvalis* (Takayama et al., 2016; Taniguchi, Kameda, et al., 2017; Taniguchi et al., 2016; Taniguchi, Saito, et al., 2017). Thus, the present study indicates that rice peptides would receive increasing attention as promising antimicrobial agents.

The periodontal pathogens are strongly associated with chronic periodontitis and initiate inflammatory response, as well as induce bone resorption, correlating with inflammatory mediators such as cytokines and chemokines. Complete elimination of periodontal pathogens and regulation of inflammation and bone metabolism are essential for successful periodontal treatment. Although anti-microbial peptide or natural products were well examined, their negative effects on immune

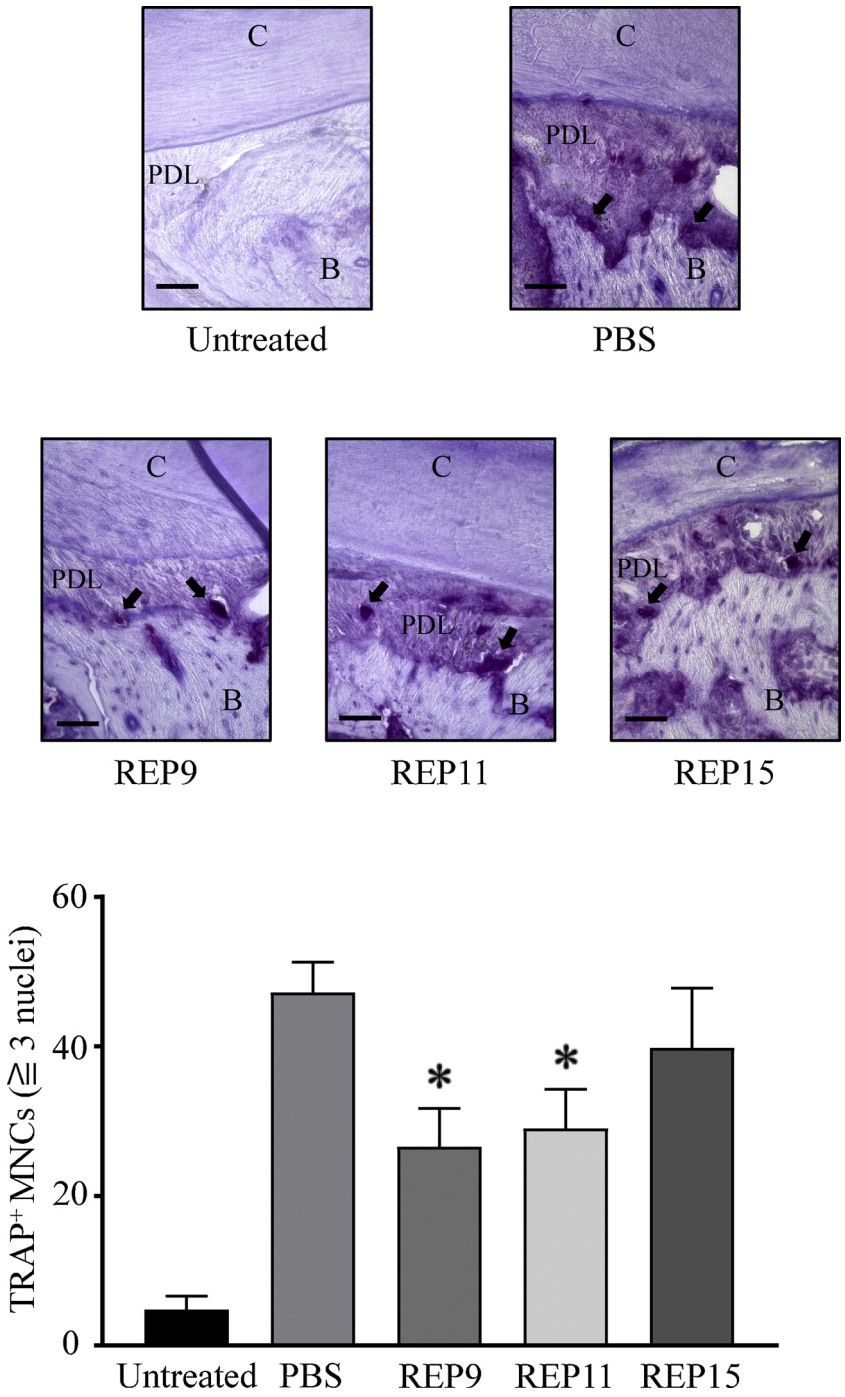

Fig. 2. Rice peptides REP9 and REP11 inhibit ligature-induced osteoclast differentiation. Representative sections with tartrate-resistant acid phosphatase (TRAP) and hematoxylin stain of maxillae at indicated groups; arrows indicate $TRAP^+$ osteoclasts. C: cementum; PDL: periodontal ligament; B: alveolar bone. In a replica experiment similar to Fig. 1, $TRAP^+$ cells were enumerated from 5 random coronal sections of the ligated molar from one male mouse and averaged with the SD from the 5 sections. *$P < 0.05$ with respect to Ligated + PBS group.

system and osteo-biology were still unknown. Therefore, in this study, we demonstrated that rice peptides can act as a suppressive compound on osteoclastogenesis and inflammation. First, we showed that administration of rice peptide REP9 and REP11 mechanistically inhibited bone resorption in a ligature-induced periodontitis model. Importantly, rice peptide suppressed gene expressions of various inflammatory cytokines and osteo-related molecules in mouse periodontal gingival tissue. The pro-inflammatory cytokines, such as IL-6, IL-1β, and TNF are necessary for initiation of the inflammatory process. Several groups have reported that other natural products have anti-inflammatory effect using in vitro and in vivo studies (Cai et al., 2015; Mah et al., 2014). Moreover, in this study, we reported that rice peptide REP9 and REP11 attenuate not only pro-inflammatory cytokines but also osteoclast-related molecules, such as Nfatc1, Bcl6, RANK, IL-1β, IL-6, and TNF. It has been implicated in the activation of the bone metabolism and expression of RANK and bone phenotype (Kwan Tat, S., M., S., & Fortun, 2004). Collectively, the number of TRAP-positive osteoclasts was less in the REP9- and REP11-administered group compared to that in the control group. We also showed that REP9 suppresses Nfatc1 and RANK expression in the presence of RANKL. Similarly, natural products such

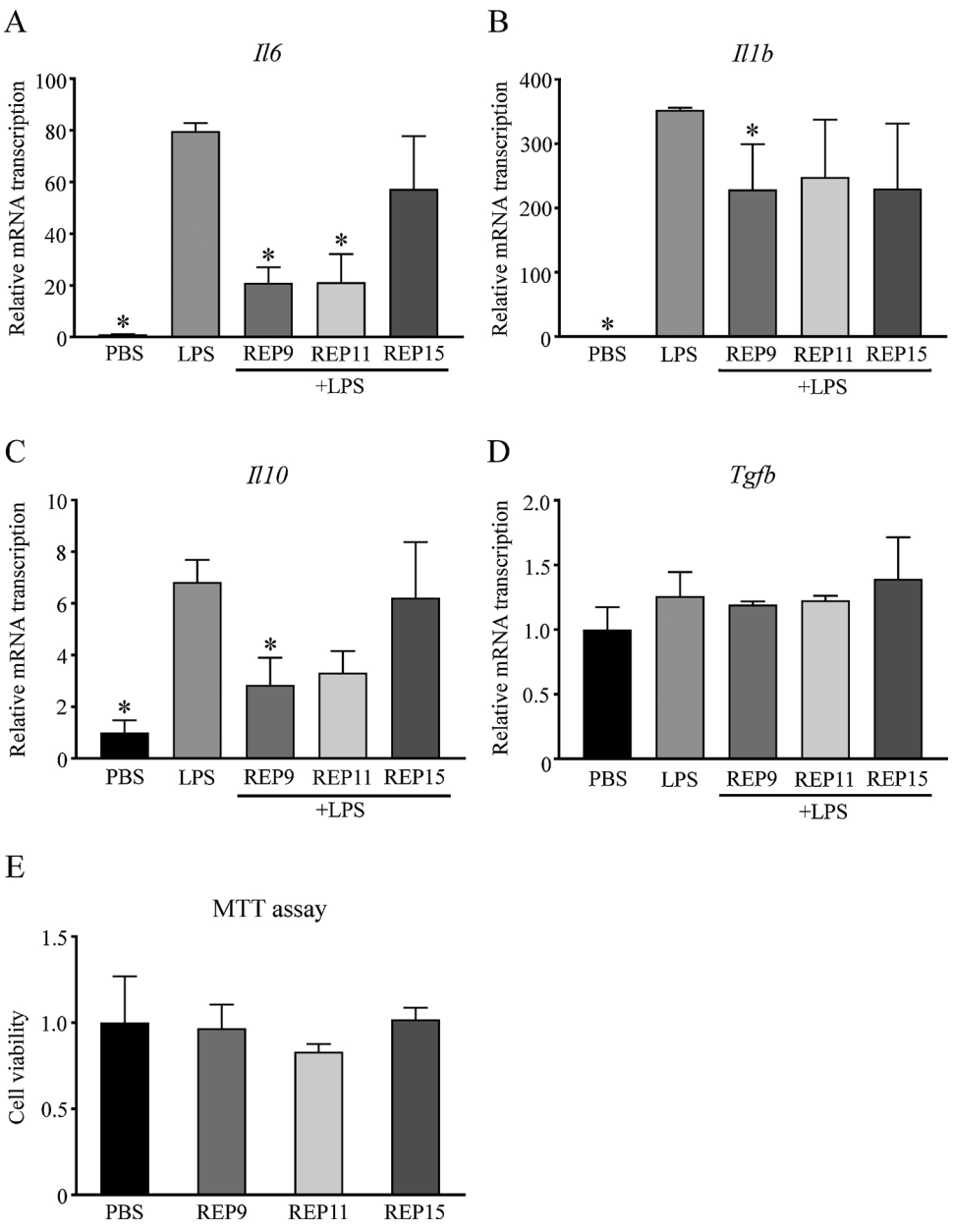

Fig. 3. Effect of rice peptides (REP9, REP11 and REP15) on the expression of inflammatory cytokines in RAW264.7 cells. Cells were pre-treated with 50 μg/mL rice-peptide (REP9, REP11 and REP15) for 1 h and then stimulated with 100 ng/mL *Porphyromonas gingivalis* lipopolysaccharide (Pg-LPS) for 4 h or only stimulated only with Pg-LPS for 4 h. Messenger RNA (mRNA) expression of (A) *Il6*, (B) *Il1b*, (C) *Il10* and (D) *Tgfb* was determined by real-time quantitative polymerase chain reaction. Results were normalized against that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Cell viability was assessed using the MTT assay; cells were treated for 5 h. (*E*) Data are means + SD (n = 4 sets of cultures). *$P < 0.05$ with respect to LPS-only group.

as Cinnamoyloxy-mammeisin from geopropolis, alpinia oxyphylla, or gentiopicroside directly regulate RANKL-induced osteoclast differentiation by reducing the expression of osteoclastogenic markers, including that of Nfatc1 and cathepshin K (Chen et al., 2018; da Cunha et al., 2017; Ha et al., 2014). Consistent with those findings, we demonstrated that local administration of REP9 regulates bone loss in periodontitis model in mice, suggesting that these peptides suppress osteoclast differentiation through down-regulating RANK and Nfatc1 expression, and up-regulating *Bcl6* expression locally in periodontal tissue. On the other hand, we showed that REP11 had an effect of significantly decreasing the expression of pre-inflammatory cytokines. In addition, REP11 tended to down-regulate the expression of *Acp5* and *Ctsk* mRNA. Furthermore, it was important that REP11 tended to up-regulate expression of *Bcl6* mRNA, which suppressed osteoclast differentiation, in the RAW264.7 cell and mouse periodontitis model. By combining these effects, REP11 could have caused reduction of bone resorption and differentiated osteoclasts, in the periodontitis tissue.

Secretion of inflammatory cytokines is associated with the recruitment of inflammatory cells involved in inflammation, such as monocyte/macrophages, neutrophils, and T-lymphocytes. In particular, since LPS is a potential stimulator of mononuclear cells, such as monocytes and macrophages, we focused on the effect of REP9 and REP11 on the inflammation induced by LPS in mice macrophage, Raw cells. This study found that LPS induced IL-6 and IL-1β gene expressions in Raw cells were significantly decreased when the cells were pre-treated with rice peptide REP. These data correlated with the observation of gingival tissue of mouse periodontitis model. In our previous studies, we showed that CL and enzymatic hydrolysates of rice exhibit antimicrobial and LPS neutralizing activities (Takayama et al., 2015; Takei et al., 2013; Taniguchi, Ochiai, et al., 2017). Further, it has been reported that rice bioactive peptides prevent cell injury through binding with TLR4 (Liang et al., 2018). In this study, REP9 and REP11 may attenuate LPS stimulation by neutralizing its effect. However, since REP9 and REP11 suppressed inflammatory gene expressions even after LPS stimulation, they may work in a way independent of the neutralizing effect.

5. Conclusions

In summary, in vivo and in vitro evidence found in the present study suggested that rice peptides could inhibit activation of pro-

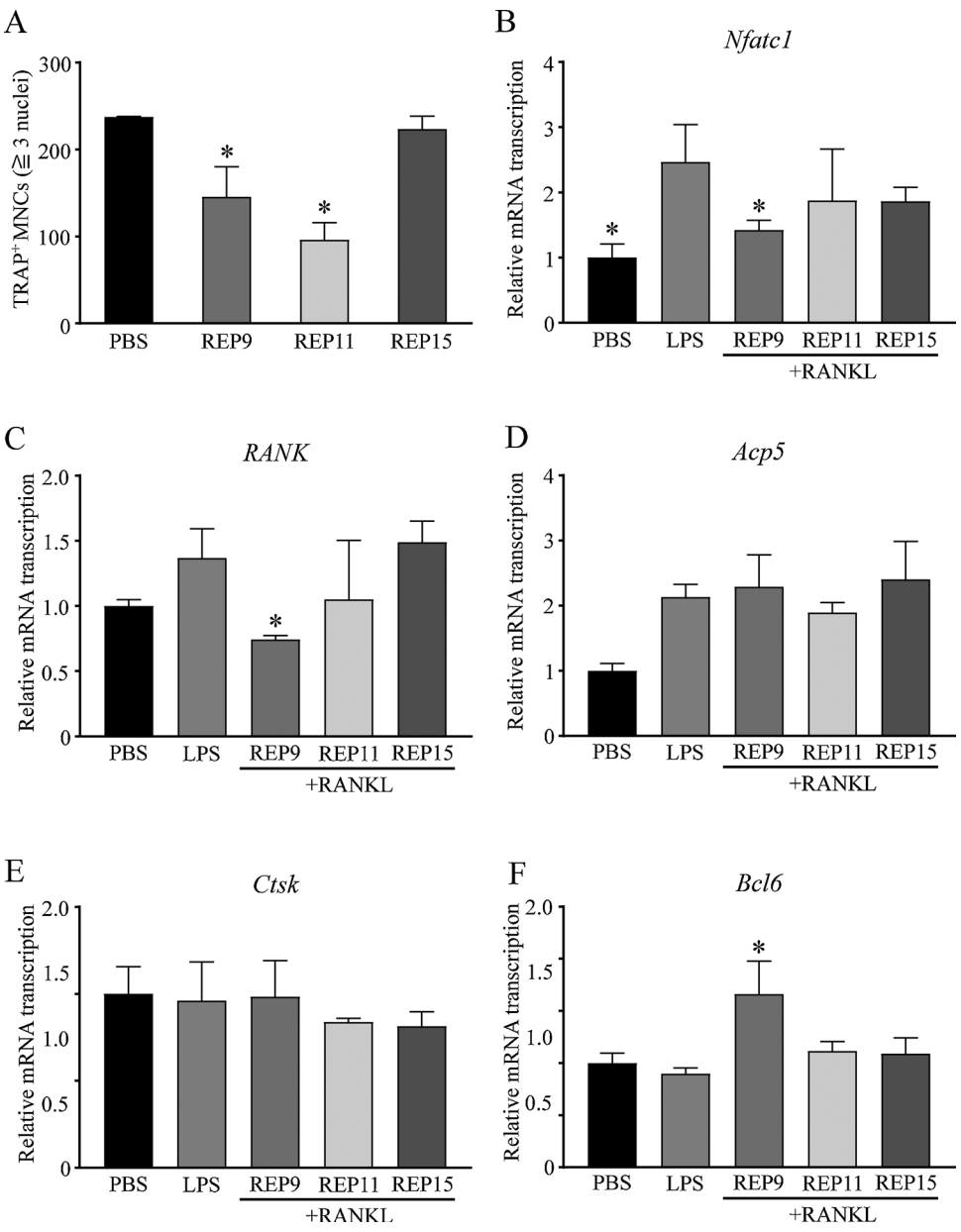

Fig. 4. Effect of Rice peptides (REP9, REP11 and REP15) on the expression of osteoclast differentiation-related factors in RAW 264.7 cells. (A) Cells were incubated in culture medium with control or 50 μg/mL rice-peptide (REP9, REP11 and REP15) and stimulated with RANKL. After 7 days, TRAP-positive MNCs were enumerated. (B, C) Cells were stimulated with RANKL and treated with control or 50 μg/mL rice-peptide (REP9, REP11 and REP15) for 24 h. The messenger RNA (mRNA) expression of (B) *Nfatc1*, (C) *RANK* (D) *Acp5*, (E) *Ctsk* and (F) *Bcl6* was determined by real-time quantitative polymerase chain reaction. Data were normalized against GAPDH mRNA. Data are means + SD (n = 4 sets of cultures). *P < 0.05 between RANKL-only group.

inflammatory gene expression that may regulate osteoclastogenesis in periodontal tissue, leading to a decrease in bone resorption that is involved with chronic inflammatory diseases, including periodontitis. Prevention of oral inflammatory disease-periodontitis by rice peptide administration could contribute to reducing the risk for certain systemic conditions, such as rheumatoid arthritis and atherosclerosis(Potempa, Mydel, & Koziel, 2017; Sabharwal, Gomes-Filho, Stellrecht, & Scannapieco, 2018). Rice peptides have a potential as locally administered therapeutics for the treatment of periodontitis.

Conflicts of Interest

There are no conflicts to declare.

Author contributions

H. Tamura performed experiments and statistical analysis, interpreted data, and wrote the paper. T. Maekawa contributed to conception, design, data acquisition, analysis, and interpretation, drafted and wrote the paper. H. Domon, T. Hiyoshi, K. Nagai, D. Yonezawa and A. Ochiai performed animal experiments and statistical analysis, M. Taniguchi contributed to data acquisition, analysis, and engineered and purified rice peptides. K. Tabeta and T. Maeda provided guidance and interpreted the data. Y. Terao contributed to conception, design, and data interpretation and critically revised the manuscript.

Acknowledgments

This work was supported by grants from JSPS KAKENHI-JAPAN (16H06272E, 17K19747) to T.M, (17H04367, 18K19638) to Y.T.Takeda Science Foundation-JAPAN, Nakajima Foundation-JAPAN and Young Investigator Research Seed Grant from JSP-JAPAN to T.M.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.archoralbio.2018.11.021.

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