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Local administration of ReveromycinA ointment suppressed alveolar bone loss in mice

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ABSTRACT

ReveromycinA (RMA) was developed and is a unique agent for inhibiting osteoclast activity. In a previous study, we experimentally induced periodontal disease in a high-turnover osteoporosis osteoprotegerin-knockout mice (OPG KO) model and found that intraperitoneal administration of RMA inhibited alveolar bone resorption. We prepared a novel RMA-containing ointment for topical non-invasive administration in the oral cavity, in preparation for possible future clinical application. And we investigated whether this ointment can inhibit alveolar bone resorption in an experimental mouse model of periodontal disease. We examined wild-type (WT) and OPG KO mice ligated with wire around contact points on the left first and second molars to cause food impaction and induce experimental periodontal disease. RMA was administered three times a day. Using micro-computed tomography, we measured the volume of alveolar bone loss and also performed histological analysis. Our findings showed that localized administration of RMA containing ointment resulted in suppressed alveolar bone resorption, reduced osteoclast count, and lower immunostaining scores of inflammation sites compared with controls in both OPG KO and WT mice. Localized application of the specific osteoclast suppressor RMA in ointment form in the oral cavity could be a novel treatment for periodontitis that inhibits alveolar bone resorption locally.

1. Introduction

Periodontal disease affecting the gingiva, alveolar bone, and other periodontal tissue is common in adult and elderly patients, and these patients often also have systemic diseases like osteoporosis.[1] In normal periodontal tissue, alveolar bone homeostasis is maintained by bone-resorbing osteoclasts and bone-forming osteoblasts; disequilibrium in either of these cell populations disrupts bone homeostasis. In orthodontic treatment, mechanical forces acting on the teeth induce bone resorption mainly by osteoclasts on the pressure side and osteogenesis and apposition mainly by osteoblasts on the tension side, with localized selective alveolar bone modeling and remodeling. Patients with systemic disease such as osteoporosis or localized disease with potential systemic effects may have impaired bone homeostasis, and the already vulnerable periodontal tissue may be susceptible to substantial bone resorption.[2]

Osteoclasts are important in bone homeostasis and are the only cells that resorb bone. Osteoclast differentiation, maturation, and function are strictly controlled by receptor activator of NF-κB ligand (RANKL), which is expressed on osteoblast and bone marrow stromal cell membranes. Osteoclasts and their precursors recognize RANKL and differentiate into mature osteoclasts. Furthermore, osteoblasts produce osteoprotegerin (OPG), a decoy receptor for

Abbreviations: RMA, ReveromycinA; OPG KO, osteoprotegerin-knockout mice; WT, wild-type mice.

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RANKL that inhibits osteoclast formation. OPG strongly inhibits RANKL–RANK interaction, suppressing osteoclast differentiation and functional expression. *In vivo*, OPG-overexpressing mice develop severe osteopetrosis with reduced bone resorption.[3–6] In contrast, OPG knockout (KO) mice have normal bone at birth, but osteoclast activity increases with age. Adult OPG KO mice have sparse trabeculation and low bone mineral density, displaying severe high-turnover osteoporosis[7,8] with utility for alveolar bone studies of osteoporosis and periodontal vulnerabilities that is useful for studies of osteoporosis and periodontal vulnerabilities in alveolar bone.[9,10]

Periodontal disease is an inflammatory condition caused by periodontopathic bacterial infection of periodontal tissue. The progression of periodontal disease can be suppressed by mechanical cleaning such as brushing, scaling, and root planing, as well as localized drug administration, including antibacterial and anti-inflammatory drugs.[11,12] One study highlighted the importance of bone resorption for better understanding and treating periodontal disease.[13]

Reveromycin A (RMA) was developed as a specific suppressor of osteoclast activity. RMA is an acid polyketide compound isolated from *Streptomyces reveromyceticus*; it is not taken up by normal cells but is selectively taken up under acidic conditions.[14,15] RMA is thus selectively taken up by activated acid-secreting osteoclasts, which dissolve bone mineral, and induces apoptosis in these cells, inhibiting bone resorption.[16]

In a previous study, we experimentally induced periodontal disease in a high-turnover osteoporosis OPG KO mouse model and found that intraperitoneal administration of RMA inhibited alveolar bone resorption.[17] In the present study, we prepared a novel RMA-containing ointment for topical non-invasive administration in the oral cavity, in preparation for possible future clinical application. We investigated whether this ointment reduces the number of osteoclasts and inhibits alveolar bone resorption in an experimental mouse model of periodontal disease.

2. Materials and methods

2.1. Animals

A total of 64 eight-week-old male WT (C57BL/6J) and OPG KO mice (CLEA Japan, Tokyo, Japan) were used in this study and housed in the animal experimentation laboratory of the School of Dentistry, Aichi Gakuin University, Japan. Room temperature and humidity were maintained at 22 ± 2 °C and 50 ± 10%, respectively, and a 12-hour light/dark cycle was established. Mice had free access to solid food (CE-2; CLEA Japan) and tap water. Animal care and experimental procedures were in accordance with the Guidelines for Animal Experiments of the School of Dentistry, Aichi Gakuin University and all experiments were approved by the Animal Experimental Committee of the School of Dentistry, Aichi Gakuin University (AGUD 331).

2.2. Topical application experiment

Experimental periodontal disease model mice were prepared through ligation by placing a 0.1-mm-diameter stainless steel wire (Nilaco Corporation, Tokyo, Japan) around the contact point between the left maxillary first molar and second molar to cause food impaction (Fig. 1-A, B, C).[18] Before ligating, the mice were anesthetized by intraperitoneal administration of three medicines: midazolam (Astellas Pharma, Tokyo, Japan), dexmedetomidine hydrochloride, and butorphanol tartrate (Meiji Seika Pharma, Tokyo, Japan).[19] A silicone-based ointment containing 1.0% RMA Na salt prepared by the Chemical Biology Research Group, RIKEN CSRS was used as the test drug. Next, 8-week-old mice were fitted with ligature wire and 1.0% RMA-containing ointment (1.6 mg) was applied just supragingivally to the proximal contact point between the first and second molars as well as to the surrounding buccal and palatal gingivae three times daily (Fig. 1-D). The same quantity of base ointment without RMA was used in the control group. The experimental groups were WT and OPG KO mice without ligature or RMA (WC, OC), WT and OPG KO mice with ligature but without RMA (WR− n = 8, OR− n = 8), and WT and OPG KO mice with both ligature and RMA (WR + n = 8, OR + n = 8). WC and OC were tested with the right side in WR− and OR−.

2.3. Micro-computed tomography scanning

The mice were sacrificed 8 weeks after ligature fitting. The ligatures were immediately cut with a pin cutter during maxillary bone sampling and extracted, with care taken not to damage the samples, which were examined using micro-computed tomography (μ-CT) (Rigaku Corporation, Tokyo, Japan). Images were captured at an X-ray tube voltage of 90 kV, tube current of 150 μA, image acquisition time of 2 min, and picture element size of 20 × 20 × 20 μm. Alveolar bone volume measurement and analysis were performed using TRI/3D-BON software (Ratoc System Engineering, Tokyo, Japan). Alveolar bone resorption was measured according to a modified version of the method reported by Park et al.[20] Following the method of Mizuno et al.,[17] the total alveolar bone space was defined as the space from the cementoenamel junction (CEJ) line to the root apex line. The percentage of remaining alveolar bone was calculated by dividing the alveolar bone volume by the total alveolar bone space (Fig. 1-E).

2.4. Pathological observations

Maxillae were collected 8 weeks after ligature fitting and fixed in 10% neutral buffered formalin, then decalcified in 10% EDTA (pH 7.2) at 4 °C for approximately 4 weeks. Paraffin blocks were prepared according to standard methods and cut into 5-μm-thick mesiodistal serial sections. Tissue observation sites were selected at points where all molar roots could be observed; hematoxylin-eosin (H&E) staining was performed. The attachment level of the first molar mesiodistal plane was determined using the distance between the CEJ and the bottom of the gingival sulcus and expressed as a percentage of the distance between the CEJ and root apex (Fig. 1-F). Tartrate-resistant acid phosphatase (TRAP) staining was performed using an acid phosphatase leukocyte kit (Sigma–Aldrich, St. Louis, MO). Osteoclast counts expressed as the number of osteoclasts per bone surface (n/mm) were determined for the alveolar process surface at the alveolar septum between the first and second molar. TNF-α and IL-1β immunostaining was performed using Histofine Simple Stain Mouse MAX-PO® and Histofine Simple Stain DAB substrate kits (Nichirei Biosciences, Tokyo, Japan) with anti-TNF α (ab6671, TNF-α: 1/200; Abcam, Tokyo, Japan) and anti-IL-1β (H-153), Human, Rabbit-Poly (SC-7884, IL1-β: 1/150; Santa Cruz Biotechnology, Santa Cruz, CA). The immunostaining observation site used for scoring was located on the alveolar process surface of the alveolar septum between the first and second molars. As described by Rogers et al.,[21] stained sections of alveolar septum were scored 1, 2, 3, or 4, indicating 0%–20%, 21%–40%, 41%–60%, and >60% positive staining, respectively. Scores were used for between-group comparison. Evaluation of the slide images was performed by two independent examiners.

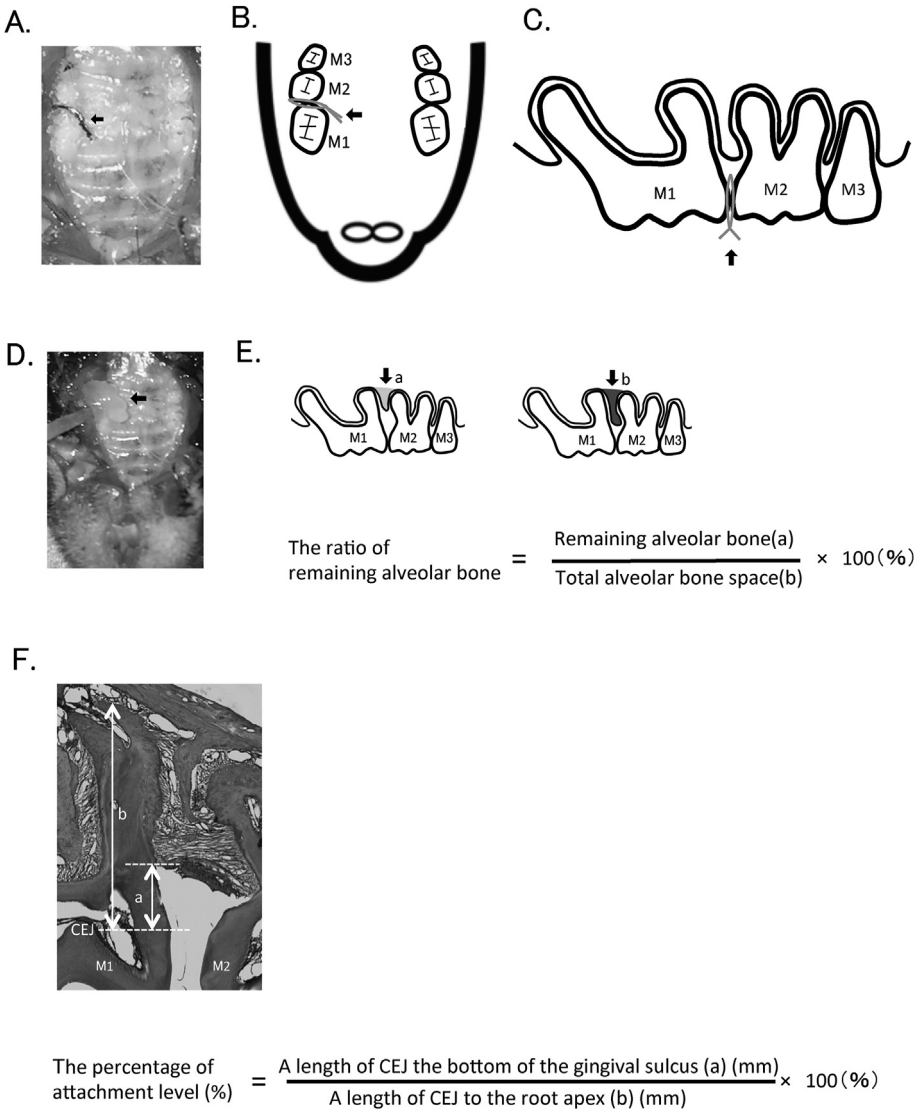

Fig. 1. Position of ligature wire placement in mice and schematic diagram. (A) Ligature wire (arrows) was tied around contact point between first molar and second molar. (B) Position of ligature wire (arrows) placement, occlusal view. (C) Position of ligature wire (arrows) placement, buccal view. Abbreviations: M1, first molar; M2, second molar; M3, third molar; ↑: ligature wire. (D) Topical ointment (arrows) application. Abbreviations: ↑: ointment. (E) Schematic diagram of the space between the remaining alveolar bone (arrows a). Schematic diagram of the total alveolar bone space (arrows b). Formula for calculating the percentage of remaining alveolar bone. Abbreviations: M1, first molar; M2, second molar; M3, third molar. (F) Schematic diagram of the percentage of attachment level. Formula for calculating the percentage of attachment level. Abbreviations: (a) length of CEJ the bottom of the gingival sulcus; (b) length of CEJ to root apex; M1, first molar; M2, second molar; CEJ, cementoenamel junction.

2.5. Serum bone metabolism marker measurement

Next, we investigated the systemic effects of swallowing excess ointment. We created four groups (ointment free, peroral administration, local administration with non-RMA ointment, and local administration with RMA ointment) to evaluate the systemic effects of ingesting RMA ointment. In this experiment, 8-week-old male WT (C57BL/6J) and OPG KO mice were used in each of the ointment-free groups (WT n = 8, OPG KO n = 8) and peroral administration groups (WT n = 8, OPG KO n = 8). The same mice used in the topical application experiment were used for the local administration groups with RMA or non-RMA ointments. We administered the ointments to WT and OPG KO mice and collected blood samples. Blood TRAP concentration was measured using an ELISA kit (Immunodiagnostic Systems, Ontario, Canada). Serum alkaline phosphatase activity was measured using Liquitech ALP (Roche Diagnostics K.K., Tokyo, Japan).

2.6. Statistical analyses

Data are expressed as means ± SEM. Normality was examined using the Shapiro–Wilk test; statistical significance was determined using Tukey's multiple comparison test. Statistical analyses were performed using Graph Pad Prism v.7 (Graph Pad Software, San Diego, CA); $P < 0.05$ was considered significant.

3. Results

3.1. Comparison of the remaining alveolar bone percentage by μ-CT imaging

There was no difference in the percentage of remaining alveolar bone between WC and OC 8 weeks after the start of the experiment. Compared with their counterparts without ligature, both WR– and OR– with ligature had a lower percentage of remaining alveolar

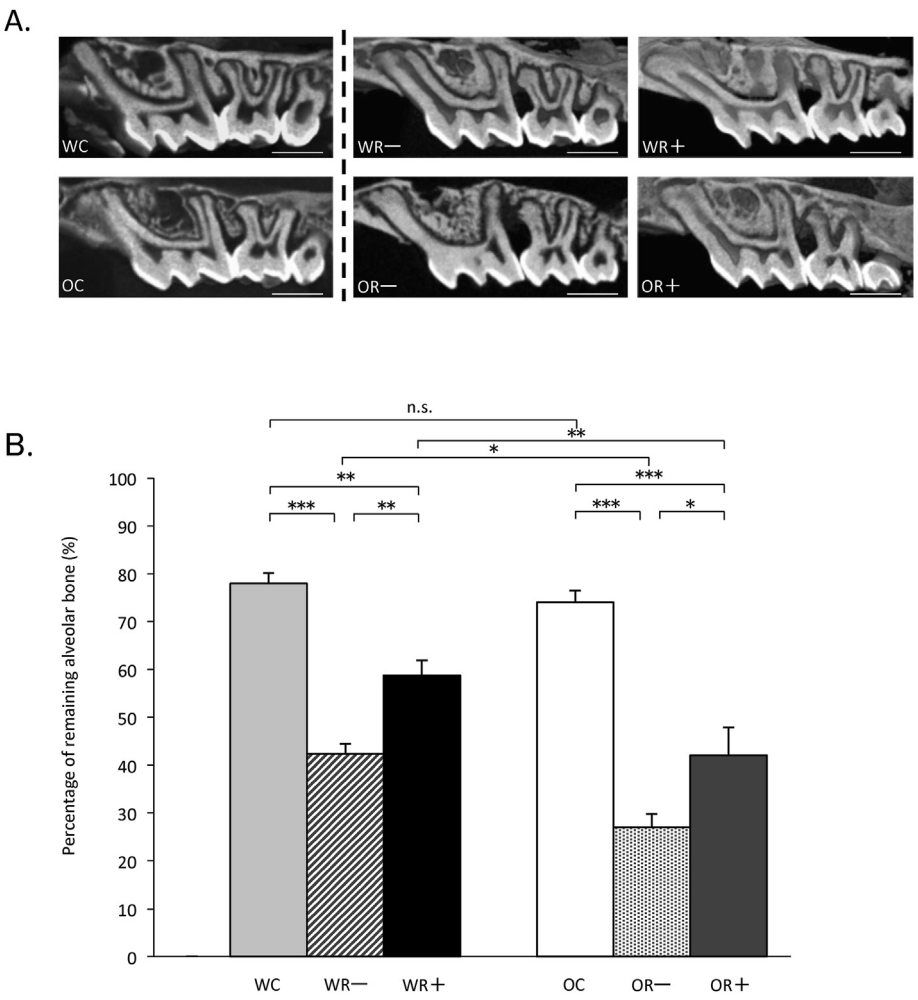

Fig. 2. Microfocus x-ray CT findings between M1and M2 in mice. (A) Representative micro-computed tomography images of the alveolar bone of test and control maxillae at 8 weeks after ligature wire was placed (buccal view), Scale Bar = 1000 μm. (B) Remaining alveolar bone (%), n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001, Abbreviations: WC, wild-type mice without ligature or RMA; OC, OPG knockout mice without ligature or RMA; WR-, wild-type mice without RMA; OR-, OPG knockout mice without RMA; WR+, wild-type mice with both ligature and RMA; OR+, OPG knockout mice with both ligature and RMA.

bone. OR− had a lower percentage of remaining alveolar bone compared with WR−. Both WR+ and OR + had a higher percentage of remaining alveolar bone compared with their RMA− counterparts (Fig. 2-A, B).

3.2. H&E staining of periodontal tissue and attachment level

No difference in the percentage of attachment was found between WC and OC 8 weeks after the start of the experiment. WR− and OR− with ligature had a larger loss of attachment compared with their counterparts without ligature. Furthermore, attachment loss was larger for OR− than for WR−, and WR + and OR + had smaller attachment loss compared with WR− and OR− (Fig. 3-A, B).

3.3. Osteoclast count

The osteoclast count was higher in OC than in WC 8 weeks after the start of the experiment. Compared with their counterparts without ligature, WR− and OR− mice with ligature had higher osteoclast numbers. Osteoclast count was also higher for OR− than for WR−. Compared with their counterparts without RMA, both WR+ and OR + had lower osteoclast counts (Fig. 3-C, D).

3.4. Immunohistochemical scores of inflammatory cytokines for TNF-α and IL-1β

TNF-α scores were not different in terms of immunostaining scores between WC and OC 8 weeks after the start of the experiment. Scores were higher for the ligated WR− and OR− mice compared with their non-ligated counterparts. Scores were also higher for OR− mice than for WR− mice. Compared with their counterparts without RMA, both WR + and OR + had lower scores (Fig. 4-A, B, C, D).

3.5. Serum bone metabolism marker measurement

In the absence of the ointment, OPG KO mice scored higher for blood TRAP and serum ALP values than WT mice. There was no difference in blood TRAP and serum ALP values between mice that received non-RMA ointment and those that received RMA-

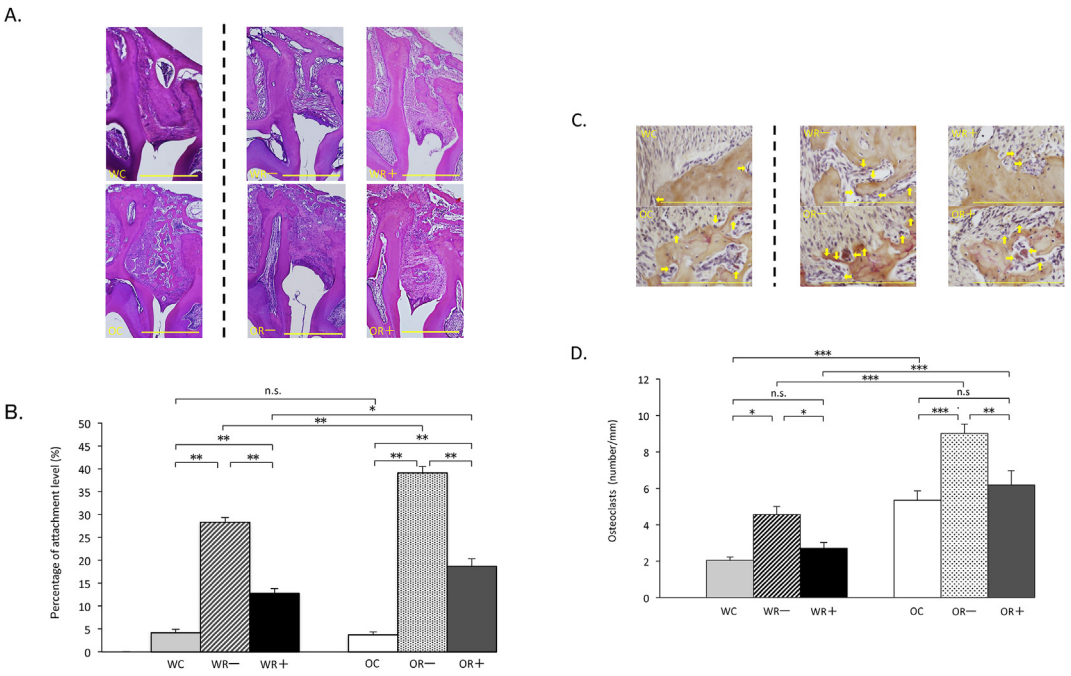

Fig. 3. Pathological observations between M1and M2 in mice. (A) Representative hematoxylin-eosin stained periodontium between the first and second molars at 8 weeks after ligature wire was placed. Bar = 500 μm, original magnification × 100. (B) The percentage of attachment level from cementoenamel junction to the apex of the root 8 weeks after placement of ligature wire (%), n.s., not significant; *p < 0.05; **p < 0.01, Abbreviations: WC, wild-type mice without ligature or RMA; OC, OPG knockout mice without ligature or RMA; WR-, wild-type mice without RMA; OR-, OPG knockout mice without RMA; WR+, wild-type mice with both ligature and RMA; OR+, OPG knockout mice with both ligature and RMA. (C) Osteoclasts (arrows) along the bone surface between the first and second molars at 8 weeks after ligature wire was placed (Bar = 200 μm; original magnification, 400×; ↑:osteoclasts). (D) Osteoclast count (number/mm), n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001, Abbreviations: WC, wild-type mice without ligature or RMA; OC, OPG knockout mice without ligature or RMA; WR-, wild-type mice without RMA; OR-, OPG knockout mice without RMA; WR+, wild-type mice with both ligature and RMA; OR+, OPG knockout mice with both ligature and RMA.

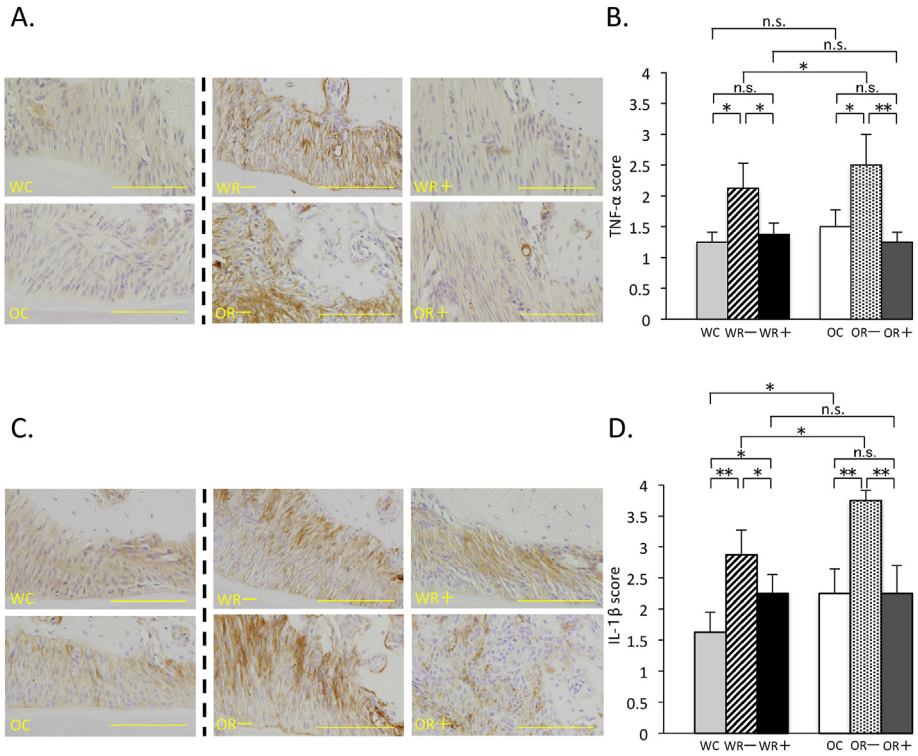

Fig. 4. Immunohistochemical observations between M1and M2 in mice. (A) Immunostaining of TNF-α, (B) Immunohistochemical scores of TNF-α, (C) Immunostaining of IL-1β, (D) Immunohistochemical scores of IL-1β at 8 weeks after ligature wire was placed. n.s., not significant; *p < 0.05; **p < 0.01, Bar = 100 μm, original magnification × 400. Abbreviations: WC, wild-type mice without ligature or RMA; OC, OPG knockout mice without ligature or RMA; WR-, wild-type mice without RMA; OR-, OPG knockout mice without RMA; WR+, wild-type mice with both ligature and RMA; OR+, OPG knockout mice with both ligature and RMA.

containing ointment orally, RMA-free ointment topically, and RMA-containing ointment topically, regardless of whether they were WT or OPG KO (Fig. 5-A, B).

4. Discussion

4.1. Experimental design

Periodontal disease is considered a lifestyle disease. It is harmful to the whole body, and thus the general well-being of an organism is closely associated with periodontal health; this relationship has led to the establishment of the field of periodontal medicine. In a systematic review of the relationship between localized alveolar bone resorption caused by periodontal disease and whole-body bone mass reduction in osteoporosis, Martínez et al. report a connection between periodontal disease and osteoporosis.[22]

The OPG KO mice used in our experiment were genetically devoid of OPG, and thus exhibited notable osteoclast activity due to osteoporosis as they matured. Compared with WT mice, these KO mice had lower bone density due to cortical and trabecular bone porosity; hence, they are considered a useful model of periodontal disease.

Bacteria in the gingival sulcus and periodontal pocket are deeply involved in the pathology of periodontal disease; thus, the aim of treatment is bacterial control.[23] The main methods of treatment are mechanical cleaning such as brushing, scaling, and root planing, with localized administration of drugs such sustained-release antibacterial agents via local drug delivery systems to act directly on the pathogens. Ointments such as minocycline hydrochloride are sometimes used to treat periodontal disease. Other such drugs are being studied[24,25]; however, these studies investigated the effects of the drugs but not the optimal formulation for clinical administration. We thus decided to use an ointment formulation, which would be most practical for local application clinically in the future. Conventionally, transdermal permeation tests with ointments[26] are performed with hairless mice, which have high transdermal permeability and absorption.[27] However, because our study aimed at local delivery to the oral cavity mucous membrane rather than skin, we opted for normal mice (C57BL/6J).

4.2. Effect of RMA-containing ointment on bone metabolism

The findings that OR− mice had lower remaining alveolar bone percentage and higher osteoclast count compared with WR− mice indicate that OPG KO mice have naturally vulnerable bone structure. Presumably under conditions favoring alveolar bone resorption such as ligature wire fitting, osteoclast activity was promoted even more, leading to loss of bone homeostasis. This suggests that periodontal disease and osteoporosis are closely interrelated. In our study, we used attachment level as a measure of periodontal disease severity (i.e., a greater attachment level corresponds to more advanced bone resorption).[28] Given that WR+ and OR + mice both had smaller attachment loss than WR− and OR−, the application of RMA-containing ointment appeared to inhibit alveolar bone resorption and alleviated periodontal disease progression. Moreover, comparison of inflammatory cytokine scores by immunostaining showed that RMA-containing ointment reduced immunostaining scores for TNF-α and IL-1β, indicating localized suppression of inflammation. Indeed, administration of neutralizing antibodies of TNF-α and IL-1β to experimentally induced periodontal disease animal model results in 80% reduction of inflammatory cell infiltration and 60% reduction of bone resorption.[29] Both TNF-α and IL-1β levels increase in the early phase of inflammation; TNF-α between 1 and 24 h[30] and IL-1β between 1 min and 1 h[31] after the onset of inflammation. Kapoor et al.[32] found TNF-α and IL-1β to be indices of tooth movement in gingival crevicular fluid during orthodontic treatment, and that inhibiting their production could lead to inhibition of periodontal tissue destruction. Although other reports describe reduced inflammatory cytokines leading to suppressed alveolar bone resorption, there is no evidence of inhibiting of local inflammation by suppression of bone resorption. As in the study by Mizuno et al.,[17] where specific osteoclast inhibitor RMA was administered intraperitoneally to periodontitis model mice, in the present study, RMA suppressed localized inflammation and alveolar bone resorption, and decreased the osteoclast count to a similar degree. However, further investigation is necessary to determine how and why TNF-α and IL-1β expression was inhibited.

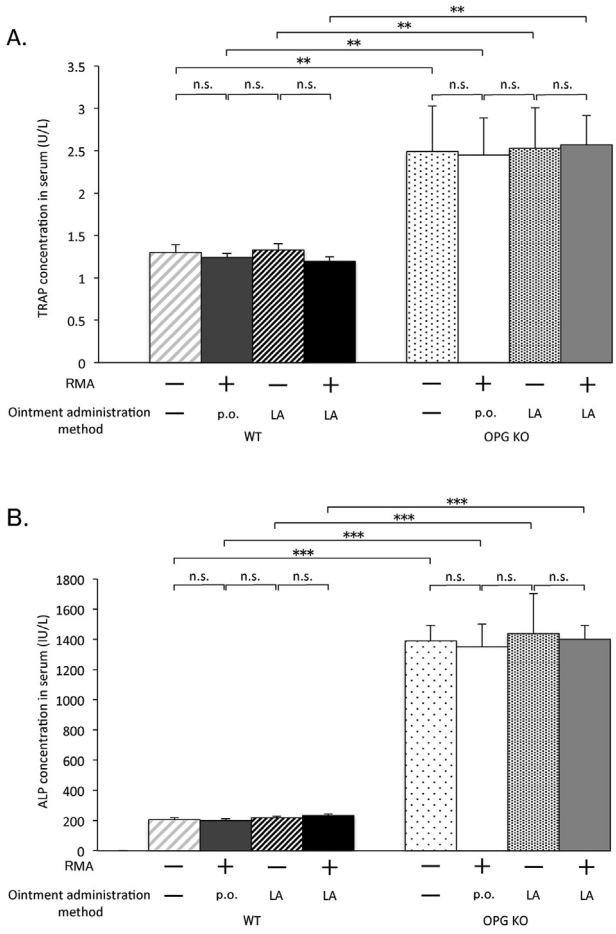

Fig. 5. Serum bone metabolism marker measurement. (A) TRAP concentration in serum (U/L). (B) ALP concentration in serum (IU/L). n.s., not significant; **p < 0.01; ***p < 0.001, Abbreviations: WT, wild-type mice; OPG KO, OPG knockout mice; RMA+, given reveromycin A with ligature; RMA−, not given reveromycin A with ligature; p.o., per os (peroral administration); L.A.,Local Administration.

Our findings showed that localized administration of RMA-containing ointment resulted in suppressed alveolar bone resorption, reduced osteoclast count, and lower immunostaining scores of inflammation sites compared with controls in both OPG KO and WT mice. The effectiveness of RMA ointment in OPG KO mice suggests its effectiveness in treating patients with vulnerable periodontal tissue due to periodontitis and patients with high-turnover osteoporosis.

4.3. RMA and other bone resorption inhibitors

A periodontitis study revealed that in addition to inflammation, bone resorption control is also important in understanding and treating periodontitis.[13] However, conventional drugs with bone resorption inhibitory effect similar to RMA have possible side effects. Bisphosphonates (BP) are already in clinical use for treatment of osteoporosis, and the mechanism of action involves adherence to bone surface to induce osteoclast dysfunction and apoptosis, reducing the osteoclast count and suppressing bone resorption. However, BP-related osteonecrosis of the jaw was reported in 2003.[33] The current incidence in overall BP-treated cases is 0.05–0.1%, with higher incidence in patients with malignancy and cases of tooth extraction during BP treatment.[34] There are also racial differences in susceptibility, with a higher incidence in the Japanese population compared with people of European descent.[35] Denosumab was thus introduced as a new drug for osteoporosis treatment. Denosumab is a human IgG2 monoclonal antibody that targets RANKL and suppresses osteoclast precursor cell differentiation into osteoclasts.[36] Unlike BP, denosumab has a short half-life of about 1 month, does not accumulate in bone, and does not induce osteoclast apoptosis,[37] thus it was expected not to cause osteonecrosis of the jaw. Contrary to expectation, denosumab was found to cause a similar condition, denosumab-related osteonecrosis of the jaw, almost at the same incidence as BP-related osteonecrosis of the jaw.[38] It was therefore considered necessary to take the utmost care not to elicit this side effect when using these drugs for periodontitis treatment. Accordingly, we shifted our attention to RMA, the novel specific inhibitor of osteoclast activity.

RMA is an acid polyketide compound isolated from *S. reveromyceticus*. It is ineffective as drug therapy against premature precursor cells and inactive osteoclasts that have no bone resorption function, and specifically induces apoptosis in active osteoclasts with bone resorption function. Because it is a tricarboxylic acid-containing compound, it is selectively taken up in an acidic environment-dependent manner by active osteoclasts that are secreting acid to dissolve bone. Moreover, RMA has a remarkably short half-life of about 1 h and is ineffective when administered orally because its half-life is too short for it to be absorbed in the digestive tract. Hematologic findings in our study showed that both oral administration and ointment application did not affect osteoclast and osteoblast activity elsewhere in the body. Further studies are required to better understand this, but given that localized application of the RMA ointment in the oral cavity had no notable systemic effect, it is considered to reduce the osteoclast count and inhibit alveolar bone resorption exclusively at the inflammation site. Together with the study by Mizuno et al.,[17] our data suggest that RMA could be applied clinically as a novel non-invasive treatment for periodontitis.

In summary, our data suggest that localized administration of RMA ointment in the oral cavity may prevent periodontitis in patients with high-turnover osteoporosis and highly vulnerable periodontal tissue, as well as in healthy individuals. Hematologic findings in our study showed that topical RMA ointment had no remarkable systemic effect but is considered to reduce the osteoclast count and inhibit alveolar bone resorption exclusively at the inflammation site. Therefore, localized application of the specific osteoclast suppressor RMA in ointment form in the oral cavity could be a novel treatment for periodontitis that inhibits alveolar bone resorption locally without risk of systemic side effects.

Authors' contributions

K. Miyazawa, M. Tabuchi, contributed to conception and design, acquisition, analysis, and interpretation, drafted the manuscript; Y. Asano, contributed to acquisition, analysis, and interpretation, drafted the manuscript; S. Kako, contributed to acquisition, analysis, critically revised the manuscript; M. Kawatani, H. Osada, contributed to acquisition, critically revised the manuscript; H. Maeda, contributed to interpretation, critically revised the manuscript; S. Goto, contributed to conception and design, interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Declaration of competing interest

The authors declare that they have no competing interests.

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