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Original article

Local administration of calcitonin inhibits alveolar bone loss in an experimental periodontitis in rats

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ABSTRACT

Calcitonin (CTN), a calcium regulatory hormone, promotes calcium diuresis from the kidney and suppresses bone resorption. The objective of this study was to evaluate whether the topical and intermittent application of CTN inhibits alveolar bone resorption using ligature-induced experimental periodontitis in rats. Experimental periodontitis was induced by placing a nylon ligature around maxillary molars of 8-week-old male Wistar rats for 20 days. Thirty-two rats were divided into four groups: basal sham control group, periodontitis group, periodontitis plus 0.2 U CTN (low dose), and periodontitis plus 1.0 U CTN (high dose) group. To investigate the effects of CTN on alveolar bone resorption, CTN was topically injected into the palatal gingivae every 2 days after ligature removal (day 0). Micro-computed tomography (CT) analysis was performed for linear parameter assessment of alveolar bone on day 5 and day 14. Periodontal tissues were examined histo-pathologically to assess the differences among the study groups. Micro-CT images showed that alveolar bone resorption was induced statistically around the molar of ligatured rats on day 5 and day 14. The amount of bone resorption was more severe on day 14 than that on day 5. On day 5, only high-dose CTN treatment significantly suppressed bone resorption. In addition, both doses of CTN significantly suppressed bone resorption on day 14. Histological examination clarified that there were fewer TRAP-positive cells in the CTN treatment groups than in the periodontitis group on day 5. Local administration of CTN decreased alveolar bone resorption by regulating osteoclast activation in rats with periodontitis.

1. Introduction

Periodontitis is a polymicrobial infectious disease and may result in loss of teeth by inflammation-mediated bone resorption [1]. It is well-known that periodontal inflammation arising from bacterial infection exacerbates bone destruction. Bone homeostasis is controlled by a balance between osteoblastic bone formation and osteoclastic bone resorption [2]. An imbalance between the interaction of osteoblasts and osteoclasts leads to the progression of periodontitis.

Osteoclast activation and maturation is regulated by the receptor activator of nuclear factor-κB (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) [3]. Excessive activation of osteoclasts in periodontitis lesions might lead to severe alveolar bone resorption. Recently, Kim et al. reported that curcumin, a potent antioxidant drug, suppressed ovariectomy-induced bone loss, at least in part, by reducing RANKL signaling [4]. Furthermore, Nakamura et al. suggested that green tea catechin suppressed lipopolysaccharide-induced bone resorption by inhibiting IL-1beta production or by directly inhibiting osteoclastogenesis [5]. Drugs with a capacity to modulate inflammatory responses including bone resorption might be useful clinically because these drugs may have new therapeutic effects.

Calcitonin (CTN), which is released from parafollicular cells (C cells) in the thyroid gland, is a hormone involved in bone homeostasis and the regulation of calcium metabolism [6]. In bone tissues, CTN binds to osteoclasts exclusively, exhibiting the highest expression of calcitonin receptor (CTR), and causes cessation of osteoclast activity [6,7]. Granholm et al. reported that CTN inhibits osteoclast formation in mouse hematopoietic cells by regulating RANK signaling [8]. In addition, CTN has been used for Paget's disease, hypercalcemia of malignancy, osteogenesis imperfecta, and post-menopausal osteoporosis [9]. Although these findings suggest that bone loss can be attenuated by CTN administration, few studies have investigated the efficacy of CTN in the treatment of periodontitis.

Parathyroid hormone (PTH), an anabolic agent targeting bone, is

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known as an endocrine hormone secreted by parathyroid glands [10], and many studies have shown that PTH is effective in reversing bone loss due to its effect in increasing osteoblast number and activity [11,12]. Furthermore, CTN and PTH are complementary hormones involved in bone metabolism [6]. Previously, we reported that topical and intermittent administration of PTH recovered alveolar bone loss in experimental rat periodontitis [13]. *In vivo* experimental periodontitis is useful to clarify the pathogenesis and treatment strategies for periodontal disease. In the present study using micro-computed tomography (micro-CT) and histology, we explored the inhibitory effects of CTN on the alveolar bone resorption of rats with experimental periodontitis.

2. Materials and methods

2.1. Animals

Thirty-six male Wistar rats (200–250 g, 8 weeks-old) were purchased from CLEA Japan (Tokyo, Japan). Rats were fed with standard rodent chow and water *ad libitum*, and housed in a temperature-controlled room (23 ± 1 °C, 60 ± 5% humidity) under a 12-h light-dark cycle. Prior to the surgical procedures, all animals were allowed to acclimatize to the laboratory environment for 1 week. The experimental protocol was approved by the Animal Research Control Committee of the Tokushima University Graduate School. The study complied with the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines developed by the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) [14].

2.2. Experimental periodontitis

For experimental periodontitis induction, the cervical area of the right second molar of the rat maxilla was ligatured with sterilized nylon thread (5–0: Natsume Corporation, Tokyo, Japan) under anesthesia with sodium pentobarbital for 20 days according to a previous report [13]. The ligature was knotted on the mesial in order to confirm the ligature remained during the experimental period, and the ligature was checked every 2 days to ensure subgingival placement, and was replaced when necessary.

CTN used as Elcitonin was a gift from Asahi Kasei Pharma (Tokyo, Japan). Elcitonin is a calcitonin derivative that is transformed from eel calcitonin by modifying the S—S bond into a stable C—N bond. Thirty-six rats were randomly divided into three groups as follows: ligature placement and administration of saline (N = 12), ligature placement and administration of low-dose of CTN (N = 12, 1.0 U/kg), and ligature placement and administration of high-dose of CTN (N = 12, 5.0 U/kg). The left maxillae without ligature was used as a non-ligatured control (basal sham control) and selected randomly (N = 12). CTN was administered into the subperiosteum at the right buccal and palatal gingivae of the maxillary second molar every 2 days in a volume of 50 μL for 5 or 14 days, and the injection level of CTN was used according to a previous report [15]. Solutions were prepared just before administration. The number of animals was justified using a sample size calculation based on the primary outcome variable [16].

2.3. Micro-CT examination

The maxillary specimens were evaluated by micro-CT imaging using a micro-CT scanner (Hitachi Medico, Tokyo, Japan). To maintain the longitudinal position of the samples, the maxillae were oriented vertically in a sample holder. Scans were performed by determining the angle based on the position of buccal and palatal cusps in the second molar. The micro-CT setting was as follows: 1024 × 1024 pixel size, 14 μm slice thickness, 8× magnification, 50 kV voltage, and 0.1 mA electrical current. Three dimensional images were produced using computer software TRI/3D-BON (Ratoc Systems Inc., Tokyo, Japan). The distance from the palatal cementum-enamel junction (CEJ) to the alveolar bone crest (ABC) of the second molar was measured using computer software.

2.4. Histological analysis

On day 5 and day 14, six rats from each group were sacrificed, and the maxillae were collected, dissected, and immediately fixed in 4% paraformaldehyde (Wako Pure Chemical Industries Ltd., Osaka, Japan). After fixation, samples were decalcified with 10% EDTA for 28 days and embedded in paraffin (Paraplust Plus, Sigma, St Louis, MO). The frontal sections, parallel with the mesial root of the second molar, were cut into 4 μm-thick sections. All sections were routinely stained with hematoxylin and eosin. Vertical alveolar bone loss of the second molar of the maxilla was assessed for inflammation and alveolar bone destruction was assessed by measuring the length (mm) of the CEJ to ABC between the first and second molars of the maxilla as described previously [13]. In brief, five sections were randomly obtained from each rat to observe histological changes and the measured area was determined at the square (200 × 200 μm at a magnification of 400×) of the buccal site around the alveolar bone crest. The length was measured at five sites in the square and the mean value was determined. Next, infiltrating cells were designated as inflammatory cells, including macrophages, lymphocytes, and neutrophils, based on shape and size. The total number of inflammatory cells represented the inflammatory status of the connective tissues, and the mean of the histological data was calculated as described previously [17]. For osteoclast analysis, sections were stained with a tartrate-resistant acid phosphatase (TRAP) kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. The stained sections were observed under an optical microscope (Microphoto V series, Nikon, Tokyo, Japan), and the TRAP-positive multinucleated cells were defined by cells with greater than three nuclei under a light microscope and counted according to a previous report [18]. Five sections of each group were analyzed and each measurement was conducted by an independent examiner in a blind manner. The results from each group were expressed as mean ± SD.

2.5. Statistical analysis

Statistical analyses were performed with SPSS Statistics version 20 (Armonk, NY). Significant differences among each group were analyzed using one-way ANOVA with Scheffe *post hoc* test or Tukey-Kramer HSD test, because the data were not normally distributed. In all statistical analyses, P values of less than 0.05 were considered significant.

3. Results

3.1. A volumetric quantitative analysis of alveolar bone loss

No clinical signs of systemic infection or mortality were noted in any of the rats at any time.

The micro-CT images show decreased alveolar bone height of the molars in ligatured rats compared to that of non-ligatured rats on both day 5 and day 14 (Fig. 1A). We confirmed that significant alveolar bone loss was found in the group of experimental periodontitis. The alveolar bone loss was inhibited by application of high-dose CTN on both day 5 and day 14. In case of low-dose CTN application, no significant difference was observed in bone loss compared to that of the periodontitis group on day 5, but significant inhibition of bone loss was observed by low-dose CTN application on day 14.

The distance between CEJ and ABC represents the degree of bone resorption, and the lengths were measured in each group and quantitatively analyzed. As shown in Fig. 1B, the CEJ-ABC length of the ligatured rat group increased significantly compared to that of the non-ligatured group (day 5: ligatured, 442.6 ± 67.3 μm, non-ligatured, 280.8 ± 52.4 μm, $P = 0.0017$; day 14: ligatured, 585.6 ± 59.4 μm, non-ligatured, 258.3 ± 21.3 μm, $P < 0.0001$, one-way ANOVA-