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Parathyroid hormone administration may modulate periodontal tissue levels of interleukin-6, matrix metalloproteinase-2 and matrix metalloproteinase-9 in experimental periodontitis

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Background and Objective: Intermittent administration of the parathyroid hormone (1–34) has an anabolic effect on bone and it has been shown to reduce alveolar bone loss in experimental periodontitis models. The aim of the present study was to investigate the effect of parathyroid hormone on tissue degradation-related factors in an experimental periodontitis model in rats.

Material and Methods: Periodontitis was induced in seventy-six male Wistar rats using ligature around the lower right first molars. The animals were then treated with parathyroid hormone (1–34) (T-group) or vehicle (C-group), three times a week for 15 d (C15, T15) or 30 d (C30, T30). At each experimental time-point, the 19 rats were killed in each group and the gingival tissue around the first lower molar was removed and prepared for the following analyses: mRNA expression of interleukin-1beta, interleukin-6, matrix metalloproteinase (MMP)-2 and MMP-9, and gelatinolytic activity of MMP-2 and MMP-9. Hemimandibles were decalcified, and serial sections were processed and analyzed for interleukin-6 immohistochemistry. Samples were also histochemically stained by tartrate-resistant acid phosphatase (TRAP) to evaluate the number of osteoclasts present.

Results: Parathyroid hormone-treated samples showed decreased of levels of mRNA for interleukin-6 in the T30 group ($p < 0.01$) and of MMP-2 in the T15 and T30 groups ($p < 0.05$). Zymography assays demonstrated that treatment with parathyroid hormone led to a decrease in MMP-9 activity ($p < 0.01$). TRAP staining of alveolar bone revealed that osteoclasts were present in higher numbers ($p < 0.05$) in the groups not treated with parathyroid hormone.

Conclusion: These data suggest that intermittent administration of parathyroid hormone can down-regulate the expression of biomarkers responsible for connective tissue breakdown and bone resorption, and potentially affect alveolar bone resorption activity.

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Bone resorption is a major pathological factor in chronic inflammatory diseases such as periodontitis and arthritis. It is known that cytokines, including interleukin-1, interleukin-6 and tumor necrosis factor-alfa, regulate the onset and progression of bone loss by initiating a cascade of cellular signals, resulting in the differentiation and activation of bone-resorbing osteoclasts (1–3). Together with bone resorption, extracellular matrix degradation in periodontal disease has been reported to occur in periodontal tissues owing to elevated activity of the matrix metalloproteinases (MMPs) (4). Pathologically elevated levels of active MMP-2 (gelatinase A-type IV collagenase) and MMP-9 (gelatinase B-type IV collagenase) have been found in the gingival crevicular fluid, gingival tissue and salivary samples from patients with chronic periodontal disease (5–7). Recently we have reported that intermittent administration of human parathyroid hormone was able to protect against periodontitis-associated bone loss in a murine model (8,9). Parathyroid hormone functions as a major mediator of bone remodeling and as an essential regulator of calcium homeostasis. Continuous infusion of parathyroid hormone is known to regulate plasma calcium levels by stimulating osteoclast activity, involving physiological or hyperparathyroidism conditions. However, exogenous intermittent administration of parathyroid hormone was reported to increase both the number of osteoblastic cells and the bone mass (10). Daily doses of parathyroid hormone can reduce the incidence of fracture in postmenopausal women, in elderly men and in women with glucocorticoid-induced osteoporosis (11,12).

The anabolic effect of parathyroid hormone has also been extensively demonstrated in mice and rats (13). Although the effect of intermittent administration of parathyroid hormone in inflammatory conditions has been poorly studied, favorable effects on bone metabolism by increasing bone formation were reported in animal models for arthritis. Moreover, studies on elderly women have indicated the anti-inflammatory effect of recombinant human parathyroid hormone (1–34) (14,15). For example, intermittent parathyroid hormone administration can activate bone formation, resulting in increased bone mineral density, which consequently improves the mechanical properties in rats with collagen-induced arthritis (16). Furthermore, a bone-protective effect has been reported, resulting from an antagonist effect to the receptor activator of nuclear factor-κβ ligand (RANKL) or tumor necrosis factor-alfa, following administration of parathyroid hormone (17).

As the mechanisms controlling the effect of parathyroid hormone on inflammatory diseases (such as periodontitis) are not understood, the aim of this study was to test the hypothesis that parathyroid hormone may modulate the expression of pro-inflammatory cytokines (interleukin-1β, interleukin-6) and of MMPs (MMP-2 and MMP-9), and the activation and number of osteoclasts in the periodontium of rats subjected to periodontal disease.

Material and methods

Animals and treatment

Seventy-six, 4-wk-old male Wistar rats, mean weight 78.4 ± 7.1 g, were included in this study. For inducing experimental periodontitis, the rats were anesthetized with ketamine (10 mg/kg) and xylazine (5 mg/mL), and then a cotton ligature was placed around the lower right first molar of the animals. On the same day, 38 rats (T, the treated group) started to receive 40 μg/kg of human parathyroid hormone (1–34) (Sigma, St Louis, MO, USA) three times a week subcutaneously, and 38 rats (C, thecontrol group) received the parathyroid hormone vehicle, 1% acetic acid, following the same regimen (8). After 15 d of treatment, 19 rats of each group were killed (the C15 group and the T15 group). The remaining animals were killed on day 30 (the C30 group and the T30 group). Gingival tissue around the lower right first molars was removed: gene expression in the tissue was analyzed using the reverse transcription–polymerase chain reaction (RT-PCR) (five animals per group); and the presence of MMP was established using immunoprecipitation and zymography (four animals per group). Histological evaluations, using tartrate-resistant acid phosphatase (TRAP), a marker for osteoclasts (five animals per group), and immunohistochemistry (five animals per group), checking for interleukin-6 expression and location, were performed after decalcification of the right hemimandibles. The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

RNA purification and semiquantitative RT-PCR

Total RNA was obtained from the gingival tissue around the ligated teeth using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The integrity of the extracted RNA was analyzed on 1.2% agarose gels stained with ethidium bromide. RNA samples were treated with DNAse I (Amplification Grade; Invitrogen), and cDNA synthesis was performed using the Superscript III RT enzyme (Invitrogen) in reactions containing 1 μg of RNA, following the manufacturer's recommendations. Specific primers were designed for MMP-2, MMP-9, interleukin-1beta, interleukin-6 and beta-actin, and the transcripts were amplified in 50-μL reaction mixes containing 2 μL of cDNA, 2 U of Platinum Taq DNA Polymerase (Invitrogen), 0.5 μM dNTPs, 0.2 mM each primer and 1× buffer containing 1.5 mM $MgCl_2$. In order to validate the semiquantitative RT-PCR technique to compare the gene expressions in different groups, a specific number of cycles of the PCR reaction for each gene studied was defined at the exponential phase of amplification. The reaction products were resolved on 1.8% agarose gels stained with ethidium bromide, and bands were quantified by densitometric analysis using the GS-700 Densitometer and Molecular Analyst Software (Bio-Rad, Hercules, CA, USA). RT-PCR reactions were performed using the following primer sequences and amplification profiles [temperature (°C)/cycles]: beta-actin: (forward)

5′-TGACATCCGTAAAGACCTCT-3′, (reverse) 5′-AGATGTGATCAGCAAGCAGCAG-3′, - 48/15; MMP-2: (forward) 5′-GAGTTGGCAGTGCAATACCT-3′, (reverse) 5′-GCCGTCCTT CTCAAAGTTGT-3′ - 54/30; interleukin-6: (forward) 5′-TAGAGTCACAGAAGGAGTGG-3′, (reverse) -3′ 5′-GCCAGTTCTTCGTAGAGAAC-3′ - 48/40; and interleukin-1beta: (forward) 5′-TCCATGAGCTTTGTACAAGG-3′, (reverse) 5′-GGTGCTGATGTACCAGTTGG-3′-60/40; MMP-9: (forward) 5′-CATGGCCTATCCCACA-3′, (reverse) 5′-TCCTCCGTGATTCGAG AAC-3′-52/35.

Zymography

Gingival tissues were washed in phosphate-buffered saline, weighed and homogenized. Then, 0.8 mL of Dulbecco's modified Eagle's minimal essential medium (Sigma Chemical Co.) containing 50 μg/mL of ampicillin (Garamicina; Schering-Plough, Duque de Caxias, Rio de Jeneiro, Brazil) was added to each ± 0.05 g of gingival tissue. The samples were incubated at 37°C for 18 h and centrifuged. The supernatants containing the secreted proteins were collected, and protein concentrations were determined according to the Bradford method. Samples were mixed with nonreducing sample buffer and resolved in 10% sodium dodecyl sulfate–polyacrylamide gels copolymerized with 1.6 mg/mL of gelatin (Sigma) as substrate. Protein denaturation was performed by incubation of the gels in 2% Triton X-100 (Sigma), and then the gels were immersed in activation buffer (50 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$) for 16 h at 37°C. Gelatinolytic activity was detected after staining with Coomassie Brilliant Blue R250 (Bio-Rad). To confirm that the bands found were related to MMP activity, a control reaction was made to inhibit the gelatinolytic activity by adding 2 mM of 1.10-phenanthroline (Sigma), a nonselective zinc chelator, to the activation buffer, confirming the specificity of the reactions. The intensities of the bands were determined using the GS-700 imaging densitometer (Bio-Rad) and the software MOLECULAR ANALYST 1.5 (Bio-Rad).

Immunoprecipitation

Samples containing the secreted proteins used for zymography assays were subjected to an immunoprecipitation reaction with rabbit anti-rat MMP-2 (Labvision NeoMarkers, Fremont, CA, USA) immunoglobulin G, and rabbit anti-rat MMP-9 (Labvision) immunoglobulin G. The immunocomplexes were precipitated with protein A–Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) for 14 h at room temperature (25°C). After washing nonspecific Sepharose-adsorbed material with 50 mmol/L Tris, pH 7.4, containing 200 mmol/L NaCl, the immunoprecipitated material was eluted with nonreducing sample buffer for 10 min at 70°C, then assayed using gelatin zymography (18,19).

Enzymohistochemistry

An assay for TRAP was carried out to localize osteoclasts (20). The right hemimandibles were fixed in 2.5% cacodylate-buffered glutaraldehyde (pH 7.4) containing 7% sucrose. The samples were decalcified in 10% EDTA (Merck, Darmstadt, Germany), dehydrated and embedded in paraplast (McCormick, St Louis, MO, USA). Bucco-lingual sections, of 3 μm thickness, were obtained of the right first lower molar regions. The sections were incubated at 37°C for 15 min with a mixture of 4 mg of naphthol AS-BI phosphate (Sigma), as substrate, and 24 mg of red violet salt (Sigma) diluted in 30 mL of acetate buffer (pH 5.2) containing 0.3 mmol/L tartrate (Sigma) pH 5.0. For control slides, the substrate was omitted. Sections were counterstained with Harris hematoxylin, and the TRAP-positive cells (preosteoclasts and osteoclasts) were counted on the linear surface of the alveolar bone crest (adjacent to the cotton ligature and the furcation area), in five sections per animal of all experimental groups, by two calibrated

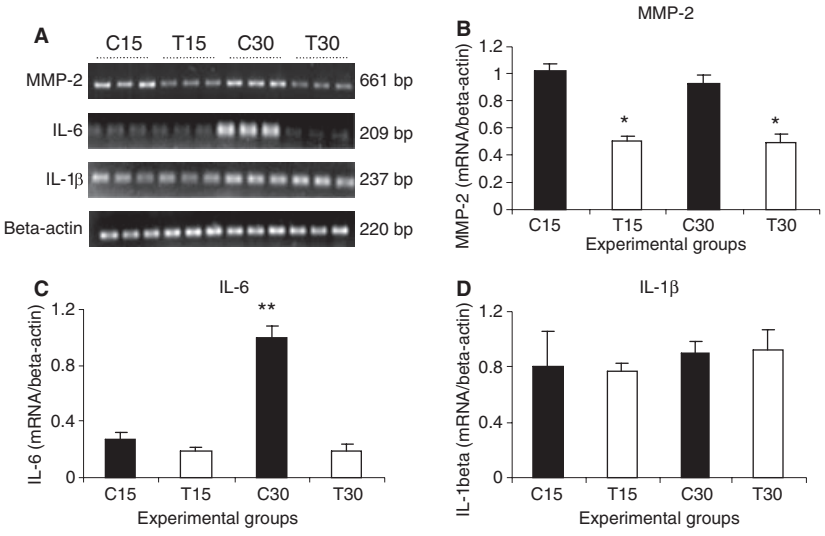

Fig. 1. Equal amounts of reverse transcription–polymerase chain reaction products (10 μL) (A), in triplicate, of gingival tissue from control (C15, C30) and parathyroid hormone-treated (T15, T30) groups of rats (five animals per group) were resolved in 1.8% agarose gels that were stained with ethidium bromide to visualize bands corresponding to matrix metalloproteinase-2 (MMP-2), interleukin-6, interleukin-1beta and beta-actin. Data represent the densitometric analysis, normalized to beta-actin, of the total reverse transcription–polymerase chain reaction products of MMP-2 (B), interleukin-6 (C) and interleukin-1beta (1D) in the experimental groups (C15, C30, T15, T30). Note that treatment with parathyroid hormone decreased the expression of MMP-2 mRNA on experimental days 15 and 30 (*) ($p < 0.05$) and also the expression of interleukin-6 mRNA (**) ($p < 0.01$) but only on experimental day 30. C15, control group, day 15; C30, control group, day 30; T15, parathyroid hormone treatment group, day 15; T30, parathyroid hormone treatment group, day 30; IL-6, interleukin-6; IL-1β, interleukin-1beta.

blinded examiners using KS400 2.0 software (Kontron Electronics, Eching, Germany).

Immunohistochemistry

Right hemimandibles were fixed in modified Karnovsky solution (2% paraformaldehyde, 0.5% glutaraldehyde and 0.2% picric acid), decalcified in 4.13% EDTA (Merck), dehydrated and embedded in paraplast. Sections, 3 μm thick, of the bucco-lingual region were obtained from the right first lower molars. Endogenous peroxidase was inhibited by treatment with 3% H_2O_2. Enzymatic digestion was performed using trypsin (Sigma), then samples were treated with rabbit serum and incubated overnight at 4°C with mouse anti-rat interleukin-6 (Serotec, Oxford, UK) the immunoglobulin isotype is IgG diluted in phosphate-buffered saline (pH 7.4). For control slides, the substrate was omitted. Sections were incubated with secondary antibody (LSAB 2; Dako Corporation, Glostrup Denmark) and then with a diaminobenzidine solution (Sigma). Myer's hematoxylin was used for counterstaining, and five sections per animal were analyzed using light microscopy (Leica DMLP; Leica, Wetzlar, Germany) to localize interleukin-6 in the periodontal tissues.

Statistical analysis

The results found for the RT-PCR (in triplicate), zymography and enzymohistochemstry analyses are presented as means ± standard deviation, and were subjected to one-way analysis of variance and Tukey's multiple comparison at a 5% level of significance.

Results

Matrix metalloproteinase analysis

The gingival tissue samples from the sites of induced periodontitis were evaluated for MMP-2 and MMP-9 activities and also for mRNA expression. MMP expression was evaluated using RT-PCR, which showed expression of MMP-2 in all groups analyzed, with MMP-2 down-regulation observed as an effect of intermittent parathyroid hormone treatment, on days 15 and 30 ($p < 0.05$) (Fig. 1A,B). The expression of MMP-9 mRNA could not be confirmed in all groups. In order to ensure the specificity of the primer sequences to MMP-9, PCR analysis for MMP-9 was performed with rat genomic DNA (data not shown). Next, the effect of parathyroid hormone treatment on MMP-2 and MMP-9 activities was assessed using zymography. The gelatin zymography assay demonstrated that MMP-2 was expressed in its precursor (~ 72 kDa) and active (~ 69 kDa) forms in all samples from all groups. Optical densities of the gel bands indicated that total MMP-2 activity (both pro and active forms) decreased ($p < 0.05$) from experimental day 15 to day 30, regardless of parathyroid hormone treatment (Fig. 2A and 2B). In addition, no effect was observed on MMP-2 activity by parathyroid hormone treatment. Although mRNA expression for MMP-9 was not found, MMP-9 was detected at a very low concentration in groups T15, C30 and T30, but at a high concentration in group C15, as shown by zymography. It was observed that treatment with parathyroid hormone decreased MMP-9 activity ($p < 0.01$), measured 15 d after ligature placement (Fig. 2A and 2C). The immunoprecipitation assay was performed with samples from group C15 (Fig. 2D), and showed the 69 kDa form of MMP-2 (i.e. the active form), a 72 kDa form representing inactive zymogen MMP-2 and 28a form of ~ 86 kDa corresponding to MMP-9.

Interleukins and TRAP analyses

Data analysis demonstrated that treatment with parathyroid hormone significantly decreased interleukin-6 mRNA levels in the gingival tissues 30 d after ligature placement compared with the nontreated group ($p < 0.01$)

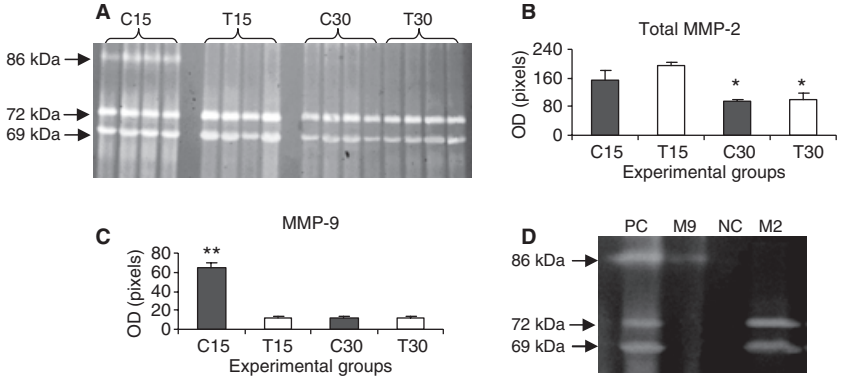

Fig. 2. Equal amounts of protein (0.20 μg), secreted by gingival tissue from all experimental groups (C15, T15, C30 and T30), were resolved in 10% sodium dodecyl sulfate gels copolymerized with 1.6 mg/mL of gelatin, and bands corresponding to matrix metalloproteinase (MMP)-2 (at ~69 and 72 kDa) and to MMP-9 (at ~86 kDa) were detected after incubation of the gels in the MMP-activating buffer (A). Data represent the densitometric optical density (OD) analysis of total MMP-2 (B) and MMP-9 (C), from all experimental groups (C15, C30, T15 T30) and are displayed as mean ± standard deviation. Note that MMP-2 activities decreased 30 d after ligature placement independently of parathyroid hormone treatment (*), $p < 0.05$. Treatment with parathyroid hormone decreased MMP-9 activity 15 d after ligature placement as compared with the nontreated group (C15) (**) $p < 0.01$, $n = 16$ animals, four to each group. Gelatin zymography (D) from immunoprecipitated MMP-2 (at ~69 and 72 kDa) and MMP-9 (at ~86 kDa) from the C15 sample. C15, control group, day 15; C30, control group, day 30; T15, parathyroid hormone treatment group, day 15; T30, parathyroid hormone treatment group, day 30; OD, optical density; M2, MMP-2-immunoprecipitated material; M9, MMP-9-immunoprecipitated material; NC, negative control containing no conditioned medium; PC,positive control containing conditioned medium.

(Fig. 1A,C). Treatment with parathyroid hormone did not affect the expression of mRNA for interleukin-1beta (Fig. 1A,D) at any experimental time-point. Immunohistochemistry analysis revealed the presence of interleukin-6 in tissues from all groups, localized mainly in osteoblasts on the alveolar bone surface adjacent to the ligature (Fig. 3D). TRAP-positive cells were also detected in a region similar to that where interleukin-6 was found(-Fig. 3C). Quantitative analysis showed that treatment with parathyroid hormone reduced the number of TRAP-positive cells ($p < 0.05$) 30 d after the induction of periodontitis (Fig. 3A).

Discussion

Recently, many studies in periodontal research have focused on elucidating the interactions between bone and the immune system in an attempt to identify the etiologies, and new therapies of immune-associated bone disorders (as occur in periodontal disease) were found (21,22). The results of the present study corroborate previously published data (8,9), supporting the view that parathyroid hormone, an important hormone related to bone metabolism, can attenuate periodontal tissue breakdown in experimental periodontitis.

Within the periodontal tissue, a variety of cell types (including T cells, B cells, endothelial cells, osteoblasts, macrophages and fibroblasts) have been found to express the interleukin-6 gene. High levels of expression of interleukin-6 mRNA in inflamed gingiva from periodontitis sites, with minimal expression in healthy gingival tissue, have been reported (23). Our findings show a down-regulation of the interleukin-6 gene in inflamed gingival tissue as a result of treatment with parathyroid hormone. Even though there was no response reported of interleukin-6 expression by intermittent administration of human parathyroid hormone (1–34) under inflammatory conditions, it has been demonstrated that intermittent administration of human parathyroid hormone(1–34) induces a rapid selective increase in interleukin-6 protein in trabecular bone cells, as well as in hematopoietic cells, predominantly localized to osteoblasts, osteocytes and megacaryocytes (24). The rapid expression of interleukin-6 protein underscores its importance as an early mediator of parathyroid hormone action in bone, as has been suggested by previous studies based on mRNA analysis (25).

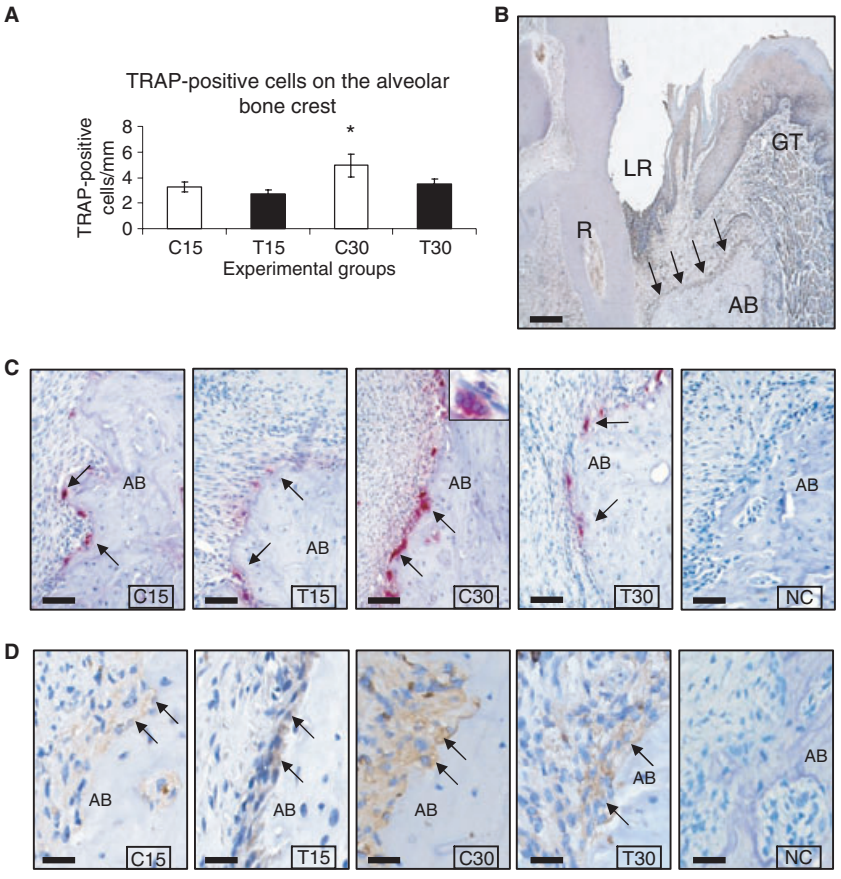

Fig. 3. (A) Data represent the means ± standard deviation of tartrate-resistant acid phosphatase (TRAP)-positive cells on the alveolar bone crest of all experimental groups (C15, C30, T15 and T30). Note that the C30 group had more TRAP-positive cells than the other experimental groups (*) ($p < 0.05$). (B) Photomicrography at low magnification (bar, 200 μm) from a molar tooth with ligature-induced periodontitis, indicating (arrows) the area where representative images of the TRAP (C) and immunohistochemistry (D) reactions were captured. AB, alveolar bone; GT, gingival tissue; LR, ligature region, R, root. (C) Photomicrographs showing enzymohistochemistry to TRAP on the alveolar bone surface in the the experimental groups (C15, T15, C30 and T30). The arrows indicate TRAP-positive cells. Note a TRAP-positive osteoclast shown in high magnification (C30 panel). AB, alveolar bone; NC, control group (no primary antibody); bar, 80 μm. (D) Photomicrographs showing immunohistochemical reactions to interleukin-6. The arrows indicate the areas where cells interleukin-6-positive cells were observed. Note that all experimental groups (C15, C30, T15 and T30) were interleukin-6 positive at a similar location (mainly osteoblasts). AB, alveolar bone; NC, negative control; bar, 50 μm. For TRAP and immunohistochemistry analyses, five slices per animal and five animals per group were used. C15, control group, day 15; C30, control group, day 30; T15, parathyroid hormone treatment group, day 15; T30, parathyroid hormone treatment group, day 30

Parathyroid hormone controls bone formation and bone resorption, primarily through modulation of the osteoprotegerin/ receptor activator of nuclear factor-κB/RANKL system. In response to parathyroid hormone, immature cells in the osteoblast lineage increase the expression of membrane-bound cytokines, including interleukin-6

and interleukin-6 Soluble Receptor, and these factors activate the protein kinase C pathway, resulting in RANKL expression and consequently osteoclast formation (26,27). In our study, intermittent treatment with parathyroid hormone represents a consistent correlation between the down-regulation of interleukin-6 in gingival tissue and less bone resorption (TRAP-positive cells). TRAP is a biochemical marker that is considered to be relatively specific for osteoclasts (20). In our study, TRAP-positive cells were found mainly on the alveolar bone surface, a region where interleukin-6 expression was also observed, here expressed basically by osteoblasts, as detected by immunohistochemistry (Fig. 3D). We also investigated quantitative changes in TRAP-positive cells in the groups evaluated. As shown in Fig. 3A, it was possible to verify a larger number of TRAP-positive cells in the C30 group than in the other groups ($p < 0.05$), and such a finding was concomitant with the high levels of interleukin-6 gene expression observed in gingival tissue from the C30 group ($p < 0.01$).

The members of the MMP family that have been implicated in periodontal tissue destruction include MMP-1, MMP-8, MMP-9 and MMP-2 (28). MMP-2 has been demonstrated to play an important role in the turnover or degradation of soft connective tissue (29). The findings observed in this study indicate that high levels of MMP-2 gene and protein are expressed in the gingival tissues of all groups, and it was demonstrated that MMP-2 expression decreases from day 15 to day 30 regardless of parathyroid hormone treatment ($p < 0.05$). In addition, we demonstrated that intermittent administration of parathyroid hormone down-regulated the expression of MMP-2 mRNA, although the peptidase activity was not changed (Fig. 2).

MMP-9 is the predominant gelatinase found in oral fluids and inflamed gingival tissues and it is reported that the MMP-9 immunoreactivity in gingival tissue becomes more intense as the number of inflammatory cells increase (4,30,31). Although it is known that parathyroid hormone can affect the expression levels of MMPs (32–34), it was unclear how treatment with parathyroid hormone reduced the activity of MMP-9 in the present study. As MMP-9 expressed in inflamed gingiva is derived from degranulating neutrophils activated by bacterial plaque (4,30,31), the decrease in MMP-9 activity might be related to a reduction in the number of inflammatory cells in the gingival tissue caused by treatment with parathyroid hormone, in concordance with findings previously published (8).

In summary, these findings from an animal model indicate that intermittent administration of parathyroid hormone may modulate factors related to connective tissue and collagen degradation (interleukin-6, MMP-9 and TRAP), thus suggesting that parathyroid hormone (1–34), the only Food and Drug Administration approved anabolic agent for osteoporosis treatment in the USA, may also exert a potential therapeutic benefit in local bone metabolism in individuals with periodontitis-related bone loss.

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