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Topical and intermittent application of parathyroid hormone recovers alveolar bone loss in rat experimental periodontitis

K. Tokunaga, H. Seto, H. Ohba, C. Mihara, H. Hama, M. Horibe, S. Yoneda, T. Nagata
Department of Periodontology and Endodontology, Institute of Health Biosciences, University of Tokushima Graduate School, Tokushima, Japan

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Background and Objective: Periodontitis is characterized by periodontal tissue inflammation and alveolar bone loss. The intermittent administration of parathyroid hormone (PTH), a major regulator of bone remodeling, has been demonstrated to stimulate osteoblastic activity. Although the systemic administration of PTH has been reported to protect against periodontitis-associated bone loss, the effect of the topical administration of PTH is unclear. In this study, the effect of intermittent administration of PTH on osteoblastic differentiation was examined in cultured calvaria cells and then the effect of topical and intermittent administration of PTH was determined by measuring the recovery of alveolar bone loss after inducing experimental periodontitis in rats.

Material and Methods: Alkaline phosphatase activity and bone nodule formation were measured in fetal rat calvaria cells. Experimental periodontitis was induced by placing nylon ligature around rat maxillary molars for 20 d. After ligature removal (day 0), PTH was topically injected into buccal gingiva three times a week for 10 wk. Micro-computed tomography analysis and histological examination were performed on days 35 and 70.

Results: Intermittent exposure of PTH in calvaria cells increased alkaline phosphatase activity and bone nodule formation by 1.4- and 2.4-fold, respectively. Ligature procedures induced marked alveolar bone loss around the molars on day 0 and greater bone recovery was observed in the PTH-treated rats on day 70. An increase in osteoid formation on the surface of alveolar bone was detected in the PTH-treated rats.

Conclusion: Intermittent treatment with PTH stimulated osteoblastic differentiation in fetal rat calvaria cell cultures, and topical and intermittent administration of PTH recovered alveolar bone loss in rat experimental periodontitis.

Toshihiko Nagata, DDS, PhD, Department of Periodontology and Endodontology, Institute of Health Biosciences, University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8504, Japan
Tel: +81 88 633 7343
Fax: +81 88 633 7345
e-mail: nagata@dent.tokushima-u.ac.jp

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Periodontitis induces the destruction of connective tissue and alveolar bone, and is a major cause of tooth loss in adults (1). In order to recover the lost tissues, some periodontal therapies, such as guided tissue regeneration, autogenous bone grafting and growth factor application, have been investigated. As potent growth factors, enamel matrix derivative and fibroblast growth factor have been used in clinical investigations (2,3). Although these

surgical procedures are important as a direct treatment to periodontal lesions, nonsurgical treatment, such as medication, is also desirable to stimulate periodontal regeneration.

Parathyroid hormone (PTH) is a major regulator of bone remodeling and calcium homeostasis. PTH has been reported to exhibit both anabolic and catabolic activities in bone tissues (4,5). When the effect of PTH was determined in calvaria cell cultures, a useful model for investigating osteoblastic differentiation (6), both anabolic and catabolic effects were reported in newborn rats (7) but only a catabolic effect was identified in fetal rats (8). The intermittent administration of PTH stimulated osteoblastic bone formation not only *in vitro* (7) but also *in vivo* (9). In addition, clinical studies have shown a positive effect of treatment with PTH on patients with osteoporosis (10,11). As periodontitis is a bone-destructive disease, the application of PTH may be effective for the recovery of alveolar bone. In fact, Barros *et al*. (12) reported that the systemic intermittent administration of PTH protected against alveolar bone loss in experimental periodontitis in rats. However, the effect of topical application of PTH has not been fully elucidated. In this study, we examined the effect of intermittent administration of PTH on osteoblastic differentiation of fetal rat calvaria cells in culture, and then investigated the effects of topical and intermittent administration of PTH by measuring the recovery of alveolar bone loss after inducing experimental periodontitis in rats.

Material and methods

Cell cultures

Rat calvaria cells were prepared from calvariae of 21-d-old fetal Wistar rats (Charles River Laboratories, Yokohama, Japan), as described previously (8,13,14). Briefly, calvariae dissected from the fetal rats were digested sequentially in an enzyme mixture containing 3.0 mg/mL of collagenase, 4.5 units/mL of elastase, 0.12 mM chondroitin sulphate and 21.3 mM Tris–HCl (pH 7.4) at 37°C for 10, 20, 30, 50 and 70 min. Cells obtained from the last four digestion steps were pooled, suspended in α-minimum essential medium (α-MEM; Dainippon Sumitomo Pharma, Osaka, Japan) containing 10% fetal calf serum (Biological Industries, Western Galilee, Israel) and antibiotics, and plated in T-75 flasks (Sumitomo Bakelite, Tokyo, Japan). The cells were maintained at 37°C under a 5% CO_2 atmosphere. After 24 h, the cells were trypsinized and seeded at a density of 5000 cells/cm^2 into α-MEM containing 10% fetal calf serum, 50 μg/mL of ascorbic acid, 2 mM β-glycerophosphate and 10 nM dexamethasone, in 12-well plates (Sumitomo Bakelite). The administration schedule and dose for intermittent treatment with PTH were determined according to the method of Ishizuya *et al*. (7). The cells were exposed to 50 ng/mL of PTH (1–34) (Asahi Kasei Corporation, Tokyo, Japan) for the first 6 h of each 48-h incubation cycle. A concentration of 50 ng/mL of PTH(1–34) corresponds to approximately 12 nM PTH(1–34). After exposure for 6 h, the cells were cultured in the absence of PTH for a further 42 h. In the control culture, vehicle solution [phosphate-buffered saline (PBS)] was added at the time of PTH treatment of the test culture. The regular medium change was performed every 48 h. Repeating these procedures, cells were maintained for up to 28 d. In order to compare the effect of continuous exposure of PTH, the PTH-containing medium was changed every 48 h.

Determination of alkaline phosphatase activity and bone nodule formation

To assess alkaline phosphatase (ALP) activity, cells were scraped into 50 mM Tris–HCl buffer (pH 7.4), sonicated and centrifuged at 2000 *g* for 10 min at 4°C. The enzyme activity in the supernatant was determined using *p*-nitrophenyl phosphate as the substrate, according to Lowry's method (15). To assess bone nodule (BN) formation, cells were washed in PBS and stained using the Alizarin Red S technique (16). The area of BN stained as red dots was determined using computer software (NIH image version 1.61; NIH, Bethesda, MD, USA).

Animal procedures

Thirty male 8-wk-old Fischer rats (Clea Japan Inc., Tokyo, Japan) were housed in individual wire cages in a temperature- and humidity-controlled room (23 ± 1°C and 60 ± 5% relative humidity) with a 12-h light/12-h dark cycle. All animal experiments complied with the guidelines approved by the Animal Research Control Committee of Tokushima University Graduate School. A powdered form of PTH(1–34) was dissolved in PBS containing 0.1% rat serum albumin. A high dose of PTH (5 μg in 50 μL of solution) and a low dose of PTH (1 μg in 50 μL of solution) were used. The PTH was administered to the rat buccal gingiva by injection, and the same volume of vehicle solution was also administered to control rats. The solution was injected, using a 26-gauge needle, into the subperiosteum at the gingival tissue (mucogingival line) corresponding to the alveolar bone crest of the buccal central site in the second molars.

Experimental design

The cervical area of the right second molar of the rat maxilla was ligatured with nylon thread (No. 5-0; Natsume Corporation, Tokyo, Japan) in rats under anesthesia with sodium pentobarbital. Twenty days after placement of the ligature, six rats were killed and their maxillae were collected as the baseline. These samples were defined as the control on day 0, and the presence of alveolar bone loss induced by ligature placement was confirmed. In another 24 rats, the nylon thread was removed on day 0 and the rats were divided into three groups: (i) the ligatured control group (12 rats) in which PBS was injected from day 0; (ii) the low-dose PTH-treated group (six rats), in which 1 μg of PTH was injected from day 0; and (iii) the high-dose PTH-treated group (six rats), in which 5 μg of PTH was injected from day 0.

The solution containing 1 or 5 μg of PTH or PBS plus rat serum albumin was injected into the subperiosteum at the buccal gingiva of the maxillary second molar three times a week for 35 or 70 d. Alternatively, the left maxillae without ligatures were used as the nonligatured control samples. The administration schedule and dose for treatment with intermittent PTH were based on the study of Barros *et al.* (12), who demonstrated, in a rat model, that systemic intermittent administration of 40 μg/kg of PTH had a protective effect against periodontitis-associated alveolar bone loss.

Micro-computed tomography analysis

Micro-computed tomography (micro-CT) scans (Hitachi Medico, Tokyo, Japan) of maxillae were obtained. To correctly maintain a longitudinal position of 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 was set as follows: pixel size, 1024×1024; slice thickness, 12 μm; magnification, 10×; voltage, 50 kV; and electrical current, 0.1 mA. Three-dimensional images were produced using computer software, TRI/3D-BON (Ratoc Systems Inc., Tokyo, Japan). The distance from the buccal cemento–enamel junction to the alveolar bone crest of the second molar was measured as a marker of the bone height using computer software (Scion Image; Scion, Frederick, MD, USA). When the three-dimensional images were reconstructed, the length of three portions (mesial, central and distal sites) on the second molar was measured and the mean value was determined as the experimental data of the distance from the cemento–enamel junction to the alveolar bone crest.

Histomorphometric analysis

For preparing undecalcified sections, the maxillae on day 70 were embedded in methylmethacrylate resin. All samples were stained with Villanueva bone stain before embedding (17). The frontal sections, paralleled with the mesial root of the second molar, were prepared to a thickness of approximately 20 μm. To correctly maintain a longitudinal position of samples, the cutting procedures were carefully carried out at every step of section preparation. Histological observation was performed using a light microscope (Microphoto V series VFD; Nikon, Tokyo, Japan) at 100× and 400× magnifications. The thickness of the osteoid, which was stained a dark violet color, was measured using computer software (Scion Image). As bone formation occurred from the buccal site, 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. When the width of the osteoid layer was measured, the layer showing intense staining (dark violet or purple colors) was evaluated as the osteoid layer. The area stained weak violet was excluded from the measurements. The width was measured at five sites in the square and the mean value was determined as experimental data.

Statistical analysis

All values in the figures are expressed as mean ± standard deviation. Significance between the groups was estimated using one-way analysis of variance and Fisher's protected least significance test. A $p < 0.05$ was considered significant.

Results

ALP activity and BN formation in cultured rat calvaria cells

Figure 1 shows the effects of intermittent and continuous exposure of PTH (50 ng/mL) on the ALP activity of fetal rat calvaria cells. Although after several days in culture, ALP activity showed a marked increase in the cells of the control group and in the cells of the group intermittently exposed to PTH, the ALP activity induced by intermittent treatment with PTH was significantly higher than that in the control on days 5, 9, 17 and 21, showing a 1.3- to 1.4-fold increase in activity. On the other hand, when PTH was administered continuously, ALP activity was markedly suppressed on days 9, 13, 17 and 21. Figure 2 shows the effect of PTH (50 ng/mL) on BN formation after 28 d of culture of fetal rat calvaria cells. The intermittent exposure of cells to PTH induced a significant increase in the mineralized area of BN compared with that in the control, expressed as a 2.4-fold greater formation of BN in PTH-treated cells. Continuous exposure of cells to PTH resulted in the marked suppression of BN formation.

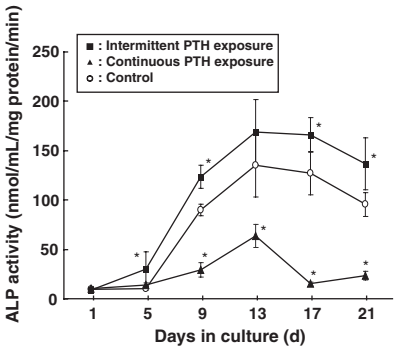

Fig. 1. Effects of treatment with parathyroid hormone (PTH), administered intermittently and continuously, on alkaline phosphatase (ALP) activity in cultured fetal rat calvaria cells. In the intermittent treatment, the cells were exposed to 50 ng/mL of PTH for the first 6 h of each 48-h incubation cycle. In the continuous treatment, the cells were treated with 50 ng/mL of PTH during the whole experimental period. In the control culture, vehicle solution (phosphate-buffered saline) was added. Cells were maintained in culture for 21 d and ALP activity was measured on the indicated days. Data are expressed as the means ± standard deviation of six separate cultures. *, $p < 0.05$ compared with the control value.

Micro-CT image analysis in rat experimental periodontitis

The right panel in the upper row of Fig. 3 shows three areas measured on the second molar. The three-dimensional images from micro-CT on day 0 (Fig. 3A and 3B) revealed a clear decrease in the buccal alveolar bone height of the second molar in the ligatured group compared with that in the nonligatured control group, indicating

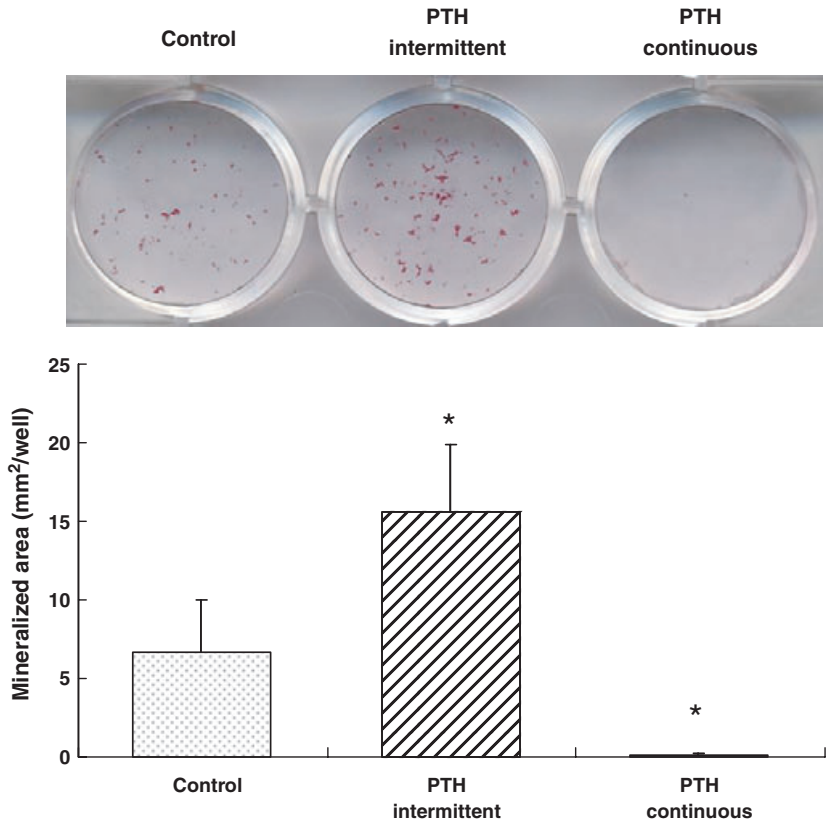

Fig. 2. Effects of treatment with 50 ng/mL of parathyroid hormone (PTH), administered intermittently and continuously, on bone nodule (BN) formation in cultured fetal rat calvaria cells. After 28 d of culture, BNs formed in the flask were measured using Alizarin Red staining and the area was determined using NIH image software. Data are expressed as the mean ± standard deviation of four separate cultures. *, $p < 0.05$ compared with the control value.

that 20 d of ligature placement disclosed the buccal furcation area on day 0. Similar profiles of alveolar bone loss were observed on days 35 and 70 in the ligatured control group (Fig. 3C, 3D, 3G and 3H). On the other hand, the decreased alveolar bone level appeared to show partial recovery in the PTH-treated groups on days 35 and 70 (Fig. 3E, 3F, 3I and 3J), suggesting that the intermittent administration of PTH recovered the ligature-induced alveolar bone loss.

Figure 4 shows a comparison of the distance from the cemento–enamel junction to the alveolar bone crest among nonligatured control, ligatured control and PTH-treated rats. Bone height showed significant recovery in PTH-treated rats on day 70. The values in the nonligatured control group were 200 ± 29, 307 ± 57 and 282 ± 17 μm on days 0, 35 and 70, respectively, indicating that there was no significant difference in the distance from the cemento-enamel junction to the alveolar bone crest in the nonligatured control during the experimental period. In contrast, the values in the ligatured control group (699 ± 51, 743 ± 111 and 622 ± 45 μm on days 0, 35 and 70, respectively) were greater than in the nonligatured control group, indicating that ligature-induced bone loss was still present on day 70 and that there was no improvement in the bone loss during the experimental period. On day 35, the values in the low-dose and high-dose PTH-treated groups did not show a significant difference compared with that in the ligatured control group, with values of 606 ± 76 μm (low-dose PTH), 603 ± 117 μm (high-dose PTH) and 743 ± 111 μm (ligatured control). On day 70, the values in the low-dose and the high-dose PTH-treated groups showed a significant decrease compared with those in the ligatured control group, with values of 474 ± 127 μm (low-dose PTH), 380 ± 33 μm (high-dose PTH) and 622 ± 45 μm (ligatured control). There was no significant difference in the distance from the cemeto-enamel junction to the alveolar bone crest between low-dose and high-dose PTH-treated groups. These results demonstrate that PTH treatment for 70 d partially recovered the ligature-induced alveolar bone loss. From the bone height data on day 70 (nonligature = 282 μm; ligature = 622 μm; low-dose PTH = 474 μm; and high-dose PTH = 380 μm), 56% and 29% of the total bone loss were reversed by treatments with low-dose and high-dose PTH, respectively.

Histological analysis in rat experimental periodontitis

As shown in Fig. 5A, the low magnifications on Villanueva bone staining on day 70 revealed the ligature-induced alveolar bone loss, indicating that the distance from the cemento–enamel junction to the alveolar bone crest was increased. In the bone-loss area, irregular collagen fibers were observed; however, inflammatory cells and osteoclasts were not observed around the bone crest zone (Fig. 5A and 5D). On the other hand, the height and the width of alveolar bone increased in both low-dose and high-dose PTH-treated groups (Fig. 5B and 5C) compared with those in the ligatured control (Fig. 5A). The newly formed bone structure in the PTH-treated groups showed a rough surface at the buccal site and it was stained dark violet, indicating the presence of osteoid. When the tissues were examined in high-magnification sections, inflammatory cells could not be observed around the newly formed bone, and the osteoid layer that was stained dark violet increased at the buccal site in the low-dose and high-dose PTH-treated groups (Fig. 5E and 5F). Such bone additions were observed less in the ligatured control group (Fig. 5D). When the osteoid width was measured, the thickness was significantly greater

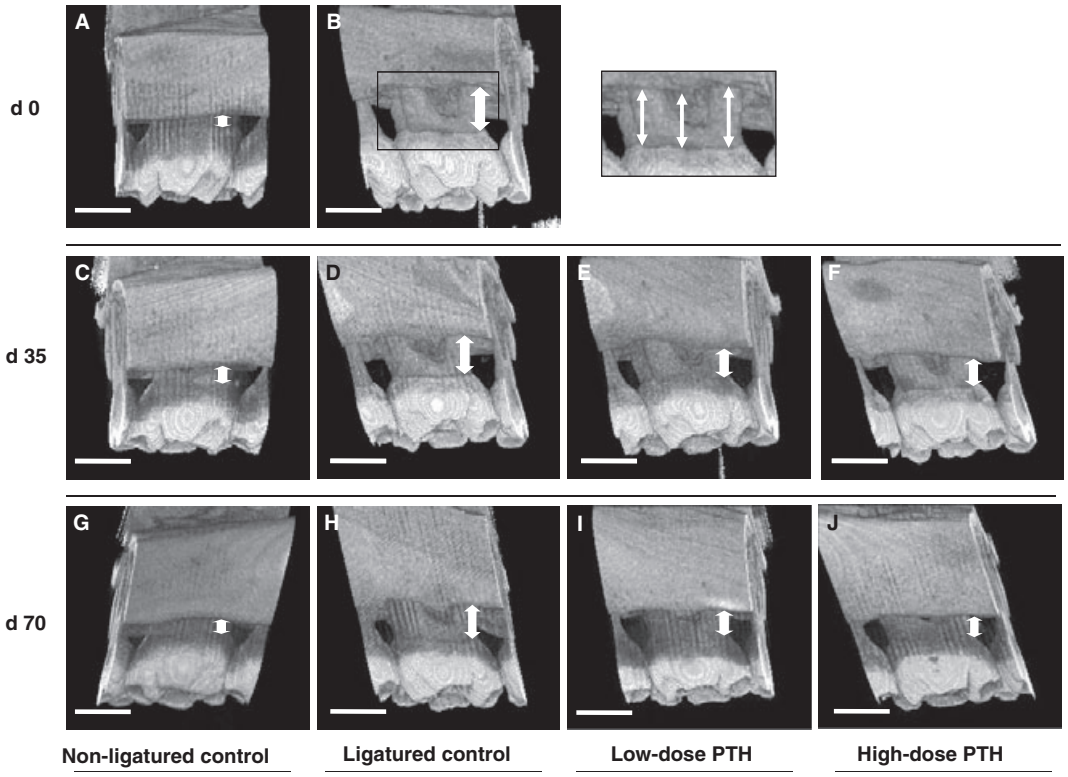

Fig. 3. Micro-computed tomography (micro-CT) images of the frontal sections of the maxillary secondary molars of rats. (A) Day 0: nonligatured control group. (B) Day 0: ligatured control group. (C) Day 35: nonligatured control group. (D) Day 35: ligatured control group. (E) Day 35: low-dose parathyroid hormone (PTH)-treated group. (F) Day 35: high-dose PTH-treated group. (G) Day 70: nonligatured control group. (H) Day 70: ligatured control group. (I) Day 70: low-dose PTH-treated group. (J) Day 70: high-dose PTH-treated group. At the right panel in the upper row, the arrows show the distance from the cemento–enamel junction to the alveolar bone crest as a marker of the alveolar bone height; the length of three areas (mesial, central and distal sites) was measured, and the mean value was determined as experimental data. In the high-dose PTH-treated group, a high dose of PTH (5 μg in 50 μL of solution) was administered intermittently. In the low-dose PTH-treated group, a low dose of PTH (1 μg in 50 μL solution) was administered intermittently. Scale bar: 1.0 mm.

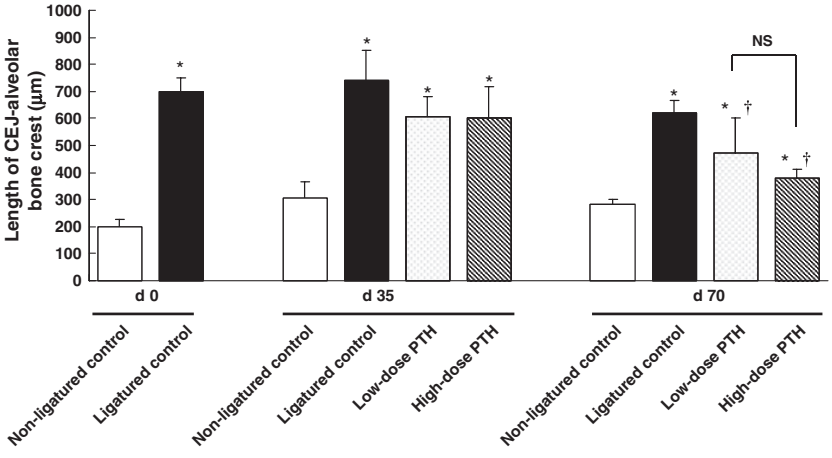

Fig. 4. Length from the cemento–enamel junction to the alveolar bone crest measured as a marker of the bone height in a micro-computed tomography (micro-CT) image. The length was measured at the three areas (mesial, central and distal sites in the first molars) shown in Fig. 3 and the mean value was determined as experimental data from each rat. Data show the mean ± standard deviation from three or six rats per group. *$p < 0.05$ compared with the nonligatured control group. †$p < 0.05$ compared with the ligatured control group. NS, not significant.

in the low-dose and high-dose PTH-treated groups than in the ligatured control, being 4.8- and 4.0-fold higher in low-dose and high-dose PTH treatments, respectively (Fig. 6). There was no difference in the osteoid width between the groups treated with low-dose and high-dose PTH.

Discussion

In this study, we used a primary osteoblastic cell culture system from fetal rat calvariae that was obtained by sequential enzyme digestion. The cells are composed of heterogeneous cell populations, and express osteoblast phenotypes that include the ability to form BN *in vitro* (6,8). Using this culture system, we reported the effect of several factors on osteoblastic differentiation (14,18–20), and Bellows *et al.*

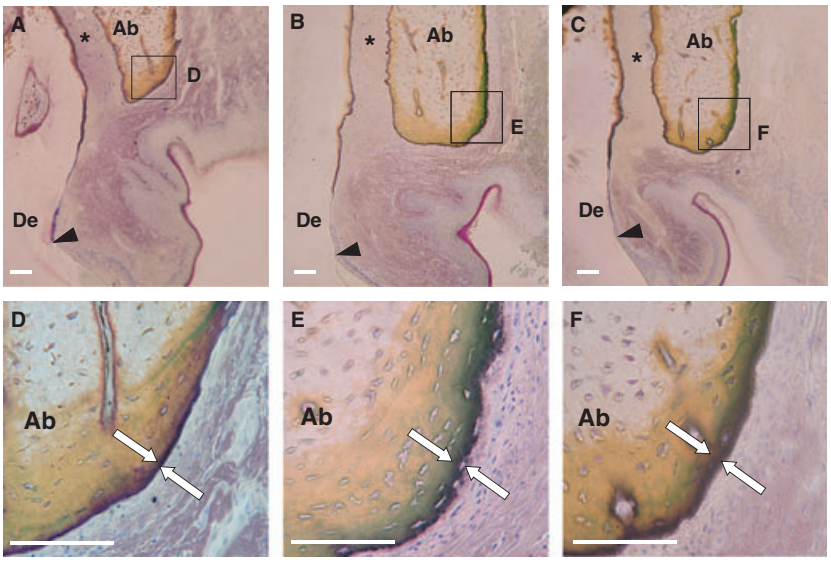

Fig. 5. Microscopic observations after Villanueva bone staining. Low and high magnifications of undecalcified frontal sections in the maxillary second molar from rats on day 70 in the ligatured control group (A, D), low-dose parathyroid hormone (PTH)-treated group (B, E) and high-dose PTH-treated group (C, F). Scale bars: 200 μm (A–C) and 100 μm (D–F). D, E, and F show magnified view of the boxed region in A, B, and C, respectively. Ab, alveolar bone; De, dentin; E. The asterisks indicate the periodontal ligament tissues, the black arrowheads indicate the position of the cemento–enamel junction and the white arrows indicate osteoid.

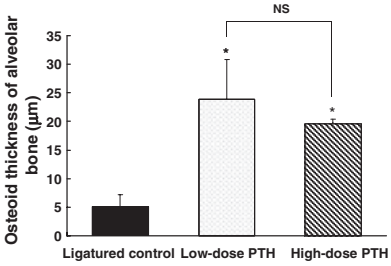

Fig. 6. Osteoid thickness of newly formed alveolar bone. The width was measured at five sites in the square shown in Fig. 5 and the mean value was determined as experimental data from each rat. Data are presented as the mean ± standard deviation from six rats per group. $*p < 0.05$ compared with the ligatured control group. $\dagger p < 0.05$ compared with the nonligatured control group. NS, not significant.

(8) reported that PTH suppressed the differentiation of osteoprogenitor cells into functional osteoblasts. In this study, we found that intermittent exposure of PTH stimulated osteoblastic differentiation in fetal rat calvaria cells. Ishizuya *et al.* (7) reported the stimulatory and inhibitory effects by intermittent and continuous exposure of PTH, respectively, in cultured newborn rat calvaria cells. In their experiments, the cells derived from 1-d-old rat calvariae and the high concentration of 10 mM β-glycerophosphate were selected, whereas we used fetal rat calvariae and the lower concentration of 2 mM β-glycerophosphate in this study. They showed 1.2- and 1.7-fold increases in ALP activity and BN numbers, respectively, after 6 h of exposure to 50 ng/mL of PTH. The present study showed higher PTH responses, of 1.4- and 2.4-fold increases in ALP and BN, respectively. However, Ishizuya *et al.* (7) also showed that the 1-h intermittent exposure of 50 ng/mL (12 nM) of PTH induced a marked decrease in BN formation. On the other hand, Bellows *et al.* (8) reported that 1 and 10 nM PTH (4.2 and 42 ng/mL) suppressed osteoblastic differentiation, and the detailed effect was determined using 1 nM PTH in various experimental conditions (short-term exposure to PTH, and PTH readdition at different times after its removal), although they did not investigate the effect of intermittent exposure to PTH. These findings suggest that the action of PTH on osteoblasts varies with cell origin and experimental conditions and that the cells from fetal rat calvaria may be more sensitive to intermittent exposure to PTH. As only an inhibitory effect of PTH was reported in fetal rat calvaria cells by Bellows *et al.* (8), the present study added the data that the cells derived from fetal rats also exhibited a stimulatory effect of PTH on osteoblastic differentiation.

Analysis of micro-CT scans was able to provide accurate figures of hard periodontal tissues by constructing three-dimensional images via a computer, and clarified the existence of a stimulatory effect of PTH on alveolar bone recovery. Previous analysis of micro-CT scans showed the effect of milk whey protein and simvastatin on alveolar bone in rat experimental periodontitis (21,22). The ligature-placement method has been accepted as a useful experimental model of periodontitis with alveolar bone resorption (23–25). Bezerra *et al.* (24) and de Lima *et al.* (25) reported that ligature placement from 4 to 7 d induced the severe resorption of alveolar bone. They also showed that inflammation and the appearance of osteoclasts and lymphocytes peaked from 7 to 11 d. In the present study, the inflammatory cells and osteoclasts had already disappeared beneath the nylon thread after 20 d of ligaturing (i.e. on day 0). It is thought that acute inflammation resulting from ligature placement induced severe bone loss during days 7–11, and that inflammation decreased within 20 d in the experimental periodontitis in rats. In addition, this alveolar bone loss in the controls did not recover in the 35–70 d after removal of the ligature. These data indicate that this experimental model is useful for evaluating the net effect of a bone-forming agent for periodontitis.

Leaffer *et al.* (26) reported that intermittent administration of PTH stimulated differentiation from lining to osteoblast-like cells. These results suggest that the intermittent administration of PTH may directly reactivate lining cells to resume their matrix-synthesizing

function, and that the anabolic effect of intermittent PTH may be derived from the initiation of osteoblastic bone formation without osteoclastic bone resorption. In the present study, PTH treatment for 70 d recovered the ligature-induced alveolar bone loss and increased osteoid in the buccal alveolar bone of the second molar. We speculate that the intermittent administration of PTH directly activated the function of osteoblasts and osteocytes present in alveolar bone, resulting in increases in the height and the width of osteoid. Fermor *et al*. (27) reported that PTH receptors were expressed on osteoblasts and osteocytes exhibiting high-level ALP activity in the cortical bone of growing rats. Podbesek *et al*. (28) reported that the intermittent administration of PTH increased osteoblast function, the formation of osteoid and plasma ALP activity. These results may support our findings that alveolar bone increase by PTH was associated with osteoid increase. It was also reported that the intermittent administration of PTH promoted the healing of fracture (29) and increased the bone mineral density and mechanical strength of new bone-forming cells (30). These results suggest that the intermittent administration of PTH may actually induce the formation of new bone. In addition, Okimoto *et al*. (9) reported that the systemic intermittent administration of PTH increased the cortical bone mass of the femur by promoting periosteal bone apposition. On the basis of these findings, the recovery of ligature-induced bone loss on day 70 may be caused by periosteal alveolar bone apposition through the increase in osteoblastic activities induced by the intermittent administration of PTH.

The effect of topical administration of compounds on periodontal tissues has been reported in various studies using rat-based experimental models. It is thought that topical administration is more effective and results in fewer side effects compared with systemic administration. The topical administration of bisphosphonates protected against alveolar bone loss and decreased the number of osteoclasts in experimental periodontitis in rats (31,32). We also reported that the topical administration of simvastatin recovered alveolar bone loss and promoted low-mineralized alveolar bone formation in experimental periodontitis in rats (22). In addition, the topical injection of bisphosphonates was found to prevent tooth movement and root resorption after applying an orthodontic force and to inhibit relapse of the tooth after removal of the orthodontic appliance in rats (33,34). In conclusion, our findings suggest that the topical and intermittent administration of PTH can increase the alveolar bone mass and may be effective for periodontal tissue regeneration.

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