Intermittent Administration of Parathyroid Hormone Ameliorates Periapical Lesions in Mice

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Abstract

Introduction: Intermittent administration of parathyroid hormone (PTH) promotes oral osseous wound healing and protects against ligature-induced alveolar bone loss. However, its therapeutic value on periapical periodontitis is unknown. The goal of this study was to determine the effect of intermittent PTH administration on the progression of periapical periodontitis. **Methods:** Seven lymphotoxin alpha–deficient mice received pulp exposures of mandibular first and second molars. Exposed pulp in the right mandible was covered with plaque-contaminated fibrin, whereas exposed pulp in the left mandible was left open. After 4 weeks, the periapical tissues were examined to determine the effect of plaque-contaminated fibrin to induce periapical lesions. Fourteen mice received pulp exposure covered with plaque-contaminated fibrin. PTH (40 μg/kg/d) was administered intermittently to half of the mice for 3 weeks beginning 1 week after pulp exposure. The remaining half received saline injections as the vehicle control. At sacrifice, mandibles and tibiae were harvested and processed for histologic examination. Evaluation of neutrophils and blood vessels was performed after staining with immunofluorescence, and periradicular bone was histomorphometrically analyzed. **Results:** The exposed pulp covered with plaque-contaminated fibrin resulted in significantly larger periapical lesions compared with the control. Intermittent PTH administration reduced the size of periapical lesions significantly. Significantly less neutrophil infiltration around the root apex was found in PTH-treated animals compared with the control. **Conclusions:** PTH treatment suppressed periapical inflammation by reducing neutrophil infiltration and protected against tissue destruction by periapical periodontitis. *(J Endod 2015;41:646–651)*

Key Words

Lymphotoxin alpha, neutrophil infiltration, parathyroid hormone, periapical periodontitis

The parathyroid hormone (PTH) increases bone mass when administered intermittently. Teriparatide, a recombinant form of human PTH (1–34), is used for the treatment of osteoporosis. Although the mechanisms for the bone anabolic action of intermittent PTH are unclear, it is known to activate both osteoblasts and osteoclasts in favor of osteoblastic bone formation over osteoclastic resorption, resulting in an increase in total bone mass (1). PTH is effective in promoting fracture healing (2, 3). Such an enhanced osseous healing by PTH is seen in craniofacial bones as well where PTH administration promotes bone fill and growth in tooth extraction sockets (4, 5). Barros et al (6) reported that intermittent PTH administration protected ligature-induced bone loss in a rat model of periodontitis. In that study, the numbers of inflammatory cells were significantly reduced in the PTH-treated group compared with the control. Likewise, we have discovered that intermittent PTH administration promotes tooth extraction socket healing in rodents (7, 8). PTH-promoted tooth extraction wound healing was accompanied with decreased inflammatory cell infiltration. These findings imply that intermittent PTH administration may have protective effects against inflammation-induced bone loss in the oral cavity.

Periapical periodontitis is an inflammatory disease resulting from root canal infections by mainly anaerobic microorganisms (9–11). Such microorganisms, their endotoxins and enzymes together, stimulate periapical tissues and alert the host defense mechanism (12, 13). Neutrophils play a crucial role in orchestrating the host defense system starting from the early phase of periapical infection. Neutrophils are phagocytes that fight against microorganisms but also cause host tissue breakdown because of their release of proinflammatory cytokines, chemokines, and proteinases (14, 15). Therefore, suppression of the intensity of inflammatory responses in periapical tissues, which is orchestrated by neutrophils, would lead to reduced damage in the host tissues. In this study, we hypothesized that the intermittent administration of PTH protects against tissue damage caused by apical periodontitis. Using lymphotoxin alpha (LTA)-deficient mice, which exhibit defects in secondary lymphoid structures and therefore in adaptive immune responses (16), periapical periodontitis was induced, and the effect of PTH administration on the disease progression of periapical periodontitis was determined.

Materials and Methods

Experimental Design

A breeding pair of mice homozygous mutant for LTA (B6.129S2-Lta^{tm1Dch}/J) was obtained from Jackson Laboratory (Bar Harbor, ME). Twenty-one offspring at the age of 8 weeks were used. Seven mice were subjected to pulp exposure of the mandibular molars to assess the effect of plaque contamination and confirm the development of

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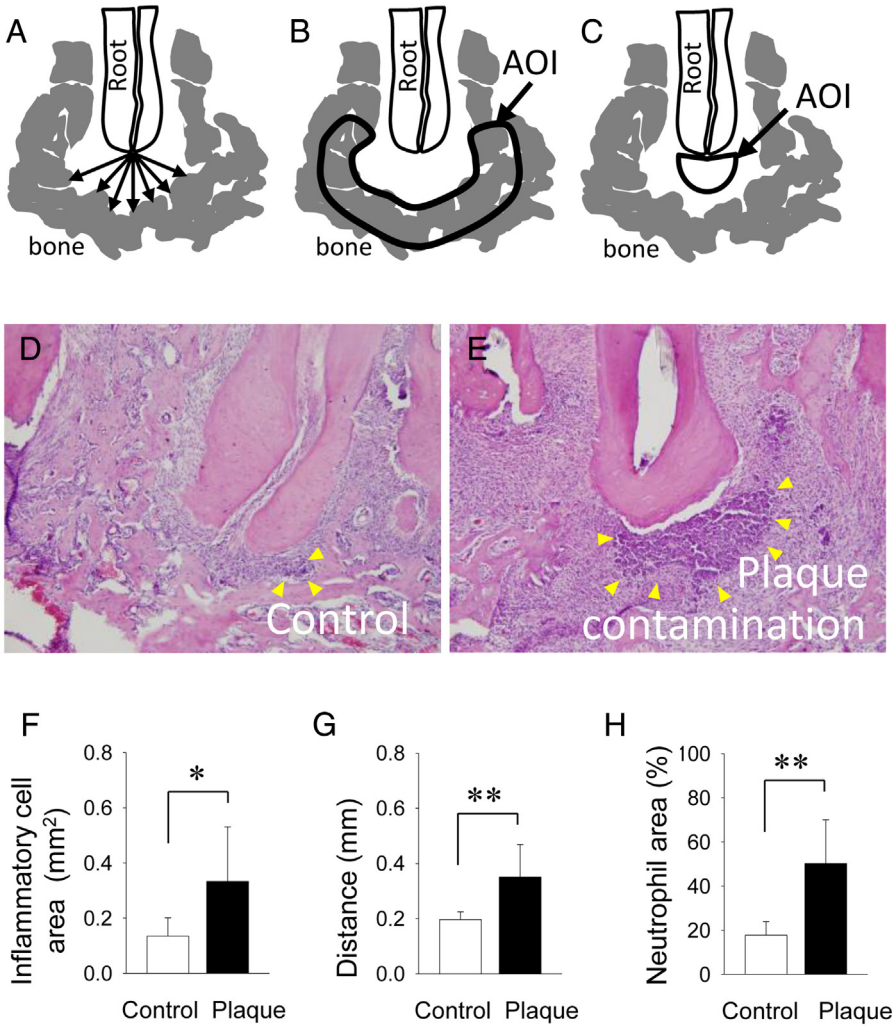

Figure 1. Exposed pulp covered with plaque-contaminated fibrin induced large periapical lesions. (*A*) The distance between the root apex and the surface of periradicular bone was measured at 7 points, and the results were averaged. The average was used as a representative distance to estimate the size of the lesion. (*B*) The bone tissue within 0.4 mm of the bone surface was defined as the AOI for the measurements of the numbers of bone fragments, bone area/tissue area, and Oc.N/BS. (*C*) The periapical soft tissue area in a semicircle with a radius of 0.6 mm was defined as the AOI for the assessment of inflammatory cells and neutrophils. (*D* and *E*) Representative photomicrographs of hematoxylin-eosin–stained sections of periapical lesions (original magnification, ×100). *Arrowheads* indicate inflammatory cell infiltration. (*F*) Tissue areas occupied by inflammatory cells were measured. A significantly larger inflammatory cell area was noted in the plaque-contaminated fibrin group than control. (*G*) The size of the periapical lesions was assessed by measuring the distance between the apex and the bone surface. Significantly larger periapical lesions were found in the plaque-contaminated fibrin group than control. (*H*) Ly6G(+) cells, which represent neutrophils, were assessed next to the apex. Significantly more neutrophils were observed in the plaque-contaminated fibrin group than the control (n = 7/group; paired t test; $^*P < .05$, $^{**}P < .01$).

periapical lesions (Supplemental Figure S1). Fourteen mice were subjected to pulp exposure with plaque contamination to induce periapical lesions. Subsequently, daily injections of either PTH or saline were performed for 3 weeks to evaluate the therapeutic value of PTH treatment on periapical lesions (Supplemental Figure S2). The experimental protocol was approved, and all animals were treated in accordance with the guidelines of the University Committee on Use and Care of Animals.

Mouse Model of Periapical Lesions

Mice were subjected to ligature placement (5/0 Silk) around each of the maxillary second molars 5 days before pulp exposure (17). On the day of pulp exposure, the ligatures were removed, and 1 ligature was placed in a tube with 1.0 mL saline and vortexed while the other was either used as a backup or discarded. Half of the plaque/saline-mixed solution (0.5 mL) was transferred to another tube and centrifuged. The pelleted plaque was mixed with 10 μL fibrinogen solution (Sigma-Aldrich, St Louis, MO) and placed on ice until use. The pulp exposure of the mandibular first and second molars were conducted with a dental handpiece and 1/4 round bur in 7 mice. The exposed pulp in the left mandible was left open to the oral environment. The exposed dental pulp in the right mandible was covered with 2 μL plaque/fibrinogen mixture followed by 5 μL thrombin (Sigma-Aldrich). Mice were euthanized 4 weeks after pulp exposure to assess the effect of plaque-contaminated fibrin on the development of periapical lesions.

Injections and Euthanasia

Fourteen mice received pulp exposure covered with plaque-contaminated fibrin as described previously to induce periapical lesions. Daily subcutaneous injections of either PTH (1–34) (40 μg/kg/d; Bachem, Torrance, CA) or an equivalent volume of 0.9% saline

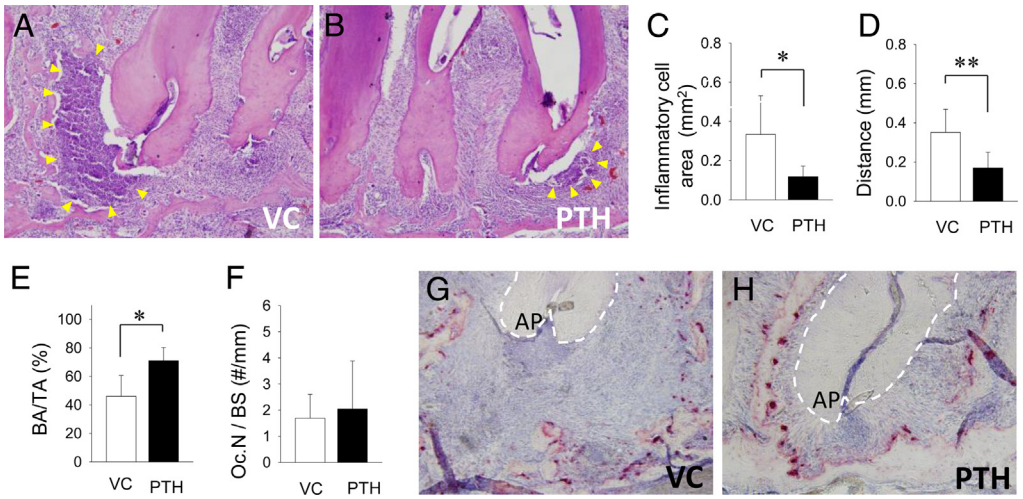

Figure 2. PTH treatment protected periapical tissues from destruction. (*A* and *B*) Representative photomicrographs of hematoxylin-eosin–stained sections of periapical lesions (original magnification, ×100). VC, vehicle control (saline) treatment. *Arrowheads* indicate inflammatory cell infiltration. (*C*) The inflammatory cell area was significantly smaller in PTH-treated animals versus the control. (*D*) The size of the periapical lesions was assessed by measuring the distance between the apex and the bone surface. Significantly smaller periapical lesions were found in the PTH compared with the VC group. (*E*) The periradicular bone was significantly denser in the PTH group compared with the VC group. (*F*) The numbers of osteoclasts per linear bone perimeters were measured. There was no difference between PTH-treated animals versus the control. (*G* and *H*) Representative photomicrographs of TRAP-stained sections of periapical lesions (original magnifications, ×100) (n = 7/group; Student's t test; *$P < .05$, **$P < .01$).

as a vehicle control (VC) for 3 weeks were performed starting 1 week after pulp exposure. The PTH dose of 40 μg/kg/d is a commonly used subcutaneous dose to study bone anabolism by PTH *in vivo* (18, 19). Mice were euthanized with CO_2 asphyxiation. The mandible and tibiae were harvested and fixed in 4% paraformaldehyde.

Histology

The mandibles and tibiae were decalcified in 10% EDTA solution. The mandibles were processed for cryosectioning at 10 μm. The tibiae were paraffin embedded and sectioned at 4 μm. Hematoxylin-eosin staining and tartrate-resistant acid phosphatase (TRAP) staining were performed on the mandibles and tibiae to identify periapical lesions, bone area, and osteoclasts. A commercial kit (Sigma-Aldrich) was used for TRAP staining following an adapted protocol (20). Immunofluorescent staining to visualize neutrophils and blood vessels was performed in the mandibular frozen sections. The sections were rehydrated, blocked with 10% serum, and incubated with primary antibodies overnight at 4°C. Primary antibodies used include anti-Ly6G (Abcam, Cambridge, MA) and anti-CD31 (Abcam). After incubation, fluorescent-conjugated secondary antibody (AlexaFluor-594; Invitrogen, Carlsbad, CA) was applied. DAPI (4′,6-diamidino-2-phenylindole) was used for nuclei visualization.

Histomorphometric Analysis

Stained sections were photomicrographed and histomorphometrically analyzed using Image-Pro Premier (Media Cybernetics, Bethesda, MD). The distance from the apex to the surrounding bone was measured at 7 different points at 10° apart (Fig. 1*A*). The average distance was used to estimate the size of the periapical lesion. Bone quality around the root apex was analyzed to study the periapical pathosis in the local environment. The area of interest (AOI) was defined as an area within 0.4 mm of the bone surface facing the apex (Fig. 1*B*). Within this AOI, the bone area/tissue area, the numbers of bone fragments, and osteoclast numbers per linear bone perimeter (Oc.N/BS) were measured. Neutrophils were assessed by measuring the distribution of Ly6G(+) cells around the apex. The AOI for this measurement was defined as a semicircle with a radius of 0.6 mm below the apex (Fig. 1*C*). CD31(+) cells were histomorphometrically examined to understand the blood vessel formation in the lesions. The bone area/tissue area and Oc.N/BS were also determined in the proximal tibiae.

Statistical Analysis

The paired sample t test was performed to assess the effect of the plaque-contaminated fibrin on the development of periapical lesions. The Student's t test was used to determine the effect of PTH treatment on periapical lesions. Statistical analysis was performed with SYSTAT (Systat, Chicago, IL). An alpha level of 0.05 was used. Results are presented as the mean ± standard deviation.

Results

Plaque Contamination Induced Severe Periapical Lesions

The effect of plaque contamination of exposed pulp on the development of periapical lesions was determined. The inflammatory cell area was used as a surrogate for inflammatory cell numbers because cell aggregation was intensive. When exposed pulp was covered with plaque-contaminated fibrin, an increased inflammatory cell area (Fig. 1*D–F*) and significantly larger periapical lesions (Fig. 1*G*) were noted compared with the control in which the exposed pulp was left open. Immunohistochemical detections of Ly6G(+) cells revealed that significantly more neutrophil aggregation to the apex was observed in the plaque-contaminated group compared with the control group (Fig. 1*H*).

PTH Treatment Suppressed Tissue Destruction and Neutrophil Infiltration

The effect of PTH treatment on periapical lesions was assessed on the teeth with plaque-contaminated pulp. Figure 2*A* and *B* shows the hematoxylin-eosin-stained sections of periapical lesions. A significantly less inflammatory cell area was noted in the PTH-treated animals compared with the VC (Fig. 2*C*). The average size of periapical lesions was found significantly smaller in the PTH-treated animals versus the VC

(Fig. 2*D*). Significantly denser periradicular bone was noted in the PTH-treated animals compared with the VC (Fig. 2*E*). Consistently, the numbers of bone fragments in the AOI was significantly less in the PTH-treated animals than the VC (4.8 ± 5.0 vs 20.3 ± 16.6). There were no differences in osteoclast numbers per linear perimeters between PTH and VC animals (Fig. 2*F*). Figure 2*G* and *H* shows TRAP-stained sections of periapical lesions. Osteoclasts were mostly observed on the bone surfaces facing the root apex in both treatment groups.

To better understand periapical pathosis, neutrophils were stained for Ly6G. As shown in Figure 3*A* and *B*, intense Ly6G(+) cell aggregation was observed next to the root apex in the VC animals, whereas minimal Ly6G(+) cells were noted in the PTH-treated animals. The neutrophil (Ly6G[+] cell) area was significantly smaller in the PTH-treated animals compared with the VC (Fig. 3*E*). Blood vessels were abundant around the periapical lesions but not inside the lesions (Fig. 3*C* and *D*). The numbers and areas of blood vessels were quantitated in the tissue area surrounding the lesion (0.05-mm band width). Significantly fewer blood vessels were noted in the PTH group than the VC group (Fig. 3*F*). Consistently, the total blood vessel area was significantly less in the PTH group versus the VC (Fig. 3*G*).

PTH Treatment Increased Bone Mass in Long Bone

The effect of intermittent PTH administration on the skeleton of LTA-deficient mice was examined in the proximal tibiae. PTH treatment significantly increased the bone area compared with the VC (Fig. 4*A–C*). Significantly larger osteoclast numbers per bone perimeters were noted in the PTH-treated animals compared with the control (Fig. 4*D*).

Discussion

The most striking and clinically relevant finding is that PTH treatment protected periradicular tissues from destruction caused by periapical periodontitis. It was further found that neutrophil aggregation next to the root apex was greatly limited by PTH treatment compared with the control, indicating reduced inflammation in the PTH-treated animals. These findings may suggest that intermittent PTH administration augments host innate immune responses. A link between PTH and immune function has been suggested because immune cells such as T and B lymphocytes and leukocytes express PTH receptors (21, 22). In fact, neutrophils from patients with hyperparathyroidism exhibit defects in chemotaxis, migration, and phagocytosis (23–25), and these defects are rescued when parathyroidectomy is performed (26). Thus, the concept that PTH is a negative modifier of the host immune system has been established (27, 28). Contrary to this concept, our finding is that PTH may be a positive modifier in the immune system. The discrepancy may lie in a difference in systemic PTH levels. These studies discuss PTH effects in the context of hyperparathyroidism in which systemic PTH levels are continuously elevated. In this study, PTH was administered intermittently, and the peak of systemic PTH levels was for a moment a day. Thus, the curve of systemic PTH levels in our study would be quite different from those in patients with hyperparathyroidism. Barros et al (6) investigated the effect of intermittent PTH administration on bone loss induced by ligature-induced periodontitis and found that intermittent PTH protected against periodontitis-associated bone loss. Bashutski et al (29) reported that intermittent PTH administration resulted in significantly

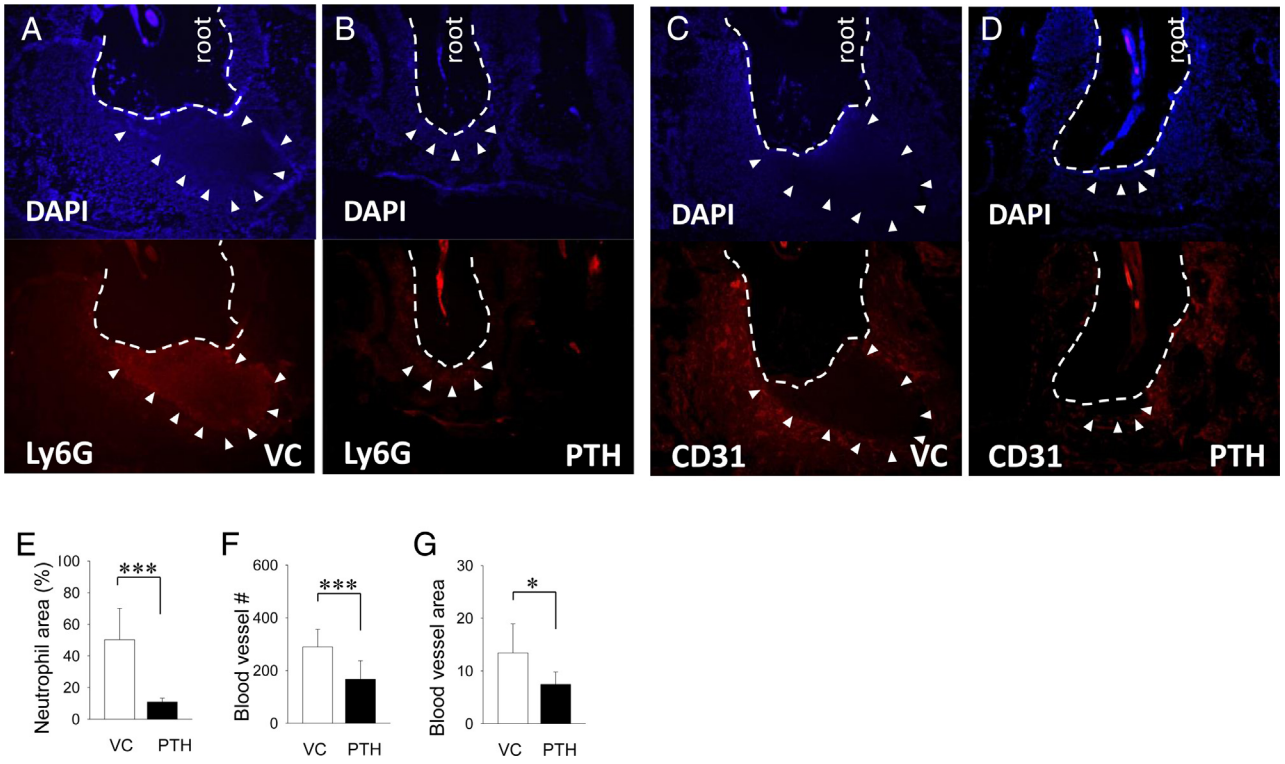

Figure 3. PTH treatment resulted in reduced neutrophil infiltration and blood vessels. (*A* and *B*) Representative immunofluorescent photomicrographs of Ly6G-stained sections of periapical lesions (*bottom*). DAPI staining was used for nuclei visualization (*top*) (original magnification, ×200). The dotted line indicates the tooth root near the apex. *Arrowheads* indicate inflammatory cell infiltration. (*E*) A significantly smaller neutrophil area was found in the PTH-treated group compared with the VC group. (*C* and *D*) Representative immunofluorescent photomicrographs of CD31-stained sections of periapical lesions (*bottom*). DAPI staining was used for nuclei visualization (*top*) (original magnification, ×200). No blood vessels were observed within the lesions. (*F* and *G*) The numbers and areas of blood vessels around the lesions were significantly less in the PTH-treated group than the VC (n = 7/group; Student's t test; *P < .05, ***P < .001).

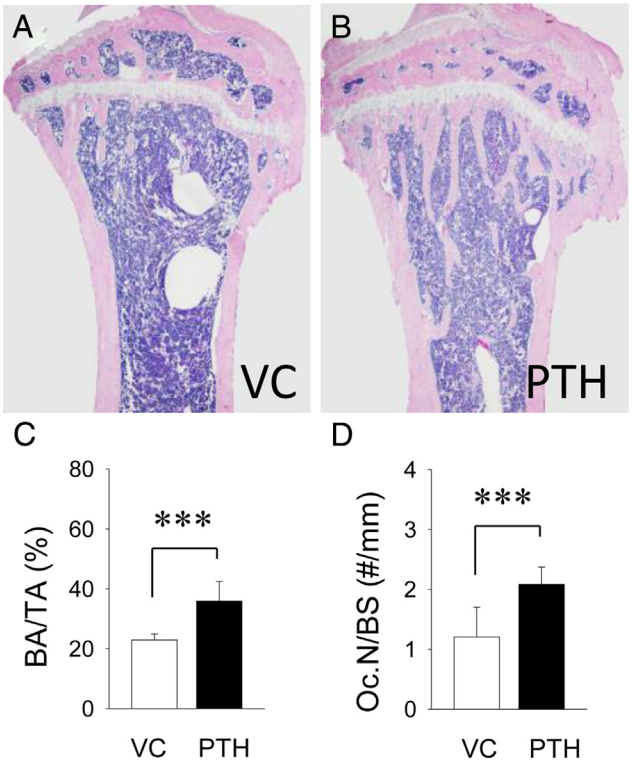

Figure 4. PTH treatment increased bone area in long bones of LTA-deficient mice. (*A* and *B*) Representative photomicrographs of hematoxylin-eosin–stained sections of proximal tibiae (original magnification, ×40). PTH treatment for 3 weeks significantly increased bone mass in (*C*) the proximal tibia with significantly more osteoclasts compared with (*D*) the VC (n = 7/group, Student's t test, ***P < .001).

less attachment loss compared with the control after periodontal surgery. Kuroshima et al (7) studied the effect of intermittent PTH administration on oral osseous wound healing and found that intermittent PTH rescued bisphosphonate-associated impaired wound healing in rats. These studies support our finding that intermittent PTH protected against tissue destruction caused by periapical periodontitis. It is known that the effect of PTH on the skeleton is a double-edged sword; it is catabolic when administered continuously as seen in hyperparathyroidism, whereas it is anabolic when administered intermittently. We speculate that it is possible that the impact of PTH on the immune system could also be different depending on the administration regimen.

The bone anabolic effect of PTH was observed in LTA-deficient mice, indicating that LTA is dispensable for PTH bone anabolic actions. PTH augmented bone mass with a significant increase in osteoclast numbers (Oc.N/BS) in tibiae. This indicates that PTH stimulated both bone formation and resorption in favor of formation. Different from tibiae, however, Oc.N/BS was similar in the periapical tissues between the PTH-treated and control animals. This is likely caused by the induced severe periapical inflammation in the VC. Because significantly more periapical bone resorption was noted in the VC than the PTH-treated animals, more osteoclasts were involved in the VC; hence, there were increased osteoclast numbers in the VC, which resulted in no differences in periapical Oc.N/BS between the PTH-treated and control animals.

Conventionally, the dental pulp is exposed and left open to induce periapical lesions (30) or inoculated with specific pathogens such as *Actinomyces viscosus* to secure the development of periapical lesions (31, 32). Although the specific anaerobic bacteria inoculation is an excellent method, it requires culturing specific bacteria. We set out to establish a simple and natural yet secure method to induce periapical lesions. In this study, we covered exposed dental pulp with plaque-contaminated fibrin. Silk ligatures were used to isolate plaque. Plaque buildup begins soon after ligature placement. Although younger plaque is composed of mainly aerobic species, anaerobic species increases as plaque matures into biofilm. A study that analyzed the composition of bacteria species in silk ligatures placed in mouse molars showed a time-dependent accumulation of anaerobic bacteria (17). A peak of an accumulation of anaerobic bacteria in a ligature occurs at 5 days after ligature placement. Hence, in the current study, silk ligatures were set on molars for 5 days presurgery to establish biofilm in which aerobic and obligatory anaerobic bacteria coexist. In this study, significantly larger periapical lesions were observed in the plaque-contaminated fibrin group compared with the simple pulp exposure group, confirming that our method is useful to study periapical lesions.

It was considered in this study that simple pulp exposure alone would also trigger the development of periapical periodontitis (33). To confirm this, the average distance between the apex and the surrounding alveolar bone was assessed on the intact third molars and then compared with that on the pulp-exposed molars without plaque contamination. We found a trend that simple pulp exposure resulted in mild periapical bone destruction, but the degree of the destruction did not reach a statistical difference when compared with the apical tissues of the intact molars (Supplemental Figure S3). This finding indicates that simple pulp exposure may not be a predictable method to induce periapical periodontitis in LTA-deficient mice. The method used in this study (ie, pulp exposure with bacterial inoculation) proved to be a better protocol to study periapical periodontitis in these specific mice.

In a preliminary study, PTH effects on periapical periodontitis were investigated in wild-type mice. Daily PTH treatment for 3 weeks significantly suppressed disease progression (Supplemental Figure S4). It is generally known that activated PMNs are involved in acute inflammation, which results from innate immune responses to bacterial infection, whereas lymphocytes play a role in chronic inflammation. Recent literature, however, indicates that not only neutrophils but also great numbers of lymphocytes participate in innate immunity (34). To focus on the PTH effects on neutrophil-dominant inflammation, in this study, LTA-deficient mice were used. LTA-deficient mice are fertile, devoid of lymph nodes, and exhibit defects in adaptive immune responses (35). Accordingly, the influence of lymphocytes on periapical inflammation is miniscule, and inflammatory cell infiltration observed next to the root apex is neutrophil dominant. Therefore, our result is consistent with previous findings that intermittent PTH administration suppressed neutrophil infiltration in acute inflammation (7).

Because periapical periodontitis is caused by chiefly anaerobic bacteria and bacterial products such as endotoxins and enzymes in contaminated root canals, no cure was expected by PTH treatment. However, the findings of this study suggest that intermittent PTH administration can be considered to promote periapical tissue healing adjunct to conventional endodontic treatment. In summary, this work indicates that intermittent administration of PTH protects against tissue destruction by periapical periodontitis. Reduced neutrophil infiltration is partially responsible for this protective effect of PTH treatment.

Acknowledgments

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The authors deny any conflicts of interest related to this study.

Supplementary Material

Supplementary material associated with this article can be found in the online version at www.jendodon.com (http://dx.doi.org/10.1016/j.joen.2014.12.008).

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