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Original

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(Accepted for publication, September 3, 2018)

Abstract: Intermittent administration of parathyroid hormone (PTH) is known to increase bone mass for the treatment of osteoporosis. The aim of this study was to evaluate the benefits of local intermittent administration of PTH for bone regeneration in rats with cranial bone defects. Cranial bone defects were induced under anesthesia using a trephine bur (diameter, 4.3 mm) in 8-week-old male Wistar rats, which were then divided into four groups for further treatment. In the PTH-3 and PTH-1 groups, animals received PTH at 14.1 μg/kg in an absorbable collagen sponge placed near the bone defect. Animals in the collagen group received saline at 0.05 ml using the same method, while control animals received only sham surgery. Following surgery, PTH-3 animals received two subcutaneous injections of PTH (14.1 μg/kg) at the experimental site, while the PTH-1, collagen, and control animals each received saline (0.05 ml). All animals were sacrificed at 21 days after surgery. The ratio of new bone mineral content to total defect volume (BMC/TV) at the experimental sites was evaluated using micro-computed tomography. Tissue sections were analyzed by hematoxylin and eosin staining and immunohistochemistry with anti-alkaline phosphatase (ALP), anti-dentin matrix protein 1 (DMP1) and anti-Osterix antibodies. The BMC/TV ratio was significantly higher in the PTH-3 and PTH-1 groups than in the collagen group. The ratio of new bone to defect area (N/D%) was significantly higher in PTH-3 animals than in controls. ALP-positivity was more widely distributed in new bone in PTH-3 animals than in the other groups, and regions of DMP1-positivity and Osterix-positivity were also more widespread in these animals. These findings suggest that local intermittent administration of PTH enhances bone regeneration in rats with cranial bone defects.

Key words: Bone, Cranial bone defects, Local administration, Parathyroid hormone, Rat

Introduction

Efforts to regenerate lost or injured tissue, such as the clinical regeneration of the periodontium, as a therapeutic strategy in those with periodontal defects, is an area of current medical interest. Tissue engineering approaches using stem cells, scaffolds, and signaling molecules were proposed in the 1990s, while guided tissue regeneration and bio-regeneration approaches have been applied to clinical situations. In particular, the application of growth factors, such as bone morphogenetic protein (BMP), basic fibroblast growth factor (b-FGF) and platelet-derived growth factor (PDGF) have been investigated for their involvement in periodontal regeneration.

Parathyroid hormone (PTH) is a peptide regulator of calcium and phosphate metabolism. Although continuous exposure to PTH promotes bone resorption by stimulating osteoclast activity, its intermittent administration promotes new bone formation by stimulating osteoblast differentiation. Systemic administration of recombinant human PTH (rhPTH) has been shown to significantly stimulate local osteoblast formation in a rat calvarial defect model, while clinical studies have shown that rhPTH therapy significantly increases spine bone mineral density (BMD), total hip BMD, and femoral BMD in adults with a mild form of osteoporosis. The clinical benefits of rhPTH thus reflect its ability to stimulate bone formation and thereby increase bone mass and strength, and the hormone appears to be effective in preventing fractures in osteoporosis. The application of rhPTH may also be effective for the treatment of oral defects, with reports that intermittent systemic administration of rhPTH is able to protect against periodontitis-associated bone resorption in rodents, and resolve bisphosphonate-associated osteonecrosis of the jaw. However, the effect of local intermittent rhPTH administration is not sufficiently clear. The aim of this study was to evaluate the benefits of local intermittent administration of rhPTH for bone regeneration in rats with cranial bone defects.

Materials and Methods

Animal procedures

This study was conducted under an approved protocol, and animal care was administered in accordance with guidelines established by the Institutional Animal Care and Use Committee at the University of Asahi (protocol 15-021). Ten 8-week-old male Wistar rats were anesthetized by isoflurane inhalation and intraperitoneal injection of xylazine (5 ml/kg). After induction, the skull of each animal was shaved and an incision made through the skin and periostea, with the full-thickness flap reflected. Two bone defects were created within the calvarium using a trephine bur (4.3 mm in external diameter). Animals were then randomized into four groups. In the PTH-1 group (n=2) and PTH-3 group (n=3), rhPTH (14.1 μg/kg) was placed in an absorbable collagen sponge (ACS) in the defect site, and the wound was closed with non-absorbable nylon sutures. The collagen group (n=3) received saline (0.05 ml) at the wound site using the same collagen sponge method, while control ani-
mals (n=2) received sham surgery. Following surgery, control, collagen, and PTH-1 animals received subcutaneous injections of saline (0.05 ml) once a week for 2 weeks, while the PTH-3 group received subcutaneous injections of rhPTH (14.1 μg/kg) once a week for 2 weeks. Animals were fed regular chow and sterile water throughout the experimental period, and were euthanized at 3 weeks post-surgery using CO₂. The calvariae were then removed and fixed in 4% neutral formalin.

**Micro-computed tomography assessment**

All samples were radiographed using micro-computed tomography (μ-CT) scans (Scanmate-Rb090SS150; Comscan, Yokohama, Japan). The settings for the μ-CT were 74.5 kV, 45.5 mA, and 2.7x magnification. Three-dimensional images were produced using the computer software 3D-BON (RATOC, Tokyo, Japan), and were used to calculate the ratio of new bone mineral content to total defect volume (BMC/TV).

**Histochemical preparation**

After radiographic analysis, all specimens were demineralized using 10% EDTA, embedded in paraffin, and sectioned at 5 μm thickness. The center of selected defect sections was stained histochemically using hematoxylin and eosin (HE), and immunohistochemically using anti-alkaline phosphatase (ALP) (Rabbit Monoclonal Antibody; Abcam, Woburn, MA), anti-dentin matrix protein 1 (DMP1) (Anti-Dentin Matrix Protein 1 polyclonal antibody; TaKaRa Biomedicals, Shiga, Japan) and anti-Osterix (Rabbit Anti-Osterix Polyclonal Antibody; Bioss, Burlingam, CA) antibodies. Sections were deparaffinized with xylene, and were immersed into descending grades of ethanol from 100% to 95%, and 3% H₂O₂ included methanol was used to block endogenous peroxidase activity. After blocking, each primary antibody was allowed to react overnight at 4°C, and secondary antibodies (simple stain mouse MAX-

![Figure 1. μ-CT analyses of bone regeneration. (a) control group, (b) collagen group, (c) PTH-1 group, (d) PTH-3 group. The yellow line shows the border between native bone and new bone. Samples from the PTH-3 group exhibited greater bone fill than the other groups.](image1)

![Figure 2. The ratio of new bone mineral content to total defect volume (BMC/TV ratio) was significantly higher in the PTH-3 and PTH-1 groups than in the collagen group (*p < 0.05).](image2)

PO(R), Nichirei Bioscience, Tokyo, Japan) were used for detection of primary antibodies. For visualization of reactive products, diaminobenzidine (ImmPACT™ DAB; Vector Laboratories, Burlingame, CA) was used as a chromogen substrate, followed by counterstaining with hematoxylin. Using imaging software (Image J), histomorphology was analyzed in HE sections, with the area of new bone formation (N) to the area of defect (D) expressed as a percentage (N/D%).

![Figure 3. Photomicrographs of representative histological sections stained with hematoxylin and eosin. (a) control group, (b) collagen group, (c) PTH-1 group, (d) PTH-3 group. Arrows represent the border between native bone and new bone. Collagen sponge was evident in the collagen, PTH-1, and PTH-3 animals. Scale bars, 2.0 mm.](image3)

**Statistical analysis**

The BMC/TV ratio and N/D% were evaluated using Scheffe’s test to determine differences between groups, with p < 0.05 considered statically significant. All values are expressed as means ± standard deviation.
Results

Micro-CT analyses of bone regeneration

Representative μ-CT radiographs are shown in Fig. 1, with the quantification of BMC/TV ratios shown in Fig. 2 (control group, 981.6 ± 37.0 mg/cm³; collagen group, 786.3 ± 61.7 mg/cm³; PTH-1 group, 1154.0 ± 107.1 mg/cm³; PTH-3 group, 1218.0 ± 59.2 mg/cm³). The PTH-3 and PTH-1 groups had significantly greater BMC/TV ratios than the collagen group (p < 0.05; Fig. 2).

Histological observations

Photomicrographs of representative HE-stained sections are shown in Fig. 3, and the N/D% for the different groups is shown in Fig. 4. The N/D% was 20.6 ± 1.1% in controls, 33.1 ± 3.7% in the collagen group, 40.3 ± 9.6% in the PTH-1 group, and 42.0 ± 2.5% in the PTH-3 group. The PTH-3 animals had a significantly higher N/D% when compared to controls.

Immunohistochemistry

ALP-positive immunostaining was associated with osteoblasts (Fig. 5), with ALP-positive osteoblasts present at the new bone surface of all groups. DMP1 expression was intense in the regions surrounding osteocytes (Fig. 6), with PTH-3 animals having greater numbers of DMP1-positive cells than the other groups. Finally, Osterix-positive osteoblasts were present at the new bone surface in the connective tissue (Fig. 7). Many Osterix-positive osteoblasts were observed in the samples from the PTH-3 and PTH-1 groups, but there were few in the samples from the collagen and control groups.

Discussion

In this study, we evaluated the effects of local administration of rhPTH for bone regeneration in rats with cranial bone defects. We created 4.3-mm diameter cranial osteotomy defects, because 3-mm diameter cranial osteotomy defects do not fully close for 3 weeks14). The intermittent PTH schedule and dose used in the present study were based on previous studies by Eduardo-de-Paula et al15). Previous work has shown that rhBMP-4 delivered with an ACS in this rat calvarial defect model results in partial ACS resorption and new bone formation at 2 weeks, but at 8 weeks, the ACS is completely resorbed and defects are almost completely filled with lamellar bone16). In the present study, an ACS was used as a scaffold in the collagen, PTH-1, and PTH-3 groups, and at 3 weeks post-surgery, the ACS partially remained. The collagen group
showed a lower BMC/TV ratio than the control group, indicating that the ACS might inhibit new bone formation.

PTH is an 84-amino acid polypeptide, with the active component consisting of the 34 amino acids from the N-terminus. This shorter peptide forms the basis of the rhPTH teriparatide. In our study, anti-ALP and anti-Osterix immunostaining revealed osteoblasts at the new bone surface. Teresita et al. reported that intermittent administration of rhPTH attenuates osteoblast apoptosis, thereby increasing osteoblast number, the rate of bone formation, and bone mass. Intermitent administration of PTH directly acts on osteoblasts to arrest cell cycle progression from G1 to S phase, and increase differentiation. Based on the results of the present study, we suggest that the intermittent administration of rhPTH directly activates osteoblasts, resulting in an increase in osteoblast number, rate of bone formation, differentiation, and bone mass. The transcription factor Osterix is required for osteoblast differentiation, with Osterix-null mice having no bone formation, and rhPTH is essential for expression of this transcription factor. Finally, Yuske et al. reported that the expression of DMP1 is mainly confined to osteocytes. In our study, DMP1 immunostaining was mainly seen in the regions surrounding osteocytes in areas of new bone.

John et al. reported that long-term treatment of rats with rhPTH caused focal bone proliferative lesions, including osteosarcoma. Our findings indicate that local administration of rhPTH enhances bone formation, but we have not verified potential systemic effects. Therefore, further work is required to confirm the optimal rhPTH administration, dose level, and treatment duration at sites of osseous wound healing in order to maximize benefits and minimize systemic effects. In conclusion, local and intermittent administration of rhPTH in rats with cranial bone defects significantly enhanced bone regeneration. Consequently, we suggest that local and intermittent administration of rhPTH is effective for periodontal regeneration.

Acknowledgements
This work was supported by Miyata Research Grant A (2017).

Conflict of Interest
The authors have declared that no conflict of interest exists.

References
17. Qin L, Li X, Ko JK and Partridge NC. Parathyroid hormone uses multiple mechanisms to arrest the cell cycle progression of osteoblastic cells from G1 to S phase. J Biol Chem 280: 3104-3111, 2005


