Bone-Healing Pattern on Critical-Sized Defects Treated by rhPTH

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Abstract: Intermittent Recombinant Human Parathyroid Hormone (rhPTH 1-34) is an effective treatment for improving bone mass in patients with osteoporosis; however, its effects on bone regeneration are still unclear. The objective of this study was to evaluate the potential toxicity systemic rhPTH, as well as its ability to regenerate critical-sized defects (CZD) in bone. We used 43 female Wistar rats (body weight, 150 ± 50 g). Critical-sized bone defects in rat calvaria received vehicle alone (Control Group, CG) or daily rhPTH (20 μg/Kg/day) by subcutaneous injection (Experimental Group, EG). We evaluated bone healing obtained at the 1st, 3rd, and 6th wks post-surgery by biochemical, soft x-ray, histological, and morphometric studies. In the EG, at the 1st and 3rd wks, many areas of focal osteoblast hyperplasia were found on parietal bone. At the 3rd wk, woven and/or lamellar bone, in an organized interconnected trabecular network, showed disrupted mineralization. At the 6th wk, looped bone was found to have formed patterns on parietal bone. New bone formed in the EG showed significant statistical differences (p = 0.023) at the 6th wk. Systemic rhPTH at the dose of 20 μg/Kg/day was able to stimulate bone formation on rat CZD. Also, pre-existing and new bone showed non-proliferative forms of bone hyperostosis (increased non-neoplastic bone).

Key Words: rhPTH, Critical-sized defect, Bone regeneration, Proliferative bone lesions, Bone hyperostosis.

Introduction

Endogenous Parathyroid Hormone (PTH 1-84) plays a key role in calcium homeostasis and bone metabolism ⁷. A continuous measure of exogenous PTH increases bone resorption, whereas its periodic administration stimulates new bone formation ⁷. Increased osteoblast numbers have been shown in adult rats after daily PTH administration, by the proliferation of osteoprogenitors from lining cell differentiation ⁷. Moreover, PTH stimulates osteoprogenitor cell proliferation and differentiation from bone marrow. PTH binds osteoblast and osteocyte receptors and influences increased expression of early genes (c-fos, c-myc, c-jun, and IL-6) involved in cell proliferation ⁷. Also, in vitro, PTH showed a mitogenic effect mediated by transforming growth factor β (TGFβ) ⁷. Moreover, improved insulin growth factor-I synthesis and secretion from osteoblast-like cells have been demonstrated ⁶.

In 2002, recombinant human parathyroid hormone (rhPTH 1-34) was approved by the US Food and Drug Administration (FDA) for osteoporosis treatment. It has the same N-terminal amino acid sequence–biologically active region–as full-length Human Parathyroid Hormone ⁷. It is assumed that the biological activity of intact PTH (hPTH 1–84) resides in the N-terminal sequence; many studies have used the 34-amino-acid peptide hPTH(1–34), now named “Teriparatide”. In contrast, Hodsman et al. ⁸ reported that it is conceivable that whole PTH may have slightly different biological actions compared with those of Teriparatide. The aim of this study was to determine the capacity of rhPTH, administered systemically, to regenerate critical-sized defects in bone. Of the limited research available, none addresses the action of Teriparatide. In this regard, Nozaka et al. ⁹ used PTH 1-34 in osteoporotic animals with non-critical-sized bone defects, and demonstrated enhanced bone repair in tibial osteotomies. Komatsu et al. ¹⁰ had similar results in femoral circular defects. Bone models in normal animals with critical-sized defects (CZD) are selected for the study of bone therapies, because such defects are
unable to heal spontaneously \cite{11,12,13,14} treated subcutaneously with intermittent PTH (1-34), combined with osteoconductive replacement material, polytetrafluoroethylene, and β tricalcium phosphate scaffolds, respectively, showed enhanced mechanical strength and increased bone deposition. However, the systemic administration of rhPTH without matrices for the treatment of critical-sized defects remains to be elucidated. In this regard, whether rhPTH induces de novo bone formation has not been demonstrated. Therefore, we hypothesized that the intermittent systemic administration of rhPTH, 20 μg/Kg/day, induced bone formation on CZD to repair large bone defects.

**Materials and Methods**

**Animals**

Forty-three female *Rattus norvegicus* var. Wistar rats (150 ± 50 g), approximately 9 wks old, were obtained from the Animal Research Center (Medical School, Tucumán University, Argentina). Animals were housed in pairs, in a specific pathogen-free environment, with a temperature of 22.4°C to 23.8°C, relative humidity of 45% to 62%, and a 12-hour light-dark cycle. A standard commercial diet and tap water were available *ad libitum*. During the study, animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals 8th Edition (National Research Council, 2011) from the National Academies Press (Washington, DC, USA), and The current examination was performed under the guidelines for use of experimental animals with permission of the Animal Experimental Committee of Okayama University.

**Experimental design**

The research protocol was submitted to and approved by the local Ethical Committee for Animal Research (Tucumán National University & CONICET). Critical-sized defects were created in calvariae as previously described \cite{11,12}. Briefly, defects were created manually with the use of a 5-mm-diameter ad hoc punch with smooth edges. Animals were randomly assigned to one of two groups: (A) The Control Group (CG-18n) received only vehicle (saline solution), and (B) the Experimental Group (EG- 25n) was treated with 20 μg/kg/day of rhPTH (Forteo-Teriparatide, Eli Lilly) via subcutaneous injection. The dose used in this study was based on Vahle et al. \cite{15} who demonstrated that rhPTH 5-30 μg/kg/day was able to induce bone formation without side-effects. After 1, 3, or 6 wks post-surgery, animals were killed, and bone samples were obtained, fixed in 20% buffered formalin phosphate for 24 hrs, and stored in 70% alcohol prior to analysis.

**Assays for serum concentration of rhPTH and bone metabolic markers**

At 10 min, 60 min, and 24 hrs after subcutaneous rhPTH injection, blood samples were collected from six animals and placed in ethylenediaminetetraacetic acid (EDTA) tubes, to determine the rhPTH plasma levels by human electrochemiluminescence immunoassay. From all animals, blood samples were obtained by tail bleeding and analyzed for total alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP), and serum calcium. Urine samples were collected by metabolic cages through 24 hrs for identification of calcium levels.

**Soft X-ray by high-resolution films**

All samples were subjected to soft x-rays with high-resolution Kodak film (mammography type, 18 x 23 cm; GBA Mamograf HF brand equipment, Buenos Aires, Argentina). Exposure time was 0.8 sec, at 27.5 kV and 7.0 mA \cite{16}.

**Histopathology**

The specimens were decalcified with modified Morse solution (Okayama University Dental School) and embedded in paraffin in a routine manner. A serial 4-μm central section per defect was selected and stained with Hematoxylin & Eosin (H&E), Masson-Goldner, Periodic-Acid Schiff (PAS), Roque’s trichrome, or Sudan Black, and examined by light microscopy. A single pathologist evaluated all tissues. Subsequently, another pathologist performed an independent review to verify the microscopic observations. The reported results reflect the mutually-agreed-upon diagnoses by both pathologists, last certified by the Argentina Health Ministry (Nº 31455). The terminology and diagnostic criteria used to interpret the bone lesions were consistent with the Standardized System of Nomenclature and Diagnostic Criteria Guides for Toxicologic Pathology \cite{17}. The criteria used to classify proliferative changes in bones were based on those used in the Teriparatide study \cite{15,18}.

**Histometric studies**

Photomicrographs were taken from slides from each specimen by means of a Sony digital camera adapted to an Olympus CH30 microscope. The photos obtained by Soft Pinnacle Studio 9.4 with 116.7X magnification were evaluated by Image Pro Plus Version 4.5.0.29 (Windows 1998/NT/2000, 1993-2001, Media Cybernetics, Inc.). A total area of CZD and 2 mm of surrounding parietal bone was determined. The parameters selected were: parietal bone thickness (from periosteal, pre-existing, and endosteal bones) at 300 pixels from the edge of CZD, evaluated in μm; and new bone formed at the edges and center of CZD, expressed as a percentage of the total defect area.

**Statistical analysis**

Data are presented as mean ± standard deviation, as indicated in the Figs. Biochemistry and histomorphometry results were evaluated by the Mann-Whitney test (p < 0.05).
Bone metabolic markers

The variations from bone metabolic markers are summarized in Figs. 1B-1D. Alkaline phosphatase (ALP) at the 1st wk showed a minimal increase of 146 U/L in the EG compared with 127 U/L in the CG. This increasing trend continued in treated rats, reaching 1.7 times and 2.9 times at 3 and 6 wks, respectively, in contrast to control group values. Tartrate-resistant acid phosphatase (TRAP) values showed a further 2.5x reduction at the 1st wk, although there was an increase at the 3rd wk, and at the 6th wk, the values decreased again. There was no statistically significant difference between groups at both markers (p < 0.005, Mann-Whitney test). Serum calcium levels increased throughout all study periods, and renal excretion decreased relative to urine calcium (d). Data subjected to statistical analysis showed differences from serum calcium levels at all study periods (p = 0.01, 0.02, and 0.04, respectively), as well as urine calcium at 6 wks (p = 0.04), indicated by * (p < 0.05, Mann-Whitney test).

Results

Concentrations of serum rhPTH

Serum levels of rhPTH increased immediately after a single subcutaneous injection, and maximum value was higher than 207.9 pg/mL at 10 min, decreasing to 89.2 pg/mL at 1 hr, and to 18.8 pg/mL at 24 hrs (Fig. 1A).
results: rounded radiolucent areas on parietal bone, representing CZD healing by fibrous connective tissue. There were some internal radiopacities, which increased in size and numbers throughout the study, especially in the EG (Figs. 2A, 2B).

**Histopathology and histometric observations**

In the control group, at the 1st wk post-surgery, the defect area exhibited granulation tissue and congestion in the superior sagittal sinus. At the 3rd and 6th wks, the CZD area showed fibrous connective tissue healing. In all 3 periods, there was almost no regenerated bone in the defects except in the immediate vicinity of the surgical margins (Fig. 3A). In the experimental group, at the 1st wk, the CZD area showed granulation tissue and congestion of the superior sagittal sinus, as in the CG (Fig. 3B). At the edges, the bone formed showed swirling cemental basophilic lines resembling disrupted mineralization (Fig. 3C). Osteosclerosis was observed with cortical bone apposition on existing surfaces. Focal osteoblast hyperplasia was found on parietal bone surfaces, showing many active osteoblasts without connections to osteoclasts. Limited focal proliferation of spindle-shaped stromal cell areas with fibrous connective tissue and vessels was observed (Fig. 4A). Within the critical-sized defect area, rare woven bone islands were detected, covered with many lining cells surrounded by fibrous connective tissue with stromal cells, so-called “fibrous osteodystrophy” (Fig. 3D). At the 3rd and 6th wks, there was a significant increase (p=0.01 and 0.02, respectively) in bone thickness, and many sutures were healed. Disrupted mineralization was also observed (Fig. 4B). Periosteal and endosteal bone formation organized in a looping or networking pattern was diagnosed as hyperostosis (Fig. 4C). Osteocytes from new bone or pre-existing bone were prominent, granular, and eosinophilic, showing intracellular hyaline body formation (IHB) and cell death (Fig. 4D).

The Table shows the results of histometric analysis. The new bone formed in the EG at 1 wk (0.20% ± 0.02) and 3 wks (0.65% ± 0.02), compared with that formed in the CG (0.14% ± 0.1 and 0.40% ± 0.2, respectively), showed no significant statistical differences. At 6 wks, the EG showed significant bone formed (2.81% ± 0.6) compared with the CG (1.06% ± 0.2) (p = 0.023).
Clinical and experimental evidence supports the use of recombinant human parathyroid hormone for severe osteoporosis treatment, based on anabolic success with daily administration. This led us to believe that rhPTH may also have an anabolic effect in other bone loss lesions. The potential of systemic intermittent rhPTH administration to promote bone regeneration in large defects is unknown.

We selected a well-established critical-size defect model using rat calvaria bone, because its biological properties are similar to those of the human maxilla. Both are flat bones, with intramembranous ossification and two hard outer layer of cortical bone containing bone marrow. Further, CZD were selected because they are unable to undergo spontaneous bone regeneration. The results in a CZD rat model were obtained with a 20 μg/kg/day intermittent subcutaneous dose from 1 to 6 wks. We followed the regeneration process up to the sixth week to restrict the study to the maturity period of the animal, avoiding the old age phase during which the biological response is reduced. The doses were selected based on previous studies where rhPTH 5-30 μg/kg/day stimulated bone formation without side-effects. In our results, bone volume at edges and center of CZD in the EG was limited to 2.81 ± 0.6% at the 6th wk, while the value from the CG was 1.06 ± 0.2%. Because bone formation was observed on defect perimeters, CZD regeneration did not occur. However, pre-existing bone thickness increased in all study periods, and was statistically significant at 3 (p = 0.01) and 6 (p = 0.02) wks. From these results, it is reasonable to consider that the slight bone formation around the CZD was based on the short rhPTH half-life, enough to trigger pre-existing bone multicellular units, but insufficient to activate “determined osteoblasts” available in the dura mater. Rather than fully filling the bone defect, rhPTH generated few woven bone ossicles, surrounded by fusocellular lining cells in fibrous connective tissue that resembled fibrous osteodystrophy. Bone non-neoplastic proliferative lesions, like the focal osteoblast hyperplasia and focal stromal hyperplasia found in this research, have been previously described, but with high doses of rhPTH (30, 50, and 75 μg/kg/day). This is the first report on the use of rhPTH (20 μg/kg/day) for systemic treatment alone for CZD rats without any osteoconductive matrices, that formed a small amount of inductive new bone. The results of the present study seem to be in disagreement with those from the study of CZD animal models where PTH and some biomaterials were used. In those studies, a significant amount of bone was formed, based on the osteoconductive ability of various bone replacements acting like a scaffold, and on the differences between whole PTH and recombinant hPTH biological action. Moreover, this is the first report showing non-proliferative forms of hyperostosis and osteopetrotic-like alterations at an rhPTH dose of 20 μg/kg/day. The diagnosis of non-proliferative hyperostosis was based on the following histopathologic lesions: bone sclerosis, closed suture space, disrupted mineralization, and periosteal-endosteal globular bone formation. New intracortical bone was organized in a looping or networking design. Based on AFIP guidelines, these considerations could be included as typical-atypical proliferative hyperostosis. Moreover, osteoblasts, osteocytes, and osteoclasts showed different sizes and shapes. The lacunar osteocytes showed many irregular shapes. Many presented an intracellular eosinophilic body resembling hyaline-like bodies (IHB), demonstrated by Roque’s staining, acting as histological and potential markers of disease progression. Others showed some degree of autophagic cell death, shown by Sudan black staining. We considered that rhPTH produced stress toxicity on bone cells, forming from IHB to cell death. This could explain why many osteocytes apparently experienced an autophagic, caspase-independent, “type II” cell death, as observed by immunohistochemistry, and also possibly explained the increase in bone mass. In conclusion, systemic administration of rhPTH induced a small amount of new bone and acted aggressively on bone cells. Also, 20 μg/Kg/day produces protein damage or injury.
a possible reason for programmed cell death (type I, II, or III) (Kroemer et al. 2009 29), and may determine bone non-neoplastic proliferative lesions with disrupted mineralization.

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