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Enhancement of recombinant human bone morphogenetic protein-2 (rhBMP-2)-induced new bone formation by concurrent treatment with parathyroid hormone and a phosphodiesterase inhibitor, pentoxifylline

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Abstract We investigated the enhancement of new bone formation elicited ectopically by recombinant human bone morphogenetic protein-2 (rhBMP-2), using parathyroid hormone (PTH) and a phosphodiesterase inhibitor (PDEi), pentoxifylline (PTX), in an animal model. Collagen sponge sheet discs containing rhBMP were implanted onto the back muscles of mice. PTX alone (200 mg/kg body weight [BW]), PTH(1–34) (10 μg/kg BW), PTX plus PTH (200 mg/kg BW and 10 μg/kg BW, respectively), or vehicle (control) were injected subcutaneously daily for 3 weeks after implantation. At the end of this period, rhBMP-2-induced ectopic ossicles were harvested from each group of animals. Ossicles from the PTX-treated group were significantly larger in size, with unchanged bone mineral density (BMD), as compared with the ossicles from the controls. In contrast, the ossicles from the PTH-treated group had significantly higher BMD, but showed no difference in size when compared with those from the control animals. The ossicles of the PTX + PTH treatment group were significantly larger than those of the control and PTH treatment groups. In addition, the BMD of the harvested tissues from the PTX + PTH treatment group was significantly higher than that of tissues from the control and PTX treatment groups. Although the calcium content of ossicles was significantly higher in the PTX-, PTH-, and PTX + PTH-treated groups than in the control group, the Ca content of ossicles from the PTH + PTX-treated group was highest (two times that of controls), followed by the PTH- and PTX-treated groups.

Key words bone morphogenetic protein (BMP) · ectopic bone formation · parathyroid hormone · phosphodiesterase inhibitor (PDEi)

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Introduction

Parathyroid hormone (PTH) is a major regulator of osteoblastic function through its specific G-protein-coupled receptor and the protein kinase A (PKA) kinase cascade [1,2]. A number of animal studies have demonstrated that intermittent PTH injection in animals exerts anabolic effects on bone formation [3–7], although the precise mechanism of this anabolic action remains unknown. From a clinical perspective, the anabolic action of this hormone highlights its potential value in the treatment of bone diseases associated with suppressed bone formation or bone loss, such as osteoporosis and non-union fractures.

Phosphodiesterase inhibitors (PDEis), e.g., pentoxifylline (PTX) and rolipram, have also been shown to enhance bone-forming potential in in vivo situations [8–10], probably through elevating intracellular levels of cyclic adenosine monophosphate (cAMP) in osteoblasts and enhancing the responsiveness of young mesenchymal cells to BMP-2 or BMP-4 [11,12].

The intracellular level of cAMP is upregulated by G protein-coupled adenylcyclase [13] and degraded by phosphodiesterases (PDEs) [14], a family of enzymes that catalyze the hydrolysis of cAMP and cyclic granosine monophosphate (cGMP). Thus, both PDEis and PTH may lead to the elevation of intracellular cAMP following activation via the PKA cascade. However, based on previous experimental studies, the mechanisms of action of these agents appear to be different. Pentoxifylline (PTX), a non-selective PDE inhibitor, promoted larger bone morphogenetic protein (BMP)-induced ossicles [9] with identical bone mineral density (BMD) when compared to non-treated control ossicles. In contrast to the effects of the PDEi, the BMP-induced ossicles from PTH-treated animals had an identical size and higher BMD when compared with the controls. These experimental data invoke the possibility that concurrent treatment with PDEi and PTH may act

in a complementary manner to maximize BMP-induced new bone mass. Clearly, this could have significant therapeutic value in the clinical setting. The present experimental study was designed to address this hypothesis.

Materials and methods

Preparation of BMP-containing collagen pellets

Recombinant human (rh)BMP-2 was produced by the Genetics Institute (Cambridge, MA, USA) and donated to us through Yamanouchi Phamaceutical (Tokyo, Japan). The rhBMP-2 was provided in a buffer solution (5 mM glutamic acid, 2.5% glycine, 0.5% sucrose, and 0.01% Tween 80) at a concentration of 1 μg/1 μl after filter sterilization. To prepare one collagen disc implant sample, 5 μl (5 μg of rhBMP) of the rhBMP-2 solution was added to 20 μl of 0.01 N HCl solution and blotted into a porous collagen disc (6 mm in diameter, 1 mm in thickness) fabricated from commercially available bovine collagen sheets (Helistat; Integra Life Sciences, Plainsboro, NJ, USA), freeze-dried, and kept at −20°C until implantation into mice. All procedures were carried out under sterile conditions.

Pentoxifylline and PTH(1–34)

Pentoxifylline (PTX) was obtained from Research Biochemical International (Natick, MA, USA) and dissolved in physiological saline prior to use. Human (h)PTH(1–34) was synthesized and donated to us by Asahi Chemical (Tokyo, Japan). The hPTH(1–34) was dissolved in vehicle containing 0.1% bovin serum albumin (BSA).

Experimental protocols

Forty male ddy mice, at 4 weeks of age, were purchased from Nippon SLC (Shizuoka, Japan). The mice were housed in cages with free access to food and water from 1 week before the start of the experiment until 3 weeks after implantation. The mice were randomly assigned to four groups (10 mice for each group): (1) control group: vehicle alone, (2) PTX 200 mg/kg (body weight) BW, injected daily, (3) PTH 10 μg/kg BW, injected daily, and (4) PTX 200 mg/kg BW + PTH 10 μg/kg BW, injected daily. The doses of PTH and PTX were optimized based on data from our previous study [9] and preliminary experiments.

Before surgery to implant the samples, the animals were anesthetized with diethyl ether. The uniformly-sized implants were placed into the left dorsal muscle pouches (one implant per animal).

The implants were harvested and processed for radiological, chemical, and histological examinations. All of the harvested tissues were measured and then radiographed with a soft X-ray apparatus (Sofron; Sofron, Tokyo, Japan) and fixed in 10% neutral pH formalin solution. The volume of each ossicle was determined based on three mutually orthogonal measurements (A,B,C) of the ossicle, done with a caliper, and the ossicle volume was calculated by the formula, $V = ABC \times \pi/6$ [10,15,16]. The BMD of each ossicle (g/mm^2) was measured by single-energy X-ray absorptiometry (SXA), using a bone mineral analyzer (DCS-600R; Aloka, Tokyo, Japan). After measurement of these parameters, three of the ten samples from each group were defatted with chloroform, decalcified with 10% EDTA, and embedded in paraffin wax. Sections 5 μm in thickness were cut, stained with hematoxylin and eosin (H&E), and examined under a light microscope. The remaining seven samples from each group were decalcified in 5 ml of 0.6 N HCl in separate sealed glass bottles and stirred for 48 h. The calcium content of each bottle was measured by the orthocresolphthalein complexone (OCPC) method (Calcium C-test kit, wako; Wako Pure Chemical Industries, Osaka, Japan), as previously described [9,17,18]. All the animal experiments were carried out in compliance with the requirements of the Institutional Animal Care Committee at Shinshu University.

Statistical analysis

The data were assessed by Kruskal-Wallis analysis of variance and the Mann-Whitney *U*-test. A P value of 0.05 was used to delineate significant differences between groups.

Results

Volume of the harvested tissue

Figure 1 shows the mean volumes of the harvested tissue from each of the four groups 3 weeks after implantation. The mean volume of the harvested tissue of the

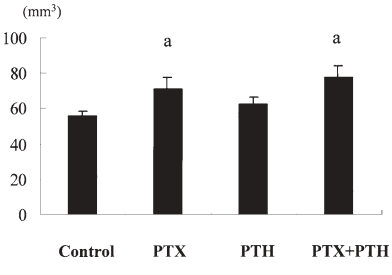

Fig. 1. The ossicles of the PTX alone and PTX + PTH groups were significantly larger than those of the control group. However, there was no significant difference between the volumes of the PTH-alone and control groups ($n = 10$). Data values are means ± SD. [a] $P < 0.05$ vs control

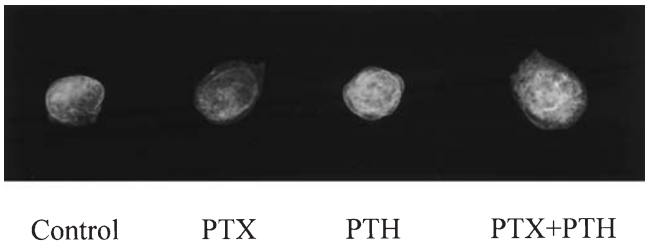

Fig. 2. Soft X-ray photographs of the ossicles at 3 weeks after implantation. A typical ossicle from each group is shown

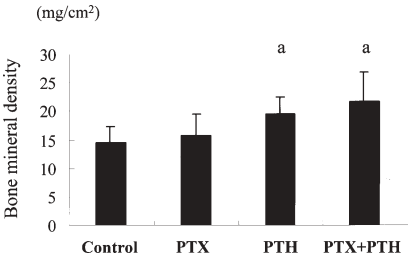

Fig. 3. The bone mineral density (BMD) values of the harvested tissues from the PTH-alone and PTX + PTH group were significantly higher than that in the control group (n = 10). Data values are means ± SD. [a] $P < 0.05$ vs control

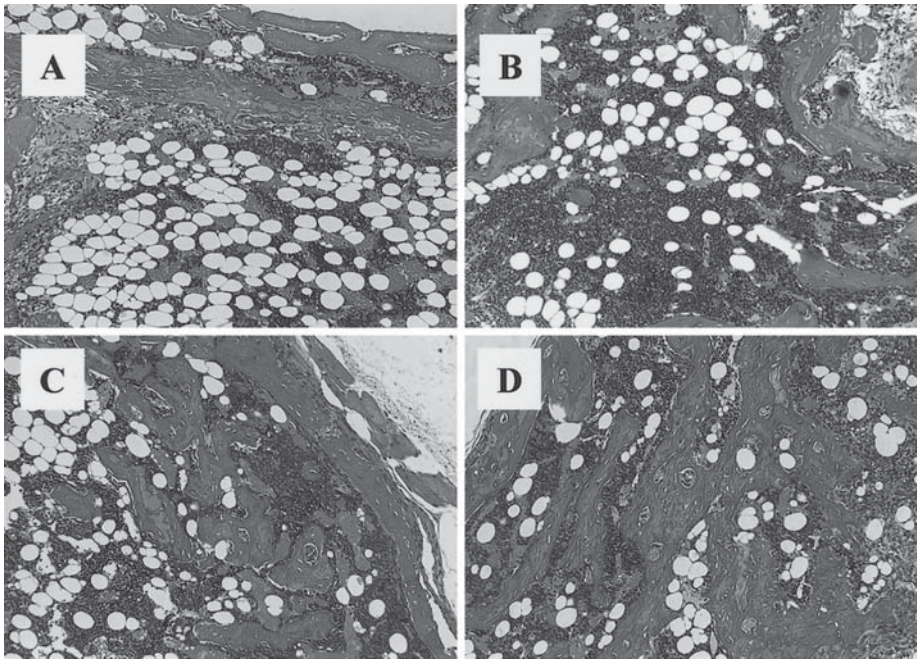

Fig. 4. Photomicrographs of the harvested tissues at 3 weeks after implantation. **A** Control; **B** PTX; **C** PTH; **D** PTX + PTH. New bone formation with hematopoietic bone marrow and trabeculae is visible in the tissue. In the PTH-alone treatment group and the PTX + PTH treatment group, there were visible increases in the number and thickness of bony trabeculae when compared to the PTX and control group. **A**–**D** H&E, ×100

control group was 55.6 ± 2.5 mm³; that of the PTX group was 70.9 ± 6.3 mm³; and that of the PTX + PTH group, 77.3 ± 6.5 mm³. The values for the PTX and PTX + PTH groups were significantly higher than that recorded for the control group ($P < 0.05$). However, there was no significant difference between the controls and the PTH group (62.1 ± 3.5 mm³).

Radiographic findings

The radiographic appearances of the harvested tissues 3 weeks after implantations are shown in Fig. 2. All of the pellets harvested 3 weeks after implantation showed radiographic evidence of calcification and calcified trabeculae.

Bone mineral density (BMD) of the ossicles

Figure 3 shows the mean BMD of ossicles from each group, measured with SXA. There was no significant difference in BMD between the PTX group (15.7 ± 3.8 mg/cm²) and the control group (14.5 ± 2.7 mg/cm²). However, the BMDs of the PTH group (19.4 ± 3.1 mg/cm²) and the PTX + PTH group (21.8 ± 5.2 mg/cm²) were significantly higher than those of the control group and the PTX group.

Histology

Ossicles from all of the four groups revealed normal bone histology, with hematopoietic marrow and bony trabeculae (Fig. 4). Small amounts of collagen carrier remnants were seen in the centers of all the ossicles.

Calcium content in the ossicles

The mean calcium content in ossicles harvested from each group is shown in Fig. 5. The calcium content in

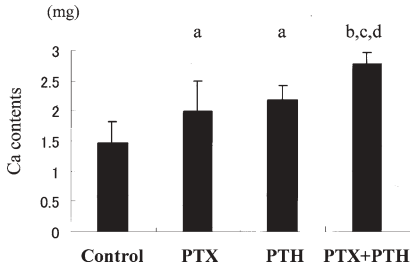

Fig. 5. Ca content of the harvested tissues at 3 weeks after implantation. Ca content was measured by OCPC method (Calcium C test kit; Wako) ($n = 7$). The Ca content of the ossicles from the PTX group and the PTH group was significantly higher than that in the control group. The Ca content of the ossicles from the PTX + PTH concurrent treatment group was significantly higher than that in the ossicles from the control, PTX-alone, and PTH-alone treatment groups. Data values are means ± SD. [a] $P < 0.05$ vs control; [b] $P < 0.01$ vs control; [c] $P < 0.05$ vs PTX group; [d] $P < 0.05$ vs PTH group

ossicles from the PTX (1.99 ± 0.51 mg), PTH (2.18 ± 0.24 mg), and PTX + PTH groups (2.78 ± 0.18 mg) was significantly higher than the value in the control group (1.46 ± 0.36 mg). The Mean calcium content in the ossicles of the PTX + PTH group was significantly higher than that in the PTH and PTX groups ($n = 7$).

Discussion

The results of the present experimental study revealed additive anabolic effects of the daily injection of active fragment of human PTH and a nonselective PDEi (PTX) on endochondral ossification elicited ectopically by rhBMP-2 in mice. The additive and complementary effects of both agents on endochondral bone formation were postulated from the results of our previous studies using the same model, indicating two aspects of the enhancement of bone formation, i.e., increased mass of the new bone and increased density of the new bone. Daily injection of PTX increased the mass of BMP-induced ectopic bone without changing the BMD [9], and PTH increased the BMD without changing the bone mass. In the initial phase of new ectopic bone formation in the BMP-retaining collagen pellets, the pellet is resorbed and replaced by a cartilage shell at the periphery of the pellet. This shell is then thought to be replaced by new bone through endochondral ossification, with the shell determining the final size of the BMP-induced ossicle [19]. In previous studies of ours, it was radiographically determined that the calcified rings in tissues harvested from mice treated for 1 week with PTX and other PDEi agents were larger than those observed in the control group [9,10]. Because the chondro-osseous differentiation of undifferentiated mesenchymal cells is initiated when these cells come into contact with BMP at the periphery of the BMP-retaining disk, this increase in the bone mass may indicate greater sensitivity and earlier initiation of the response to BMP-2 induced by PTX compared with the control. PTX therefore appears to have a stimulatory effect on the early stage of bone formation during BMP-induced osteogenesis. In an in vitro study, PTX has been shown to enhance BMP effects predominantly in less differentiated cells which have the potential for osteogenic or chondrogenic differentiation [20]. PTX may target undifferentiated mesenchymal cells that come into contact with BMP in the initial phase of ossification and may increase their responsiveness to BMP. This, is turn, may lead to the earlier initiation of chondrogenic differentiation, resulting in a larger cartilagenous anlage to be replaced later by new bone tissue, and leading to an increase in implant size. However, our study did not find an increase in the size of the harvested tissue as a result of PTH treatment. This indicates that PTH may not affect the cartilage formation phase in this model. The tissues harvested from mice treated with PTH for only the first week were not significantly different from the control, either in size or in BMD (data not shown). The BMD of the tissue harvested from the PTH-treatment group at 3 weeks after implantation was significantly higher than that of the tissue from the control group and the group treated with PTX alone. These findings suggest that PTH may affect a late rather than an early phase of this BMP-induced endochondral ossification process. The mechanisms of the anabolic effect of PTH remain unknown, however. Several studies have reported that the receptor for PTH is expressed in the cells of both chondrocytes and osteoblasts [21,22]. However, this claim does not seem to support the findings of the present study, in that the anabolic effects of PTH were observed at a later phase of the BMP-induced endochondral ossification process. Further studies to clarify this point therefore appear to be necessary.

Thus, PTX and PTH appear to act on distinct phases of the osteogenic process. Therefore, we hypothesized that the two agents would work additively when used concurrently. Our data seemed to confirm this hypothesis, with concurrent PTX + PTH treatment resulting in the induction of new ossicles of a larger size and with a higher BMD than the controls.

Several studies have reported that the cortical area of the tibia or femoral diaphysis increased significantly after injections of 50–200 μg/kg BW per day of PTH(1–34) [23–28]. However, other studies found that injection at doses of 1.5–10 μg/kg BW had an anabolic effect on bone formation [29–30]. We performed preliminary experiments in the model used in the present study. The injection of 4, 10, and 40 μg/kg BW per day of PTH (1–

34) resulted in a significant increase in the BMD of the harvested tissues compared with that produced by the vehicle alone. However, it should be noted that the increase in BMD generated by the 40 μg/kg-dose (18.5 ± 3.9 mg/cm^2) was less than that resulting from the doses of 4 μg/kg (20.5 ± 3.3 mg/cm^2) and 10 μg/kg (21.0 ± 3.5 mg/cm^2). The dose of 10 μg/kg was therefore considered optimal, and a low dose of PTH(1–34) seemed to have an effect on this ectopic bone-formation model.

The mechanisms underlying the anabolic effects of PTH and PTX on bone formation are not fully understood. PTH appears to increase the bone-forming activity of osteoblasts, and it may increase the rate of maturation of pre-osteoblasts into osteoblasts, or it may increase the bone-forming activity of osteoblasts [31]. Bone mass can be increased by intermittent PTH administration, but the mechanism of this phenomenon is not known. In the study presented here, PTH treatment was found to increase the BMD and the calcium content of BMP-2 induced ectopic new bone, but it did not increase bone volume. These results indicate that intermittent administration of PTH is likely to have an anabolic effect on BMP-2-induced ectopic new bone formation. Further studies are needed, however, to clarify the mechanisms involved.

The exact mechanism by which a PDEi stimulates BMP-induced bone formation also awaits elucidation. Elevation of intracellular cAMP level by a PDEi, coupled with intracellular signaling through the PKA cascade by PTH, may stimulate bone formation [20]. For the future, it will be important to study the crosstalk between BMP, BMP receptors, Smads, Cbfa-1, and the PKA signaling cascade. There is considerable evidence in the literature to suggest that the anabolic effects of PTH are mediated by cAMP [1,2,32] and, by extrapolation, PDEs [11,20].

A recent study aimed at further understanding of the anabolic actions of PTX on bone formation has also implicated crosstalk between BMP signaling and PKC signaling cascades [12]. However, that report mentioned that PDEis, including PTX, could promote osteoblast differentiation by a mechanism independent of PKA activation. We speculate that this mechanism may be one of the reasons why PTH and PDEi have different effects on osteoblast differentiation. Future studies to investigate the molecules and signal pathways by which PTH and PDEis mediate osteoblast differentiation should contribute to an understanding of their anabolic effect on bone.

In conclusion, the present study has confirmed that daily injections of PTH and PTX enhance rhBMP-2 induced endochondral new bone formation in an additive and complementary manner in an animal model of bone induction. These agents may provide a new approach to enhancing the clinical efficacy of BMP-mediated new bone formation for the treatment of fracture and the correction of bone defects.

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