Local application of alendronate controls bone formation and β-tricalcium phosphate resorption induced by recombinant human bone morphogenetic protein-2

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Abstract

This study examined the ability of local alendronate (ALN) administration to control β-tricalcium phosphate (β-TCP) resorption as well as the induction of bone formation by recombinant human bone morphogenetic protein-2 (rhBMP-2). A 15-mm critical-sized bone defect was created in the diaphysis of rabbit ulnae. Nine female rabbits (4 to 5 months-old) were divided into 3 groups. Group 1 (n = 6 ulnae) animals received implants consisting of β-TCP granules and 25 μg of rhBMP-2 in 6.5% collagen gel. Group 2 (6 ulnae) and Group 3 (6 ulnae) animals received the same implants, but with 10^{-6} M and 10^{-3} M ALN-treated TCP granules, respectively. Two weeks post-surgery, tartrate-resistant acid phosphatase-positive cell counts, new bone formation, and residual β-TCP were evaluated. This study showed that a high dose of ALN strongly reduced osteoclastic resorption of β-TCP induced by rhBMP-2, resulting in decreased bone formation. In contrast, a low dose of ALN slightly reduced the bone resorptive effect but increased bone formation. These results suggest that osteoclast-mediated resorption plays an important role in bone formation and a coupling-like phenomenon could occur in the β-TCP-implanted area, and that administration of a low dose of ALN may solve clinical bone resorptive problems induced by rhBMP-2.

Key Words: Tricalcium phosphate; Bisphosphonate; Bone morphogenetic protein; Bone

resorption; Bone regeneration

INTRODUCTION

Treatment strategies for large bone defects (e.g., revision arthroplasty, tumor surgery, or nonunion operations) frequently rely on the use of autologous bone, allografts, or artificial bone substitutes for bone transplantation. Autologous bone is generally considered the best available graft material because it functions as an osteoconductive scaffold and also contains progenitor cells and growth factors, which promote osteogenesis.[1] However, this approach has several disadvantages, including limited availability of graft material, prolonged operative times, persistent pain, nerve damage, and donor-site morbidity, including esthetic aspects.[2,3] Allografts, in contrast, are more readily available in substantially larger quantities and various shapes. They also have variable osteoinductive and osteoconductive properties but lack viable cells.[4,5] Bone tissue engineering has attracted considerable attention as an approach to overcome these limitations. Bone tissue engineering relies on the individual or combined use of osteogenic cells, various growth factors, and biocompatible scaffolds to induce bone regeneration.[6-8]

Bone morphogenetic proteins (BMPs) comprise a family of growth factors that influence signaling cascades involved in skeletal development, homeostasis, and regeneration, and their

use can thus replace that of bone grafts and enhance osteogenic drive.[9-11] Molecular cloning studies have revealed that BMPs comprise a sizeable subfamily within the broader transforming growth factor-β superfamily.[12,13] The mechanism of action of BMP-2 involves both osteoinductive signaling as well as the regulation of several gene-expression pathways associated with mesenchymal progenitor cell recruitment and their subsequent differentiation into osteoblasts.[14-17] Clinical studies and research in animal models have utilized various surgical procedures to reveal the osteoinductive effects of recombinant human BMPs (rhBMPs); these procedures include fracture repair, spinal fusion, and the healing of bone defects at critical sizes. Furthermore, both rhBMP-7 and rhBMP-2 have been approved for clinical use.[18-20] As BMPs are known to promote bone resorption by directly and indirectly stimulating activation of mature osteoclasts and osteoclast formation,[21-25] new bone formation requires a reduction of BMP-stimulated osteoclastic resorption.

Bisphosphonates (BPs), including alendronate (ALN), have broad applications in the treatment of diseases that involve bone resorption. BPs interfere with isoprenoid biosynthesis and subsequently inhibit protein prenylation in osteoclasts.[26] ALN was initially used to treat osteoporosis, but has since been used in various orthopedic fields. Treatment with BPs is known to improve clinical outcomes in several metabolic bone disorders characterized by excessive bone resorption, including osteoporosis.[27-29] Studies utilizing critical-sized bone defects in animal models have also shown that local and systemic application of BPs can effectively prevent newly formed bone from being resorbed.[30-34] These studies suggest that BPs may also affect BMP-induced bone resorption.

β-tricalcium phosphate (β-TCP) has gained interest as a possible bone graft substitute owing to its biodegradability and biocompatibility.[35-39] Resorption of β-TCP involves both cell- and solution-mediated disintegration. Previously, we reported that TRAP-positive cells have a substantial role in β-TCP bioresorption, highlighting the importance of β-TCP resorption in the formation of bone.[35,39-41]

Chappard et al. have recently shown that both osteoclasts and activated macrophages act together to resorb β-TCP. It has been shown that these macrophages are also positive for the TRAP reaction.[42,43] Furthermore, there are many studies that bisphosphonates are effective in macrophage depletion.[44,45]

We have shown that ALN administered locally at a concentration of 10^{-2} to 10^{-6} M reduced the number of TRAP-positive cells on the surface of β-TCP blocks implanted in the femoral cancellous bone defects in a dose-dependent manner.[41]

This study was conducted in order to determine whether different concentrations of ALN administered locally can control both the resorption of β-TCP and bone formation induced by rhBMP-2.

MATERIALS AND METHODS

β-TCP granules

β-TCP used in this study was provided by Olympus Terumo Biomaterials Co. Ltd. (Tokyo, Japan). Fine β-TCP powder was synthesized by wet milling (a mechanochemical method). $CaHPO_4/2H_2O$ and $CaCO_3$ at the molar ratio of 2:1 were mixed into a slurry with pure water and balls of zirconium, in a pot mill, for 24 h, and dried at 80°C. Afterward, calcium-deficient

hydroxyapatite was obtained. This crystal was converted to β-TCP by calcination at 750°C for 1 h. A 50 wt% of β-TCP aquous-slurry was prepared with the poly (ammonium acrylate) and alkyl-phenyl ether as the surfactants. The slurry was whipped for 5 min. and dried at the room temperature for 1 week. After sintering of dried β-TCP at 1050°C for 1 h, a porous β-TCP block, with a mean macropore size of 200 μm and a porosity of 75%, was obtained. In this study, the β-TCP block was crushed, using a ceramic bowl, into granules ranging in diameter from 250 to 500 μm.

Collagen

Atelocollagen (6.5%), a highly purified type I collagen that is isolated from calf dermis and has been digested by pepsin to reduce the antigenicity of its telopeptide, was purchased from Koken Co., Ltd. (Tokyo, Japan).

Recombinant human bone morphogenetic protein-2

The *Escherichia coli*-derived recombinant human bone morphogenetic protein-2 (rhBMP-2) was purchased from Osteopharma, Inc. (Osaka, Japan). Lyophilized rhBMP-2 was diluted to a concentration of 5 μg/μl by adding distilled water just before implantation. **Alendronate**

ALN solutions were purchased from Teijin Pharma Co., Ltd. (Tokyo, Japan).

Implant preparation

For 2 days, β-TCP granules were immersed in 10^{-6} M and 10^{-3} M ALN solutions, after which excess ALN was absorbed using sterilized filter paper. To prepare the injectable complex, 0.2 g of ALN-treated or untreated β-TCP granules, 0.2 ml of 6.5% atelocollagen, and 25 μg of rhBMP-2 were mixed in a dish aseptically, achieving a total complex volume of approximately

0.3 ml.

Surgical procedure

Nine female New Zealand White rabbits (age, 4–5 months; weight, 3.0–3.2 kg) were obtained for use in this experiment. After administration of general isoflurane (3%) anesthesia and intravenous pentobarbital (20 mg/kg), a 5-cm longitudinal incision was made in the skin covering the lateral aspect of the ulna. A 15-mm-long critical segmental bone defect was then created in the diaphysis of the rabbit ulnae using a dental burr. The bone defects were filled with 0.3 ml of a complex of 6.5% atelocollagen gel containing 25 μg of rhBMP-2 and β-TCP granules with or without ALN-treatment (Fig. 1). The skin was closed in layers after the fascia and muscle were repaired. Following surgery, all animals were permitted to move about freely within their cages without joint immobilization and were supplied with their ordinary food and water regimen.

The nine experimental rabbits were separated into three groups and were treated as follows. The bone defects in Groups 1, 2, and 3 (for each group, $n = 6$; i.e., both ulnae from each of the three rabbits) were filled with a complex of 6.5% atelocollagen gel containing 25 μg of rhBMP-2 and non-ALN treated, 10^{-6} M ALN-treated, or 10^{-3} M ALN-treated β-TCP granules, respectively.

Two-weeks after surgery, the rabbits were sacrificed by pentobarbital overdose. For histological examination, forelimb specimens were harvested *en bloc*.

The animal experiment protocols, which conformed to the Guidelines for the Proper Conduct of Animal Experiments of the Science Council of Japan, were reviewed and approved by the Institutional Animal Care and Use Committee of the Jikei University (No. 2015-045).

Histological analysis

Both β-TCP resorption and bone regeneration were histologically assessed. The forelimbs containing the treated ulnae were fixed with 70% ethanol for 1 week. After glycol methacrylate (GMA) embedding, 3-μm-thick serial histological sections were cut parallel to the coronal plane. Non-decalcified sections cut in the center of ulnae were used for tartrate-resistant acid phosphatase (TRAP) staining; 20 mg of naphthol AS-BI phosphate (Wako Pure Chemical Industries, Osaka, Japan), 1.2 ml of 4% pararosaniline (Wako Pure Chemical Industries), and 0.05M L(+)-tartaric acid (330mg; Wako Pure Chemical Industries) were dissolved in 35.6 ml 0.1M sodium acetate buffer (pH 5.0). The serial 2 sections were covered with this solution and incubated for 30 min. at room temperature. The reaction was stopped by adding water. Nuclei were stained with Mayer's Hematoxylin (Muto Pure Chemicals, Tokyo, Japan).

The surface area of the TRAP-positive cells within the defects was determined from images digitally captured images using an Olympus DP 70 (Olympus Corporation, Tokyo, Japan). To eliminate errors from measurement of the implant margin, the total area was defined as a 1-mm square within the bone defect at 3 mm from the ulna end and 5 mm from the radius, and the number of TRAP-positive cells was calculated within this area (Fig. 2). The surface area of newly formed bone and the residual β-TCP were measured using WinROOF image analysis software (Mitani Co., Tokyo, Japan). Cortex bone was excluded, and results were expressed as percentages of the defect area (Fig. 3).

Statistical analyses

All statistical analyses were performed with SAS statistical software, version 9.4 (SAS Institute

Inc., Cary, NC), and a $p < 0.05$ statistical significance threshold was applied. Group means were compared using analysis of variance followed by the Tukey-Kramer post-hoc test for multiple comparisons.

RESULTS

Histological findings

TRAP staining of samples from Group 1, in which the β-TCP was not pre-treated with ALN, revealed the resorption of most β-TCP, particularly at the center of each defect, and that fatty marrow had already formed. The formation of new bone was observed on the periosteum of the radius and at both ends of the ulna. Regeneration of cortex-like bone was found at the opposite side of the radius (Fig. 2A). In Group 2, many of the β-TCP granules, which had been pre-treated with 10^{-6} M ALN, still remained in the defect. New bone formation was observable at the periphery of the defect, as observed in Group 1 (Fig. 2B). However, in Group 3, most of the 10^{-3} M ALN-treated β-TCP granules still remained in the defect, as observed in Group 2. In contrast, minor new bone formation was found only at the ends of the ulna. Furthermore, poor cortex-like bone formed at the opposite side of the radius (Fig. 2C).

TRAP-positive cells were observable under higher magnification on the surface of β-TCP granules in all groups. TRAP-positive cells were reduced in number by locally administered ALN in a dose-dependent manner (Fig. 2D, E, and F).

Measurement of TRAP-positive cells

The mean numbers of TRAP-positive cells for β-TCP granules that had received no ALN treatment (Group 1), 10^{-6} M ALN treatment (Group 2), and 10^{-3} M ALN treatment (Group 3)

were 117, 87, and 47, respectively. There were significantly fewer TRAP-positive cells in the 10^{-3} M ALN-treated group (Group 3) relative to the non-ALN-treated and 10^{-6} M ALN-treated groups (Groups 1 and 2, respectively; Fig. 4).

Measurement of newly formed bone and residual β-TCP area

Without ALN-treated β-TCP granules (Group 1), the mean rate of new bone formation in the defects was 5.50%, whereas the rates of groups with 10^{-6} M (Group 2) and 10^{-3} M (Group 3) ALN-treated β-TCP granules were 8.50% and 3.43%, respectively. A statistically significant difference was found between the 10^{-6} M and 10^{-3} M ALN-treated groups (Fig. 5). The mean residual β-TCP in the defects without ALN treatment was 12.50%, whereas the residual β-TCP percentages for those treated with 10^{-6} M and 10^{-3} M ALN were 17.67% and 19.83%, respectively. There was a statistically significant difference in residual β-TCP granules between the non-ALN-treated and 10^{-3} M ALN-treated groups (Fig. 6).

DISCUSSION

In our previous study using rabbit tibial cortical defects, a β-TCP granule and collagen complex did not promote reconstruction of the cortical bone defect. The cortical bone defect was only reconstructed when the complex was supplemented with fibroblast growth factor-2 (FGF-2), though the effect occurred 12 weeks post-implantation.[36] A complex of β-TCP granules and either hyaluronic acid or collagen promoted cancellous bone formation.[35] Relative to cancellous bone, cortical bone regenerates less easily.[36,39] FGF-2 promotes undifferentiated cell proliferation and vascularization but does not promote differentiation to osteogenic cells.[36] In this study, we used rhBMP-2, which is well known to stimulate osteogenesis.

The results of this study showed that new bone formation had already occurred on the periosteum of the radius and both ends of the ulna by 2 weeks. Furthermore, regeneration of cortex-like bone was found at the opposite side of the radius. In this model, cells with the potential to differentiate into osteoprogenitor cells were present in periosteum, bone marrow, and muscle tissues.

Notably, β-TCP resorption was prominent in the center of defects in Group 1 (rhBMP-2 alone). When cancellous bone defects were filled with β-TCP, the formation of new bone and resorption of β-TCP occurred in the periphery towards the center. This bone formation is attributable to the invasion of cells and blood flow from the periphery.[41] In the case of cortical bone defects, cell invasion could also occur from the periphery. However, β-TCP resorption was dominant in the center. This may be explained by the strong resorptive effect of rhBMP-2 possibly creating a cavity in the center of the implanted material.

BMP-2 affects not only osteogenic differentiation but also adipogenesis.[17,46-48] Normal cortical bone consists of cortical bone and bone marrow. BMP-2 enhanced β-TCP resorption by osteoclasts directly or indirectly to establish a cavity and to create bone trabeculae and fat tissue. Differences were observed in the sensitivity of individual animals to rhBMP-2. In this study, one rabbit showed ballooning of the defects without ALN administration. A similar phenomenon was reported in clinical cases as bone cyst formation. Recently, adverse effects of BMP therapy for spinal fusion, such as local bone resorption were reported.[49,50] Although BMP therapy for spinal fusion holds promise, the concern remains that BMPs may have a side effect of promoting bone resorption when cellular recruitment conditions favor osteoclasts over osteoblasts. It is known

that this phenomenon depends on the concentration of BMP-2.[48,51] This issue was highlighted by the research of McClellan et al., who retrospectively investigated the incidence of vertebral bone resorption in patients treated by transforaminal lumbar interbody fusion (TLIF) with rhBMP-2.[49] Based on the high rate of bone resorption observed, they concluded bone resorption within the vertebral body can be caused by the osseous remodeling potential of rhBMP-2. Lewandrowski et al. assessed a series of five patients who had been treated by TLIF with rhBMP-2 and subsequently exhibited osteolysis at the L5-S1 border.[50] They inferred that potentially rhBMP-induced inflammatory effects observed following the spinal fusion may have been caused by dose-dependent cellular cascade activation of osteoclasts over osteoblasts. Resorptive activity by osteoclasts may be higher in these cases. These examples indicate that mistimed catabolism can occur following rhBMP-2 administration and that this critical adverse effect, which may be prevented by BP administration, requires further investigation. ALN is well known to be a drug that acts on mature osteoclasts and inhibits bone resorption.[29,52-54] BPs are clinically used as therapies for bone loss that occurs with osteoclast-mediated bone resorption. Typical systemic BP treatment achieved via intravenous injection or oral administration can, however, have both low bioavailability and side effects. Local administration was therefore attempted to avoid these adverse effects.[55] Systemic or local administration of BPs has been reported by several previous studies to promote bone formation and reduce BMP-induced catabolic effects.[30,31,33,34,56,57] We previously reported a dose-dependent effect in which there were fewer TRAP-positive cells on the surface of β-TCP blocks when ALN was locally administered at concentrations ranging from 10^{-2} to 10^{-6} M.[41]

It has been recently reported that both osteoclasts and activated macrophages resorb β-TCP, and these macrophages are positive for TRAP staining.[42,43] It has been also reported that macrophages play a key role in β-TCP induced osteogenesis and favor osteoblastogenesis from stromal cells present in the bone marrow.[58]

ALN has been frequently reported to have an anabolic effect on osteoblasts that is distinct from their inhibition of osteoclastic bone resorption. BPs promote mesenchymal stem cell differentiation into osteoblasts.[59-62] However, some researchers have reported that ALN can cause osteoblast apoptosis, thus inhibiting osteoblast growth.[63] This contradiction may be explained by osteogenic cell growth and differentiation being promoted under relatively low ALN concentrations, with inhibitory effects occurring at higher concentrations,[41,64] which is in agreement with this study. At a lower concentration of ALN, the bone formation rate in Group 2 was higher than that in Group 1 (i.e., under rhBMP-2 + ALN 10^{-6} M treatment versus rhBMP-2 alone treatment). This may be accounted for by not only inhibition of osteoclast resorption in newly formed bone, but also by ALN-promoted osteogenic cell differentiation. At a higher concentration of ALN (10^{-3} M), there were significantly fewer TRAP-positive cells and the formation of new bone was significantly lower. This was explained by the inhibition of osteoclasts and activated macrophages by ALN, resulting in decreased bone formation. Together, these results suggest that osteoclast-mediated resorption is involved in bone formation and that a coupling-like phenomenon may occur in the β-TCP implanted area.

As mentioned above, ALN at relatively low concentrations promotes osteoblastogenesis, but inhibits adipogenesis. Osteoporosis, local bone resorption, and bone cysts are the result of aging,

reduced osteogenesis, and increased adipogenesis. BMP-2 promotes both osteogenesis and adipogenesis. Thus, the effects of BMP-2 and ALN on adipogenesis are controversial. Administration of ALN at low concentrations may inhibit the effects of both adipogenesis and osteoclastogenesis caused by BMP-2, thereby reducing the adverse effects of BMP-2 in clinical settings. Furthermore, low dosages of-ALN and BMP-2 may increase osteogenesis.

LIMITATIONS

There are some limitations of this study. First, BMP-2 and ALN are often used in middle-aged and older patients. However, in this study, ulnar segmental defects were created only in young female rabbits, so differences in age and sex were not investigated. Second, we investigated bone formation and β-TCP resorption using only nine rabbits at a one-time endpoint to limit the number of rabbits used, so the sample size was low. Third, after implantation of β-TCP and BMP-2, the bone defect became inflamed. Many kinds of cells, cytokines, and growth factors participate in early-stage tissue repair; inflammation is therefore a sign that bone repair has been initiated. However, we investigated only TRAP-positive β-TCP resorbing cells at 2 weeks, because β-TCP resorption is essential for new bone formation during the repair process.

CONCLUSIONS

We investigated whether different concentrations of ALN administered locally could control the resorption of β-TCP and rhBMP-2-induced bone formation. Accordingly, the present study showed a combination of rhBMP-2 and lower concentrations of ALN may promote the formation of new bone while inhibiting both the resorption of newly formed bone as well as fat formation, thereby preventing the adverse bone resorptive effects of BMP-2 observed in spinal fusion.

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CONFLICT OF INTEREST

The β-TCP granules used in this study were provided by Olympus Terumo Biomaterials Co. (Tokyo, Japan). The authors declare that they have no competing financial interests. No benefit of any kind has been or will be received directly or indirectly by any of the authors.

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Figure Legends

Figure 1. Macroscopic image of segmental defects. (A) A 15-mm critical-sized defect was created in rabbit ulnae. (B) Defects were filled with a complex of 6.5% collagen gel containing 25 μg of rhBMP-2 and β-TCP granules (75% porosity) that had or had not been treated with

ALN. U, ulna; dashed box, radius; double ended arrow, bone defect area.

Figure 2. Photomicrographs of TRAP staining. Histological sections of rabbit ulnar defects 2 weeks after the application of collagen, β-TCP, and rhBMP-2 (A); collagen, rhBMP-2, and β-TCP pretreated with 10^{-6} M ALN (B); or collagen, rhBMP-2, and β-TCP pretreated with 10^{-3} M ALN (C). The scale bar shows 2 mm. U, ulna; R, radius; yellow arrows, new bone; red arrows, cortex-like bone. Higher magnification photomicrographs of the white boxed areas are shown in the row below. The scale bar shows 200 μm. The number of TRAP-positive cells in the white boxed areas was measured 3 mm from the ulna end and 5 mm from the radius. TRAP-positive cells were present on the surface of β-TCP in all groups (D, E, and F).

Figure 3. An image analysis of an ulna bone defect. (A) The original image. (B) The surface area of newly formed bone and the remaining β-TCP were measured using an image analyzer, WinROOF (Mitani Co., Tokyo, Japan). The colored area was used for evaluation of the residual amount of β-TCP and newly formed bone. The scale bar shows 2 mm. Blue, β-TCP; green, newly formed bone; red, cortex bone; U, ulna; R, radius.

Figure 4. The mean number of TRAP-positive cells. The number of TRAP-positive cells in the 10^{-3} M ALN-treated group was significantly lower than that for other groups ($*p < 0.05$).

Figure 5. New bone formation rate. The mean rate of new bone formation in the 10^{-6} M

ALN-treated group was significantly increased compared to the 10^{-3} M ALN-treated group (*$p < 0.05$). There was no statistically significant difference between the non-ALN-treated group and the other groups.

Figure 6. The area of residual β-TCP. The mean remaining area of β-TCP in the non-ALN-treated group was significantly lower than that in the 10^{-3} M ALN-treated group (*$p < 0.05$).