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Manipulating the Anabolic and Catabolic Response in Bone Graft Remodeling: Synergism by a Combination of Local BMP-7 and a Single Systemic Dosis of Zoledronate

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ABSTRACT: Remodeling of a bone graft can be influenced both by anabolic substances, such as a bone morphogenic protein (BMP) and by anticatabolic substances, such as the bisphosphonates. BMPs are potent bone anabolic substances, but also boost catabolism and cause resorption. Bisphosphonates inhibit osteoclast function and can be used to postpone resorption. In the present study a combination of both drugs was explored in a rat bone chamber model. Cancellous bone grafts were treated with either BMP-7 or saline and placed in a bone chamber implanted in the proximal tibia. After 2 weeks, an injection of either zoledronate 0.1 mg/kg or saline was given subcutaneously. The rats were killed after 6 weeks, and bone ingrowth distance into the graft and graft resorption were measured by histomorphometry. BMP-7 significantly ($p = 0.007$) increased new bone ingrowth distance into the graft from 2.0 mm (SD = 0.98 mm) in the controls to 3.1 mm (SD = 0.93 mm). If bisphosphonate was not given, most of the newly formed and old graft bone was resorbed. A single injection of zoledronate significantly ($p < 0.001$) increased the trabecular volume/total volume to 40% (SD = 9%) compared to 14% (SD = 10%) in the nonbisphosphonate treated. In total, the net amount of bone increased by 400% when BMP-7 and zoledronate combined was compared to saline. A bone graft can be treated with BMP-7 to increase new bone formation and at the same time be protected against premature catabolism by a single dose of a bisphosphonate. This combination might be useful in various conditions in orthopedic reconstruction. © 2008 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 26:1245–1249, 2008

Keywords: anabolism; catabolism; bone graft remodeling; bisphosphonates; bone morphogenic protein (BMP)

Healing and normal physiological skeletal repair involve a complex set of regulated signaling pathways that control the formation of new bone matrix and the resorption of damaged bone at the disease or injury site. These pathways are mediated by a number of effector cells, primarily the catabolic bone-resorbing osteoclasts and the anabolic bone-forming osteoblasts, which produce new bone tissue to restore skeletal integrity. A balance between them is required for normal fracture repair and bone graft remodeling. Delayed healing or non-union can be a failure of the anabolic response, an increased or premature catabolic response, or sometimes both.[1] The anabolic and catabolic responses can be manipulated with pharmaceuticals, and in the bone conduction chamber,[2] we can study how drugs, separately or in combinations, influence bone formation and resorption.

Bisphosphonates reduce osteoclastic catabolic activity in experimental studies. Circulating bisphosphonates bind to bone mineral, and, when the bone is resorbed by osteoclasts, bisphosphonates are internalized and interfere with cell metabolism leading to osteoclast apoptosis.[3] Systemic bisphosphonate treatment can postpone resorption of a bone graft.[4] In fractures, postponing the catabolic response and in consequence the resorption of the new-forming callus, leads to a stronger callus.[5]

Bone morphogenic proteins (BMPs) bind to specific cell receptors, starting a signaling cascade that leads to both recruitment and differentiation of mesenchymal progenitor cells into osteoblasts. However, resorption can also be increased.[6,7] In bone chambers, BMPs increase bone ingrowth rate in nonvital bone grafts, but also cause an almost simultaneous resorption of newly formed bone.[8] The combination of a local bisphosphonate (clodronate) and BMP-7 caused both increased ingrowth and increased bone density in impacted grafts in the bone conduction chamber model.[9]

Both BMPs and bisphosphonates are approved for clinical use. Use of bisphosphonates in orthopedics in conditions other than osteoporosis and bone metastases is limited. BMP-7 is used in non-unions and spine fusion, but the clinical spread is limited. The costs of BMPs are high in relation to the clinical effect on fracture or pseudartrosis healing, where the compound has been found equivalent to autograft in a few major controlled and randomized studies.[10,11]

Bisphosphonates given systemically can be administered as a single dose, at least with the most potent ones like zoledronate.[12] The optimal time-point for administering a systemic bisphosphonate, to maintain the BMP-7-mediated callus, is 2 weeks as shown in a critical defect rat model.[13] The aim of our study was to examine if the combination of BMP-7 and a potent nitrogen-containing bisphosphonate given systemically, even as a single dose, will lead to both increased ingrowth distance into a bone graft and retained or increased total bone volume.

MATERIAL AND METHODS

Study Design

We used a model with cancellous grafts in bone conduction chambers in rats. Bilateral chambers were inserted with BMP-7 treated graft on one side and saline-treated on the other in 24 rats. After 2 weeks, 12 rats were given a bisphosphonate

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injection; the remaining 12 were injected with saline. After 6 weeks, the rats were killed, and the chambers and grafts harvested. The ingrowth distance of new bone into the graft and the bone density in the remodeled area were measured by histomorphometry. The study was approved by the local animal ethics committee.

The Chamber

The chamber consisted of a threaded titanium cylinder, formed out of two half cylinders and held together by a hexagonal screw cap (Fig. 1). The interior is 7 mm long and 2 mm in diameter. One end of the implant is screwed into the proximal tibia. At this end there are two ingrowth openings where tissue can grow in from the subcortical bone. The other end of the chamber extends out into the subcutaneous tissue and can be palpated through the skin, but the interior is sealed off from soft tissue by the screw cap. Ingrowing mesenchymal tissue will grow into the graft from the bottom and will replace a part but not all of the graft in 6 weeks.

Grafts and BMP Treatment

Pairs of structurally intact cancellous bone grafts were harvested from 24 female Sprague-Dawley rats (ca. 200 g). A cylindrical 2×6 mm bone rod was resected in the axial direction from the knee joint with a hole cutter. The epiphysis and growth plate were excised. The grafts were kept sterile and freeze-dried for 24 h at -70°C to enable absorption of a BMP-7 (Osigraft, Stryker, Malmö, Sweden) solution before insertion. BMP-7 was delivered as 0.875 mg/mL sterile solution and diluted in 0.9% saline to a BMP-7 concentration of 0.125 μg/μL. Each graft was soaked with 8 μL of this dilution corresponding to 1 μg BMP-7 per graft just before implantation. The control grafts were soaked with 8 μL buffer alone.

Surgical procedure and bisphosphonate regime: 24 male Sprague-Dawley rats (320–360 g) received bilateral chambers. The rats were anesthetized with a peritoneal injection of 0.6 to 0.7 mL of a solution containing pentobarbital (15 mg/mL) and diazepam (2.5 mg/mL). Under aseptic conditions, longitudinal incisions were made bilaterally over the anteromedial aspect of the proximal tibial metaphysis. After incising and raising the periosteum, the medial and posterolateral cortices were pierced with a 1 mm awl just anterior to the insertion of the medial collateral ligament. The hole created in the medial cortex was enlarged manually with a 2.7 mm drill. The chambers were then screwed into position so that the bone ingrowth holes were placed at the level of the cortical bone. Each animal received a chamber containing BMP-7 treated bone graft in one leg and an untreated graft in the other.

At day 14, the animals in group 1 were given a subcutaneous injection of 0.4 mL NaCl, and the animals in group 2 were given a subcutaneous injection of zoledronate 0.1 mg/kg. All animals were killed at day 42 with an overdose of pentobarbital.

Evaluation and Statistics

Specimens were fixed in 4% formalin, decalcified, dehydrated, and embedded in paraffin. They were cut parallel to the long axis of the chamber with a microtome and stained with hematoxylin and eosin. Three sections from the middle, each at 300 μm distance from the other, were used for histological and histomorphometric analyses. All slides within each experiment were examined in random order and blinded. The area of the new ingrown bone was measured by circumscribing it on a digitizing table using the Videoplan (Kontron GmbH, Germany) equipment at 40× screen magnification. This area includes marrow cavities and graft remnants that had been surrounded by new bone.

The mean ingrowth distance in each slide was calculated by dividing the new bone area with the width of the specimen. In all cases, fibrous tissue had penetrated into the chamber ahead of the new bone. The total tissue ingrowth distance, that is, the distance from the ingrowth end of the chamber to the fibrous ingrowth border, was measured in the same way as bone ingrowth. We measured the bone density of the remodeled graft by manual point counting of an area of interest ranging from the bottom of the chamber (the ingrowth end) to the advancing new bone formation frontier, but comprising only the central third of the bone so that bone close to the titanium side walls was excluded. Points superimposing new bone or dead graft were counted and recorded as "bone points." The total number of "bone points" on the slide was divided by the sum of total measured points, and the mean for the slide calculated. The mean of all three slides was then used to determine the final value for each chamber.

The results were tested for significance using paired and unpaired Student's *t*-test. The net amount of retained graft and new-formed bone within the remodeled graft was determined by calculating the volume of the remodeled bone cylinder (the radius^2 of the interior of the chamber $\times \pi \times$ the ingrowth distance of new bone) times the BV/TV in percentage.

RESULTS

Histology

No infection occurred. In all specimens, macrophages phagocytized the marrow cells in the bone graft, and a cell depleted fibrotic marrow had replaced the marrow

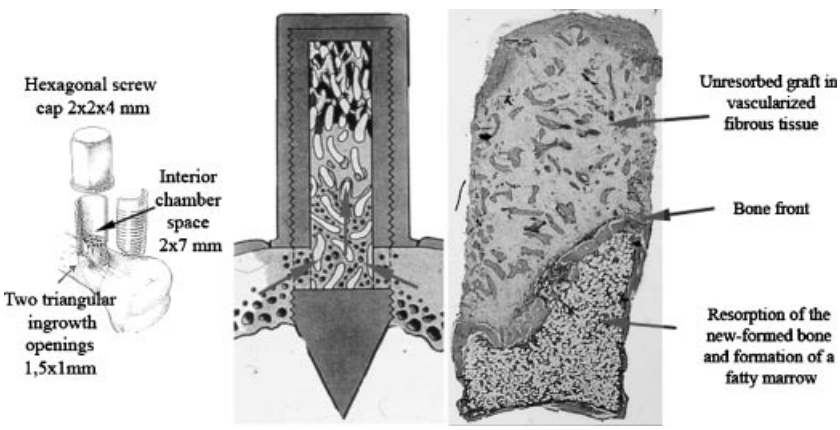

Figure 1. The bone conduction chamber in situ in the proximal tibia (T). The graft (G) is placed in the chamber and mesenchymal tissue grows in from the bottom upwards into the bone graft, which subsequently remodels. Arrows point at ingrowth openings. (Reproduced with permission from Eur J Exp Musculoskel Res 1993:2;70).

cells at the upper part of the specimen. This area was revitalized and revascularized but showed no signs of bone remodeling. Below this zone an ingrowth front of active bone formation was seen, where the primary bone formation occurred. Immediately above and below this front, osteoclasts were seen, and the newly formed bone was replaced by a fatty marrow. In the grafts in the rats given zoledronate, the graft and the new formed bone underneath the active bone formation front remained intact with new bone lining the graft trabeculae, leaving little space for the marrow (Figs. 2C and 3). In the nonbisphosphonate treated specimens, most of both newly formed and graft bone were resorbed and replaced by a fatty marrow regardless of treatment with BMP-7 or not (Figs. 2B,D and 3).

Histomorphometry

The bone ingrowth distance in the BMP-7 treated specimens was significantly increased ($p = 0.007$), measuring 3.14 mm (SD = 0.93 mm) compared to 2.04 mm (SD = 0.98 mm) in their controls. In the group given zoledronate, ingrowth distance in the BMP-7 treated side was 3.64 mm (SD = 1.21 mm), compared to 2.69 mm (SD = 1.04 mm) in the control side (Table 1), though the increase was not significant ($p = 0.08$).

The total amount of bone in the BMP-7 specimens was 40% (SD = 9%) in the zoledronate treated group compared to 14% (SD = 10%) in the nonbisphosphonate treated group, a significant increase ($p < 0.001$). In the specimens without BMP-7, the total amount of bone was 39% (SD = 11 %) in the zoledronate treated group compared to 14% (SD = 7%) in the nonbisphosphonate treated group, again a significant increase ($p < 0.001$). Dividing the total amount of bone into newly formed and remaining graft bone, the total amount of retained graft bone in the BMP-7 specimens was 7% (SD = 4%) in the zoledronate treated group compared to 0.6 (SD = 0.7%) in the nonbisphosphonate treated group, a significant difference ($p = 0.002$). The amount of new bone was 33% (SD = 8%) in the zoledronate treated group compared to 14% (SD = 10%) in the nonbisphosphonate treated group, a significant increase ($p < 0.001$).

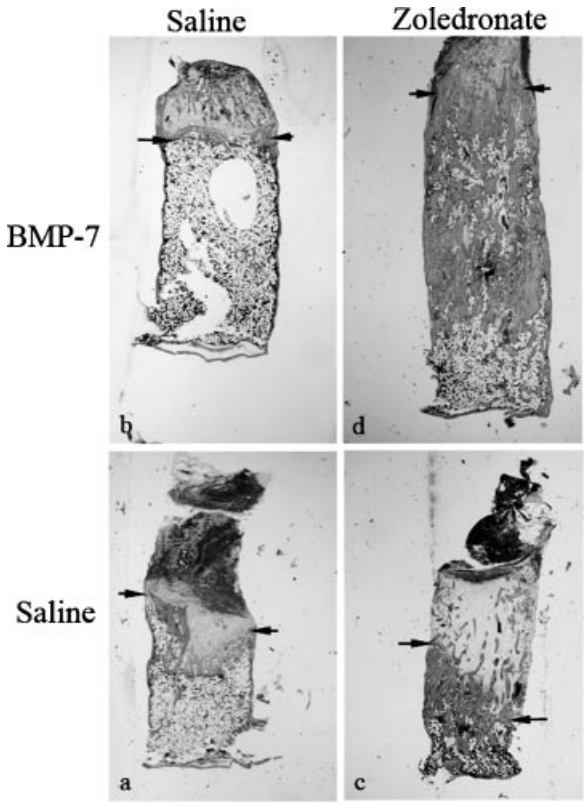

Figure 2. (A) Untreated chamber specimen with remodeled bone graft after 6 weeks in the chamber. Host tissue can enter the graft through small holes in the bottom of the chamber and ingrowth/remodeling occurs from the bottom. The bone ingrowth front is marked between arrows, and the tissue below has been remodeled. (B) In the BMP-7 treated specimens, the bone ingrowth front (arrows) reached 50% to 100% further into the graft compared to controls. Most newly formed bone below the bone ingrowth front had already been resorbed and replaced by fatty marrow. (C) In the zoledronate treated specimens, the remodeled bone below the ingrowth front contained three to four times more bone than in the controls. In (D), where both BMP-7 and zoledronate were given, both an increased ingrowth distance and increased bone retention occurred. Hematoxylin/eosin stain; original magnification, ×20.

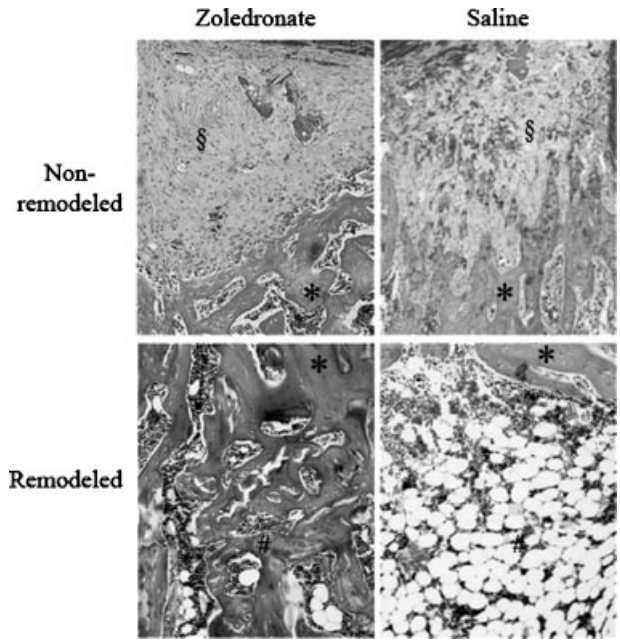

Figure 3. The remodeled zone beneath the bone ingrowth (*) front and the revascularized but not yet remodeled bone graft (§) above the bone ingrowth front (*). The nonremodeled graft in the upper panels appeared similar in both the zoledronate- and saline-treated specimens with a vascularized fibrotic neo-marrow. The remodeled bone in the lower panels, however, appeared different with retention of the majority of the newly formed bone in the zoledronate-treated graft to the right compared to an almost complete resorption in the nonbisphosphonate treated specimen to the left.

DISCUSSION

Bone healing and bone graft remodeling can be influenced by adding both an anabolic or anticatabolic drug like BMP or bisphosphonates. Sometimes, when the anabolic response is a minor problem, such as in a low energy fracture or when using autograft, decreasing or stalling the catabolic response may be enough.

Table 1. Ingrowth Distance of New Bone into the Grafts as Equivalent of Bone Anabolism and the Bone Volume Fraction in Remodeled Graft Bone as Equivalent of Bone Catabolism

	Group 1 Anabolic ($n = 12$)			Group 2 Anabolic + Anticatabolic ($n = 12$)		
Parameter	Right Leg BMP-7	Left Leg Control	p Value	Right Leg BMP-7 + ZA	Left Leg ZA	p Value
Bone ingrowth distance (mm)	3.14, SD 0.93	2.04, SD 0.98	=0.007	3.64, SD 1.21	2.69, SD 1.04	=0.08
	14.2, SD 10			40.5, SD 9		<0.001
BV/TV (%)		14.3, SD 7			39.0, SD 11	<0.001

Simply by an increase in the amount of retained forming callus, faster and stronger healing will be achieved.[5] In other situations with few cells at hand, like in a critical defect or using an allograft, an anabolic drug can be used to increase the recruitment and/or the differentiation of mesenchymal osteoprogenitor cells.[11] In some fractures, an anabolic deficiency, such as a genetic defect like congenital pseudartrosis of the tibia or a necrosis of the bone ends, is combined with increased or premature catabolism, due to stress shielding or instability, and the combination of both an anabolic and an anticatabolic compound seems attractive.

In the bone chamber, bone healing can be divided into and measured separately as equivalents of both anabolism and catabolism. Catabolism, visualized as resorption (Fig. 2A,B) of the graft and the newly formed bone can be quantified as a decreased BV/TV fraction. Also an equivalent to bone anabolism can be visualized and quantified as an increased bone ingrowth distance or speed into the graft (Fig. 2B,D). The catabolic drive in the chamber is high, given the stress-shielded microenvironment into which the healing tissue enters. Bone forms by default, but is almost immediately resorbed (Fig. 1). Decreasing the catabolism by treating the bone graft itself[14] or the forming bone[10] with a bisphosphonate increases the retention of the graft and newly formed bone between three to five times. The anabolic response to the trauma, caused by the insertion of the chamber, must be considered to be sufficient as the chamber is inserted into metaphyseal bone with its rich, well vascularized marrow with abundant osteogenic cells and access to growth factors and cytokines. Also, the anabolic response within the chamber could be manipulated by pharmaceuticals, for example, by adding a BMP to the bone graft. In the BMP-7 treated graft the newly forming bone grows into the dead graft at almost twice the speed compared to the untreated controls.[8] However, increasing the anabolism pharmacologically leads to unexpected consequences. BMPs are differentiating paracrine proteins acting on a variety of cells in bone remodeling, not only bone forming cells but also osteoclasts.[6,15] In the chamber, the results of the osteoclast activity can clearly be seen as the ingrowth front sweeps into the dead graft (Fig. 2A,B). The newly formed bone and the graft are immediately resorbed if not treated by an antiresorptive agent (Fig. 2C,D).

The resorption might explain some of the shortcomings of BMP-7 in several previously published clinical studies, and the resorption might be stalled by combining BMP-7 with an antiresorptive drug as in the present experiment. A large increase in retained callus volume can be expected when combining the two drugs (Figs. 2D and 4). If BMP-7 increases the volume of remodeled/newly formed bone by about 100% by increasing the formation rate, and simultaneously a bisphosphonate increases the retention by 200% to 300 %, then theoretically four to six times as much callus could be expected in the chamber. In the present series, the amount of bone formed and retained in the BMP-7 + zoledronate group was increased by 400% compared to the untreated controls (Fig. 4).

In the clinical situation of a healing fracture or bone graft, the balance between catabolism and anabolism is modulated by the mechanical situation. In analogy to our stress-shielded chamber, a low stress environment like a rigidly fixed fracture would cause a resorptive stimulus, which then would be further boosted by the BMP-7. In another bone chamber study in rabbits allowing motion, the response to BMP-2 switched from net resorption to net formation when micromotion was added.[16] In a multicenter randomized study of 450 open tibial fractures,[13] the fractures treated with intramedullary nail fixation and an implant containing BMP-2 had reduced

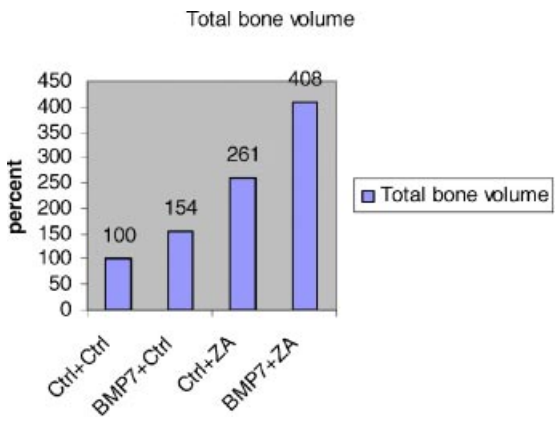

Figure 4. The net amount of bone in the remodeled part of the grafts as a function of ingrowth distance × radius × BV/TV for the drug treated grafts relative to the untreated controls.

frequency of secondary interventions, accelerated fracture and wound healing, and reduced infection rate compared to those treated with intramedullary nail fixation and routine soft-tissue management. The rate of union, however, was similar to autograft. In another large study, using BMP-7 for tibial non-unions, the healing rate was 85% and on par with autograft.[12] Fixation of a fracture or pseudarthrosis using an intramedullary nail, especially if locked and nondynamized, would increase the catabolic stimulus. BMP-7 might, in fractures with an already adequate anabolic drive, only boost catabolism, and the addition of an anticatabolic drug could even increase the proportion of healed fractures.

At the other end of the spectrum of mechanical stimulation, a high stress environment, for example, in the vicinity of a semistabile prosthesis or an unstable fracture, would cause an increased resorptive stimulus, maybe further boosted by BMP-7. In a weight-bearing noncemented knee prosthesis model in rabbits, treatment of impacted bone grafts by BMP-7 did not increase bone density, probably because instability caused increased resorption of both old graft and newly formed bone.[17] In a human series, radical resorption and implant loosening were found in 4 out of 10 acetabular components using BMP-7 in morselized and impacted allograft for hip revisions.[18] The combination of BMP-7 and another bisphosphonate, clodronate, given locally, was investigated in the chamber model using impacted grafts as in hip revisions. Clodronate decreased the ingrowth distance into the impacted grafts, which could be compensated by BMP-7.[9]

In the present study, in nonimpacted grafts, a single dose of the more potent zoledronate given as a single dose after 2 weeks did not imply a reduction of the anabolism. Rather, a tendency to an increased ingrowth distance was found in the zolendronate treated grafts, both in the BMP-7 treated group and saline treated controls. It appears logical to give the bisphosphonate after the initial callus has formed; 2 weeks is optimal.[11] Maybe an anabolically negative effect, as found in vitro[19] can thereby be avoided. We believe that the combination of an anabolic and an anticatabolic drug might be a potent bone graft enhancer and a powerful tool to treat nonunions or to prophylactically prevent delayed or nonunion in some difficult, compromised fractures.

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