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Interaction between Living Bone Particles and rhBMP-2 in Large Segmental Defect Healing in the Rat Femur

Fangjun Liu^{1,2,6}, James W. Wells^{1,2,†}, Ryan M. Porter^{1,2}, Vaida Glatt^{1,2,†}, Zhenxin Shen¹, Martina Schinhan^{1,3}, Alan Ivkovic^{1,∞}, Mark S. Vrahas^{2,4}, Christopher H. Evans^{1,*,2,§}, and Elisabeth Ferreira^{1,2}

¹Center for Advanced Orthopaedic Studies, Beth Israel Deaconess Medical Center, Harvard Medical School, 330, Brookline Avenue RN-115, Boston, MA 02215, USA ²Collaborative Research Center, AO Foundation, Davos, Switzerland ³Department of Orthopaedic Surgery, Medical University of Vienna, Vienna, Austria ⁴Department of Orthopaedic Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

Abstract

Orthopaedic surgeons sometimes combine recombinant, human BMP -2 with autograft bone when dealing with problematic osseous fractures. Although some case reports indicate success with this off-label strategy, there have been no randomized controlled trials. Moreover, a literature search revealed only one pre-clinical study and this was in a cranial defect model. The present project thus examined the consequences of combining different doses of BMP-2 with particles of living bone in a rat femoral defect model. Human bone particles were recovered with a reamer-irrigator-aspirator. To allow acceptance of the xenograft as surrogate autograft, rats were administered an immunosuppressive cocktail that does not interfere with bone healing. Implantation of 200 µg living bone particles generated a small amount of new bone and defects did not heal. Graded amounts of BMP-2 that alone provoked no healing (1.1 µg), borderline healing (5.5 µg) or full healing (11 µg) were added to this amount of human bone particles. Addition of BMP-2 (1.1 µg) increased osteogenesis, and produced bridging in 2 of 7 defects. The quantitative effects of BMP-2 (5.5 µg) and bone particles were additive at best, but made healing more reliable and advanced the maturation of the regenerate. Bone formation with BMP-2 (11 µg) and bone particles was less than

*To whom correspondence should be addressed: evans.christopher@mayo.edu.

⁶Present affiliation: Institute of Plastic and Reconstructive Surgery, WeiFang Medical University, Shandong, China

[†]Present affiliation: Queensland University of Technology, Institute of Health and Biomedical Innovation, Brisbane, Australia

[‡]Present affiliation: The University of Queensland Diamantina Institute, The University of Queensland, Brisbane, Australia

[§]Present affiliation: Rehabilitation Medicine Research Center, The Mayo Clinic, Rochester, MN

[∞]Present affiliation: Department of Orthopaedic Surgery, University Hospital SvetiDuh, Zagreb, Croatia

Correspondence and Reprint Requests: Chris Evans, Rehabilitation Medicine Research Center, Mayo Clinic, Rochester, MN 55905, evans.christopher@mayo.edu

Author contributions

FL, JWW, RMP, VG, ZS, MS, AI, EF: Rat surgery, X-ray

MSV, EF: Harvest, isolation of human bone particles

VG: Micro-CT, mechanical testing, DXA

ZS: Histology

JWW: Design of immunosuppression protocol

FL: Manuscript preparation

CHE: Study design, data interpretation, manuscript preparation

All authors: Manuscript review and refinement

additive, and the major effect was to improve the maturation of the newly formed bone. The data suggest that the combination of autograft and BMP-2 can be helpful clinically under conditions where a healing response is present, but suboptimal.

INTRODUCTION

Bone healing is sometimes problematic, under which conditions it is common for orthopaedic surgeons to implant autologous bone grafts (autografts) into the defect. Although effective, enthusiasm for autograft is tempered by its limited availability and donor site morbidity^{1,2}. Recombinant bone morphogenetic protein (BMP) -2 and -7 provide alternative osteogenic stimuli and have been approved for clinical use in certain applications where it is necessary to form bone. However, outcomes have generally been less spectacular in human patients than in pre-clinical animal models, and BMPs are widely considered to be roughly equivalent in effectiveness to autograft³⁻⁵. Combining autograft with a BMP could achieve a better outcome than either provides by itself, and allow the treatment of larger defects than autograft alone can fill.

There have been a number of reported clinical cases where BMPs have been used in combination with autograft to try to improve bone healing^{6,7}. Although promising results have been published⁶⁻⁸, such uses are off-label with the clinical studies being descriptive and lacking controls. No randomized, controlled trials have been performed. The only published, pre-clinical investigation of the effectiveness of BMP-2 and autograft in combination is that of Issa et al.⁹ who used a rat cranial defect model. Their data suggest that a combination of BMP-2 and autograft induces more newly formed bone than either alone, but the effect is less than additive. No published, pre-clinical studies have been performed to examine this matter in long bone healing. The present study was designed to evaluate the interaction between living human bone and human, recombinant BMP-2 using a rat segmental defect model.

Autograft is typically harvested from the iliac crest, but the reamer -irrigator-aspirator (RIA) provides an alternative source¹⁰⁻¹². This option is particularly attractive when intramedullary reaming is required for stabilization of the injured long bone. Bone harvest using the RIA also eliminates the issue of persistent donor site pain in the ileum. In the present study, particles of living human bone recovered by the RIA were implanted as surrogate autograft into large segmental defects in the femora of immunosuppressed rats¹³, in the presence or absence of different amounts of recombinant, human BMP-2.

The point of this study was to determine whether BMP-2 could enhance the effectiveness of autograft bone and vice-versa. To address this, a fixed amount of living bone particles was added to the defect and this was titrated against doses of BMP-2 that ranged from ineffective to fully effective.

MATERIALS AND METHODS

Study Design

Because a thymic rats have been shown to mount immune responses to xenoantigens, thereby inhibiting bone healing¹³, particles of living human bone were implanted into Fischer rats under 3-weeks' immunosuppression with a combination of FK506 (Tacrolimus) and SEW2871. We have shown that this immunosuppressive regimen permits xenografting without affecting bone healing¹⁴. A total of 52 Fischer F344 rats underwent surgery to create 5 mm femoral defects and were randomly divided into 8 experimental groups, as detailed in Table 1. To minimize any effect of human donor variability, each patient provided bone for one rat in each bone group. Defects of all animals were monitored weekly using a dental X-ray unit (Sirona Dental System GmbH, Bensheim, Germany). At 8 weeks, all animals were euthanized and the defects compared by histology, micro-computed tomography (μ CT), dual-energy X-ray absorptiometry (DXA), and mechanical testing.

Animals

Male Fischer F344 rats were purchased from Charles River Laboratory (Wilmington MA). All animals were 12 weeks of age at the time of surgery. Rats were housed in a central animal care facility with 12-hour light cycles and were given chow and water *ad libitum*. Animal care and experimental protocols were followed in accordance with National Institutes of Health guidelines and approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Specimen collection and implant preparation

Upon obtaining informed consent, reaming materials generated using the RIA system were collected from 9 patients (5 males; 4 females) with a mean age of 52.1 ± 16.3 years (range: 31–80 years), undergoing hemiarthroplasty for femoral neck fracture at Massachusetts General Hospital. The surgical protocol was pre-approved by the Partners institutional review board. After reaming, filtered bone particles were transferred to a laminar flow hood and processed under aseptic conditions. After washing five times in phosphate-buffered saline (PBS; Sigma, St. Louis, MO), bone particles from each patient were weighed (mean $\pm$ standard deviation: 3.5 ± 2.5 g; range: 1.5–9.6 g) and divided into equal 200 μ g fractions for each defect implantation as surrogate autograft. In parallel, absorbable collagen sponges (ACS) (Medtronic Inc., Minneapolis, MN) pre-soaked for 30 min with various doses of rhBMP-2 (Medtronic Inc., Minneapolis, MN) were placed inside the defects. The volume of the ACS remained constant for each rat; the dose of BMP-2 was determined by the volume of BMP-2 solution added to the ACS. The immune-suppressive cocktail consisting of 1.0 mg/kg FK506 and 0.5 mg/kg SEW2871 (Cayman Chemical, Ann Arbor, MI) was delivered once a day subcutaneously, starting on the day of implantation¹³.

Surgical Procedure

Animals were divided randomly into experimental groups (Table 1). A 5 mm, critical-sized, mid-femoral defect was created in the right hind limb of each rat. Under sterile conditions, a 4 cm incision was made on the posterolateral thigh. The lateral intermuscular septum with

respect to the femur was dissected to expose the diaphysis of the femur. The periosteum was removed on the anterolateral femur. Using a demo-fixator as a guide, four predrilled pin - holes were made in the central portion of the femoral shaft with a sterile 0.79 mm drill bit. The fixator (axial stiffness 185 N/mm¹⁵; RI System AG, Davos, Switzerland) was then secured in position by screwing 4 precise pins into both cortices of the rat femora through these predrilled holes and the fixator. A saw guide was used to position and create the 5 mm segmental defect using a 0.22 mm wire Gigli saw. After the defect was created, the saw guide was removed and pre-treated collagen sponges were press-fit into the defect. For those animals receiving human bone xenograft, 200 µg of pre-weighed particles were packed around the collagen sponge within the defect core. The wound was closed in layers with 3-0 Vicryl sutures and the skin incision closed over the fixator using 9-mm Autoclip wound clips.

Radiographic Evaluation

Serial, weekly radiography using a digital dental X-ray unit was performed under general anesthesia to evaluate bone regeneration in the defects. Rats were ventrally positioned and the X-ray sensor was placed under the defect area of each femur. Formation of bone in response to BMP-2 was scored by the semi-quantitative scale of Pensak et al¹⁶, which ranges from 0 (no bone formation) to 5 (defect filled with bone). The 8-week X-rays were also scored for bone formation in the defect and whether healing had occurred (yes/no).

Micro-Computed Tomography (µCT)

The architecture of newly formed bone in the rat segmental defects was examined with a desktop micro-tomographic imaging system (µCT 40, Scanco Medical AG, Bassersdorf, Switzerland) equipped with a 10 mm focal spot microfocus X-ray tube. Femoral defects were scanned using a 20 µm isotropic voxel size, at 55 keV energy, 200 ms integration time, with approximately 500 µCT slices per specimen. Evaluation of only a 4 mm (~220 slices) central region of the defects was used to exclude any pre-existing intact cortical bone. Total volume of the callus size of the defect (TV, mm³), bone volume (BV, mm³), and BV/TV fraction were calculated from µCT images. Images were thresholded using an adaptive - iterative algorithm and morphometric variables were computed from the binarized images using direct, 3D techniques that do not rely on any prior assumptions about the underlying structure.

Dual-Energy X-Ray Absorptiometry (DXA)

DXA measurements (Bone mineral content, BMC (grams)) of the defect area were obtained with PIXImus2 apparatus (GE-Lunar, Madison, WI, USA). Briefly, each femoral defect was placed on a lucite block during scanning to simulate soft tissue. The scans were acquired using small animal high-resolution mode. All specimens were evaluated at 8-weeks in the area corresponding to the region of the critical-sized bony defect.

Ex Vivo Torsion Testing

Specimens were tested to failure in torsion to evaluate the mechanical properties of the healed defect in shear. Before the test, both ends of each specimen were embedded in

polymethylmethacrylate (Arkema Inc., Exton, PA) to provide a reproducible gripping interface with the testing fixture. All femora were tested to failure under regular deformation control and at a constant deformation rate of 5 rad/min. Angular deformation and applied load data were acquired at 10 Hz using a Synergie 200 (MTS Systems, Eden Prairie, MN). The torque and rotation data were used to calculate the torsional stiffness and strength of the healed defect.

Histology

Right femora were removed from euthanized rats and fixed in 4% ice cold paraformaldehyde for 48 h at 4°C before decalcification with 20% EDTA in 0.1 M phosphate buffer (pH 7.4). Extracted femora were maintained in EDTA solution for 4 weeks and tested with a needle as decalcification proceeded. The specimens were subsequently dehydrated in graded ethanol and embedded in paraffin. Serial 5 µm paraffin sections were placed on poly-L-lysine-coated slides, and dried overnight. Sections were stained with either hematoxylin-eosin or safranin orange-fast green, and then examined and photographed under light microscopy.

Statistical Analysis

Comparisons of continuous variables between two treatment groups were performed using a two-tailed Student's t-test, and between three groups by analysis of variance (one way ANOVA). If the difference between the contralateral, intact femora and the treatment groups was significant, a *post-hoc* test (Tukey) was performed. Data are presented as means ± standard deviation (SD), unless otherwise noted as means ± standard error of a mean (SEM). The results were taken to be statistically significant at a probability level of $p < 0.05$.

RESULTS

rhBMP-2 dose response

The dose-response experiment (figure 1) revealed complete healing of defects at 8 weeks with 11 µg BMP-2 but no healing with 1.1 µg BMP-2. A dose of 5.5 µg BMP-2 provoked copious bone deposition, but bridging was sometimes interrupted by a radiolucent line at the proximal end of the defect (figure 1, Table 2). Semi-quantitative scoring of the X-rays confirmed that the onset of new bone formation began within the first week, and was most rapid with 11 µg BMP-2; the maximum rate of bone formation occurred between weeks 1 and 3 (figure 2).

The µCT data (figure 3, figure 4), mechanical testing data (figure 4) and histological sections (figure 5) are consistent with the X-ray images. With 1.1 µg BMP-2 only small amounts of bone were deposited in the defect, largely at the distal and proximal ends. Little bone was formed in the center of the defect and there was no bridging. All quantitative measures of bone formation were low (figure 4) and histology (figure 5) detected only fibrous tissue. The corresponding femora were too fragile to support mechanical testing.

With 5.5 µg BMP-2 substantial amounts of bone were deposited, as visualized by X-ray (figures 1, 2; Table 2) and µCT (figure 3), confirmed by quantitative analysis (figure 4). Bone mineral content (figure 4A) and the mechanical properties (figure 4 E, F) of the

regenerate were also restored close to those of the contralateral femur. Histology, however, revealed residual cartilage (figure 5) that was seen on X-ray (figure 1) as a transverse, radiolucent line in certain specimens. With 11 μ g BMP-2, there was no residual cartilage and more advanced cortication (figures 1, 3, 5), while the stiffness of the regenerate was closer to normal values (figure 4E). However, there was no increase in bone mineral content or bone area above that produced by 5.5 μ g BMP-2, and the ultimate strength of the regenerate was equal with both of these doses of BMP-2 (figure 4).

Response to bone particles

It was difficult to monitor healing under the influence of the human bone particles alone by X-ray (figure 1), because of the radio-opacity of the implanted material. However, it appears from the μ CT images (figure 3) that, although the implantation of 200 μ g bone particles led to bone deposition within the defect, this did not lead to union (Table 2). This conclusion is corroborated by the quantitative μ CT and DXA data (figure 4) and histology (figure 5).

Interaction between bone particles and BMP-2

Although the 200 μ g dose of human bone particles implanted into the defect provoked little bone healing by itself, the particles influenced the actions of exogenous BMP-2. The quantitative and qualitative aspects of these effects differed, depending on the dose on BMP-2.

At the lowest dose of BMP-2, the primary combined effect was quantitative with an increase in the amount of bone formed. Both the X-ray (figure 1) and μ CT images (figure 3) are consistent with greater bone formation when 1.1 μ g BMP-2 and bone particles are combined. There was an increase in bone mineral content (figure 4A) and bone area (figure 4B) when 1.1 μ g BMP-2 and bone particles were combined, contributing to an increased incidence of radiologic union from 0/6 in the absence of bone particles to 2/7 in their presence (Table 2). Histology (figure 5) was consistent with these observations. The combination of bone particles and 1.1 μ g BMP-2 did not result in the formation of tissue robust enough to withstand mechanical testing (figure 4 E, F).

With 5.5 μ g BMP-2, the bone particles increased the total amount of bone formed and the size of the callus. This is clearly seen on the X-ray (figure 1) and μ CT images (figures 3) and confirmed by the quantitative analysis (figure 4). The combined effect of this amount of BMP-2 with bone particles was additive, or less, with regard to bone mineral density and the μ CT data (figure 4). The most striking effect, however, was to make healing more reliable. There were no areas of residual cartilage (figure 5), and the radiolucent zone, seen on X-ray in the absence of bone particles, was no longer present (figure 1; Table 2). The healed bone was already remodeled into a continuous tubular bone structure with less trabecular bone and more uniform neocortices, with well-formed bone marrow visible inside the new bone cavity. The flawless continuity between neocortices and intact bone was prominent in the regenerates (figure 4C). The greater maturation of the bone formed in the presence of bone particles is reflected in reduced stiffness at 8 weeks, closer to that of normal bone (figure 3E).

At the 11 μg dose of BMP-2, the effects of adding the human bone particles were again both quantitative and qualitative. X-ray (figure 1) and μCT (figure 3) images show greater bone deposition when this amount of BMP-2 is combined with bone particles, confirmed by quantitative μCT (figure 4). Quantitative assessment of combining 11 μg BMP-2 with bone particles shows the two were approximately additive for bone mineral content (figure 4A), but less than additive for bone volume (figure 4B) and total volume (figure 4C). Histology (figure 5) provided evidence of greater maturation, seen as more trabecular bone, thicker cortices and a more developed marrow. This had the effect of increasing the strength of the healed bone, restoring it to the normal value (figure 4).

Heterotopic ossification was not seen in any of the rats in the presence or absence of bone particles.

DISCUSSION

Pensak et al.¹⁶ have drawn attention to the need for pre-clinical evaluation in relevant animal models when contemplating off-label combinations of FDA-approved biological agents. Their study demonstrated that a combination of parathyroid hormone and demineralized bone matrix failed to enhance healing of a critical sized murine femoral defect, despite the intuitive attractiveness of this approach.

The present study explored possible synergies between autologous bone graft and rhBMP-2 in a rat, critical sized segmental defect model. Autograft and BMP-2 are both used clinically, but the former is available in limited quantities, whereas the clinical effectiveness of the latter is modest. The ability of BMP-2 to provide “graft expansion”⁸ would address an important clinical need, which might substitute for long and painful procedures, such as distraction osteogenesis, when treating large segmental defects.

Our data suggest that the combination of BMP-2 and autograft indeed leads to greater bone formation than either achieves alone. It also improves bone maturation. Its clinical applicability could reside in those cases where the healing process, although robust, is not quite sufficient for reliable union. For example, the combination could be used for patients with indications predicting a poor response to autograft, such as type 2 diabetes or a history of smoking. In the model used here, this occurred with 5.5 μg BMP-2. Although the osteogenic response to this amount of BMP-2 by itself almost filled the defect with new bone, a cartilaginous zone persisted at the proximal end of the defect in many animals. Supplementation of the BMP-2 with living bone particles led to reliable full union and more mature bone lacking any residual cartilage.

At higher or lower concentrations of BMP-2, addition of bone particles had less pronounced effects. The lowest dose of BMP-2 (1.1 μg) elicited very little bone formation and none of the defects healed. The addition of bone particles increased this such that healing was observed in 2 of 7 rats. The highest dose of BMP-2 (11 μg) healed the defects very effectively by itself. Addition of bone particles, however, improved the maturation of the regenerate, leading to thicker cortices, more advanced marrow formation and greater strength. Our data do not indicate whether cells derived from the implanted human material

are incorporated into the newly formed bone. There are well-established techniques for identifying human cells after xenotransplantation, so such studies will be possible in future work.

These observations are interesting in light of the data of Issa et al.⁹ who, in a similar type of study using a rat cranial defect model, noted that the increment in new bone formation when combining 5 µg BMP-2 with autograft was less than additive. These authors also found a quantitatively similar increment when allograft or xenograft bone was combined with BMP-2. Because the xenograft bone was lyophilized, a process that would kill its cells, the role of the cellular component of the bone graft is called into question. It would be a straightforward future experiment to devitalize the human bone particles used in the present project to determine the effects on bone healing in the presence of BMP-2. In the work of Issa et al, it is possible that the devitalized bone particles potentiated the action of BMP-2 by, for instance, adsorption onto the mineral surface followed by slow release into the defect. Clinical success when combining BMP-2 or BMP-7 with allograft human bone has been reported^{17,18}.

These data need to be interpreted in light of the greater osteogenic response of rats to recombinant, human BMP-2 compared to humans. Using the INFUSE system, the recommended human dose is 1.5 mg/ml of graft volume¹⁹. The radius of the femur of the Fischer rat at the site of the defect is 0.25 mm. Thus the volume of the 5 mm, femoral defect in the Fischer rat is almost 1 ml. In this case, 11 µg BMP-2 in the 5 mm rat defect gives a concentration of around 11 µg/ml. This is over two orders of magnitude less than the human dose. This seems to represent a fundamental biological difference between rats and humans. This could reflect species differences in the biochemistry of cell responses to BMP-2²⁰ or mechanical influences, given the configuration of the rat femur.

Although the model used in the present study implants human bone into rats under transient immunosuppression, we have confirmed that the immunosuppressive drugs do not impair bone healing in this model¹³. Nevertheless, the experiment bears repeating using donor bone particles recovered from syngeneic rats. While only a limited range of BMP-2 concentrations was tested and only one dose of bone particles was used, the data nevertheless suggest that the combination of autograft and BMP-2 can be helpful under conditions where a healing response is present, but sub-optimal. The data further suggest that combining living bone particles and BMP-2 in this way does not induce heterotopic ossification.

Acknowledgments

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Clinical significance

These data support the clinical use of recombinant, human BMP-2 combined with autograft bone when treating large segmental osseous defects. The combination leads to greater bone formation than either provides alone, and accelerates the maturation of the regenerate.

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Figure 1. Radiographs of femoral defects at different time points after treatment with different doses of rhBMP2 in the presence or absence of bone particles

ACS = absorbable collagen sponge. White arrows in 1 week and 8 week ACS radiographs indicate the original defect boundaries. Arrow in 5.5 µg BMP-2, 8 week panel indicates a radiolucent zone. Scale bar in bottom right hand panel = 1.0 mm.

Figure 2. Bone formation within defects at different time points after treatment with different doses of rhBMP-2

Bone formation was estimated from the X-ray images and scored according to the semi-quantitative scale of Pensak et al ¹⁶

Figure 3. Micro-computed tomography (μ CT) scans of femoral defects treated with human living bone particles and different doses of rhBMP-2

Critical-sized segmental femoral defects in immunosuppressed Fischer F344 rats were treated with doses of rhBMP-2 from 0–11 μ g with or without human living bone particles. The defects were harvested for μ CT scan 8 weeks following surgery. (A) Representative μ CT images taken from the femoral defects treated with different doses of rhBMP-2. ACS = absorbable collagen sponge. (B) Representative μ CT images from the femoral defects treated with both 200 μ g human living bone particles and different doses of rhBMP-2. (Bottom right hand panel Scale Bar = 1.0 mm)

Figure 4. Physical properties of the defects 8 weeks after implantation of human living bone particles and rhBMP-2

(A) Bone mineral content (B) Bone Area (BA) (C) Total callus volume (CV) (D) BV/TV fraction (E) Stiffness (F) Ultimate strength. The dashed line indicates the value for each parameter in the contralateral, unoperated femora; the shaded area indicates the standard deviation for each value. Coll = absorbable collagen sponge. * denotes significant difference (p<0.05) when compared to no bone particles group at each dose of rhBMP-2; † indicates significant difference (p<0.05) relative to intact, contralateral femur.

Figure 5. Histological appearance of defects 8 weeks after implantation of human living bone particles and rhBMP-2

Critical-sized segmental femoral defects in immunosuppressed Fischer F344 rats were treated with doses of rhBMP-2 from 0–11 μ g with or without human living bone particles. The defects were harvested 8 weeks following surgery. Row A shows representative images of sections stained with hematoxylin and eosin under low magnification (Right hand panel scale bar = 1 mm). The boxed regions shown in row A are shown at high magnification (Right hand panel scale bar = 0.2 mm) in row B and stained with safranin orange-fast green (Right hand panel scale bar = 0.2 mm) in row C.

Arrowheads in the second panel of row A indicate the pin holes of the external fixator. “N” denotes new cortical bone within the defects, “S” denotes absorbable collagen sponge in the defects, “P” denotes living bone particles, “T” denotes trabecular bone, “M” denotes bone marrow, and “F” denotes fibrous repair tissue, “C” denotes cartilage tissue within the defects.

Table 1**Experimental design**

Groups of rats were assigned to experimental groups as indicated. Recombinant human BMP-2 protein (3 different doses) was loaded onto an absorbable collagen sponge (ACS) and implanted into surgically created femoral defects with or without human living bone particles obtained from “reamings” recovered by the RIA system. All animals were subjected to weekly X-rays and euthanized after 8 weeks. Healing of the defects was analyzed in all animals by DXA and μ CT. The femora of two animals from each group were used for histology, and the remainder subjected to mechanical testing.

Defect contents	Number of rats
Bone (0.2 mg) + rhBMP-2 (11 μ g)	7
Bone (0.2 mg) + rhBMP-2 (5.5 μ g)	7
Bone (0.2 mg) + rhBMP-2 (1.1 μ g)	7
Bone (0.2 mg) + ACS alone	7
rhBMP-2 (11 μ g)	6
rhBMP-2 (5.5 μ g)	6
rhBMP-2 (1.1 μ g)	6
ACS alone	6

Table 2
Radiographic scoring of bone healing in response to BMP-2 and bone particles

Numbers of rats from each group that formed bone within the defect or healed the defect 8 weeks after implantation of test materials. Values are not given for bone formation in the presence of implanted bone particles because the latter are radiopaque and cannot be distinguished from newly formed bone.

Treatment	Bone formation at 8 weeks	Bone healing at 8 weeks
ACS alone	0/6	0/6
1.1 µg	6/6	0/6
5.0 µg	6/6	6 [*] /6
11 µg	6/6	6/6
ACS + bone		0/7
1.1 µg + bone		2/7
5.0 µg + bone		7/7
11 µg + bone		7/7

* These rats healed, but had a radiolucent discontinuity on the X-ray (see figures 1, 5).