Fig. E-1

Positioning stand used for mechanical testing of the femoral neck in bending mode. The metal hook (solid arrow) engages the greater trochanter to limit bending of the femoral shaft during testing. The metal platen of the mechanical testing actuator (dashed arrow) is free to slide across the femoral head during testing.

Fig. E-2

Fluoroscopic appearance of the 18-gauge spinal needle used to administer rhBMP-2/CPM; the needle has been preplaced into the proximal part of the femur prior to the injection (Fig. E-2A). Radiodense CPM carrier is visible fluoroscopically immediately after the injection (closed arrows, Fig. E-2B).
Six months after treatment, histological appearance of the diaphysis of the proximal part of a femur that was treated with 1.5 mg/mL rhBMP-2/CPM (Figs. E-3A and E-3B). Periosteal thickening (Fig. E-3B, closed arrow) was primarily the result of woven subperiosteal trabecular bone formation (double-headed black arrow in Fig. E-3C, which is a magnification of the area within the solid box in Fig. E-3B). Distinct yellow tetracycline label (open arrowheads) on the preexisting periosteal surface and diffuse yellow tetracycline label within the woven cores of subperiosteal woven trabecular bone (closed arrowheads) indicate that mineralization of the subperiosteal new bone was ongoing between day ten and day twenty-eight. The darker regions with central bright fluorescent circles (between dashed arrows) represent primary osteons that are filling in the spaces between newly formed woven trabeculae. The fluorescent circles within the darker regions are calcein-stained bone that appear white with use of the tetracycline fluorescent filter. Endosteal thickening (Fig. E-3B, open arrow) was primarily the result of lamellar appositional bone formation (double-headed white arrow in Fig. E-3D, which is a magnification of the area within the dashed box in Fig. E-3B). Two green calcein labels (open and closed arrows) indicate that mineralization of the endosteal new bone was ongoing between fourteen days and three days prior to euthanasia at six months. The lack of tetracycline label indicates mineralization did not initiate until after twenty-eight days. (Goldner trichrome stain for Figs. E-3A and E-3B and unstained, ultraviolet fluorescence for Figs. E-3C and E-3D; original magnification, ×0.5 for Fig. E-3A, ×4 for Fig. E-3B, and ×10 for Figs. E-3C and E-3D)
Histological appearance of the contralateral, untreated proximal femoral diaphyseal cortex at six months in the same animal pictured in Fig. E-3 (Figs. E-4A and E-4B). Minimal appositional or subperiosteal trabecular bone formation (Fig. E-4B, closed arrow) was confirmed by the shorter length of the region between the double arrow in Fig. E-4C, which is a magnification of the area within the solid-outlined box in Fig. E-4B, compared with the corresponding region in Fig. E-3C and the lack of yellow tetracycline label in this region. Ongoing endosteal appositional bone formation (Fig. E-4B, open arrow) was confirmed by the presence of a single green calcein label on the endosteal surface (closed arrows in Fig. E-4D, which is a magnification of the area within the dashed-outlined box in Fig. E-4B). However the total amount of new bone was minimal (Fig. E-4D, double arrow) compared with the corresponding region in Fig. E-3D. Regions of cortical bone outlined by two concentric tetracycline labels with a central calcein label (Figs. E-4C and E-4D, dashed arrows) represent remodeling osteons that were initiated at ten days after the start of the study and completed by six months. Smaller regions of cortical bone with single calcein circles are newer remodeling osteons that were recently initiated (Figs. E-4C and E-4D, open arrows). (Goldner trichrome stain for Figs. E-4A and E-4B; unstained and ultraviolet fluorescence for Figs. E-4C and E-4D; original magnification, ×0.5 for Fig. E-4A, ×4 for Fig. E-4B, and ×10 for Figs. E-4C and E-4D.)
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*rhBMP-2 = recombinant human bone morphogenetic protein-2, CPM = calcium phosphate matrix. †0.5 mL. ‡0.5 mL of 1.5 mg/mL rhBMP-2/CPM (0.75 mg rhBMP-2).
Appendix: Materials and Methods

Study Design

Efficacy of a single intraosseous injection of the combination of rhBMP-2/CPM or CPM alone to increase local bone structure and mechanical properties in the proximal part of the femur was evaluated in fourteen aged female cynomolgus monkeys (*Macaca fascicularis*). The monkeys used in this study were greater than fifteen years of age and had been ovariectomized five years prior to treatment. Eight animals were administered a single percutaneous, intraosseous injection of 0.5 mL of 1.5 mg/mL rhBMP-2 delivered in CPM into one femoral neck with the aid of intraoperative fluoroscopy. A single percutaneous, intraosseous injection of 0.5 mL of CPM alone was also administered into one femoral neck in six additional animals. The contralateral femoral neck was not treated in either group. Two animals in the rhBMP-2/CPM group were killed at an earlier time point (one at one month and the other at three months after treatment) to evaluate the histological time course of bone induction and resorption of the CPM carrier. The remaining animals in both groups were killed at six months. Radiography, peripheral quantitative computed tomography (pQCT), microcomputed tomography (µCT), biomechanical testing, and histological analysis were used to evaluate the efficacy of the rhBMP-2/CPM and CPM-alone treatments. The Institutional Animal Care and Use Committee approved all of the protocols. All procedures were performed according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International guidelines.

rhBMP-2/CPM Formulations

A Chinese hamster ovary (CHO) cell expression system was used to produce the secreted, glycosylated, dimeric rhBMP-2 protein (Wyeth BioPharma [now part of Pfizer], Andover, Massachusetts)\(^1\). The rhBMP-2 was administered in a calcium phosphate matrix (CPM) (ETEX, Cambridge, Massachusetts). Rheology, setting time, and mechanical properties of CPM were similar to the reported data for a rapidly resorbing calcium phosphate paste (α-BSM)\(^2\). The 1.5 mg/mL rhBMP-2/CPM combination was formulated immediately prior to injection by adding 0.8 mL of 2.65 mg/mL rhBMP-2/buffer solution to the sterile 1.25-g bulbs of CPM powder (liquid-to-powder ratio of 0.64). The buffer solution contained 5.0 mM L-glutamic acid, 5.0 mM NaCl, 2.5% glycine, 0.5% sucrose, and 0.01% polysorbate 80. The final concentration of 1.5 mg/mL rhBMP-2/CPM was based on a 1.4-mL final paste volume following the addition of the rhBMP-2/buffer solution. The CPM alone and the 0.5 mg/mL and 4.5 mg/mL rhBMP-2/CPM combinations were formulated by adding 0.8 mL of 0.0 mg/mL, 0.7 mg/mL, and 8.0 mg/mL rhBMP-2/buffer solution, respectively, to the 1.25-g bulbs of CPM powder. Sufficient volumes of rhBMP-2/CPM or CPM alone required for treatment were then
transferred from the bulbs to a 1-mL syringe. Injections were performed within ten minutes of formulation.

Animal Husbandry

The animals were housed in a limited access AAALAC-accredited facility. On arrival, the animals were quarantined for a minimum of thirty days prior to the initiation of the study. All of the animals were weighed and had routine physical examinations performed prior to the start of the study. Laboratory screening tests performed prior to enrollment in the study included a baseline complete blood cell count, serum chemistry profile, fecal bacteriology and parasitology screen, tuberculosis test, and virus titer screens (herpes B virus, measles, simian retrovirus, simian immunodeficiency virus, and simian T-cell leukemia virus). They were fed monkey chow from an approved supplier and were given supplemental fruits, and they had access to water ad libitum. The animals also participated in a behavioral enrichment program. Prior to surgery, the animals were examined and their presurgical blood work was evaluated to ensure that each animal was in good health at the time of surgery.

Sedation, Presurgery Treatment, General Anesthesia, Postsurgery Treatment, and Euthanasia

The animals were preanesthetized with intramuscular atropine (0.04 mg/kg) and sedated with intramuscular Telazol (tiletamine/zolazepam) (2 to 6 mg/kg) for radiographic evaluations. For the surgical procedure, the food was withheld for eighteen to twenty-four hours. The animals were administered intramuscular antibiotics (cefazolin, 17 mg/kg) and analgesics (buprenorphine hydrochloride, 0.01-0.03 mg/kg) prior to surgery. Following sedation with intramuscular Telazol (3 to 5 mg/kg) and preanesthetic intramuscular atropine (0.04 mg/kg), the animals were intubated and general anesthesia was maintained with isoflurane in conjunction with oxygen. Bland ophthalmic ointment was placed in both eyes to prevent corneal desiccation. Lactated Ringer solution (10 mL/kg/hr) was administered during surgery through an intravenous catheter placed in the brachial vein. Hypothermia was prevented with use of heating pads, insulated blankets, and a warm air unit. Intraoperative monitoring of vital signs was continued until the animal recovered from anesthesia and became ambulatory. The animals were monitored in a temperature-controlled intensive care unit during recovery from anesthesia. Intramuscular buprenorphine (0.01 to 0.03 mg/kg) was administered twice a day for one day after surgery and as needed thereafter on the basis of the level of discomfort observed during the immediate postoperative period. The animals were administered an intramuscular injection of tetracycline (25 mg/kg) at ten and twenty-eight days after treatment. Intramuscular calcein (5 mg/kg) was administered fourteen and three days prior to euthanasia. These fluorochromium labels were administered to determine the time course of new bone formation over the six-month study period. The animals were killed with an intravenous bolus of sodium pentobarbital (100 mg/kg).
Surgery

Sterile preparation and draping were performed with the animals positioned in the appropriate lateral recumbency. Using fluoroscopic guidance, a Kirschner wire was inserted through the skin into the proximal part of the femur, with use of a motorized drill. The Kirschner wire was inserted centrally into the femoral neck. The Kirschner wire was then advanced to within 5 mm of the femoral articular cartilage (proximal part of the femur). An 18-gauge, 3-in (7.62 cm) spinal needle was placed over the Kirschner wire and guided through the lateral cortex into the femoral neck. The Kirschner wire was then removed and the appropriate volume of rhBMP-2/CPM or CPM alone was injected into the proximal part of the femur as the needle was withdrawn. The stylet was placed into the spinal needle to extrude any remaining material prior to removal of the needle.

Radiography

Standard radiographs of the proximal part of the femora were made prior to and immediately after surgery. Additional radiographs were made at one, two, four, and six months after treatment. Each set of radiographs included a craniocaudal and lateromedial view containing a gray-scale indicator to standardize the exposures. Care was taken to ensure uniformity of film-focus distances, positioning, and exposure.

Specimen Harvest

Following euthanasia, the legs were disarticulated, the appropriate limb segments were dissected out, and the soft tissues were removed. The proximal femoral specimens were cut from the femora approximately 5 cm distal from the top of the greater trochanter with use of a band saw. The specimens were stored in 10% formalin for two weeks as a precaution against potential exposure to herpes B virus. Following the initial two-week period, the specimens were transferred to 70% isopropyl alcohol and stored at 4° C for subsequent evaluation.

Peripheral Quantitative Computed Tomography (pQCT)

Ex vivo pQCT imaging was performed on bone specimens placed in polypropylene conical tubes filled with saline solution (XCT3000; Stratec, Pforzheim, Germany). Contiguous slices, perpendicular to the femoral neck axis, were made for the femoral specimens, starting at the femoral head and ending in the trochanteric region. The specimens were repositioned and imaged perpendicular to the long axis of the proximal part of the femoral diaphysis. Image slices were 2.5 mm thick and were made at 2.0-mm intervals with an in-plane voxel size of 0.2 mm × 0.2 mm. Standard contour and peel-mode thresholding methods were used to distinguish soft tissue (169 mg/cm²) from cortical and trabecular bone (400 and 700 mg/cm², depending on the type of analysis performed). Measurements from three to four contiguous images were used to determine cross-sectional area, cortical density, cortical area, and the density of the trabecular region (density of the region within the marrow space). A circular ring model was used to
determine cortical thickness. It was not possible to obtain measurements of cortical thickness, cortical area, cortical density, and density of the trabecular region of the CPM-treated femora due to the presence of residual radiodense CPM in the pQCT images at six months.

**Biomechanical Testing**

The proximal femoral specimens were mounted vertically in a materials testing system (model 858 Mini Bionix II, 50 kN load cell; MTS, Eden Prairie, Minnesota) with the cut end of the diaphysis clamped into a metal base plate. A metal column extending vertically from the base plate and fitted with a horizontal, machined metal bar with a hooked end was positioned to engage the greater trochanter to limit bending of the femoral shaft during testing. A flat loading platen was then lowered to contact the articular surface of the femoral head, and a 50-N preload was applied. The femoral head was then loaded in compression at a constant displacement rate of 1.5 mm/sec until failure. The flat loading platen was free to move across the articular surface of the femoral head during testing. This testing configuration simulates bending of the femoral neck with a load applied to the femoral head. Maximum bending stiffness and maximum bending force were calculated from the load-versus-displacement data.

**Histology**

Histological evaluation of the femora harvested at six months was performed on specimens following mechanical testing. The fractured femoral neck sections were taped together to reestablish the appropriate anatomic orientation prior to embedding. Histological evaluation of the femora that were harvested at one and three months was performed on intact specimens, as these specimens had not been subjected to biomechanical testing. The proximal end of each specimen was marked with India ink for orientation purposes. Specimen blocks, oriented in the cross-sectional plane, perpendicular to the femoral head-neck axis or the cortex, were obtained from the femoral head, femoral neck, greater trochanteric region, and diaphysis of the proximal part of the femora with use of a low-speed saw (Buehler IsoMet, Lake Bluff, Illinois). Sagittal specimen blocks were also cut from lateral to medial for the distal part of the femora. Each specimen block was then placed into labeled processing bags and immersed into fresh 70% ethanol for fixation. All undecalcified specimens were processed through alcohol gradients (cleared in xylene) under vacuum and gentle agitation in an automatic tissue processor (LX 120 Automatic Tissue Processor; Fisher Healthcare, Houston, Texas). Specimen blocks were infiltrated and embedded in methylmethacrylate and allowed to polymerize for three to five days at room temperature following a modified method by Schenk et al. After polymerization was complete, the specimen blocks were removed from their molds and prepared for thin-sectioning. Specimens were sectioned with use of a microtome (SM 2005E Riechert-Jung Polycut microtome; Leica
Microsystems, Wetzlar, Germany). Three sections from each specimen block were stretched and pressed on gelatin-chromium-alum slides and placed in an oven at 45° to 50°C overnight (minimum time) to ensure tissue adherence to the slides. Slides were then allowed to reach room temperature prior to staining. One 7-µm thick section was stained with von Kossa stain and one 5-µm-thick section was stained with Goldner trichrome stain. A third, 8-µm-thick section was left unstained for evaluation of fluorochromium labels. The presence of tetracycline label was evaluated with use of a fluorochromium filter cube with an excitation wavelength of 370 nM and an emission wavelength of 440 nM, resulting in a yellow color. The presence of calcein label was evaluated with use of a fluorochromium filter cube with an excitation wavelength of 430 nM and an emission wavelength of 540 nM, resulting in a green color.

Qualitative evaluation was performed on both stained and unstained sections with use of a microscope equipped with both transmitted and fluorescent light. The timing of new bone formation was determined by evaluation of the presence or absence of the various fluorochromium labels administered during the course of the study. Static histomorphometry measurements for mineralized bone were performed on the von Kossa-stained sections with use of black-and-white digital images displayed on a monitor screen. Trabecular perimeter and trabecular area were measured on the basis of the black-stained regions corresponding to mineralized bone with use of image analysis software (BIOQUANT OSTEO software; Bioquant Image Analysis, Nashville, Tennessee). From these direct measurements, trabecular volume (% total mineralized bone area), mineralized trabecular thickness, trabecular number, and trabecular separation were derived using previously published methods.4,5 Osteoid surface was measured from Goldner trichrome-stained color sections displayed on a monitor screen. The osteoid surface (% of total trabecular surface covered with osteoid) was measured with use of the BIOQUANT OSTEO image analysis software to determine the length of the osteoid surfaces and the length of the total trabecular surfaces.

Data Analysis

Within-group comparisons of pQCT, biomechanical measurements, and histological measurements were evaluated with use of the Wilcoxon signed-rank test (StatPlus:mac; AnalystSoft, Alexandria, Virginia). Between-group comparisons of histological measurements were evaluated with use of the Wilcoxon-Mann-Whitney test. Nonparametric tests were chosen due to the small sample size. Tests were two-tailed, and differences were considered significant at the p < 0.05 level.
References


