Systemically Administered Bone Morphogenetic Protein-6 Restores Bone in Aged Ovariectomized Rats by Increasing Bone Formation and Suppressing Bone Resorption*

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Although recombinant human bone morphogenetic proteins (BMPs) are used locally for treating bone defects in humans, their systemic effect on bone augmentation has not been explored. We have previously demonstrated that demineralized bone (DB) from ovariectomized (OVX) rats cannot induce bone formation when implanted ectopically at the subcutaneous site. Here we showed in vitro that 17β-estradiol (E2) specifically induced expression of Bmp6 mRNA in MC3T3-E1 preosteoblastic cells and that bone extracts from OVX rats lack BMPs. Next we demonstrated that 125I-BMP-6 administered systemically accumulated in the skeleton and also restored the osteoinductive capacity of ectopically implanted DB from OVX rats. BMP-6 applied systemically to aged OVX rats significantly increased bone volume and mechanical characteristics of both the trabecular and cortical bone, the osteoblast surface, serum osteocalcin and osteoprotegerin levels, and decreased the osteoclast surface, serum C-telopeptide, and interleukin-6. E2 was significantly less effective, and was not synergistic with BMP-6. Animals that discontinued BMP-6 therapy maintained bone mineral density gains for another 12 weeks. BMP-6 increased in vivo the bone expression of Acvr-1, Bmpr1b, Smads5, alkaline phosphatase, and collagen type I and decreased expression of Bmp3 and BMP antagonists, chordin and cerberus. These results show, for the first time, that systemically administered BMP-6 restores the bone inductive capacity, microarchitecture, and quality of the skeleton in osteoporotic rats.

Bone loss during aging and after menopause in women is known to result from an imbalance between bone formation and resorption leading to altered bone microarchitecture and excess bone fragility. Inferior bone strength and increased bone fracture rate of bone in patients with osteoporosis might be associated with decreased osteoinductive and thus self-renewable bone capacity eventually due to the lower content of growth and differentiation factors including bone morphogenetic proteins in the bone extracellular matrix (1–4).

Demineralized bone matrix (DBM) induces de novo bone formation when implanted into the rat muscle (5). On the contrary, DBM from OVX animals implanted into both normal and OVX rats induces only fibrous tissues suggesting that its decreased bone inducing activity is due to abnormal composition of bone from OVX rats and not to the 17β-estradiol (E2)-deficient microenvironment (1). Lack of specific signals needed for ectopic bone induction may, at least in part, explain diminished bone potency to heal fractures in osteoporotic patients (6, 7). It has been demonstrated that fetal osteoblastic cell lines treated by E2 specifically express Bmp6 mRNA, whereas gene transcripts of other members of the BMP family are unaffected (8). A functional relationship between E2 and BMP-6 was further suggested by E2 binding to the Bmp6 gene promoter (9) and by increased BMP-6 immunostaining in bone marrow of mice treated with E2 (10).

Although numerous studies have unequivocally demonstrated that BMPs induce new bone formation locally in animals and humans, both ectopically and when implanted between bone ends in patients with delayed non-unions or acute fractures (11, 12), so far it has not been shown that a systemically administered recombinant BMP can effect the bone volume in OVX rats. In these studies we tested the effect of systemically administered recombinant BMP-6 on bone formation in aged OVX rats and showed for the first time that BMP-6 is a novel systemic bone anabolic agent and has a potential for treating bone loss in patients with osteoporosis.

EXPERIMENTAL PROCEDURES

Demineralized Bone Matrix—Bone matrix was prepared from 6-month-old Sprague-Dawley rats, 3 months following OVX. After sacrifice, diaphyses of femurs and tibiae were removed and then powdered, sieved, and demineralized as previously described (5). DBM from OVX rats was implanted subcutaneously in the pectoral region of normal and OVX rats, which were subsequently injected with 20, 50, and 100 μg/kg/day of BMP-6 intravenously (iv) from days 2 to 6 following...

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2 The abbreviations used are: DBM, demineralized bone matrix; OVX, ovariectomized; BMD, bone mineral density; CT, computerized tomography; N, newton; ELISA, enzyme-linked immunosorbent assay; OPG, osteoprotegerin; IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance; DTT, dithiothreitol; PTH, parathyroid hormone.
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implantation. Two weeks following implantation of DBM, pellets were removed and embedded in paraffin, cut, stained with toluidine blue, and examined for the presence of new cartilage and bone.

MC3T3-E1 Preosteoblastic Cell Line and Cell Culture Experiments—Stock cultures of non-confluent mouse calvarial preosteoblasts (MC3T3-E1) were grown in α minimal essential medium, supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. All culture reagents were from Invitrogen. MC3T3-E1 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were plated at 3 × 10⁴ cells/cm² in 24-multidwell culture dishes (Costar) and cultured until reaching ~90% confluence. The growth medium was replaced by Dulbecco’s modified Eagle’s medium/F-12 (without phenol red; Invitrogen) supplemented with ITS + culture supplement (Collaborative Biomedical Products, Bedford, MA) and L-ascorbic acid 2-sulfate (50 g/ml; Sigma). Cells were stimulated with E₂ (Sigma) at 10⁻⁷–10⁻³ M for 12 and 72 h. At the designated time points total cellular RNA was extracted and analyzed by semi-quantitative PCR.

Animals—Four-, 6-, or 7-month-old Sprague-Dawley rats were subjected to OVX. Animals were anesthetized with an intraperitoneal injection of thiopental at doses of 4 mg/kg body weight. Thirty animals per experiment were subjected to sham surgery during which the ovaries were exteriorized but replaced intact. Bilateral ovariectomies were performed in the remaining rats from the dorsal approach and they were left untreated for a period of 6 to 18 months following surgery to await the development of osteopenia. BMP-6 at doses of 1, 10, 25, and 50 μg/kg was injected through the rat tail vein one or three times a week for 8–12 or 30 weeks (extended protocol). In total 390 sham and OVX rats were used in three protocols as follows: A: 1, sham (n = 30); 2, OVX (n = 20); 3, OVX + BMP-6 (10 μg/kg intravenously, 3 times/week) (n = 20); 4, OVX + BMP-6 (25 μg/kg intravenously, 3 times/week) (n = 20); 5, OVX + BMP-6 (50 μg/kg intravenously, 3 times/week) (n = 20); 6, OVX + E₂ (50 μg/kg intraperitoneally, 3 times/week) (n = 20); and 7, OVX + E₂ (50 μg/kg intravenously, 3 times/week) (n = 20). Therapy started 12 months following OVX and continued for 12 weeks (Fig. 2).

Protocol B was: 1, sham (n = 30); 2, OVX (n = 20); 3, OVX + BMP-6 (10 μg/kg intravenously, 3 times/week) (n = 50) for 3 months, and then group 3 OVX + BMP-6 was divided into: 4, OVX + BMP-6 (10 μg/kg intravenously, 3 times/week) (n = 20); 5, OVX + E₂ (50 μg/kg intraperitoneally, 3 times/week) (n = 20); and 6, OVX + no therapy (n = 10) for 4.5 months. Therapy started 6 months following OVX and continued for 7.5 months including the first 3 months and second therapies (3.5 months) (Fig. 4).

Protocol C was: 1, sham (n = 30); 2, OVX (n = 20); 3, OVX + BMP-6 (1 μg/kg intravenously, 3 times/week) (n = 20); 4, OVX + BMP-6 (10 μg/kg intravenously, 3 times/week) (n = 20); and 5, OVX + BMP-6 (10 μg/kg intravenously, once/week) (n = 20). Therapy started 18 months following OVX and continued for 6 weeks (Fig. 5). All experiments and protocols were approved by the Institutional Animal Care Committee and the Ministry of Science and Technology (project 108295).

Recombinant BMP-6—Human mature BMP-6 was produced in Chinese hamster ovary cells as previously described (13, 14). Proteins were purified by using a Filtron Minisette (PallGelman) cross-flow device. pH was adjusted to 5.5 and the material was dialyzed against 0.1 M Tris, 75 mM acetic acid, 0.2 mM KH₂PO₄, 5 mM EDTA, 6 μM urea and then filtered. Subsequently, about 150 mg of protein was loaded onto a 5-ml HiTrap™ Heparin-Sepharose HP (Amersham Biosciences) column that had been pre-equilibrated with 0.1 M Tris, 125 mM acetic acid, 5 mM EDTA, and 6 μM urea containing 0.3 M NaCl. Loading was performed at a flow rate of 4 ml/min. The column was loaded with maximal 30 mg of dialyzed protein. Dimeric BMP-6 species was eluted at a NaCl concentration of 0.7 M. Homogenous fractions were pooled, dialyzed against 10 mM NH₄ acetate, pH 4.0, lyophilized, and stored until used.

Biodistribution and Pharmacokinetics of ¹²⁵I-Labeled BMP-6 (¹²⁵I-BMP-6)—Highly purified BMP-6 (15.7 mg) was radioiodinated with 5 mCi of carrier-free Na¹²⁵I using a modification of the lactoperoxidase method as described earlier (15, 16). Shortly, gel filtration on a Sephadex G-25 column was used to separate radioiodinated BMP-6 (¹²⁵I-BMP-6) from the free iodide. The column was eluted with 20 mM sodium acetate buffer, pH 4.5, containing 0.2 Tween 80 and 0.1% ovalbumin. The specific activity of the ¹²⁵I-BMP-6 preparation used in this study was 0.273 mCi/mg. Rats (n = 50) received a single injection of ¹²⁵I-BMP-6 at a dose level of 10 μg/kg with the activity of 20 μCi. Injection volume was 500 μl. Animals were sacrificed 30 min, 1, 3, 6, and 24 h following injection. Tissues were removed, weighed, and radioactivity was measured in a γ-counter. The relative uptake of ¹²⁵I-BMP-6 by tissues during the above time was expressed as nanograms of ¹²⁵I-BMP-6 per gram of wet tissue weight.

Western Blot Analysis of Bone Extracts from Sham and OVX Rats—Femur and tibia from sham and OVX rats were excised, flash-frozen in liquid nitrogen, and crushed into a fine powder with a biopulverizer (Biospec Products, Bartlesville, OK). Two hundred grams of bone powder were extracted at 4 °C with 4 M guanidine HCl and a mixture of protease inhibitors (5 mM benzamidine, 0.1 M 6-amino-6-hexanoic acid, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide). The protein extract was then ethanol-precipitated and lyophilized (17). The pellet was dissolved in 6 M urea, 50 mM Tris, pH 7.0, containing 0.15 M NaCl and purified by a heparin-Sepharose column (Amersham Biosciences). Bound proteins were eluted with 0.5, 1, and 2 mM NaCl in 10 mM sodium phosphate buffer, pH 7, at a flow rate of 1 ml/min. The protein eluted by 0.5 mM NaCl was collected and dialyzed extensively against distilled water and 30% acetonitrile, 0.1% trifluoroacetic acid at 4 °C. SDS-PAGE was run on a 10% gel according to the method of Laemmli (18). Samples were heated in boiling water for 3 min with or without dithiothreitol (100 mM) prior to electrophoresis. Following electrophoresis, the gel was blotted onto the nitrocellulose membrane and incubated with a goat polyclonal BMP-6 (Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal BMP-2 (Santa Cruz Biotechnology), and rabbit polyclonal BMP-7 primary antibodies (19). Alkaline phosphatase-conjugated anti-rabbit and anti-goat
were used as a secondary antibody. The membranes were developed with the chromogenic substrate (Invitrogen).

In Vivo and ex Vivo Bone Mineral Density (BMD) Measurement—At 6-week intervals the animals were scanned for bone density measurements by dual-energy x-ray absorptiometry (Hologic QDR-4000, Hologic, Waltham, MA) (20). At the end of the experiment, animals were anesthetized, weighed, and killed by cervical dislocation. The hind limbs and lumbar vertebrae were removed at death, prepared for histomorphometric analysis, and fixed in 70% ethanol. The right femur and tibia, and the lumbar vertebrae (L1–5) were used for determination of bone density of whole bones, proximal, and distal metaphyses and the shaft of femurs and tibiae were determined.

Peripheral Quantitative Computerized Tomography—Isolated femurs were scanned by a peripheral quantitative computerized tomography x-ray machine (Stratec XCT Research M; Norland Medical Systems, Fort Atkinson, WI) with software version 5.40. Volumetric content, density, and area of the total bone, trabecular, and cortical regions were determined as previously described (22, 23).

Micro-CT—The microcomputerized tomography apparatus (μCT 40) and the analyzing software used in these experiments were obtained from SCANCO Medical AG (Bassersdorf, Switzerland) (24). The distal femur was scanned in 250 slices, each 13-μm thick in the dorsoventral direction (25). Three-dimensional reconstruction of bone was performed using the triangulation algorithm. The trabecular bone volume (BV, mm³), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, μm), and trabecular separation (Tb.Sp, μm) were directly measured on three-dimensional images using the method described by Hildebrand et al. (26). The trabecular bone pattern factor and the structure model index were computed using software provided with the micro-CT machine (27, 28).

Histology and Histomorphometry—Animals were given a subcutaneous injection of the fluorochrome calcein at 10 mg/kg (Sigma) at 4 and 14 days before death. The femurs were removed at death, prepared for histomorphometric analysis, and quantified using a computer-aided image analysis system (Bioquant II, R and M Biometrics, Nashville, TN) as previously described by Hildebrand et al. (26). The trabecular bone pattern factor and the structure model index were computed using software provided with the micro-CT machine (27, 28).

**TABLE 1**

| Target gene | Semi-quantitative PCR | Real time-PCR |
|-------------|-----------------------|---------------|
| **IL-6**   | Forward: CAAGAAGATTCCTGAGCCCAATTCGAC | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Bmp2**   | Forward: GGAGCACTGAGAATTTTGGC | Reverse: GGCCGAGTAGACCTACATAGCGAC |
| **Mouse**  | Forward: GAGGACAGGATGCTGAGAAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
|            | Reverse: GTGGTGGCTGCTGAGCCAAATTC | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Bmp3**   | Forward: CAATTACGCTCTTCGAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Mouse**  | Forward: GGAGGCGGTCGCTGAGAAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
|            | Reverse: GTGGTGGCTGCTGAGCCAAATTC | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Bmp4**   | Forward: AGGAGACGCTGAGGAGGAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Mouse**  | Forward: GGAGGCGGTCGCTGAGAAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
|            | Reverse: GTGGTGGCTGCTGAGCCAAATTC | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Bmp6**   | Forward: ACCACCACTCTTCGAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Mouse**  | Forward: AGGAGACGCTGAGGAGGAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
|            | Reverse: GTGGTGGCTGCTGAGCCAAATTC | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Acvr1**  | Forward: CATGATGTTAGGAGGAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Mouse**  | Forward: AGGAGACGCTGAGGAGGAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
|            | Reverse: GTGGTGGCTGCTGAGCCAAATTC | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Bmpr1b** | Forward: GCTGCTCTTCGAGGAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Smad5**  | Forward: GAGGAGGAGGAGGAGGAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Alp2**   | Forward: ACCACCACTCTTCGAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Mouse**  | Forward: AGGAGACGCTGAGGAGGAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
|            | Reverse: GTGGTGGCTGCTGAGCCAAATTC | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Omp**    | Forward: GTGACTGCTGCTGCTGCTGCTG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Colla1** | Forward: TATGCTGCTGCTGCTGCTGCTG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Chrd**   | Forward: GAGGAGGAGGAGGAGGAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Cer1**   | Forward: GGGAGGATAGGGGCTGCTGCTG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Twsg1**  | Forward: CCAGAGCTCTGCCTGCTGCTG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Nog**    | Forward: AGGGAGAAGGCGCATTACAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |
| **Gapdh**  | Forward: ACCACACCTCTTCGAGGAGG | Reverse: TGGCCGAGTAGACCTACATAGCGAC |

**Histology and Histomorphometry**—Animals were given a subcutaneous injection of the fluorochrome calcein at 10 mg/kg (Sigma) at 4 and 14 days before death. The femurs were removed at death, prepared for histomorphometric analysis, and quantified using a computer-aided image analysis system (Bioquant II, R and M Biometrics, Nashville, TN) as previously described by Hildebrand et al. (26). The trabecular bone pattern factor and the structure model index were computed using software provided with the micro-CT machine (27, 28).
described (29–31). Statistical analyses were performed using StatView 4.0 packages (Abacus Concepts, Berkeley, CA). Organs were taken for histologic analyzes, embedded in paraffin, cut in 10-μm thick sections, and stained with hemalaun-eosin and toluidine blue to reveal potential therapeutic adverse effects.

Biomechanical Testing—Using a materials testing system (Model 810, MTS Systems Corp., Minneapolis, MN), two types of mechanical testing were performed on the femur. Three-point bending test of the femoral shaft was used to determine the mechanical properties of the midshaft femur. The midshaft of the femur was subjected to three-point bending to failure at a displacement rate of 0.1 mm/s, as described by Turner and Burr (32), using a 2.5 kN load cell (MTS Model 661, 14A-03). The maximal load and stiffness were calculated from the load-displacement curve. Indentation test of the distal femoral metaphysis was used to determine the mechanical properties of cancellous bone in the marrow cavity of the distal femoral metaphysis, as described previously (33, 34).

Biochemical Serum and Urine Parameters—Serum bone formation and resorption markers were measured by commercially available kits. Serum concentration of osteocalcin was measured by ELISA using rat osteocalcin EIA kits (Biomedical Technologies Inc., Stoughton, MA). Serum concentration of C-telopeptide was measured by ELISA using RatLaps ELISA kits (Nordic Bioscience Diagnostics, Herlev, Denmark). The osteoprotegerin (OPG) level in serum was measured by ELISA using the Biomedica rat OPG ELISA kit (Biomedica, Wien, Austria). Serum concentration of IL-6 was measured by ELISA using an IL-6 Quantikine ELISA kit (R&D, Minneapolis, MN) following procedures provided by the manufacturer. The minimum detectable concentration of osteocalcin, C-telopeptide, OPG, and IL-6 were 1 ng/ml, 2 ng/ml, 0.73 pmol/liter, and 10 pg/ml, respectively.

RNA Isolation and PCR Analysis—RNA was extracted with TRIzol (Invitrogen) and cDNA was synthesized from 2 μg of total RNA with Superscript II RNase H Reverse Transcriptase as indi-
Real Time PCR—Two μg of purified total RNA from each rat bone were reverse transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase RNase (BD Biosciences) using random hexamer primers (final concentration 20 pmol per sample) according to the manufacturer’s protocol. Gene expression of interest was measured using a commercial kit (LightCycler FastStart DNA Master SYBR Green, Roche Diagnostics, Mannheim, Germany) in a LightCycler instrument (Roche Diagnostics) as described (35). Sequences of primers are shown in Table 1. Expression of four housekeeping genes was analyzed and geNorm software was used to identify the most suitable reference gene. Gapdh transcripts were used.

* Significantly different from group 2 (by ANOVA Dunnett test, *p < 0.05*).

**FIGURE 3.** μCT longitudinal and horizontal as well as histological analyses of distal femurs in aged rats treated with E2 (50 μg/kg), BMP-6 (10 μg/kg), and E2 (50 μg/kg) + BMP-6 (10 μg/kg) for 3 months following 12 months of OVX. BMP-6 alone and in combination with E2 increased trabecular bone volume and connectivity. The standardized region of interest for μCT analyses began 4 mm above the intercondylar fossa and included 50 slices.
as a normalizer. Results are represented as -fold change of the comparative expression level.

**Statistical Analysis**—Distributions of quantitative data were tested with Kolmogorov-Smirnov test. Densitometric and histologic data measurement within the same time point were analyzed with one-way ANOVA with one-sided Dunnett post hoc test against OVX and sham animals. Serum values of IL-6 were analyzed with paired t test. Changes in gene expression were evaluated using one-way ANOVA with one-sided Dunnett t post hoc test against OVX and sham animals.

**RESULTS**

**Bone Matrix from OVX Rats Is Deficient in BMPs**—Bone extracts from normal and OVX rats were analyzed by Western blot analysis following reduction with DTT (Fig. 1, A–C). Unlike bone extracts from normal rats, OVX animals had...
undetectable amounts of BMP-2, -6, and -7 (Fig. 1, A–C), suggesting that E2 deficiency results with decreased amounts of BMPs in bone. This suggests that the lack of capacity of DBM from OVX rats to form new bone at an ectopic site in both normal and OVX rats may be at least in part due to BMP-deficient DBM (1).
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Intravenously Administered BMP-6 Induces Ectopic Bone Formation by Binding to Subcutaneously Implanted Extracted DBM from OVX Rats—Systemic effects of BMP-6 were tested by intravenous injections following subcutaneous implantation of DBM from OVX rats into both normal and OVX rats. New bone formation was found 2 weeks later in an amount similar to the one induced by implanting DBM from normal rats (Fig. 1, F and G). These results suggest that, following systemic administration of BMP-6, DBM from OVX rats, lacking new bone induction capacity, regained the osteogenic potential in both normal and OVX rats. A similar result was obtained when 8 μm urea extracted and thus non-osteogenic DBM from normal rats was implanted subcutaneously into male rats injected intravenously from days 2 to 5 with 100 μg/kg BMP-6 (data not shown). These results prompted us to test whether systemic administration of BMP-6 is effective in restoring bone volume in aged rats 12–18 months following OVX.

Pharmacokinetics and Biodistribution of Systemically Administered 125I-BMP-6—Prior to testing the efficacy of BMP-6 on the skeleton we tested its pharmacokinetic properties and biodistribution. High serum levels of 125I-BMP-6 were achieved immediately after intravenous injection followed by a steady decline (Fig. 1D). Approximately 60 ng of BMP-6/ml was present in circulation 1 min after injecting a dose of 10 μg BMP-6/kg. Biodistribution of 125I-BMP-6 in the rat kidney increased during the first 4 h following injection, and decreased in the following 20 h, whereas the level of 125I-BMP-6 in the liver increased immediately and decreased slowly throughout the experiment. The uptake of 125I-BMP-6 in femur was 0.15% of the total applied dose (~4.5 ng of 125I-BMP-6), reached the maximal value at 4 h, and then declined slowly to 0.05% of the applied dose at 24 h following injection (Fig. 1C). The maximal uptake of 125I-BMP-6 in the tibia was 0.12% of the applied dose (~3.6 ng of 125I-BMP-6) at 8 h following injection and declined slowly to 0.025% of the applied dose at 24 h following injection (Fig. 1E). Autoradiographic analysis demonstrated that 125I-BMP-6 remained intact in the serum, assayed at 4 h after administration (data not shown).

E2 Specifically Induces Expression of BMP-6 mRNA in MC3T3-E1 Preosteoblastic Cells in Vitro—Prior to use of E2 as a positive control in these studies we sought to confirm its specific effect on BMP6 mRNA expression in osteoblastic cells in vitro. MC3T3-E1 pre-osteoblasts express Bmp2 and Bmp4 during differentiation and in vitro bone formation (3). Addition of E2 to cell cultures suppressed the expression of Bmp2 and Bmp4, but specifically induced the expression of Bmp6 transcripts in a dose-dependent manner (Fig. 1H). The number of bone nodules on day 27 of culture were not affected (data not shown). E2-induced Bmp6 was thus capable of replacing the function of Bmp2 and Bmp4 in in vitro osteogenesis. Moreover, upon E2 treatment, the expression profile of specific BMP receptors by MC3T3-E1 cells changed. Besides bone morphogenetic protein receptor types 1A and 1B (Bmpr1a and Bmpr1b), MC3T3-E1 cells treated with E2 expressed activin A receptor type 1 (Acvrl), a specific BMP type I receptor reported to be preferentially used by BMP-6 in osteoblasts (data not shown).

BMP-6 Restores Bone Volume in OVX Rats—Twelve months following OVX rats were treated for three months with BMP-6 and the BMD was fully restored (Fig. 2). Within 6 weeks of treatment rats regained the lost BMD and at 12 weeks following therapy they had higher hind limb (femur and tibia) BMD as compared with OVX, E2-treated, and sham animals (9, 6, and 3% increase, respectively) (Fig. 2). There were no significant differences between BMP-6 doses of 10, 25, and 50 μg/kg. Within the 12-week period hind limb BMD of sham treated rats decreased by 2%. BMD of the lumbar spine showed an increase of 9% at 12 weeks following BMP-6 treatment (data not shown). Ex vivo BMD values of tibiae, femurs, and vertebrae increased 8–11% in rats treated with BMP-6, independently of a dose used. E2 was about four times less effective than BMP-6 in restoring BMD (data not shown).

Peripheral quantitative computerized tomography analyses of femurs showed that total BMD was 13.8% higher and the total femoral bone mineral content was about 18% higher in
BMP-6-treated rats as compared with OVX rats. Cortical bone mineral content was higher for 24%, cortical bone mineral area for 21%, and the cortical thickness at 28% as compared with OVX rats (Table 2). Cortical bone parameters in femurs were preferentially enhanced over the trabecular bone by BMP-6 treatment (Table 2). Analyses of the tibiae showed a similar pattern, confirming a greater effect of BMP-6 on the cortical bone of the appendicular skeleton (data not shown).

Micro-CT analyses of distal femurs showed that the bone volume (BV/TV) of rats treated with 10 μg of BMP-6 was 78% increased compared with OVX control animals and 47% increased compared with rats receiving E2 (Figs. 3 and 4A). The trabecular number was 38% higher in BMP-6 and 19% in E2-treated rats (Fig. 4B). The trabecular thickness was increased 35% in BMP-6-treated as compared with OVX rats, and was 28 and 11% higher than in E2-treated and sham animals, respectively (Fig. 4C). The trabecular separation was decreased 36% in BMP-6-treated rats (Fig. 4D). Surprisingly, BMP-6 therapy increased the connectivity density 33% (Fig. 4E) and decreased the structure model index 42% (Fig. 4F). Connectivity parameters were 38% better than in E2-treated animals. However, none of the treatments were fully effective at restoring cancellous bone architecture and volume of sham rats. Micro-CT analysis of the 5th lumbar vertebrae showed 25% increased BV/TV, 8% increased trabecular number, 16% increased trabecular thickness, and 29% decreased trabecular separation in BMP-6-treated rats (Table 3). This suggested that the trabecular bone of the lumbar vertebrae responded better, reaching almost the values of sham animals, to the systemic administration of BMP-6 as compared with long bones. The three-point bending test was used to determine the mechanical properties of the midshaft femur. Maximal load and stiffness were 32 and 23% higher in BMP-6-treated animals as compared with OVX rats. Bones from BMP-6-treated animals absorbed 33% more energy than in sham animals (Table 4). Toughness was increased by 22% in BMP-6-treated rats as compared with sham rats (Table 4). The indentation test was used to determine the mechanical characteristics of trabeculae of the distal femoral metaphyses. Direct parameters of maximal load, stiffness, and energy absorbed were increased about 3–4-fold in BMP-6-treated rats as compared with sham rats (Table 4). The ultimate strength showed the same trend. BMP-6 treatment improved the trabecular bone parameters as compared with OVX animals, but did not restore the mechanical properties to those exhibited by sham rats.

Histomorphometric analyses showed that bone volume and trabecular thickness of distal femurs were higher for 78 and 35%, respectively, in BMP-6-treated animals (Fig. 3). Dynamic bone parameters showed an increased mineral apposition rate at 48% and bone formation rate/bone volume at 18% in BMP-6-treated rats, whereas E2 decreased the bone formation rate by 39% (Fig. 5). Most importantly, BMP-6 both increased the osteoblast surface for 32% and decreased the osteoclast surface for 29%. E2 decreased the osteoclast surface for 43% but did not have an effect on the osteoblast surface (Fig. 5). Improved bone formation rates and mechanical properties of BMP-6-treated bones resulted from both increased number of osteoblasts and potentially increased extracellular matrix production over E2 therapy (Table 4, Figs. 5 and 6).

The body weights in this experiment did not change until day 375. Later, the body weight of BMP-6-treated rats was 22% higher than those receiving E2 and 11% higher than in sham animals, but was not different from OVX rats (data not shown).

Autopsy did not reveal any treatment-related gross pathology. Organ weights were normal, only rats treated with E2 or E2 and BMP-6 had increased uterine weights (data not shown). Histopathological findings of heart, lung, liver, spleen, kidney, uterus, pancreas, esophagus, stomach, intestine, lymph nodes, bladder, brain, eyes, aorta, trachea, skeletal muscle, bone marrow ( sternum), and femur were normal. Beyond calcification of the tail at the site of BMP-6 injection no adverse effects were observed.

**FIGURE 7. Serum values of osteocalcin (Oc), C-telopeptides (Ctx), and osteoprotegerin (OPG).** Three months of BMP-6 (10 μg/kg) therapy increased the levels of Oc (A) and OPG (B) and decreased the levels of Ctx (C) in serum of aged rats 12 months following OVX; O, significantly different from OVX control rats (p < 0.05, ANOVA, Dunnett test).

**BMP-6 and E2 Do Not Act Synergistically on Increasing Bone Volume in OVX Rats**—Rats treated with E2 at 12 weeks following therapy had similar femur BMD values as OVX control animals (Table 2). When both BMP-6 and E2 were given to OVX rats, BMD values at 12 weeks increased by 14%. However, BMD in rats treated with E2 and BMP-6 was not different from
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**FIGURE 8.** A, decreased serum IL-6 levels 72 h following a single injection of BMP-6 (10 μg/kg); B, semiquantitative reverse transcriptase-PCR analyses showed reduced IL-6 and Gapdh expression in the spleen of rats treated for 7 days with BMP-6 (10 μg/kg/3 times/week) as compared with OVX rats.

**FIGURE 9.** In vivo hind limb BMD in aged rats initially treated with BMP-6 (10 μg/kg) for 3 months and then subdivided into groups that continued BMP-6 (10 μg/kg) or E2 (50 μg/kg/3 times/week) treatment for the following 4.5 months and the third group that discontinued the initial BMP-6 therapy. Rats that discontinued the BMP-6 therapy maintained the BMD gains until the 24th week, whereas rats continuing the BMP-6 therapy increased BMD values for another 8%. Rats treated with E2 after 12 weeks of initial therapy with BMP-6, maintained the gained BMD until the end of experiment at week 30. O, significantly different from sham group of animals (by ANOVA Dunnett test, \( p < 0.05 \)).

rats treated with BMP-6 alone (Table 2). E2 had no effect on cortical and trabecular thickness, whereas a combination of E2 and BMP-6 increased cortical thickness by 22% without an effect on trabecular thickness (Table 2, Fig. 4). These results suggest that E2 alone did not have an effect on the bone in aged OVX rats unless combined with BMP-6.

**Effects of BMP-6 on Femoral Gene Expression**—At three months following BMP-6 therapy the expression of Acrv1 and Bmpr1b, BMP receptors, as well as Smad5, a downstream molecule in the BMP signaling pathway was increased (Fig. 6). E2 reduced the expression of Acrv1 and Bmpr1b and had no effect on Smad5 (Fig. 6). Bmp2 and Bmp3 expression was increased following OVX and suppressed by both BMP-6 and E2 therapy (Fig. 6). On the other hand, OVX reduced the expression of Bmp6 in femurs, whereas E2 increased the Bmp6 expression (Fig. 6). Bmp4 expression was unchanged with both BMP-6 and E2 treatment. Furthermore, BMP-6 increased the expression of alkaline phosphatase (Akp2) and collagen type I (Colla1), reducing the expression of osteopontin (Fig. 6). Expression of BMP antagonists, chordin (Chrd) and cerberus (Cer1), was decreased in rat femurs treated with BMP-6. The expression of twisted gastrulation (Twsg1) was decreased following OVX and increased to normal levels following BMP-6 therapy (Fig. 6). On the contrary, the expression of noggin (Nog) was increased following OVX, BMP-6 therapy further increased noggin expression, whereas E2 had no effect (Fig. 6).

**Serum Biochemical Parameters**—BMP-6 treatment at a dose of 10 μg/kg/3 times/week increased the serum osteocalcin level, a bone formation marker, as compared with OVX animals, whereas E2 did not have an effect (Fig. 7A). Serum C-telopeptide values were lower in rats treated with BMP-6 indicating that BMP-6 suppressed the activity of osteoclasts (Fig. 7B). Furthermore, BMP-6 increased the OPG serum levels as compared with OVX animals (Fig. 7C), which may correspond to lower osteoclast numbers in BMP-6-treated rats (Fig. 5). BMP-6 may, thus, increase bone formation and suppress bone resorption, which may in part explain its anabolic bone effect.

**BMP-6 Suppresses Expression of IL-6 in Spleen and IL-6 Serum Levels**—BMP-6 decreased IL-6 serum levels 72 h following a single intravenous injection (Fig. 8A). Three BMP-6 injections reduced the expression of IL-6 in the spleen to undetectable values as compared with OVX rats (Fig. 8B) suggesting an additional mechanism by which BMP-6 might mediate the osteoclast production.

**Maintenance of Bone in OVX Rats Pre-treated by BMP-6**—To further explore whether newly formed bone following BMP-6 therapy was maintained, BMP-6 was given to aged OVX rats for 12 weeks and then discontinued. Animals were divided into three groups: untreated, treated with E2, and those continuing the BMP-6 therapy. BMP-6 was then monitored at 6, 12, 18, 24, and 30 weeks (Fig. 9). BMP-6 therapy throughout the treatment period of 7.5 months increased the hind limb BMD values as compared with both OVX and sham rats that lost about 4% of BMD from the beginning of treatment (Fig. 9). Hind limb BMD values in animals treated with BMP-6 were about 8% higher at 18 weeks than at 12 weeks, and about 14% higher as compared with OVX rats, whereas at 24 and 30 weeks BMD did not change any more (Fig. 9). In rats treated with E2 following the initial BMP-6 therapy BMD gains were maintained. Animals without any therapy maintained the BMD values until the 24th week and then showed a decline to values still 10% higher than in OVX control rats (Fig. 9). We suggest that animals that discontinued BMP-6 therapy maintained the BMD gains for another 12 weeks, whereas treatment with E2 maintained the BMD until the termination of the study. Ex vivo BMD values of the excised femur, tibia, and lumbar vertebrae were similar to the in vivo results (data not shown).
Less Frequently Administered Low BMP-6 Dose Is the Most Efficacious in Increasing Bone Volume in OVX Rats—In search for the most effective BMP-6 dose, aged OVX rats 18 months following OVX were administered BMP-6 less frequently at lower doses. Six weeks following the beginning of therapy, BMP-6-treated animals showed higher BMD values of hind limbs as compared with both OVX and sham animals. BMP-6 at a dose of 1 μg/kg 3 times/week was most effective in restoring BMD. O, significantly different from OVX, group of animals (by ANOVA Dunnett test, p < 0.05).

DISCUSSION

In these studies we show that systemically administered BMP-6 in aged OVX rats has a pronounced anabolic effect significantly improving the bone quality. Both in vivo and ex vivo bone densitometric measurements revealed that BMP-6 restores trabecular bone of the axial skeleton and the cortical bone, whereas only a partial restoration of the trabecular bone occurs in long bones. The maximal load, stiffness, and energy absorbed were increased about 3–4-fold in BMP-6-treated rats. Therapy with BMP-6 reduces serum C-telopeptide and IL-6 while increasing serum osteocalcin and osteoprotegerin levels, suggesting uncoupling of bone formation from bone resorption.

Not only hormones, like E₂ and parathyroid hormone (PTH), have different effects on the peripheral and axial skeleton. For example, mutations of the cartilage-derived morphogenetic protein-1 (CDMP-1 and GDF-5) cause Hunter-Thompson and Grebe chondrodysplasia (36, 37). Both diseases are characterized by severe shortening of the limbs and multiple defects of limb joints and the normal axial skeleton (36, 37). Grebe chondrodysplasia is caused by a point mutation in a CDMP-1 gene, whereas in Hunter-Thompson chondrodysplasia there is an insertion in the mature domain leading to non-functional CDMP-1 protein.

So far the PTH is the only approved anabolic bone agent that increases trabecular bone volume, but may decrease the cortical bone mass (38) and produce cortical resorptive tunnels (39). PTH in aged OVX rats restores bone volume by thickening the existing trabeculi without increasing the trabecular connectivity (40, 41), although not influencing the trabecular mechanical strength (42). PTH also improves rigidity and stiffness of bone, but makes them more brittle, with less elasticity and less deformation before fracture (43). BMP-6 improves the trabecular microarchitecture in aged OVX rats by increasing trabecular thickness, trabecular number, and in particular, trabecular connectivity. BMP-6 also increases elasticity and energy absorbed. After discontinuation of BMP-6 therapy animals maintained the BMD value for another 12 weeks. In comparative studies utilizing aged rats with a similar post-OVX time period, PTH withdrawal results in a loss of acquired BMD within 5 weeks (44). Unlike PTH, which circulates in serum, BMP-6 circulates when bone regenerates, like in patients with multiple bone fractures. This excludes other BMPs as potential circulating candidates in the regeneration of the skeleton including osteoporosis.

BMP-6 exerts its effects on bone via promoting bone formation and reducing bone resorption thereby providing a distinct means to accumulate bone tissue mass. No known therapeutic agent achieves both effects by in vivo systemic administration. The currently available resorption inhibitors (E₂ and related compounds, bisphosphonates and calcitonin) have little effect on bone formation over prolonged periods of time (45). It has been recently shown that leptin has a direct positive effect on osteoblastic differentiation of stromal cells, and may also modulate bone remodeling by inhibiting the expression of receptor activator of nuclear factor κB ligand (46). However, the systemic effect of exogenously administered leptin on BMD has never been demonstrated. BMP-6 administration increases bone formation, and in parallel increases OPG serum levels,

FIGURE 10. In vivo hind limb BMD of 26.5-month-old rats treated with low doses of BMP-6 for 1.5 and 18 months following OVX. Six weeks following the beginning of therapy, BMP-6-treated animals showed higher BMD values of hind limbs as compared with both OVX and sham animals. BMP-6 at a dose of 1 μg/kg 3 times/week was most effective in restoring BMD. O, significantly different from OVX, group of animals (by ANOVA Dunnett test, p < 0.05).
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...uncoupling the osteoblast from osteoclast activity, leading to bone gain at both trabecular and cortical bone compartments.

IL-6 as a single molecule plays an important role in the pathophysiology of postmenopausal osteoporosis (47). We therefore studied its availability in BMP-6-treated rats and found that BMP-6 decreases the level of IL-6 in serum and more importantly its expression in the spleen, as one of important organs for IL-6 synthesis. Recently, we and others have also shown that BMP-6 significantly reduces IL-6 production from marrow stroma and the gastrointestinal system in rats (48, 49). E2 can also inhibit IL-6 expression via an E2 receptor-mediated transcriptional activity (49, 50), which is supported by the fact that E2 depletion results in IL-6 up-regulation (51).

We and others (10) have suggested that E2-induced osteogenesis is associated with increased levels of Bmp6 mRNA in mouse femurs, reflecting the emergence of clusters of BMP-6 positive stromal cells adjacent to active bone formation surface. We show that addition of E2 to BMP-6 therapy has no synergistic effect on BMD and bone volume, which, may at least in part, suggest a BMP-6 requirement for E2 bone activity. On the other hand, E2 maintained the gained BMD after discontinuation of BMP-6 prolonged therapy. It was suggested that E2 specifically up-regulates Bmp6 mRNA in osteoblastic cell lines (8). BMP-6 and E2 cross-talk might be Wnt-mediated because BMPs are essential for Wnt-induced osteoblast differentiation (52), and Wnt signaling is also critical for the effects of E2 on bone (53). Another example involves the transient up-regulation of Chfα-1 in response to BMP (54) and its affection by the E2-OPG pathway (54). It has been also shown that E2 prevents bone loss through a transforming growth factor-β-dependent mechanism, and that transforming growth factor-β signaling in T cells preserves bone homeostasis by blunting the T cell activation (56, 57). However, a systemic effect of transforming growth factor-β on bone in animal models of osteopenia has not yet been studied.

Apart from BMPs, several other factors important for osteogenesis have been investigated in animal models of osteopenia. Basic fibroblast growth factor improves trabecular connectivity in aged OVX rats by increasing osteoid accumulation on trabecular surfaces and between perforated trabecular rods (41). Like BMP-6, basic fibroblast growth factor improves trabecular connectivity (58, 59) but, as a general mitogen with serious adverse side effects, its systemic administration is not practical (42, 58). Human growth hormone and insulin-like growth factor I in a rat model of osteopenia increase bone size and mineral content but decrease BMD (59). Their effects on bone are currently investigated in clinical trials (59).

Numerous studies have demonstrated that BMPs are involved in local bone formation via stimulating proliferation and chemotaxis of bone progenitor cells, angiogenesis during development and bone repair, and endochondral osteogenesis by stimulating osteogenic compartment of bone stromal cells (2, 3, 60–63). It was never demonstrated that BMPs systematically targets bone marrow stem cells. Based on findings that BMP-6 successfully promotes restoration of bone in young and aged osteopenic rats, we suggest that it targets bone marrow stem cells that do not loose self-renewal and differentiation characteristics by aging. However, it has been demonstrated that the amount of stem cells decreases by advanced age in both rats and men (64).

Apart from bone formation at the site of injection no side effects were observed by long standing systemic administration of BMP-6. Of particular interest is the fact that systemically administered BMP-6 influences bone formation at endosteal, periosteal, and trabecular bone compartments.

Among genes analyzed Bmp3 is of particular interest. Its up-regulation following OVX and suppression by BMP-6 might be important because it has been suggested that BMP-3 is a negative regulator of in vitro and in vivo osteogenesis (65). BMP-3 inhibited alkaline phosphatase production induced by BMP-2 in vitro and BMP-3 knock-out mice had about twice the trabecular bone volume as wild-type controls (65). We have previously demonstrated that changes in Bmp3 and Acvr1 expression are reduced by mechanical loading that enables the induction of cartilage in a bone chamber (66). Treatment by BMP-6 might have inhibited Bmp3 expression and activity by formation of inactive BMP-3 heterodimers, but also inhibiting the effect of the BMP-3 homodimer. Collectively, these results show, for the first time, that systemically administered BMP-6 restores bone volume and quality in osteoporotic rats making it a feasible candidate for treating osteoporosis in patients with pronounced bone loss.

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