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Regulation of Bone Matrix Protein Expression and Induction of Differentiation of Human Osteoblasts and Human Bone Marrow Stromal Cells by Bone Morphogenetic Protein-2

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Abstract We have examined the effects of BMP-2 on the expression of bone matrix proteins in both human bone marrow stromal cells (HBMSC) and human osteoblasts (HOB) and their proliferation and mineralization. Both HBMSC and HOB express BMP-2/-4 type I and type II receptors. Treatment of these two cell types with BMP-2 for 4 weeks in the presence of β-glycerophosphate and ascorbic acid results in mineralization of their matrix. BMP-2 increases the mRNA level and activities of alkaline phosphatase and elevates the mRNA levels and protein synthesis of osteopontin, bone sialoprotein, osteocalcin, and α1(I) collagen in both cell types. Whereas the mRNA level of decorin is increased, the mRNA concentration of biglycan is not altered by BMP-2. No effect on osteonectin is observed. The effect of BMP-2 on bone matrix protein expression is dose dependent from 25 to 100 ng/ml and is evident after 1–7 days treatment. In the presence of BMP-2, proliferation of HBMSC and HOB is decreased under either serum-free condition or in the presence of serum. Thus, BMP-2 has profound effects on the proliferation, expression of most of the bone matrix proteins and the mineralization of both relatively immature human bone marrow stromal preosteoblasts and mature human osteoblasts. J. Cell. Biochem. 67:386–398, 1997. © 1997 Wiley-Liss, Inc.

Key words: human osteoblasts; human bone marrow stromal cells; alkaline phosphatase; osteopontin; bone sialoprotein; osteonectin; decorin; biglycan; type I collagen; osteocalcin; mineralization

INTRODUCTION

Bone remodeling is the result of a well-regulated sequential activation of osteoclasts and osteoblasts within basic multicellular units resulting in a cyclical succession of bone resorption and bone formation. Bone formation is a complex process that involves proliferation of osteoprogenitor cells, migration of these cells to bone surface, and their differentiation into osteoblasts, resulting in the secretion of abundant bone matrix proteins which coordinate mineralization process. Although control mechanisms which orchestrate the proliferation, migration and differentiation of osteoprogenitor cells are still conjectural, recent evidence implicates bone morphogenetic proteins (BMPs) as essential in this regard.

BMPs are members of the TGF-β superfamily. Currently, at least seven BMPs belong to this superfamily including BMP-2 to BMP-8 [1,2]. BMPs are characterized by their ability to induce endochondral bone formation when implanted intramuscularly or subcutaneously in rats [3–6]. BMP-2 may also play an important role in early limb development since it appears in precartilaginous mesenchyme during early developmental stages [7]. BMP-5, which is also functional during embryonic development, has been implicated conditioning fracture healing [8].

Several studies indicate that BMPs induce differentiation of a variety of rat and mouse mesenchymal cells into osteoblast phenotypes, since cells subjected to these morphogens respond with increments in alkaline phosphatase

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activity and synthesis of osteocalcin and collagen [9–14]. Moreover, BMPs stimulate matrix mineralization and accelerate differentiation of rodent osteoblastic cells [4,5,15–18]. In humans, BMP-3 increases alkaline phosphatase activity, osteocalcin and collagen type I synthesis in bone marrow stromal cells [19], while BMP-2 increases the clustering and colony formation in these cells [20]. Although BMPs share structural homology and induce bone formation in vivo, their potencies and specificities differ [10,21]. Conflicting results are also reported on the effect of BMP on the proliferation and expression of collagen in osteoblasts [10,11,21–27]. Furthermore, BMP-2, but neither BMP-4 nor BMP-6, stimulates the chemotactic migration of human osteoblastic cells [28]. Although many reports have shown the effects of BMPs on animal cell model systems there is a lack of information detailing the effects of BMP-2 on normal human osteoblastic cells and only the regulation of some of the bone matrix proteins (mainly alkaline phosphatase, collagen, and osteocalcin) are analyzed. Because of the future potential use of BMPs in managing human bone diseases, there is a continued need to analyze the responses of normal human osteoblasts to BMPs. Accordingly, we pursued studies designed to establish the patterns of expression of bone matrix proteins after exposure to BMP-2; and to compare the responses to BMP-2 of mature normal human osteoblasts and human bone marrow stromal "preosteoblastic" cells.

MATERIALS AND METHODS

Materials

Recombinant human BMP-2, BMP-4, BMP-6, and BMP-7 were generously provided by Genetics Institute, Cambridge, MA. Sodium ^{125}iodide (100 mCi/ml) was from ICN Pharmaceuticals (Costa Mesa, CA) and disuccinimidyl suberate (DSS) was from Pierce Chemical (Rockford, IL). Ficoll/Hypaque (Histopaque-1077, 1.077 g/cm^3), Dulbecco's Modified Eagle's Medium (DMEM), 1 : 1 mixture of DMEM and Ham's F-12 medium (DME/F-12), α-Minimum Essential Medium (α-MEM), trypsin/EDTA, and fetal bovine serum (FBS) were obtained from Sigma Chemical Co. (St. Louis, MO). Mini RiboSep mRNA isolation kit was obtained from Collaborative Biomedical Products (Bedford, MA). cDNA encoding human alkaline phosphatase was kindly provided by Dr. Paula Henthorn (University of Pennsylvania, Philadelphia, PA). cDNAs for human osteopontin, bone sialoprotein, decorin, biglycan, and osteonectin were generously supplied by Dr. Marian Young (National Institutes of Health, Bethesda, MD). cDNA for osteocalcin and cDNAs for human α1(I) and α1(II) collagen was generously provided by Dr. John Wozney (Genetics Institute, Cambridge, MA) and by Dr. William Park (Washington University, St. Louis, MO), respectively. Lipoprotein lipase cDNA was kindly provided by Dr. Jeffrey M. Gimble (Oklahoma Medical Research Foundation, Oklahoma City, OK). cDNA for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Clontech Laboratories (Palo Alto, CA). Megaprime labeling kit, Hyperfilms, ECL Western blotting analysis system, and 5′-[α^{32}P]-dCTP (3000 Ci/mmol, aqueous solution) were from Amersham (Arlington Heights, IL). 100 × Denhardt's solution, salmon testes DNA, and 50% dextran sulfate were from 5 Prime → 3 Prime (Boulder, CO). Formamide was from Oncor (Gaithersburg, MD). Rabbit anti-human osteopontin (LF-7), anti-human bone sialoprotein (LF-6), and anti-α1(I) collagen C-telopeptide (LF-67) antisera were kindly provided by Dr. Larry Fisher (National Institutes of Health, Bethesda, MD). Reagents for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were from BioRad Laboratories (Hercules, CA). Immobilon-P membranes were from Millipore Co. (Bedford, MA). The osteocalcin radioimmunoassay (RIA) kit was obtained from INCSTAR (Still Water, MN). The rest of chemicals were reagent grades and obtained from Sigma Chemical Co.

Cell Cultures

Human ribs were obtained from patients (6 males and 4 females) who were undergoing surgery. Their ages ranged from 22 to 81 years (mean ± SEM, 48.5 ± 9.4) for the male patients and from 35 to 82 years (mean ± SEM, 55.5 ± 10.0) for the female patients. Human osteoblasts (HOB) were established from trabecular bone according to procedures we have published previously [29]. Briefly, trabecular bone chips were removed from the inner surface of ribs by scraping with curette and were washed and treated with 250 U/ml collagenase and 1 μg/ml DNase in DME/F-12 for 2 h. Following digestion, bone chips were washed extensively and incubated in DME/F-12 medium containing 10% heat-inactivated fetal bovine serum

(HIFBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml ascorbic acid. After 3–4 weeks, outgrowing HOB were subcultured.

Human bone marrow stromal cells (HBMSC) were isolated as described previously [29, 30]. Briefly, human bone marrow cells were harvested by rinsing the marrow cavities of the ribs with DME/F-12 medium containing 10 U/ml heparin and 1 µg/ml DNase. The cells were pelleted, resuspended in α-MEM containing 10% HIFBS, and separated by centrifugation, using Ficoll/Hypaque density gradient. The cells in the interphase were collected, washed, and incubated in α-MEM containing 10% HIFBS. At confluence, HBMSC were subcultured. All assays were performed on first passaged cells of HBMSC and HOB after primary plating. No difference in cellular response was observed, on the basis of age or sex.

^{125}I Labeling of BMP-2 and BMP-4

Recombinant human BMP-2 and BMP-4 were radioiodinated by the chloramine T procedure [31]. Briefly, 1 µg of BMP in 20 µl of 1.5 M sodium phosphate buffer, pH 7.5, was incubated at room temperature with 1 mCi (10 µl) of Na ^{125}I and freshly prepared chloramine T (100 µg/ml, 10 µl) for 2 min. Addition of chloramine T was repeated twice but with shortened incubation period, i.e., 1.5 min and 1 min, respectively. Iodination was stopped by the addition of 40 µl of 50 mM N-acetyltyrosine and 400 µl of 60 mM KI. Labeled BMP was purified by Sephadex G-50 chromatography (13 ml column) that had been equilibrated with 15 mM phosphate buffer, pH 7.4, containing 75 mM NaCl and 0.1% bovine serum albumin (BSA). Peak fractions were pooled, aliquoted and stored at −20°C until use.

BMP Receptor Binding and Affinity Cross-linking

Confluent cultures of HBMSC and HOB in 6-well plates were washed with 2 ml/well of binding buffer (50 mM Hepes, pH 7.4, containing 128 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, and 1.2 mM $Ca.Cl_2$) followed by incubation in 0.8 ml binding buffer containing 0.2% BSA and 100-fold of indicated unlabeled competitor for 5 min. ^{125}I-labeled BMP-2 or BMP-4 (15 ng/20 µl/well) was then added and incubation was proceeded for 2–3 h at 4°C on a rocker platform. Cells were washed with 1 ml/well binding buffer twice, and cross-linking was performed at 4°C for 20 min in 0.8 ml/well binding buffer containing 10 µl of 27 mM DSS in dimethylsulfoxide (DMSO). Cells were then washed with 0.8 ml detachment buffer (10 mM Tris, pH 7.4, 0.25 M sucrose, 1 mM EDTA, and 0.3 mM PMSF) and scrapped into microfuge tubes. Cell pellets were extracted with 48 µl/tube of solubilization buffer (10 mM Tris, pH 7.4, 125 mM NaCl, 1 mM EDTA, and 1% Triton X-100) for 1 h and microfuged. Supernatants were diluted with 2× sample buffer and SDS–PAGE performed followed by autoradiography using Hyperfilm.

Cell Proliferation Assay

Proliferation was estimated by [^{3}H]-thymidine incorporation. HBMSC and HOB were seeded in 24-well tissue culture plates at a density of 20,000/well. One day later, cells were washed serum free and incubated in α-MEM for 24 h. Cells were then treated for another 24 h with or without BMP-2 (100 ng/ml) in fresh α-MEM containing 2 µCi/well of [methyl-^{3}H]-thymidine (20 Ci/mmol, 1 mCi/ml) and 10% BSA or 10% HIFBS. The quantity of isotope incorporated in proliferating cells was measured after trichloroacetic acid (TCA) precipitation and twice ethanol washing as previously described [30].

Protein Measurements

Protein in the cell layer was measured using either the BioRad protein assay kit or the Bio-Rad DC protein assay kit and BSA as standard according to the protocols supplied by the manufacturer.

Alkaline Phosphatase Assay

Cells in 24-well plates were treated with or without BMP-2 at the indicated concentration for predetermined time intervals. Cell layers were washed three times with TBS (50 mM Tris, pH 7.4, and 0.15 M NaCl) and stored at −20°C until assayed for alkaline phosphatase activities. For assay, the cell layer from each well was scraped into 0.5 ml of 50 mM Tris, pH 7.4, and sonicated with a Fisher Dismembranetor (30–40% of maximum strength). The alkaline phosphatase activities in the sonicate were measured using p-nitrophenyl phosphate (3 mM final concentration) as substrate in 0.7 M 2-amino-2-methyl-1-propanol, pH 10.3, and 6.7

mM $MgCl_2$. Protein concentration in the sonicate was measured using the BioRad protein assay kit. Enzyme activities were determined by measuring absorption at 405 nm and expressed as p-nitrophenol produced in nmol/min/mg protein.

Osteocalcin Assay

Confluent cells in 24-well plates were treated with or without BMP-2 (100 ng/ml) for 7 days with one feeding and fresh BMP-2 added on day 4. Cells were washed with serum-free medium and incubated in α-MEM containing 0.05% BSA. During this period, cells continued to receive either nothing or BMP-2 treatment with or without $1,25(OH)_2D_3$ (20 nM) as indicated. The conditioned medium was harvested after 90-h incubation and stored at −80°C until assay for osteocalcin content by RIA. Cell layers were washed three times with PBS and dissolved in 0.5 ml of 0.3 N NaOH for protein measurement using Bio-Rad protein assay kit as described above. Osteocalcin concentration in the conditioned medium was expressed as ng/100 μg cellular protein.

Western Blot Analysis for Type I Collagen, Osteopontin, and Bone Sialoprotein

Confluent cultures of HOB or HBMSC in p-100 culture dishes were treated with or without BMP-2 (100 ng/ml) for indicated period of time. Cell layer was washed three times with phosphate-buffered saline (PBS) and extracted with 1 ml of 0.5% Triton X-100 in 10 mM Hepes, pH 7.4 containing 150 mM NaCl, 0.02% sodium azide and protease inhibitors (2 mM EDTA, 2 mM EGTA, 1 mM phenanthroline, 0.12 TIU/ml aprotinin, 100 μg/ml TPCK, 40 μg/ml each of TLCK and bestatin, 50 μg/ml benzamidine, 10 μg/ml each of leupeptin, pepstatin A, antipain, soybean trypsin inhibitor, chymostatin, and iodoacetamide). The protein concentration in the extract was measured using a BioRad DC kit. Samples containing equal amount of proteins were applied to a 7.5% SDS–PAGE. After electrophoresis, proteins were blotted to an Immobilon-P membrane. Membranes were incubated with specific rabbit polyclonal antiserum (1 : 1,000) for osteopontin (LF-7), bone sialoprotein (LF-6), or type I collagen (LF-67), followed by horseradish peroxidase-conjugated anti-rabbit antibodies (1 : 5,000). Matrix protein bands were visualized by enhanced chemiluminescence using the ECL kit.

Northern Blot Analysis

Cells in p-150 culture dishes were treated with or without BMP-2 at the indicated concentrations for predetermined periods of time. Poly A+ enriched RNA preparation was isolated using Mini RiboSep mRNA isolation kit according to manufacturer's instructions. mRNAs were separated on formaldehyde-containing agarose gels and transferred to nylon membranes as previously described [29,32]. The membranes were prehybridized with 40% formamide, 5× SSC (20× SSC contains 3 M sodium chloride, 0.3 M sodium citrate, pH 7.0), 10 mM Tris–HCl, pH 7.4, 1.25× Denhardt solution and 125 μg/ml salmon testes DNA at 42°C overnight, followed by hybridization in the same solution, but with 10% dextran sulfate and [^{32}P]-cDNA probe to human bone matrix protein overnight at 42°C. [^{32}P]-cDNAs were prepared using the Megaprime Labeling kit and 5′-[α^{32}P]-dCTP according to the manufacturer's instructions. Following two washes with 2× SSC and 0.1% SDS for 15 min at room temperature and two washes with 0.2× SSC and 0.1% SDS at 52°C, the membranes were exposed to Hyperfilms at −70°C. To reprobe with another [^{32}P]-cDNA, the membranes were stripped in boiling 20 mM Tris–HCl buffer, pH 7.4, for 15 min, followed by prehybridization and hybridization. To analyze the intensity of each band on the autoradiograms, the X-ray films were subjected to image analyses using ISS SepraScan 2001 (Integrated Separation Systems, Natick, MA). The intensity of each band was normalized to GAPDH.

von Kossa Staining

For experiments designed to analyze mineralization potential, cells in 6-well culture plates were treated with or without BMP-2 (100 ng/ml) in the presence of ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM) for 4 weeks. The cells were then washed three times with TBS, fixed with 10% neutral formalin solution for 5 min, and rinsed with deionized water. Following the addition of 5% silver nitrate solution, the wells were exposed to ultraviolet (UV) light for 1 h. The plates were rinsed with deionized water and the residual silver nitrate neutralized by 5% sodium thiosulfate.

Statistics

Statistical analyses were performed using either analysis of variance (ANOVA) or Student's t-test as indicated.

RESULTS

Identification of BMP-2/-4 Receptors on Human Bone Marrow Stromal Cells and Human Osteoblasts

Since it has been demonstrated that BMP-2 and BMP-4 bind to the same receptors [33], we analyzed the types of BMP-2/-4 receptors present on HBMSC and HOB by employing affinity cross-linking using either ^{125}I-labeled BMP-4 or ^{125}I-labeled BMP-2 as ligand. As shown in Figure 1, HBMSC and HOB expressed both type I and type II receptors for ^{125}I-labeled BMP-4. In addition, a specific band at 44 kDa was also observed and could be competed out by excess amount of BMP-2 or BMP-4. At present, we do not know the identity of this band, although a BMP-7 receptor of 34 kDa has been reported in human osteosarcoma cells TE85 [34]. The combined molecular weight for BMP-4 and type I receptor was approximately 68 kDa, while that of BMP-4 and type II receptor was 86 kDa. Only BMP-2 and BMP-4 competed with ^{125}I-labeled BMP-4, whereas neither TGF-β (Fig. 1) nor IGF I (data not shown) at 100-fold excess concentration was effective. Similar results were obtained when ^{125}I-labeled BMP-2 was used as the ligand, and in separate experiments, BMP-6 and BMP-7 were also found to compete with ^{125}I-labeled BMP-4 for the receptors (data not shown).

Effect of BMP-2 on the Proliferation and Mineralization of Human Bone Marrow Stromal Cells and Human Osteoblasts

Treatment of HBMSC or HOB with BMP-2 for 1 day decreased cellular proliferation in the presence of serum or in serum free conditions (Fig. 2A,B). The inhibition, however, was less profound in the presence of serum. Similar results were also obtained after 2 days treatment

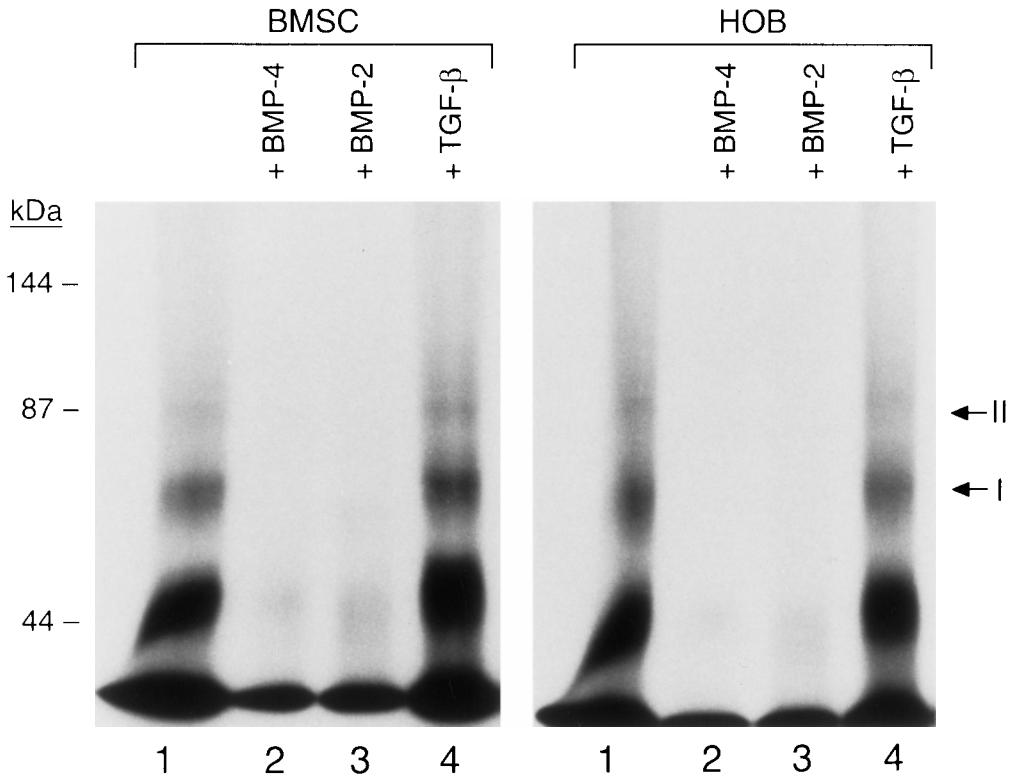

Fig. 1. Analysis of BMP-2/-4 receptors on HBMSC and HOB. Cells were incubated with ^{125}I-BMP-4 in the presence or absence of 100 fold of indicated competitor. Bound ^{125}I-BMP-4 was cross-linked to receptor by disuccinimidyl suberate and separated on SDS-PAGE. **Lane 1,** ^{125}I-BMP-4 alone; **lane 2,** ^{125}I-BMP-4 + BMP-4; **lane 3,** ^{125}I-BMP-4 + BMP-2; **lane 4,** ^{125}I-BMP-4 + TGF-β. I indicates type I receptor; II indicates type II receptor. The band at bottom was free ^{125}I-BMP-4.

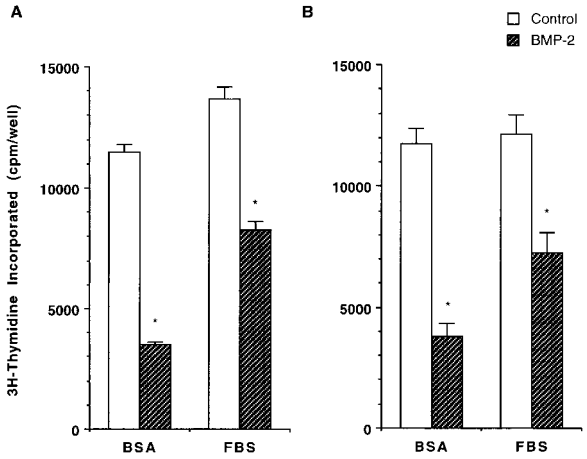

Fig. 2. Effect of BMP-2 on cell proliferation in HBMSC and HOB. HBMSC (**A**) and HOB (**B**) were seeded in 24-well plates. One day later, cells were washed with serum-free α-MEM and incubated in this medium for another 24 h, followed by treatment without (control) or with BMP-2 (100 ng/ml) in α-MEM containing either 10% BSA or 10% HIFBS and 2 μCi of [^{3}H]thymidine for 24 h. [^{3}H]Thymidine incorporation was determined after 10% TCA precipitation and ethanol washing as described in Materials and Methods. Data presented are mean ±SEM of six wells. The experiments were repeated three times and only the representative data are presented. *$P < 0.001$ compared to the corresponding control value by Student's t-test. White column, control; shaded column, BMP-2-treated cultures.

(data not shown). In the presence of β-glycerophosphate and ascorbic acid, the matrix of HBMSC was mineralized by BMP-2 (100 ng/ml) after 4 weeks of exposure (Fig. 3). No mineralization was observed in the control cultures of HBMSC. Similarly, the mineralization of the matrix of HOB was enhanced by BMP-2 (Fig. 3).

Regulation of Alkaline Phosphatase by BMP-2 in Human Bone Marrow Stromal Cells and Human Osteoblasts

BMP-2 at 100 ng/ml significantly increased alkaline phosphatase activity in HBMSC after 3 days exposure and reached 4-fold after 7 days (Fig. 4A). BMP-2 also increased alkaline phosphatase activity in HOB after 3 days treatment (Fig. 4B). The stimulation was dose dependent and required 50–100 ng/ml BMP-2 (Fig. 4C,D). HBMSC also expressed a higher level of basal alkaline phosphatase activity than that of HOB, consistent with the cell commitment to differentiate into osteoblast phenotype. Northern blot analysis indicated that the steady-state mRNA level for alkaline phosphatase was increased after 1–7 days exposure in both HBMSC and HOB (data not shown).

Effect of BMP-2 on the Expression of Type I Collagen in Human Bone Marrow Stromal Cells and Human Osteoblasts

BMP-2 increased α1(I) collagen mRNA in HOB after 4 days exposure at 100 ng/ml (Fig. 5A). BMP-2 also increased the steady-state mRNA levels of type I collagen in HBMSC in a time- and dose-dependent manner. Relatively low stimulation (30%) was observed during the first few days of exposure to BMP-2 and the stimulation was more profound after 7 days exposure (data not shown). The increase in the steady-state mRNA levels of type I collagen in HBMSC by BMP-2 was effective at 25 ng/ml and higher at 100 ng/ml (Fig. 5B). Western blot analysis indicated that the protein level of type I collagen was increased by 100 ng/ml BMP-2 in both cell types (data not shown).

Effect of BMP-2 on the Expression of Osteopontin and Bone Sialoprotein in Human Bone Marrow Stromal Cells and Human Osteoblasts

BMP-2 increased the steady-state mRNA levels of osteopontin (OPN) in HBMSC (Fig. 6A). The stimulation was effective after one day treatment and continued over 3 days. In another experiment, stimulation was maintained after 7 days exposure (data not shown). Similarly, BMP-2 increased the OPN mRNA level in HOB, although the increment was not as large as that observed in HBMSC (Fig. 6B). The stimulation of osteopontin by BMP-2 in both cell types was also evident at the protein level (Fig. 6C). Several species of OPN were recognized ranging in molecular weight from 72 kDa to 42 kDa. All of these variants were increased by BMP-2 treatment. In a comparable fashion, BMP-2 increased the steady-state mRNA levels of bone sialoprotein (BSP) in HBMSC (Fig. 7A,B) and HOB (Fig. 7C). The stimulation which was observed after 3 days exposure and was effective at 25 ng/ml and associated with increments in bone sialoprotein content of the cell layer of HBMSC and HOB (Fig. 7D).

Effect of BMP-2 on the Expression of Osteocalcin and Osteonectin in Human Bone Marrow Stromal Cells and Human Osteoblasts

BMP-2 also increased the expression of osteocalcin at steady-state mRNA level (data not shown) and protein level (Table I). The induction of the synthesis and secretion of osteocalcin in both HBMSC and HOB was effective both

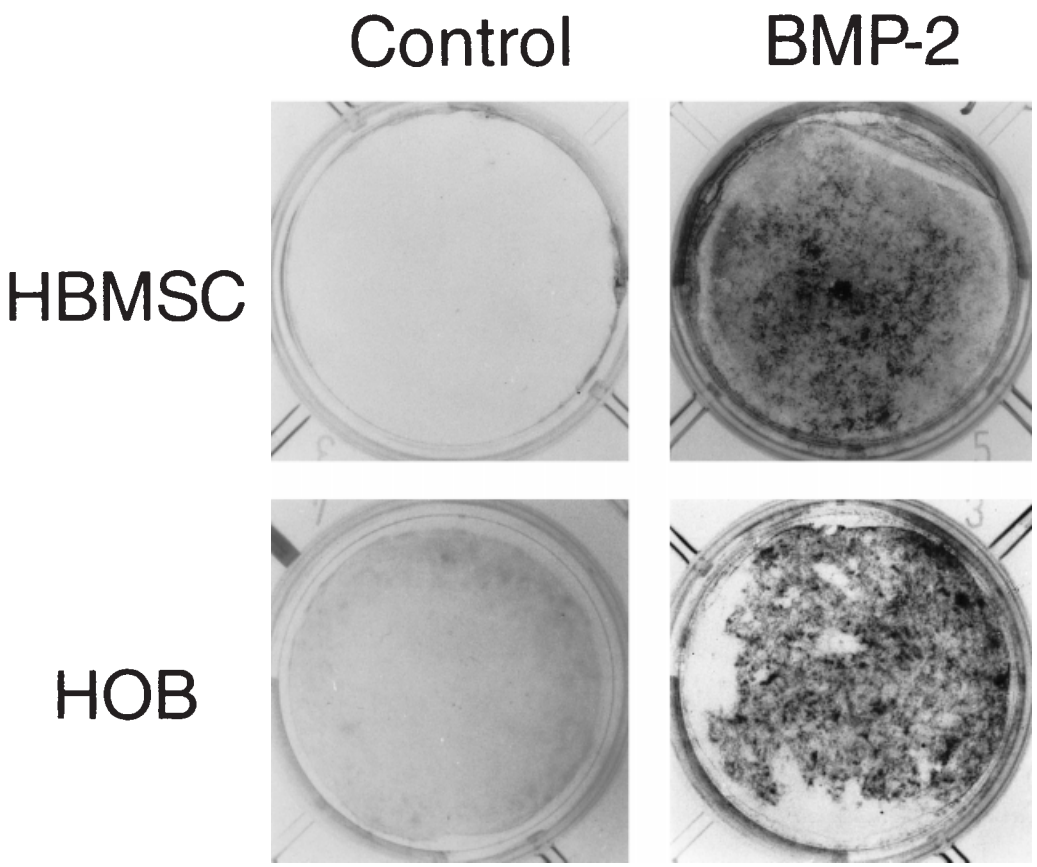

Fig. 3. Effect of BMP-2 on the mineralization of HBMSC and HOB. Cells were treated without (control) or with BMP-2 (100 ng/ml) in the presence of ascorbic acid and β-glycerophosphate for 4 weeks. Mineralization of the matrix was detected by von Kossa staining as described in Materials and Methods.

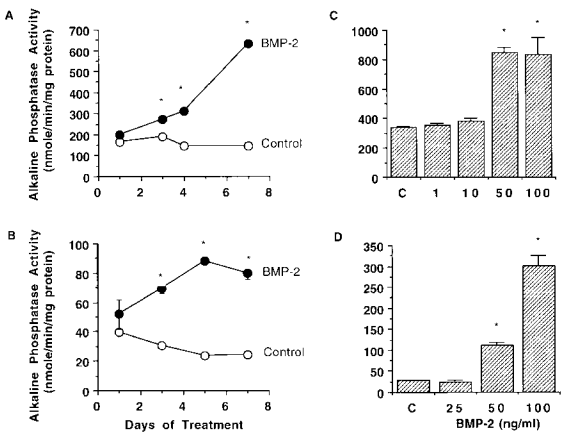

Fig. 4. Effect of BMP-2 on the alkaline phosphatase activity in HBMSC (**A,C**) and HOB (**B,D**). Confluent cells were treated without (control) or with BMP-2 at 100 ng/ml and harvested at indicated time (**A,B**) or treated at the indicated concentration for 4 days (**C,D**). Alkaline phosphatase activities in the cell layer were measured and normalized to the concentration of cellular protein as described in Materials and Methods. Each point (mean ±SEM) was derived from triplicate cultures. ○, control; ●, BMP-2. C, Control. *$P < 0.001$ compared to the corresponding control value by ANOVA.

in the absence and presence of $1,25(OH)_2D_3$ (Table 1). In the absence of $1,25(OH)_2D_3$, no osteocalcin protein was detected in the conditioned medium of control culture while BMP-2 induced the synthesis and secretion to almost the level of 20 nM $1,25(OH)_2D_3$-treated control cultures in HBMSC and to approximately half of that of $1,25(OH)_2D_3$-treated cultures in HOB. In the presence of $1,25(OH)_2D_3$, BMP-2 further increased osteocalcin protein levels to two- to three-fold of the control values in both HBMSC and HOB. By contrast, BMP-2 has very little effect on the osteonectin mRNA level or the secreted osteonectin in the conditioned medium [35] in either HBMSC or HOB (data not shown).

Effect of BMP-2 on the Steady-State mRNA Level of Small Proteoglycans in Human Bone Marrow Stromal Cells and Human Osteoblasts

The steady-state mRNA level for decorin (DCN) in HBMSC was increased after 3 days of 100 ng/ml BMP exposure (Fig. 8A), with a maxi-

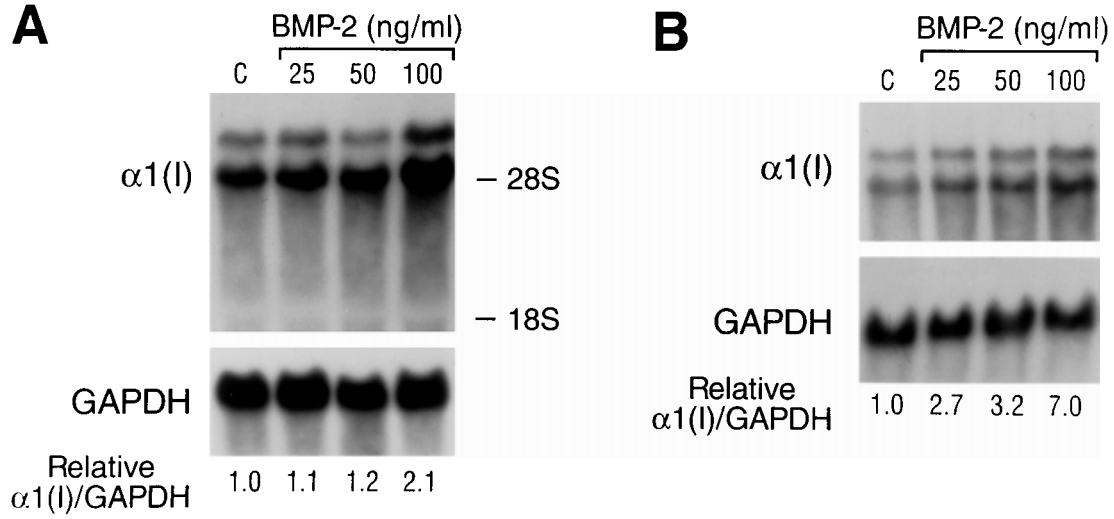

Fig. 5. Effect of BMP-2 on the expression of type I collagen in HBMSC and HOB. HOB (**A**) and HBMSC (**B**) were treated without (Control) or with indicated concentration of BMP-2 for 4 days. mRNA-enriched RNA preparations were isolated and Northern blot analysis performed as described in Materials and Methods. Membranes were probed with ^{32}P-labeled cDNA for human α1(I) collagen, followed by ^{32}P-labeled cDNA for human GAPDH and the relative α1(I) collagen mRNA concentration normalized with the GAPDH level. The relative α1(I) collagen mRNA level for the Control culture was defined as 1.0. Each illustration shown here is a representative of two experiments performed on cells derived from different patients. C, Control.

mum response noted at a concentration of 25 ng/ml of HBMSC (Fig. 8B). Similarly, BMP-2 increased decorin mRNA level in HOB, although 100 ng/ml was required (Fig. 8C). By contrast, BMP-2 was relatively ineffective on the biglycan (BGN) mRNA levels in both HBMSC and HOB (data not shown).

DISCUSSION

It has been suggested that the effect of BMP on osteoblast proliferation varies depending on the degree of cell differentiation. Thus, BMP-2 stimulated the proliferation of less differentiated calvaria-derived rat osteoblast-like cells, ROB-C26 and CFK1 osseous cells, but inhibited that of more differentiated ROB-C20 [11,24]. Our data did not support this hypothesis, but rather that BMP-2 inhibited the proliferation of both relatively immature and more mature normal human osteoblastic cells. Moreover, BMP-2 failed to affect the proliferation of MC3T3-E1 cells, mouse stromal cell line W-20-17, and SV40 large T antigen transfected normal human osteoblast-like cell line HOBIT, regardless of their differentiation statuses [21–23,26,27]. Thus, the accumulated data emphasize that the effect of BMP-2 on cell proliferation is dependent on the cell type.

Because BMP-2 has been shown to induce differentiation of mesenchymal cells into adipocytes and chondrocytes in addition to osteoblasts in vitro and in vivo [4,5,9,10,36], we considered the possibility that our cultures were contaminated with cells of adipocytic and/or chondrocytic lineage in response to BMP-2 stimulation. No adipocyte was detected in both HBMSC and HOB cultures under our experimental conditions by using three criteria, namely morphology, oil red O staining, and lipoprotein lipase mRNA expression (data not shown). Chondrocytes were also absent in our cultures as determined by the lack of α1(II) mRNA expression and the absence of Alcian blue staining (data not shown).

BMP-2 increased the expression of alkaline phosphatase, type I collagen, osteopontin, bone sialoprotein, osteocalcin, and decorin in both HBMSC and HOB. The regulation of these matrix proteins with the exception of osteopontin required exposure to BMP-2 for 3 days or longer in both cell types. The relative potencies, however, differed between these two cells. In general, the dose of BMP-2 needed for maximum stimulation was usually lower (e.g., alkaline phosphatase, type I collagen, bone sialoprotein, and decorin) and the fold of stimulation was often higher (e.g. type I collagen and osteopontin) in HBMSC than in HOB. Thus, BMP-2 is more potent in inducing the differentiation of relatively immature preosteoblastic cells and

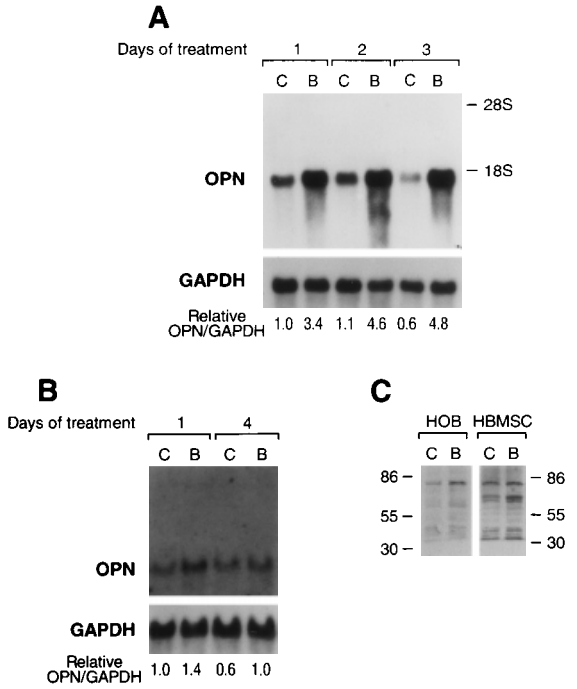

Fig. 6. Effect of BMP-2 on the expression of osteopontin in HBMSC and HOB. HBMSC (**A**) and HOB (**B**) were treated without (control) or with BMP-2 (100 ng/ml) for indicated period of time. mRNA-enriched RNA preparations were isolated and Northern blot analysis performed as described in Materials and Methods. Membranes were probed with ^{32}P-labeled cDNA for human osteopontin (OPN) followed by ^{32}P-labeled cDNA for human GAPDH. The relative OPN mRNA concentration on the autoradiograms was analyzed by densitometer and was normalized with the GAPDH level. The ratio of OPN/GAPDH for the Control culture after 1 day treatment was defined as 1.0. **C:** Protein analysis of osteopontin. Cells were treated without or with BMP-2 for 7 days. Cell layers were extracted and Western blot analysis performed as described in Materials and Methods. Membranes were probed with rabbit anti-osteopontin antiserum and visualized by enhanced chemiluminescence. Each illustration shown here is a representative of two experiments performed on cells derived from different patients. C, control; B, BMP-2.

the effectiveness is decreased once the cells become more differentiated.

The stimulation of alkaline phosphatase activity and osteocalcin expression by BMP-2 has also been well documented in a variety of osteoblastic cell systems [10,12,13,17–19,21–26,36–38]. We extended these observations to include normal human bone marrow stromal cells and normal human osteoblasts. The increase in the expression of alkaline phosphatase was consistent with the mineralization effect of BMP-2 on HBSMC and HOB. Osteopontin, bone sialoprotein, and osteocalcin have been implicated to be essential for modulating the matrix mineralization process. Their expression correlates with matrix mineralization and their concentration is high in mineralized matrix of newly formed bone [39–43]. Bone sialoprotein can serve as "nucleator" for hydroxyapatite formation [44] and stimulates the differentiation of preosteoblasts into "calcifying" osteoblasts [45], whereas osteopontin and osteocalcin modulate the elongation and growth of hydroxyapatite [46,47]. Recently Aubin et al. recorded that knock-out mice lacking bone sialoprotein expression have alterations in bone remodeling [48]. Consistent with the mineralization effect of BMP-2 on HBMSC and HOB, the expression of osteopontin, osteocalcin, and bone sialoprotein was increased by BMP-2 in these two cells.

The role of proteoglycans in the mineralization process is still ill-defined, although decorin has been shown to bind to specific sites on collagen and to regulate the fibril length of collagen [49]. No information is available regarding the effect of BMP-2 on proteoglycan synthesis in osteoblasts, although BMP-3 has been shown to stimulate proteoglycan synthesis in chondroblasts [23,50]. Our results demonstrated that BMP-2 selectively regulated the expression of a small proteoglycan, decorin, but not biglycan, in HOB and HBMSC. Although these two small proteoglycans are closely related in their primary structure, their promoters share little homology [51]. Therefore, it is not surprising to find the selective stimulation of decorin by BMP-2. The stimulation of decorin by BMP-2 is of particular interest since it has been shown that decorin interacts with TGF-β and increases TGF-β activity in MC3T3-E1 cells but decreases that in Chinese hamster ovary (CHO) cells [52,53]. Thus, BMP-2, a member of TGF-β superfamily, may regulate the activity of TGF-β in the same superfamily via the stimulation of decorin. Moreover, since both BMP-2 and TGF-β are important regulators of osteoblast activities, the decorin-mediated interaction between these two regulators will certainly "fine-tune" the function of osteoblast.

We have previously demonstrated that dexamethasone (Dex) induced mineralization of HBMSC [30]. Since long-term treatment of HBMSC with BMP-2 also resulted in mineralization, it is of interest to compare their early effects on matrix protein expression. As stated above, BMP-2 increased the expression of alkaline phosphatase, osteopontin, bone sialoprotein, osteocalcin, and decorin but had no effect on biglycan expression. In Dex-treated cultures, however, the expression of osteopontin, bone sialoprotein, osteocalcin, and biglycan were

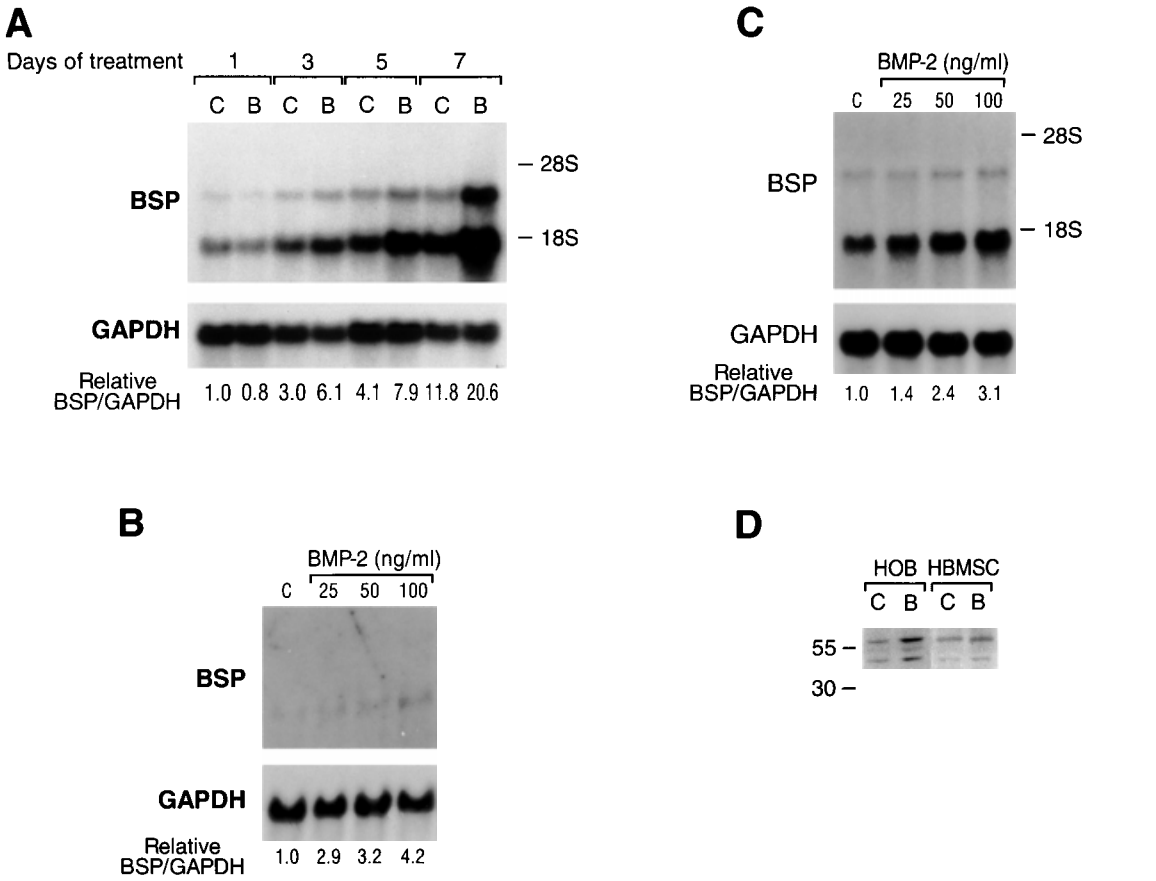

Fig. 7. Effect of BMP-2 on the expression of bone sialoprotein in HBMSC and HOB. **A:** HBMSC were treated without (control) or with BMP-2 for 1–7 days. For dose-response analysis, HBMSC (**B**) and HOB (**C**) were treated with BMP-2 at indicated concentration for 4 days. mRNA-enriched RNA preparations were isolated and Northern blot analysis performed as described in Materials and Methods. Membranes were probed with ^{32}P-labeled cDNA for human bone sialoprotein (BSP) followed by ^{32}P-labeled cDNA for human GAPDH. The relative BSP mRNA concentration on the autoradiograms was analyzed by densitometer and normalized with the GAPDH level. **A:** the ratio of BSP/GAPDH for the control culture after 1-day treatment was defined as 1.0. **B,C:** the ratio of BSP/GAPDH for the control culture was defined as 1.0. **D:** Protein analysis of BSP. Cells were treated without or with BMP-2 (100 ng/ml) for 7 days. Cell layers were extracted and Western blot analysis performed as described in Materials and Methods. Membranes were probed with rabbit anti-BSP antiserum and visualized by enhanced chemiluminescence. Each illustration shown here is a representative of two experiments performed on cells derived from different patients. C, Control; B, BMP-2.

TABLE I. Effect of BMP-2 on the Synthesis and Secretion of Osteocalcin in HBMSC and HOB[a]

	Osteocalcin (ng/100 µg protein)			
	$-1,25(OH)_2D_3$		$+1,25(OH)_2D_3$	
Cell Types	Control	BMP-2	Control	BMP-2
HBMSC	0	9.96 ± 0.60*	11.9 ± 0.65	35.77 ± 1.13*
HOB	0	2.91 ± 0.17*	6.35 ± 0.14	12.86 ± 1.67*

[a]HBMSC and HOB obtained from two different patients were treated without or with BMP-2 at 100 ng/ml for 7 days. Cells were washed serum-free, and treatment resumed in serum-free medium containing 0.05% BSA with or without the addition of 20 nM $1,25(OH)_2D_3$ for 90 h. Conditioned medium was harvested and analyzed for osteocalcin by RIA, as described in Materials and Methods. Cell layer was dissolved in 0.3 N NaOH and protein concentration measured. Osteocalcin level in the conditioned medium was normalized by the cellular protein concentration. Data presented were mean ±SEM of triplicate cultures.

*$P < 0.001$ compared to the corresponding control value by Student's t-test.

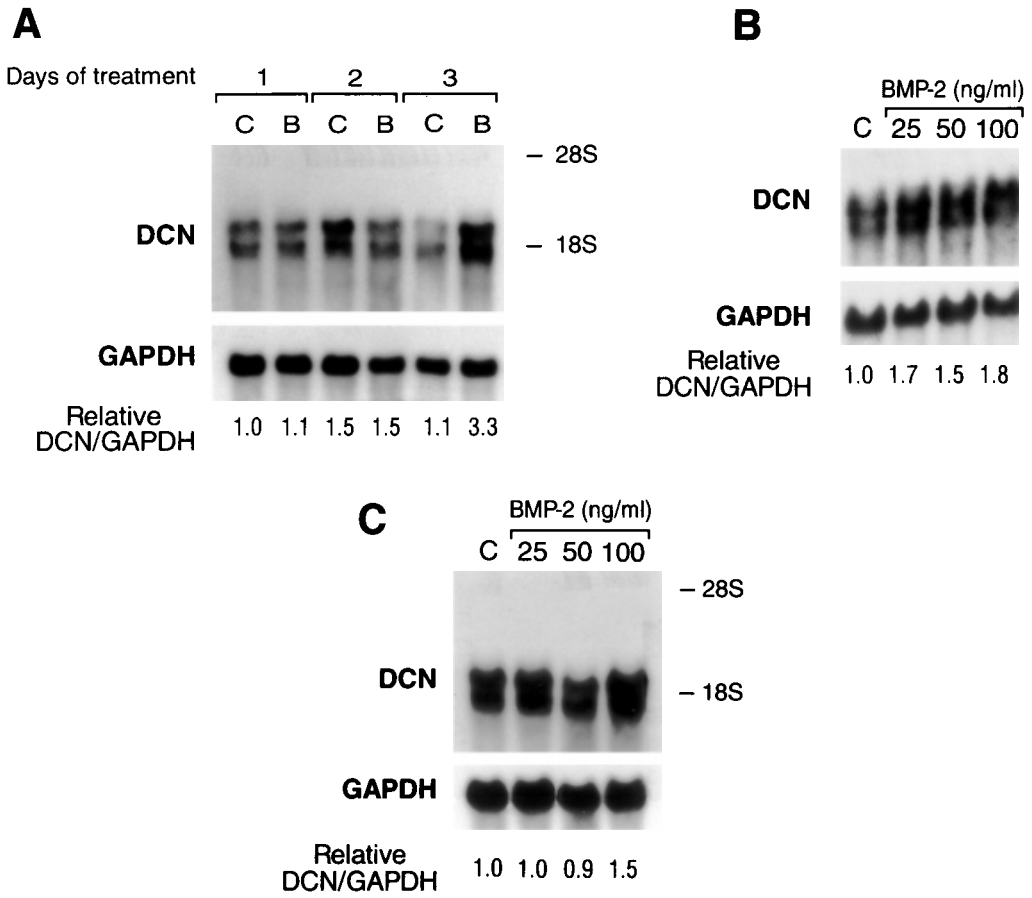

Fig. 8. Effect of BMP-2 on the steady-state mRNA levels of decorin in HBMSC and HOB. HBMSC were treated without (control) or with BMP-2 (100 ng/ml) for 1–3 days (**A**). mRNA-enriched RNA preparations were isolated and Northern blot analysis performed as described in Materials and Methods. Membranes were probed with ^{32}P-labeled cDNA for human decorin (DCN) followed by ^{32}P-labeled cDNA for human GAPDH. The relative DCN mRNA concentration on the autoradiograms was analyzed by densitometer and normalized with the GAPDH level. The ratio of DCN/GAPDH for the control culture after 1 day treatment was defined as 1.0. For dose-response analysis, HBMSC (**B**) and HOB (**C**) were treated without or with indicated concentration of BMP-2 for 4 days. The relative DCN mRNA level for the control culture was defined as 1.0. Each illustration shown here is a representative of two experiments performed on cells derived from different patients. C, control; B, BMP-2.

decreased while those of alkaline phosphatase and decorin were stimulated [29,32]. These accumulated data indicated that although BMP-2 and Dex both stimulated the mineralization of bone matrix in HBMSC, their mechanisms differed. In order for these two opposing regulatory processes to have the same matrix mineralization effect, the relative ratio of these matrix proteins appears to be more important than just a simple increase or decrease in specific matrix protein expression. The stimulation of decorin and alkaline phosphatase by both BMP-2 and Dex also suggest that these two proteins may have functional roles in regulating and/or initiating the mineralization of bone matrix in human osteoblastic cultures. Our accumulated data demonstrated that BMP-2 stimulated the differentiation of human bone marrow stromal cells toward the osteoblastic phenotype and accelerated the differentiation of mature human osteoblasts in vitro, an effect which was consistent with bone formation activities of BMP-2 observed in vivo. Since Dex has deleterious effects in vivo and no correlation is available between in vitro and in vivo studies [54], BMP-2 probably represents a more suitable paradigm with which to assess the mineralization process in vitro.

In summary, we have demonstrated that BMP-2 inhibits the proliferation but induces the differentiation of both HBMSC and HOB through binding to BMP-2/-4 receptors. By orchestrating a fine-tuned expression of bone matrix proteins, BMP-2 treatment results in min-

eralization of the matrix. Information thus obtained with normal human osteoblastic cell cultures is consistent with the current hypothesis that BMP-2 is a candidate drug for managing skeletal disorders in human. The demonstration of the upregulation of osteopontin, bone sialoprotein, and osteocalcin expression by BMP-2 also will provide good end points for future analyses of signal transduction pathways involved and in the identification of transcription factors that mediate the effects of BMP-2 in osteoblast cell systems.

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REFERENCES

1. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988): Novel regulators of bone formation: Molecular clones and activities. Science 242:1528–1534.
2. Hahn GV, Cohen RB, Wozney JM, Levitz CI, Shore RM, Zasloff MA, Kaplan FS (1992): A bone morphogenetic protein subfamily: Chromosomal localization of human genes for BMP 5, BMP 6, and BMP 7. Genomics 14:759–762.
3. Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, Israel DI, Hewick RM, Kerns KM, LaPan P, Luxenberg DP, McQuaid D, Moutsatsos IK, Nove J, Wozney JM (1990): Recombinant human bone morphogenetic protein induces bone formation. Proc Natl Acad Sci USA 87:2220–2224.
4. Reddi AH (1992): Regulation of cartilage and bone differentiation by bone morphogenetic proteins. Curr Opin Cell Biol 4:850–855.
5. Rosen V, Thies RS (1992): The BMP proteins in bone formation and repair. Trends Genet 8:97–102.
6. Urist MR, Huo YK, Brownell AG, Hohl WM, Buyske J, Lietze A, Tempst P, Hunkapiller M, DeLange RJ (1984): Purification of bovine bone morphogenetic protein by hydroxyapatite chromatography. Proc Natl Acad Sci USA 81:371–375.
7. Lyons KM, Pelton RW, Hogan BLM (1989): Patterns of expression of murine Vgr-1 and BMP-2a RNA suggest that transforming growth factor-β like genes coordinately regulate aspects of embryonic development. Genes Dev 3:1657–1668.
8. Kingsley DM (1994): What do BMPs do in mammals? Clues from the mouse short-ear mutation. Trends Genet 10:16–21.
9. Wang EA, Israel DI, Kelly S, Luxenberg DP (1993): Bone morphogenetic protein 2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells. Growth Factors 9:57–71.
10. Aberns M, Ankenbauer T, Scjrpder D, Hollnagel A, Mayer H, Gross G (1993): Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T ½ cells induces differentiation into distinct mesenchymal cell lineages. DNA Cell Biol 12:871–880.
11. Yamaguchi A, Katagiri T, Ikeda T, Wozney JM, Rosen V, Wang EA, Kahn AJ, Suda T, Yoshiki S (1991): Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. J Cell Biol 113:681–687.
12. Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T (1994): Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755–1766.
13. Katagiri T, Yamaguchi A, Ikeda T, Yoshiki S, Wozney JM, Rosen V, Wang EA, Tanaka H, Omura S, Suda T (1990): The nonosteogenic mouse pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. Biochem Biophys Res Commun 172:295–299.
14. Thies RS, Bauduy M, Ashton BA, Kurtzberg L, Wozney JM, Rosen V (1992): Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells. Endocrinology 130:1318–1324.
15. Kingsley D (1994): The TGFβ superfamily: new members, new receptors, and new genetic tests of function in different organisms. Gene Dev 8:133–146.
16. Wozney JM (1992): The bone morphogenetic protein family and osteogenesis. Mol Reprod Dev 32:160–167.
17. Chen TL, Bates RL, Dudley A, Hammonds Jr. GR, Amento EP (1991): Bone morphogenetic protein-2b stimulation of growth and osteogenic phenotypes in rat osteoblast-like cells: comparison with TGF-β_1. J Bone Miner Res 6:1387–1393.
18. Maliakal JC, Asahina I, Hauschaka PV, Sampath TK (1994): Osteogenic protein-1 (BMP-7) inhibits cell proliferation and stimulates the expression of markers characteristic of osteoblast phenotype in rat osteosarcoma (17/2.8) cells. Growth Factors 11:227–234.
19. Amedee J, Bareille R, Rouais F, Cunningham N, Reddi H, Harmand M-F (1994): Osteogenin (bone morphogenic protein 3) inhibits proliferation and stimulates differentiation of osteoprogenitors in human bone marrow. Differentiation 58:157–164.
20. Long MW, Robinson JA, Ashcraft EA, Mann KG (1995): Regulation of human bone marrow-derived osteoprogenitor cells by osteogenic growth factors. J Clin Invest 95:881–887.
21. Hughes FJ, Collyer J, Stanfield M, Goodman SA (1995): The effects of bone morphogenetic protein-2, -4, and -6 on differentiation of rat osteoblast cells *in vitro*. Endocrinology 136:2671–2677.

22. Leboy PS, Beresford JN, Devlin C, Owen ME (1991): Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J Cell Physiol 146:370–378.
23. Hiraki Y, Inoue H, Shigeno C, Sanma Y, Bentz H, Rosen DM, Asada A, Suzuki F (1991): Bone morphogenetic proteins (BMP-2 and BMP-3) promote growth and expression of the differentiated phenotype of rabbit chondrocytes and osteoblastic MC3T3-E1 cells in vitro. J Bone Miner Res 6:1373–1385.
24. Bernier SM, Goltzman D (1992): Effect of protein and steroidal osteotropic agents on differentiation and epidermal growth factor-mediated growth of the CFK1 osseous cell line. J Cell Physiol 152:317–327.
25. Rickard DJ, Sullivan TA, Shenker BJ, Leboy PS, Kazhadan I (1994): Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. Dev Biol 161:218–228.
26. Takuwa Y, Ohse C, Wang EA, Wozney JM, Yamashita K (1991): Bone morphogenetic protein-2 stimulates alkaline phosphatase activity and collagen synthesis in cultured osteoblastic cells MC3T3-E1. Biochem Biophys Res Commun 174:96–101.
27. Zheng MH, Wood DJ, Wysocki S, Papadimitriou JM, Wang EA (1994): Recombinant human bone morphogenetic protein-2 enhances expression of interleukin-6 and transforming growth factor-β1 genes in normal human osteoblast-like cells. J Cell Physiol 159:76–82.
28. Lind M, Eriksen EF, Bünger C (1996): Bone morphogenetic protein-2 but not bone morphogenetic protein-4 and -6 stimulates chemotactic migration of human osteoblasts, human marrow osteoblasts, and U2-OS cells. Bone 18:53–57.
29. Kimoto S, Cheng S-L, Zhang S-F, Avioli LV (1994): The effect of glucocorticoid on the synthesis of biglycan and decorin in human osteoblasts and bone marrow stromal cells. Endocrinology 135:2423–2431.
30. Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV (1994): Differentiation of human bone marrow osteogenic stromal cells in vitro: Induction of the osteoblast phenotype by dexamethasone. Endocrinology 134:277–286.
31. Frolik CA, Wakefield LM, Smith DM, Sporn MB (1984): Characterization of a membrane receptor for transforming growth factor-β in normal rat kidney fibroblasts. J Biol Chem 259:10995–11000.
32. Cheng S-L, Zhang S-F, Avioli LV (1996): The expression of bone matrix proteins during dexamethasone-induced mineralization of human bone marrow stromal cells. J Cell Biochem 61:182–193.
33. Yamaji N, Celeste AJ, Thies RS, Song JJ, Bemier SM, Goltzman D, Lyons KM, Nove J, Rosen V, Wozney JM (1994): A mammalian serine/threonine kinase receptor specifically binds BMP-2 and BMP-4. Biochem Biophys Res Commun 205:1944–1951.
34. Malpe R, Baylink DJ, Sampath TK, Mohan S (1994): Evidence that human bone cells in culture contain binding sites for osteogenic protein-1. Biochem Biophys Res Commun 201:1140–1147.
35. Johansen JS, Williamson MK, Rice JS, Price PA (1992): Identification of proteins secreted by human osteoblastic cells in culture. J Bone Miner Res 7:501–512.
36. Kusumoto K, Bessho K, Fujimura K, Konishi Y, Ogawa Y, Iizuka T (1995): Comparative study of bone marrow induced by purified BMP and recombinant human BMP-2. Biochem Biophys Res Commun 215:205–211.
37. Ohta S, Hiraki Y, Shigno C, Suzuki F, Kasai R, Ikeda T, Kohno H, Lee K, Kikuchi H, Konishi J, Bentz H, Rosen DM, Yamamuro T (1992): Bone morphogenetic proteins (BMP-2 and BMP-3) induce the late phase expression of the proto-oncogene c-fos in murine osteoblastic MC3T3-E1 cells. FEBS Letters 314:356–360.
38. Nakashima M, Nagasawa H, Yamada Y, Reddi AH (1994): Regulatory role of transforming growth factor-β, bone morphogenetic protein-2, and protein-4 on gene expression of extracellular matrix proteins and differentiation of dental pulp cells. Dev Biol 162:18–28.
39. Hultenby K, Reinholt FP, Oldberg Å, Heinegård D (1991): Ultrastructural immunolocalization of osteopontin in metaphyseal and cortical bone. Matrix 11:206–213.
40. Chen J, Singh K, Mukherjee BB, Sodek J (1993): Developmental expression of osteopontin (OPN) mRNA in rat tissues: Evidence for a role for OPN in bone formation and resorption. Matrix 13:113–123.
41. Stein GS, Lian JB (1983): Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocrine Rev 14:424–441.
42. McKee MD, Glimcher MJ, Nanci A (1992): High resolution immunolocalization of osteopontin and osteocalcin in bone and cartilage during endochondral ossification in the chicken tibia. Anat Rec 234:479–492.
43. Chen J, McKee MD, Nanci A, Sodek J (1994): Bone sialoprotein mRNA expression and ultrastructural localization in fetal porcine calvarial bone: comparisons with osteopontin. Histochem J 26:67–78.
44. Hunter GK, Goldberg HA (1993): Nucleation of hydroxyapatite by bone sialoprotein. Proc Natl Acad Sci USA 90:8562–8565.
45. Zhou HY, Takita H, Fujisawa R, Mizuno M, Kukoki Y (1995): Stimulation by bone sialoprotein of calcification in osteoblast-like MC3T3-E1-cells Calcif Tissue Int 56: 403–407.
46. Boskey AL (1995): Osteopontin and related phosphorylated sialoproteins: Effects on mineralization. Ann NY Acad Sci 760:249–256.
47. Romberg RW, Werness PG, Riggs BL, Mann KG (1986): Inhibition of hydroxyapatite crystal growth by bone-specific and other calcium-binding proteins. Biochemistry 25:1176–1180.
48. Aubin JE, Gupta AK, Zirngbl R, Rossant J (1996): Knockout mice lacking bone sialoprotein expression have bone abnormalities. J Bone Miner Res 11(suppl 1):S102.
49. Scott JE, Haigh M (1985): Proteoglycan-type I collagen fibril interactions in bone and non-calcifying connective tissues. Biosci Rep 5:71–81.
50. Vukicevic S, Luyten FP, Reddi AH (1989): Stimulation of the expression of osteogenic and chondrogenic phenotypes in vitro by osteogenin. Proc Natl Acad Sci USA 86:8793–8797.
51. Ungefroren H, Krull NB (1996): Transcriptional regulation of the human biglycan gene. J Biol Chem 271: 15787–15796.
52. Takeuchi Y, Kodama Y, Matsumoto T (1994): Bone matrix decorin binds transforming growth factor-β and enhances its bioactivity. J Biol Chem 269:32634–32638.
53. Yamaguchi Y, Mann DM, Ruoslahti E (1990): Negative regulation of transforming growth factor-β by the proteoglycan decorin. Nature 346:281–284.
54. Baylink DJ (1983): Glucocorticoid-induced osteoporosis. N Engl J Med 309:306–308.