Bone morphogenetic protein-2 and -9 regulate the interaction of insulin-like growth factor-I with growth plate chondrocytes

TOSHIAKI TAKAHASHI[1,3], ELISABETH A. MORRIS[2] and STEPHEN B. TRIPPEL[1,4]

[1]Orthopaedic Research Laboratories, Massachusetts General Hospital, Harvard Medical School, 55 Fruit Street, Boston, MA 02114; [2]Wyeth Research, Department of Women's Health and Musculoskeletal Biology, 87 Cambridge Park Drive, Cambridge, MA 02140, USA; [3]Department of Bone and Joint Surgery, Ehime University Graduate School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan; [4]Department of Orthopaedic Surgery, Indiana University School of Medicine, 541 Clinical Drive, Suite 600, Indianapolis, IN 46202-5111, USA

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Abstract. Insulin-like growth factor-I (IGF-I) is thought to play an important role in skeletal growth and development through its mitogenic and anabolic effects on epiphyseal growth plate chondrocytes. The bone morphogenetic proteins (BMPs) have been shown to promote endochondral osteogenesis, and some members of the BMP family, including BMP-2 and BMP-9, have anabolic effects on chondrocyte metabolism. We tested the hypothesis that BMP-2 and BMP-9 interact with IGF-I to modulate growth plate chondrocyte mitotic activity. IGF-I, but neither BMP-2 nor BMP-9, stimulated chondrocyte DNA synthesis. However, both BMP-2 and BMP-9 augmented the mitogenic action of IGF-I. BMP-2, but not BMP-9 increased IGF-I binding to growth plate chondrocytes in kinetic studies. In affinity labeling studies, ^{125}I-IGF-I predominantly labeled an Mr ~135-kDa moiety, consistent with the α subunit of the type 1 IGF receptor and an Mr ~250-kDa moiety consistent with the type 2 IGF receptor. ^{125}I-IGF-I labeling also appeared at Mr ~43 kDa, consistent with ^{125}I-IGF-I binding to insulin-like growth binding protein-3. Treatment of chondrocytes with BMP-2, but not with BMP-9, increased the intensity of the Mr ~135-kDa band and decreased the intensity of the Mr ~43-kDa band. Taken together, these data suggest that the BMPs may modulate the action of IGF-I via the type 1 IGF receptor and/or IGF binding proteins.

Correspondence to: Dr Toshiaki Takahashi, Department of Bone and Joint Surgery, Ehime University Graduate School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan
E-mail: takahast@m.ehime-u.ac.jp

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Introduction

The normal growth and differentiation of cartilage is regulated by multiple cell-signaling polypeptides. Among these are insulin-like growth factor I (IGF-I) and members of the bone morphogenetic protein (BMP) family. IGF-I is mitogenic for growth plate chondrocytes and stimulates chondrocyte synthesis of matrix macromolecules, including proteoglycan and collagen (1-3). IGF-I is at least partly responsible for skeletal growth and development *in vivo* through its actions on the epiphyseal growth plate (4). The bone morphogenetic proteins are members of the transforming growth factor-ß superfamily. In skeletal tissues they play diverse roles ranging from patterning to differentiation (5). BMP-2 and BMP-9 are capable of initiating the full sequence of endochondral osteogenesis in ectopic sites, a process that recapitulates the endochondral osteogenesis regulated by IGF-I in the growth plate. IGF-I, BMP-2 and BMP-9 have all been shown to stimulate the synthesis of proteoglycan, a major constituent of cartilage matrix (6,7).

IGF-I is thought to exert its biological effect on chondrocytes by binding to the type 1 IGF receptor, a member of the protein tyrosine kinase receptor family. This receptor exists as a heterotetrameric complex composed of disulfide-linked subunits. Two paired extracellular α subunits contain the IGF binding site and two paired transmembrane ß subunits possess the kinase domains (8). IGF-I also binds to a monomeric type 2 IGF receptor that lacks kinase activity and may serve to modulate IGF availability (9). IGF-I action is also influenced by insulin-like growth factor binding proteins (IGFBPs) (10). Of the six canonical members of the IGFBP family, IGFBP-3 is present in greatest abundance in serum where it serves to increase the half-life of IGF-I (11). The action of IGFBP-3 is context-dependent and may inhibit or augment the effects of IGF-I (12). BMP receptors are members of the serine/threonine kinase receptor family, contrasting them with the IGF-I receptor (12,13).

BMPs (14-16) and IGF-I (17) are present in cartilage. It is unlikely that IGF-I and BMP-2 or BMP-9 regulate cartilage independently. In order to understand the role of these factors

in cartilage regulation, it is perhaps as important, if not more important, to understand their interaction as it is to understand their individual effects. In the present study we tested the hypothesis that BMP-2 and BMP-9 interact with IGF-I to modulate growth plate chondrocyte function.

Materials and methods

Reagents and growth factors. Recombinant human BMP-2 and BMP-9 were purified from Chinese hamster ovary cells as previously described (17). ^{125}I-labeled IGF-I (^{125}I-IGF-I) was the kind gift of Dr L.E. Underwood and Dr J.J. Van Wyk (University of North Carolina-Chapel Hill). DMEM and MCDB-105 culture media were from Gibco Life Technologies (Grand Island, NY). Bovine serum albumin, screened by radioimmunoassay for IGF-I content <20 ng/ml, was from Sigma (St. Louis, MO), and fetal bovine serum (FBS) was from Invitrogen Corp. (Carlsbad, CA). Pluronic F-68 was from BASF Corp. (Parsippany, NJ). Collagenase was from Worthington Biochemical Corporation (Lakewood, NJ). Acrylamide gel was from Novex (San Diego, CA), and ^{3}H-thymidine was from New England Nuclear Life Science Products, Inc. (Boston, MA).

Chondrocyte isolation. Neonatal bovine distal radio-ulnar growth plate cartilage was harvested under sterile conditions, diced into ~2-mm cubes, and incubated in DMEM containing 0.1% collagenase and antibiotics overnight in spinner bottles at 37˚C, 5% CO_2. Undigested matrix was removed by filtration through 150-μm pore size nylon mesh followed by centrifugation at 700 x g for 6 min over a Ficoll-Hypaque (10/11%) gradient. The isolated cells were washed with phosphate-buffered saline solution (PBS), and re-suspended at $0.5x10^6$ cells/ml in DMEM with 2% FBS, antibiotics and 0.1% pluronic F-68 for 24 h.

Binding of ^{125}I-labeled IGF-I to growth plate chondrocytes. Cells were washed and re-suspended in DMEM containing antibiotics and graded concentrations of rhBMP-2 (0-300 ng/ml) (0-$9.4x10^{-9}$ M) or rhBMP-9 (0-100 ng/ml) (0-$3.13x10^{-9}$ M) for 24 h. For competition binding studies, cells were washed twice in binding medium consisting of bicarbonate-free DMEM with 20 mM HEPES, pH 7.8, and 1% bovine serum albumin at 4˚C, and aliquots of re-suspended cells were incubated in binding medium in the presence of 50,000 cpm/$3x10^6$ cells, ^{125}I-IGF-I (specific activity 382-427 μCi/μg) and graded concentrations of unlabeled IGF-I (0, $1x10^{-11}$, $1x10^{-10}$, $1x10^{-9}$, $1x10^{-8}$ and $2x10^{-7}$ M) in a total volume of 0.4 ml for 24 h at 15˚C in duplicate. Bound ligand was separated from free ligand by centrifugation for 1.5 min at 9500 x g followed by a wash with ice-cold binding medium and repeat centrifugation. The cell pellet was counted in a γ-spectrometer (Auto Gamma, Packard Instruments, Meriden, CT). Nonspecific binding was defined as binding in the presence of an excess ($2x10^{-7}$ M) of unlabeled IGF-I. Specific binding was defined as total binding minus nonspecific binding and normalized to that of the controls (0 M unlabeled IGF-I).

Cross-linking of ^{125}I-IGF-I to growth plate chondrocytes. Chondrocytes isolated as described above were incubated in the absence of BMP or in the presence of 300 ng/ml ($9.4x10^{-9}$ M) BMP-2 or 300 ng/ml ($9.4x10^{-9}$ M) BMP-9 in DMEM with antibiotics in a spinner bottle at 37˚C for 16 h. Cells were washed twice in ice-cold binding medium, and aliquots of $8x10^6$ cells were incubated with $0.5x10^6$ cpm of ^{125}I-IGF-I in the absence or presence of unlabeled IGF-I ($2x10^{-7}$ M) in a total volume of 0.5 ml at 4˚C. After 24 h, freshly solubilized disuccinimidylsuberate (DSS) in dimethyl sulfoxide (DMSO) was added in a final concentration of 0.5 mM. After 30 min at 22˚C, the reaction was quenched with an excess (0.25 M) of NH_4Br. The reaction mixture was diluted with 0.8 ml of PBS at 4˚C, and the cells were centrifuged at 9500 x g for 15 min at 4˚C, and washed with 1 ml of cold PBS.

Cell pellets were dissolved in 100 μl of 1X sample buffer (SDS 4 g, Tris 1.51 g, bromphenol blue 2 mg, glycerol 40 ml, and H_2O 160 ml). In the reducing condition, a 5% volume of 2-mercaptoethanol was added. In both reducing and non-reducing conditions, cells were boiled with sample buffer at 95˚C for 2 min. Samples were passed through a 22-gauge needle 15 times and subjected to SDS polyacrylamide gel electrophoresis (PAGE). The SDS PAGE was performed using 10% acrylamide TG 1.5-mm gel (Novex, San Diego, CA) without SDS. Gels were stained with Coomassie blue, destained, and dried. Autoradiograms were made on Kodak X-omat AR film in the presence of an image-intensifying screen for 7-14 days at -70˚C and developed (17).

^{3}H-thymidine incorporation. Isolated chondrocytes were washed in PBS, plated in DMEM with 10% FBS and antibiotics at $5x10^5$ cells/ml in quadruplicate in 96-well plates, and incubated for 24 h at 37˚C in an atmosphere of 5% CO_2 in air. The medium was changed to serum-free MCDB-105 medium (pH 7.4) with antibiotics (basal medium) for 24 h, replaced with basal medium containing IGF-I (0-230 ng/ml) (0-$3x10^{-8}$ M); BMP-2 (0-320 ng/ml) (0-$1x10^{-8}$ M); the combination of IGF-I (0-230 ng/ml) (0-$3x10^{-8}$ M) + BMP-2 (0-320 ng/ml) (0-$1x10^{-8}$ M); or the combination of IGF-I (0-230 ng/ml) (0-$3x10^{-8}$ M) + BMP-9 (0-320 ng/ml) (0-$1x10^{-8}$ M) for 48 h. Twenty-four hours before the end of incubation, 8 μCi/ml ^{3}H-thymidine was added. The cells were washed three times with 200 μl ice-cold PBS, twice with 200 μl ice-cold 5% trichloroacetic acid and twice with 200 μl 3:1 (volume) ethanol/ethyl ether. The samples were dissolved in 200 μl 0.3 M NaOH, neutralized with 10 μl 6 N HCI on ice and transferred to scintillation vials. Cytoscint, 4 ml/vial, was added, vortexed, and let stand at room temperature for 24 h. Incorporation of ^{3}H-thymidine was determined by liquid scintillation counting (1).

Statistical analysis. Statistical analysis was performed by the Student's t-test for IGF-I, BMP-2 or BMP-9 alone and by two- way ANOVA for combinations of BMPs and IGF-I.

Results

Binding studies. Specific binding of ^{125}I-IGF-I in the absence of unlabeled IGF-I (maximal specific binding) averaged 12.2% per $3x10^6$ cells. Non-specific binding was ~2.4%. Unlabeled IGF-I competed with ^{125}I-IGF-I binding to growth plate chondrocytes with 50% displacement at ~10^{-9} M for

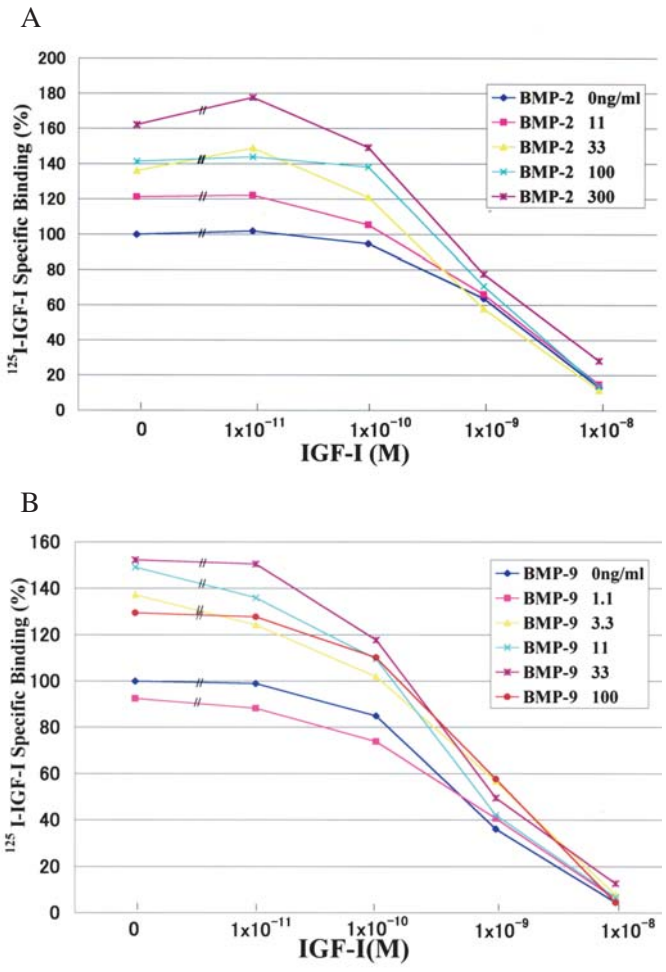

Figure 1. Specific IGF-I binding to growth plate chondrocytes cultured in the presence of graded concentrations of BMP-2 (A) or BMP-9 (B). Specific binding was normalized to the control group (no BMP-2 or -9). Graphs represent one of three independent experiments.

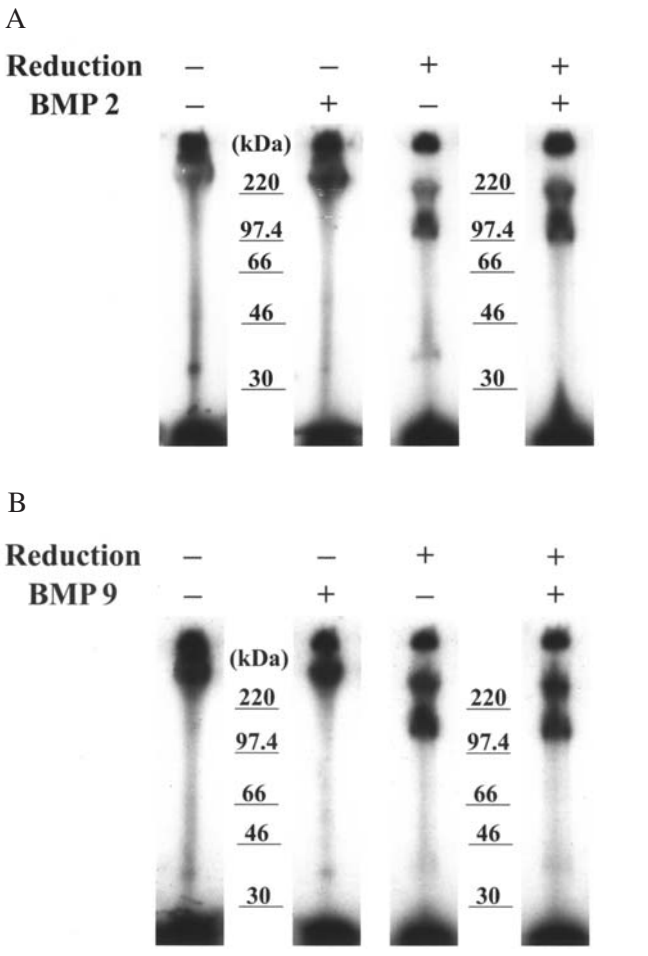

Figure 2. Affinity labeling of [125I]-IGF-I to growth plate chondrocytes with or without reduction by 2-mercaptoethanol. Labeling was without unlabeled ligand of cells treated with or without BMP-2 (A) or BMP-9 (B) and subsequently prepared with or without reduction by 2-mercaptoethanol. Molecular mass markers are shown in kDa.

control cells not exposed to BMP (Fig. 1A and B). Incubation of growth plate chondrocytes with BMP-2 for 24 h increased maximal specific binding of ^{125}I-IGF-I by 22% (p=0.627), 48% (p=0.036), 43% (p=0.259), and 78% (p=0.011) at concentrations of 11 ($3.44x10^{-10}$ M), 33 ($1.04x10^{-9}$ M), 100 ($3.13x10^{-9}$ M), and 300 ng/ml ($9.4x10^{-9}$ M), respectively (n=3). Incubation of chondrocytes with BMP-9 altered maximal specific binding of ^{125}I-IGF-I by -7% (p=0.478), 37% (p=0.101), 49% (p=0.117) 52% (p=0.067), and 30% (p=0.621) at concentrations of 1.1 ($3.44x10^{-11}$ M), 3.3 ($1.04x10^{-10}$ M), 11 ($3.44x10^{-10}$ M), 33 ($1.04x10^{-9}$ M), and 100 ng/ml ($3.13x10^{-9}$ M), respectively (n=3).

Cross-linking studies. When growth plate chondrocytes were labeled with ^{125}I-IGF-I without prior disulfide bond reduction, ^{125}I-IGF-I binding was to species migrating with an apparent Mr of >220 kDa, consistent with the unreduced forms of the type 1 and type 2 IGF receptors. Under reducing conditions, the majority of ^{125}I-IGF-I appeared at Mr ~135 kDa, consistent with the α subunit of the type 1 IGF receptor and at Mr ~250 kDa, consistent with the reduced form of the type 2 IGF receptor. Treatment with BMP-2 slightly increased the intensity of the Mr ~135-kDa band (Fig. 1A). ^{125}I-IGF-I also labeled an Mr ~43-kDa band, consistent with binding to insulin-like growth factor binding protein-3. Treatment of chondrocytes with BMP-2 decreased the intensity of the Mr ~43-kDa band. In contrast to BMP-2, BMP-9 did not increase the intensity of the Mr ~135-kDa band and either had no effect on or increased the intensity of the Mr ~43-kDa band (Fig. 2A).

^{3}H-thymidine incorporation. BMP-2 alone had no consistent effect on DNA synthesis (p>0.05). BMP-9 alone similarly did not affect DNA synthesis (p>0.05). In combination with IGF-I, graded concentrations of BMP-2 increased the stimulatory effect of IGF-I (p=0.002) (Fig. 3A). In combination with IGF-I, BMP-9 similarly augmented the stimulatory effect of IGF-I (p=0.010) (Fig. 3B).

Discussion

Several BMP family members regulate the differentiation of cartilage and the differentiated phenotype of chondrocytes. BMP-2 induces the chondrogenic differentiation of human multipotential mesenchymal cells (18), a clonal undifferentiated cell line (19) and mesenchymal cells (20). BMP-2

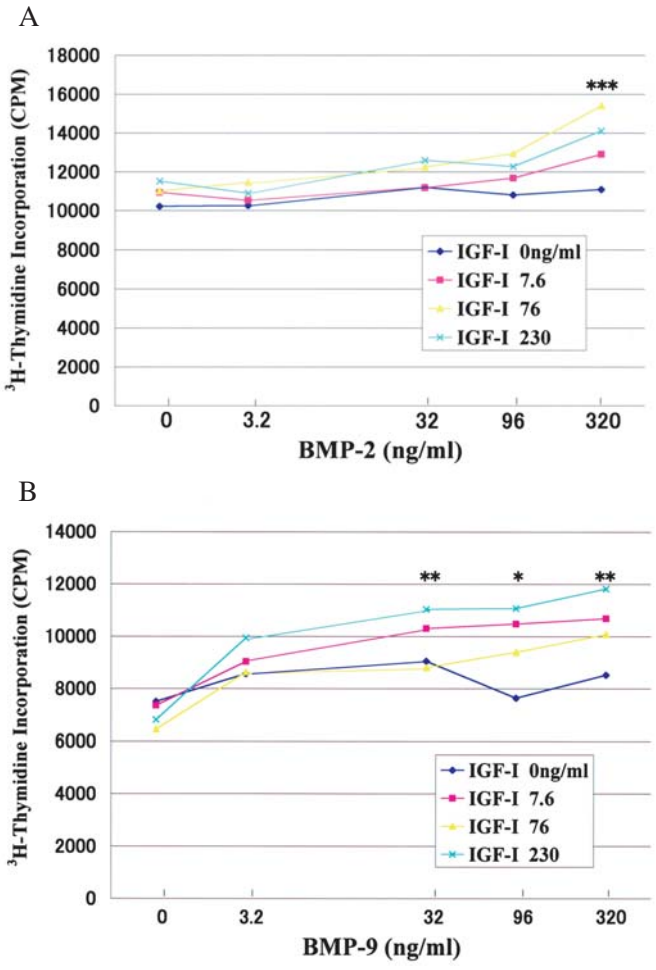

Figure 3. The effect of IGF-I with BMP-2 (A) or with BMP-9 (B) on thymidine incorporation by growth plate chondrocytes. Each data point represents the mean of three independent experiments (five replicates per experiment). Significant differences among the groups for each concentration of BMP-2 and BMP-9 are denoted by $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

and BMP-3 stimulate and maintain proteoglycan synthesis and enhance alkaline phosphatase activity in differentiated chondrocytes *in vitro* (20,21). Only a few reports have addressed the function of BMP-9 (22) in cartilage. BMP-9, like BMP-2, stimulates alkaline phosphatase activity in a murine osteoprogenitor cell line (22,23), and increases proteoglycan synthesis in bovine articular cartilage explants.

IGF-I has well-established mitogenic and anabolic effects on growth plate chondrocytes *in vitro* and has been shown to regulate skeletal growth *in vivo*. Despite the presumed importance of the BMPs and IGF-I in chondrocyte regulation, there has been, to our knowledge, no investigation of the interaction between the IGF and BMP families in the regulation of these cells. The present data suggest that BMP-2 and BMP-9 modulate IGF-I action in growth plate chondrocytes.

The cellular actions of IGF-I are mediated by binding to transmembrane IGF receptors (24). The type 1 IGF receptor is a heterotetrameric complex (~300 kDa) consisting of two extracellular α subunits (~135 kDa) and two transmembrane ß subunits (~90 kDa) joined by disulfide bonds. The complex migrates on SDS-PAGE at Mr >300 kDa under non-reducing conditions and separates into ~135-kDa α and ~90-kDa ß subunits under reducing conditions. The type 2 IGF receptor is a monomer that contains intramolecular disulfide bonds, such that it migrates at Mr ~240 and ~260 under non-reducing and reducing conditions, respectively. IGF-I binds to both receptors, though with a higher affinity for the type 1 receptor. Most of the biological effects of IGF-I are thought to be mediated by the type 1 receptor (8). In the present kinetic studies, BMP-2 significantly increased IGF-I binding at 2 of the 4 concentrations tested. Although BMP-9 increased IGF-I binding in individual experiments (Fig. 1B), this effect was not statistically significant in multiple experiments. This lack of statistical difference may be due to interexperimental variation. In the present affinity labeling studies, BMP-2, but not BMP-9 appeared to increase IGF-I binding to the type 1 and possibly the type 2 IGF receptors. This difference could also reflect, at least in part, potential differences among the electrophoresis lanes.

The actions of IGF-I are modulated by the insulin-like growth factor binding proteins (IGFBPs), a family of 6 proteins that have affinities for IGF-I that may exceed those of the IGF receptors (10). Although IGFBP-2 to -5 have all been demonstrated to be present in human cartilage, IGFBP-3 and -4 are secreted in the greatest abundance by human chondrocytes in culture (9). IGFBP-3 may inhibit IGF binding to type 1 IGF receptor (25), but under some conditions may augment the effects of IGF-I.

BMPs exert their biological effect by interactions with cell surface receptors (26). BMP-9 receptors on HepG2 cells do not bind other members of the BMP or TGF-ß families, indicating a mechanism for binding specificity for BMP-9 (22). BMP-7, another osteogenic BMP, has been shown to increase the levels of IGFBP-3 and -5 in the conditioned medium (27) of human bone cells. BMP-7, alone or in combination with IGF-I, increased IGFBP-3 mRNA levels and reduced those of IGFBP-4, -5, and -6 in osteoblastic cells (28). In the present study, the apparent decrease in IGFBP-3 observed in response to BMP-2 may reflect a difference between BMP-2 and BMP-7 or between osteoblastic cells and growth plate chondrocytes. The observation that BMP-2, but not BMP-9, reduced IGF-I binding to the ~43-kDa moiety is one of the few differences observed between BMP-2 and BMP-9.

Treatment with BMP-2, -3, or -4 has been reported to increase thymidine incorporation by cultured chondrocytes (21). In contrast, Sailor *et al* (6) reported that BMP-2 did not alter articular chondrocyte proliferation. Using periosteal cells, Mayer *et al* (29) found that BMP-2 caused a time-dependent stimulation of ^{3}H-thymidine incorporation that peaked at 20 h after treatment and was lost after 30 h. Erickson *et al* (30) reported that BMP-2 increased the incorporation of ^{3}H-thymidine by both quiescent resting zone and growth zone growth plate chondrocytes. In nonquiescent cultures, BMP-2 increased that of resting zone cells, but not that of growth zone cells. Resting zone chondrocytes were more sensitive to the regulation of proliferation, differentiation, and matrix production.

In the present study, neither BMP-2 nor BMP-9 increased DNA synthesis. However, both BMP-2 and BMP-9 augmented

the mitogenic action of IGF-I. This augmentation occurred at lower concentrations of BMP-9 than of BMP-2. In prior studies, epidermal growth factor (EGF) increased both IGF-I binding and IGF-I responsiveness in growth plate chondrocytes (31), and IGF-I enhanced the mitogenic effect of TGF-ß1 and fibroblast growth factor (FGF) on these cells (32). Taken together, these data support the view that growth factor interaction may be an important mechanism of cell regulation, including the regulation of growth plate chondrocytes.

In conclusion, these data suggest that BMP-2 and BMP-9 have regulatory effects on the IGF axis. The data further suggest that the BMPs may modulate the IGF action via the type 1 receptor and/or IGF binding proteins. Future studies will be required to elucidate the cellular and molecular mechanisms by which these growth factor interactions are regulated.

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