HEPARIN POTENTIATES THE IN VIVO ECTOPIC BONE FORMATION INDUCED BY BONE MORPHOGENETIC PROTEIN-2

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Running title: Heparin enhances in vivo induction of bone formation by BMP

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Although bone morphogenetic proteins (BMPs) are clinically useful for bone regeneration, large amounts are required to induce new bone formation in monkeys and humans. We recently found that heparin stimulated BMP activity in vitro. In the present study, we examined whether heparin can enhance bone formation induced by BMPs in vivo and attempted to determine the molecular mechanism by which heparin stimulates BMP activity using C2C12 myoblasts. Heparin enhanced BMP-2-induced gene expression and Smad1/5/8 phosphorylation at 24 h and thereafter, though not within 12 h. Heparitinase treatment did not affect the response of cells to BMP-2. In the presence of heparin, degradation of BMP-2 was blocked and the half-life of BMP-2 in culture media was prolonged nearly 20-fold. Although noggin mRNA was induced by BMP-2 within 1 h regardless of the presence of heparin, noggin failed to inhibit BMP-2 activity in the presence of heparin. Furthermore, simultaneous administration of BMP-2 and heparin in vivo dose-dependently induced larger amounts of mineralized bone tissue than BMP-2 alone. These findings clearly indicate that heparin enhances BMP-induced osteoblast differentiation not only in vitro but also in vivo. These studies indicate that heparin enhances BMP-induced osteoblast differentiation in vitro and in vivo by protecting BMPs from degradation and inhibition by BMP antagonists.

Introduction

Bone morphogenetic proteins were originally identified as an activity inducing ectopic bone formation when implanted into muscle tissue (1). Isolation of BMPs using heparin affinity column chromatography and molecular cloning of BMPs revealed that the ectopic bone-inducing activity was due to several BMPs (2,3). More than 15 BMPs have since been identified in vertebrates, and are classified into several subgroups: the BMP-2/4 subgroup, BMP-5/6/7/8 subgroup,
growth and differentiation factor-5 (GDF-5) (also termed BMP-14)/GDF-6 (BMP-13)/GDF-7 (BMP-12) subgroup, and BMP-3 subgroup (4-6). With the exception of BMP-1, BMPs are members of the transforming growth factor-β (TGF-β) superfamily, which includes multifunctional factors in vertebrates and invertebrates (3). BMPs play critical roles in skeletal development, especially in mammals (4-7).

BMPs regulate the differentiation and function of cells that are involved in bone and cartilage formation and deformation, including osteoblasts, chondrocytes, and osteoclasts (4,5,7). A cell culture system using C2C12 myoblasts can mimic part of the process of the ectopic bone formation induced by BMPs in muscle tissue (8). BMPs inhibit myogenic differentiation of myoblasts into mature muscle cells and convert their differentiation pathway into that of osteoblast lineage cells. Using this model system in vitro, a critical factor for osteoblast differentiation, termed osterix, has been cloned as a novel Zn finger transcription factor induced by BMP-2 (9). Osterix knockout mice do not have bone tissues due to lack of osteoblast differentiation from mesenchymal progenitor cells (9). Although several growth factors including TGF-β and activin inhibit the myogenic differentiation of C2C12 cells in vitro, they do not induce osteoblast differentiation (8,10). It has been reported that TGF-β itself failed to induce ectopic bone formation in vivo (2). These findings suggest that the C2C12 cell model system is useful for examining the molecular mechanisms of the TGF-β superfamily members during ectopic bone formation in muscle tissue. It should therefore be useful for examining the molecular mechanism of bone formation and osteoblast differentiation induced by BMPs.

BMP signaling is transduced by two types of transmembrane serine/threonine kinase receptors. BMPR-IA (ALK-3), BMPR-IB (ALK-6), ALK-1, and ALK-2 function as type I BMP receptors, while BMPR-II, ActR-II, and ActR-IIB function as type II receptors, which are constitutively active kinases (4,11-13). After type II receptors phosphorylate type I receptors in ligand-dependent fashion, activated type I receptors phosphorylate downstream molecules in the cytoplasm. The Smad1/5/8 transcription factors are phosphorylated by BMP type I receptors in the cytoplasm as substrates and accumulate in the nucleus within 1 h after BMP stimulation (4,11-13). The phosphorylated Smads directly regulate expression of primary target genes through binding to their promoter or enhancer elements together with Smad4 and other transcription factors (14,15).

BMP activity is positively and negatively regulated by many types of molecules both intracellularly and also in the extracellular environment (7,13,16). Various co-repressors and co-activators of Smads have been identified in the cytoplasm or nucleus in mammalian cells. Noggin, chordin, follistatin, and the DAN family proteins bind to BMPs in the extracellular space and inhibit binding of ligands to cell surface receptors (16). In contrast, heparin enhances BMP-induced osteoblast differentiation in C2C12 myoblasts in vitro (17). Other types of sulfated polysaccharides such as native heparan sulfate and synthetic dextran sulfate also stimulate BMP-2 (17). The capacity of these sulfated polysaccharides to stimulate BMP-2 appeared to depend on the size and number of their sulfated residues (17). Heparin did not enhance
osteoblast differentiation induced by direct activation of the BMP intracellular signaling pathway by overexpressing a constitutively activated BMP receptor without addition of BMPs (17). This finding suggests that heparin primarily exhibits effects upstream of the BMP receptor, including BMP ligands, rather than on the BMP intracellular signaling pathway. Heparin has been reported to enhance the activities of fibroblast growth factors (FGFs) by increasing the affinity of ligands for receptors as a co-receptor (18,19). However, heparin decreased the amount of BMP-2-receptor complex in C2C12 cells, suggesting that a novel mechanism of the heparin activity is involved in the case of BMPs (17).

The unique and specific capacity of BMPs to induce new bone formation in vivo should be useful for the development of therapeutic drugs for in vivo bone regeneration (1,20). However, it was reported that more than 100-fold larger amounts of BMPs were required to induce bone formation in higher animals, such as monkeys and humans, than in rodents (21). We speculated that simultaneous administration of BMPs with sulfated polysaccharides might reduce the amounts of BMPs required to induce clinically useful amounts of new bone in vivo. In the present study, we found that heparin stimulates the ectopic bone formation activity of BMP-2 in vivo, and propose a novel mechanism of the effects of heparin on BMP signaling in target cells.

MATERIALS AND METHODS
Recombinant proteins and heparin
Purified recombinant human BMP-2 was obtained from Astellas Pharmaceuticals Co., Ltd. (Tokyo, Japan). Recombinant human BMP-4, BMP-6, and recombinant mouse noggin/Fc chimera were purchased from R & D Systems, Inc. (Minneapolis, MN). Heparin prepared from porcine intestine and low molecular weight (LMW) heparin was purchased from Sigma Chemical Co. (St. Louis, MO). Purified heparin for injection for patients was obtained from Fuso Pharmaceutical Industries (Osaka, Japan).

Cell cultures
C2C12 myoblasts were maintained and treated with BMPs as described (8,17). Briefly, cells were inoculated at a density of 4.5x10^4 cells/cm^2 one day before treatment. The cells were treated with BMPs in the presence or absence of heparin in Dulbecco’s modified Eagle medium (DMEM) containing 2.5% fetal bovine serum (FBS). In experiments, the concentrations of BMP-2 in culture media were quantified using an enzyme-linked immunosorbent assay kit (R & D Systems, Inc.).

Alkaline phosphatase activity
Alkaline phosphatase (ALP) activity was measured as a marker of osteoblast differentiation (8). Cells treated with an acetone/ethanol mixture (50:50, v/v) were incubated with a substrate solution composed of 0.1 M diethanolamine, 1 mM MgCl₂, and 10 mg/ml of p-nitrophenylphosphate. The reaction was terminated by adding NaOH, and OD values were measured at 405 nm.

Reverse transcription-polymerase chain reaction
Levels of expression of genes were determined by reverse transcription - polymerase chain reaction
Total RNAs were extracted from C2C12 cells with Trizol Reagent (Invitrogen, Carlsbad, CA) and then reverse-transcribed using Superscript II (Invitrogen). PCR amplification was performed with Platinum Pfx DNA polymerase (Invitrogen) and the following primers: ALP, GATCATTCACGTTTTCAC and TGGCGGCGTTGCGGACCTGC; osteocalcin, CAAGTCCCACACAGCAGCTT and AAAGCCGAGCTGCCAGAGTT; Runx2 type I, ATGCGTATTCTGTAGATCC and CTACAACCTTGAGCCACGC; Runx2 types II/III, ATGCTTCATTCATTCGCCTCACAAAC and CCAAAAAGAAGCTTTGCTG; Osterix, TTAAGCTTGCGTCCTCTCTGCTTGA and TTTCTAGATCAGCTCTAGCAGGT; noggin, ATGGAGCGCTGCCCCAGCCT and CTAGCAGAAACACTTACACT; and GAPDH, TGAAGGTCGGTGTGAACGGATTGGC and CATGTAGGCCATGAGGTCCACCAC. cDNA was denatured at 94°C for 5 min followed by repeated 25 cycles of 45 sec at 94°C, annealing at 55°C for 1 min, and extension at 68°C for 1 min.

**Western blot analysis**

C2C12 cells were treated with 100 ng/ml BMP-2 in the presence or absence of 5 mg/ml heparin and then lysed in TBS (25 mM Tris-HCl and 150 mM NaCl, pH 7.5) containing 1% Triton X-100 and a 1 X protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) as described (17,23). The lysates were subjected to 8% SDS-PAGE gel electrophoresis and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Phosphorylated and total Smad1/5/8 were detected using the anti-phospho-Smad1/5/8 antibody (Cell Signaling Technology) and anti-Smad1 antibody (Upstate, Lake Placid, NY), respectively.

**Determination of amounts of BMP-2**

C2C12 cells were incubated up to 50 h with 100 ng/ml of BMP-2 in DMEM containing 2.5% FBS. At each time point, the culture medium and the cell lysate, which were prepared by extracting the cells with phosphate-buffered saline containing 1M urea for 30 min at room temperature, were obtained and kept at -80°C until use. Amounts of BMP-2 in culture media and cell lysates were determined by enzyme-linked immunosorbent assay (ELISA) using a kit (R & D Systems) according to the manufacturer’s instructions.

**Ectopic bone formation assay**

The effects of heparin on bone formation in vivo induced by BMP-2 were examined by ectopic bone formation assay in mice (24). Five μg of BMP-2 and 0, 0.25, 2.5 or 25 μg of heparin were
mixed and added to 20 ml of 0.01M HCl solution, blotted onto a collagen sponge disk (6 mm diameter, 1 mm thickness) fabricated from commercially available bovine collagen sheets (Helistat, Integra Life Sciences Corp., Plainsboro, NJ), freeze-dried, and kept at -20°C until implantation into mice. All procedures were carried out under sterile conditions. Twenty-four male ICR mice (4-weeks old; Nippon SLC, Hamamatsu, Japan) were housed and acclimated in cages with free access to food and water for 1 week, and they were divided into 4 groups (8 mice per group). The mice were anesthetized by diethyl-ether gas inhalation, and the collagen pellets prepared as described above were surgically implanted into the both dorsal muscle pouches (two pellets per animal) of the mice. At 2, 3, and 4 weeks after surgery, the mice were sacrificed, and the implants were harvested and processed for histological analysis as follows. All harvested samples were radiographed with a soft X-ray apparatus (Sofron Co., Ltd., Tokyo, Japan). The bone mineral content (BMC) (milligrams per ossicle) of each ossicle was measured by dual-energy X-ray absorptiometry (DXA) using a bone mineral analyzer (DCS-600EX, Aloka Co., Tokyo). Sections of the ossicles or tissue mass from each group were stained with hematoxylin–and-eosin, and examined under a light microscope. Experiments were carried out in strict accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals of Osaka City University. Differences in the BMD among groups were evaluated with one way analysis of variance, one way ANOVA. To adjust for multiple comparisons when ANOVA showed a significant difference between groups (P<0.05), Fisher LSD post-hoc test was used to identify which group differences accounted for the significant P value. The analyses were conducted by using the StatView 5.0 (SAS Institute, Cary, NC).

Results

Specificity of enhancing effect of heparin on induction of ALP activity by BMPs

We first compared the effect of heparin on ALP activity, a typical marker of osteoblast differentiation, induced by BMP-2, BMP-4, and BMP-6 in C2C12 cells. As we reported previously, heparin at 5 mg/ml enhanced the ALP activity induced by both BMP-2 and BMP-4 (Figs. 1A and 1B). In contrast, the enzyme activity induced by BMP-6 was suppressed by heparin (Fig. 1C). Drosophila DPP, a homolog of mammalian BMP-2 and BMP-4, failed to induce ALP activity in the presence or absence of heparin in our assay system (data not shown), though highly purified heparin had effects on BMP-2 similar to those of the heparin used throughout the present study (Fig. 1D). Low-molecular-weight (LMW) heparin, which is roughly 5-fold smaller than native heparin, also enhanced induction of ALP activity by BMP-2, but to a lesser extent than native heparin (Fig. 1D). The estimated ED₅₀ of LMW heparin and native heparin were 10 and 2 mg/ml, respectively, suggesting that the size of sulfated polysaccharides is critical for enhancement of induction of ALP activity by BMPs.

Role of endogenous heparan sulfate and pre-treatment with heparin in responses to BMPs

C2C12 cells express heparan sulfate
proteoglycans on the cell surface and/or in the extracellular space (Fig. 2A, upper panels). We next examined the role of endogenous heparan sulfate in responses to BMP-2. When C2C12 cells were pretreated with heparitinase and then further cultured with BMP-2 in the presence of heparitinase, endogenous heparan sulfate was removed from the cultures in a dose-dependent manner (Fig. 2A, upper panels). However, similar numbers of ALP-positive cells were induced by BMP-2 regardless of the presence of heparitinase (Fig. 2A, lower panels). Furthermore, the dose-dependency of effects of BMP-2 on ALP activity was nearly equivalent in the presence or absence of 0.1 U/ml of heparitinase (Fig. 2B).

We previously reported that heparin did not enhance the osteoblast differentiation induced by activation of a signaling pathway downstream from BMP receptors, suggesting that a primary target of heparin is upstream of the receptor. In agreement with this finding, pretreatment of C2C12 cells with heparin from day -1 to day 0 did not enhance the ALP activity induced by BMP-2 on day 3 (Fig. 2C). Moreover, pre-incubation of BMP-2 with heparin before treatment did not affect ALP activity (Fig. 2C). Although we can not rule out the possibility that the pre-incubation with heparin was negligible on day 3, these results suggest that heparin rapidly modified BMP-2 in culture media.

**Time-dependency of effects of heparin on BMP-2 activity**

We further examined the time-courses of change in levels of expression of genes related to osteoblast differentiation, such as ALP, osteocalcin, osterix, and Runx2 type I and types II/III (Fig. 3). Levels of Runx2 type I were not changed by BMP stimulation either with or without heparin until 48 h. The expression of the other genes was induced by BMP-2 and further stimulated by heparin after 24 h. However, the extent of induction of osterix by BMP-2 at 4 h was not enhanced by heparin (Fig. 3).

We next assessed whether heparin affected the levels of phosphorylation of Smad1/5/8 induced by BMP-2. BMP-2-induced phosphorylation of Smad1/5/8 peaked within 1 h and gradually decreased until 72 h (Figs. 4A and 4B). Interestingly, levels of phosphorylation of Smad proteins were not affected within 12 h by heparin, but were increased at 24 and 48 h (Fig. 4A). This time-dependent effect of heparin on the phosphorylation of Smads was further confirmed by immunohistochemical analysis. Accumulation of phosphorylated Smad1/5/8 in nuclei was increased by BMP-2 stimulation at 24 h and thereafter only in the presence of heparin (Fig. 4B). We further examined the time-dependency of enhancing effects of heparin on the phosphorylation of Smads induced by BMP-2. C2C12 cells were stimulated with BMP-2 in the presence or absence of heparin for 1 or 24 h on day 1 or 2 after cell inoculation to examine the effect of cell growth on the stimulatory capacity of heparin. Phosphorylated Smads were increased only in cultures treated for 24 h with BMP-2 in the presence of heparin (Fig. 4C), suggesting that the enhancement of phosphorylation of Smad1/5/8 by heparin depends on the period of treatment and not the day of incubation. We examined the effect of heparin on the stability of phosphorylated Smad proteins. C2C12 cells were pretreated for 1 or 6 h with BMP-2 to induce phosphorylation of Smads, then
cultured without BMP-2, and finally examined for levels of phosphorylated Smads at 24 h. Phosphorylated Smads were undetectable in cultures pre-treated with BMP-2 for 1 or 6 h even in the presence of heparin (Fig. 4D), suggesting that heparin enhances the levels of phosphorylated Smads induced by BMP-2 without increasing their stability.

**Heparin maintains the concentration of BMP-2 in culture medium**

Since phosphorylated Smads were increased by heparin only in the presence of BMP-2, we determined amounts of BMP-2 in culture media in the presence and absence of heparin. Surprisingly, in the absence of heparin, the concentration of BMP-2 in culture media rapidly decreased below 50% of the original concentration within 1 h and was almost undetectable after 10 h (Fig. 5A). In contrast, in the presence of heparin, more than 60% of BMP-2 remained in culture media at 12 h, and BMP-2 was still detectable at 50 h (Fig. 5A). We also determined the amounts of BMP-2 in cell layers throughout the period of culture. As shown in Fig. 5A, right panel, amounts of BMP-2 were below 10% in the presence and absence of BMP-2, suggesting that disappearance of BMP-2 from culture media was due to degradation of the ligand rather than accumulation in cell layers.

We next examined whether BMP-2 in culture media affects the differentiation of C2C12 cells in the absence of heparin. When C2C12 cells were treated for 3 days with graded concentrations of BMP-2, myogenic differentiation was almost completely inhibited by 100 ng/ml of BMP-2 and osteoblast differentiation was induced by 300 ng/ml of BMP-2 (Fig. 5B and (8)). However, these concentrations were decreased to 1/3 - 1/10 when culture media were changed every 24 h to keep the concentrations of BMP-2 in the media (Fig. 5B). These findings suggest that heparin blocks rapid decrease in BMP concentration in the culture media and that lower concentrations of BMP-2 are capable of affecting the pathway of differentiation of C2C12 cells if they are continuously stimulated by daily refreshment of ligand.

**Heparin protects BMP activity from inhibition by noggin.**

Noggin, a BMP antagonist that blocks BMP signaling in the extracellular space, is induced by BMPs as an early response gene (25). Noggin mRNA was induced within 1 h after BMP-2 stimulation in C2C12 cells (Fig. 6A). The level of induction of noggin mRNA by BMP-2 was not enhanced by heparin at 1 h but was increased at 72 h (Fig. 6A). Furthermore, exogenously added recombinant noggin dose-dependently decreased the numbers of ALP-positive cells induced by 300 ng/ml of BMP-2. In contrast, 100 ng/ml of BMP-2 with 5 μg/ml heparin induced levels of ALP activity similar to those induced by 300 ng/ml of BMP-2 alone. In this condition, large numbers of ALP-positive cells remained even in the presence of 300 ng/ml of exogenous noggin (Fig. 6B). These data suggest that heparin protects BMPs from inhibition by noggin.

**Heparin stimulates the ectopic bone formation induced by BMP-2 in vivo.**

Finally, we examined the enhancing effect of heparin on BMP-2 activity in vivo. BMP-2 induced ectopic bone formation within 2 weeks in mice. Heparin dose-dependently increased the
size of new bone induced by BMP-2 up to 4 weeks (Figs. 7A and 7B). Heparin alone preserved more bone tissue induced by BMP-2 after 4 weeks of incubation than did BMP-2 alone (data not shown). Bone mineral density (BMD) was also increased by heparin in a dose-dependent fashion (Fig. 7C). At 4 weeks, the inside of the bone pellets treated with BMP-2 alone was filled with bone marrow. In the presence of heparin, however, not only bone marrow but also much trabecular bone was observed in the pellets (Fig. 7D).

Discussion
Some groups including our own have independently reported that sulfated polysaccharides, such as heparin, heparan sulfate, and dextran sulfate, enhanced the osteoblast differentiation induced by BMPs in vitro (17,26). In the present study, we demonstrated that heparin enhances the bone formation induced by BMP-2 in vivo as well. In the presence of heparin, BMP-2 induced larger amounts of mineralized bone tissues within 2 weeks in mice. Moreover, we demonstrated that LMW heparin, which is smaller than native unfractionated heparin and is widely used clinically, also enhanced BMP-2 activity at least in vitro, although the specific activity of LMW heparin in enhancing this effect was less than that of native heparin. This confirmed our previous finding that sulfated polysaccharides stimulate BMP activities in a size–dependent fashion (17). Taken together, these findings suggested that simultaneous application of BMP-2 and sulfated polysaccharides is useful for regeneration of local bone tissues in vivo. This possibility requires examination in in vivo models of bone regeneration in higher animals.

Cultured cells express various types of proteoglycans, which consist of core proteins and polysaccharide chains including heparan sulfate (27-29). We have shown that not only heparin but also other types of sulfated polysaccharides such as native heparan sulfate and synthetic dextran sulfate enhance BMP activities (17). These proteoglycans may accumulate on the cell surface and in the extracellular matrix and regulate BMP activities. Indeed, it has been reported that cell surface proteoglycans inhibit the activity of BMP-7 in ROS17/2.8 osteosarcoma cells (30). However, removal of heparan sulfate from cultures of C2C12 cells did not affect the osteoblast differentiation that occurred in response to BMP-2. This discrepancy in findings may due to differences in cell types tested, i.e., osteosarcoma versus myoblasts, or in BMP subgroups, BMP-7 versus BMP-2. Interestingly, heparin inhibited the ALP activity that was stimulated by BMP-6 in C2C12 cells. BMP-6 is classified into the same subgroup as BMP-7, but not with BMP-2/BMP-4, based on amino acid similarity (31). It appeared that the positive or negative effects of sulfated polysaccharides on BMP activities depend on the primary structures of BMPs. Although all amino acid sequences of BMP-2 through BMP-8 were identified in the bone-inducing fraction purified from bone extracts using a heparin column, the structures of the N-terminus of BMP-2 and BMP-4, which is thought to be the heparin-binding domain, are quite different from those of BMP-6 and BMP-7 (26,31). These differences may affect the three-dimensional structures and receptor-binding abilities of ligand-heparin complexes. Indeed, different types of receptors have been identified
for the BMP-2 and BMP-7 subgroups (4, 5). These issues require further examination.

Heparin is thought to increase the binding affinity of FGF to its specific receptor and thereby enhance downstream signaling (18, 19). A similar mechanism seems unlikely to occur in the case of BMPs, since heparin did not stimulate complex formation between BMP-2 and BMPR-IA receptor on the surface of C2C12 cells (17). Moreover, heparin failed to stimulate certain early events in response to BMP-2, such as the phosphorylation of Smad proteins and the induction of early response genes including noggin and osterix. Instead, heparin required more than 12 h to stimulate the events induced by BMP-2. Interestingly, we found that heparin maintained the concentration of BMP-2 in culture medium at higher levels and protected BMP-2 from the inhibition by noggin, which was induced as part of the negative feedback loop in response to BMP-2. Based on the findings we obtained, we propose a novel mechanism for the stimulatory activity of heparin on BMPs (Fig. 8). In the absence of heparin, BMP activities are negatively regulated by the inhibitory microenvironment, e.g., immediate decreases in amounts of active ligands due to degradation, and induction of BMP antagonists are induced by BMP signaling as part of the negative feedback loop to suppress excess signaling. However, in the presence of heparin, bioactive BMPs remain in the extracellular space for a longer period and active ligands are protected from suppression by antagonists.

In conclusion, heparin stimulated not only the osteoblast differentiation induced by BMP-2 and BMP-4 in vitro but also the bone formation induced by BMP-2 in vivo. It appears that heparin stimulates BMP activities by protecting ligands from the inhibitory microenvironment around target cells rather than increasing receptor-binding affinity. These findings suggest that simultaneous administration of BMPs with sulfated polysaccharides should be clinically useful at least for local bone regeneration.

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Footnotes
*The abbreviations used are: BMP, bone morphogenetic protein; GDF, growth and differentiation factor; TGF-β, transforming growth factor-β; FGFs, fibroblast growth factors; LMW, low molecular weight; DMEM, Dulbecco’s modified Eagle medium; FBS, fetal bovine serum; ALP, Alkaline phosphatase; RT, reverse transcription; PCR, polymerase chain reaction; MHC, myosin heavy chain; BMC, bone mineral content; DXA, dual-energy X-ray absorptiometry; BMD, bone mineral density.

Figure legends
Figure 1. Specificity of effects of heparin on BMP activities in C2C12 cells
C2C12 cells were cultured for 3 days with graded concentrations of BMP-2 (A), BMP-4 (B), or BMP-6 (C) in the presence (closed circles) or absence (open circles) of 5 μg/ml heparin. (D) C2C12 cells were treated for 3 days with graded concentrations of unfractionated heparin (closed circles), highly purified heparin (closed diamonds) or LMW heparin (open circles) in the presence of 100 ng/ml of BMP-2.

Figure 2. Effects of endogenous and exogenous sulfated polysaccharides on the ALP activity induced by BMP-2.
(A) C2C12 cells were treated with heparitinase at 0.001 or 0.01 U/ml for 3 days in the presence of 300 ng/ml of BMP-2. After 3-day incubation, the cells were stained with anti-heparan sulfate antibody (upper panels) or for ALP activity (lower panels). (B) C2C12 cells were incubated for 3 days with graded concentrations of BMP-2 in the presence (closed circles) or absence (open circles) of 0.1 U/ml of heparitinase, and ALP activity was then determined. (C) ALP activity in C2C12 cells cultured in the presence and absence of BMP-2 and/or heparin. C2C12 cells were inoculated 2 days before (day -2) treatment with BMP-2 and/or heparin. The cells were treated without (Control) or with 100 ng/ml of BMP-2 (BMP2) or 5 μg/ml of heparin (Heparin) on day 0. A portion of the cultures was pre-cultured with 5 μg/ml of heparin on day -1, then incubated with 100 ng/ml of BMP-2 in the absence of heparin on day 0 (Heparin > BMP2). In other cultures, on day 0, the cells were treated with mixtures of 100 ng/ml of BMP-2 and 5 μg/ml of heparin pre-incubated for 1, 3, 6, or 9 h at room temperature in a clean bench or without pre-incubation (0 h). The ALP activity in all of the cultures was determined on day 3.

Figure 3. Time-dependent effects of heparin on the expression of genes induced by BMP-2
C2C12 cells were cultured with and without 100 ng/ml of BMP-2 in the presence or absence of 5 μg/ml of heparin. Total RNAs were harvested at 0, 1, 4, 24, and 48 hr after stimulation, and RT-PCR analysis
was performed for ALP, osteocalcin, osterix, and Runx2 type I and types II/III. GAPDH was used as an internal control. Similar results were obtained in 3 independent experiments.

**Figure 4. Heparin enhances phosphorylation of Smad1/5/8 induced by BMP-2.**

(A) Time-dependent effects of heparin on the phosphorylation of Smad1/5/8 induced by BMP-2. C2C12 cells were treated with 100 ng/ml of BMP-2 in the presence or absence of 5 μg/ml heparin up to 48 h. The cells were lysed at the indicated time points, and then phosphorylated and total Smad1/5/8 were determined by Western blotting. (B) Nuclear accumulation of phosphorylated Smad1/5/8 induced by BMP-2 in the presence of heparin. C2C12 cells were treated with 100 ng/ml of BMP-2 in the presence or absence of 5 μg/ml heparin for up to 72 h. The cells were then fixed and stained with anti-phosphorylated Smad1/5/8 antibody at the indicated time points. (C) Heparin increased phosphorylated Smad1/5/8 induced by BMP-2 after 12 h but not at 1 h. C2C12 cells were treated for 1 or 24 h with 100 ng/ml BMP-2 in the presence or absence of 5 μg/ml heparin on day 1 or day 2. Phosphorylated Smad1/5/8 were examined by Western blotting. (D) Heparin did not stabilize phosphorylated Smad1/5/8 induced by BMP-2. C2C12 cells were pretreated for 1 or 6 h with 100 ng/ml BMP-2 in the presence of 5 μg/ml heparin, and culture media were then changed to fresh media without BMP-2. Phosphorylated Smad1/5/8 were examined at 24 h by Western blotting. Similar results were obtained in 3 independent experiments.

**Figure 5. Heparin prolonged the half-life of BMP-2 in culture media.**

C2C12 cells were treated with 100 ng/ml BMP-2 at time 0 in the presence (closed circles) or absence (open circles) of 5 μg/ml heparin. The amounts of BMP-2 in the media (left panel) and cell extracts (right panel) were determined by ELISA at each time point (0, 1, 3, 10, 27, and 49 h). (B) Effect of refreshment of culture media containing BMP-2 on the differentiation of C2C12 cells. C2C12 cells were treated with graded concentrations of BMP-2 on day 0. The cells were cultured for 3 days without changing the media (upper panel) or with daily changing the media (lower panel). The cells were doubly stained for MHC (red) and ALP (blue) as markers of differentiation for mature muscle cells and osteoblasts, respectively.

**Figure 6. Heparin protects BMP-2 from the inhibitory activity of noggin.**

(A) RT-PCR analysis of noggin mRNA induced by BMP-2 in C2C12 cells. Cells were treated with 100 ng/ml BMP-2 in the presence or absence of 5 μg/ml heparin up to 72 h. Levels of expression of noggin mRNA were examined by RT-PCR. (B) Heparin protects BMP-2 from the inhibitory activity of noggin. C2C12 cells were cultured with graded concentrations of recombinant mouse noggin/Fc chimera in the presence of 300 ng/ml of BMP-2 alone or 100 ng/ml of BMP-2 plus 5 μg/ml of heparin. ALP activity was stained on day 3 as a marker of osteoblast differentiation. Similar results were obtained in 3 independent experiments.
Figure 7.  Heparin enhances the ectopic bone formation induced by BMP-2 in vivo.  
Five μg of BMP-2 and 0, 0.25, 2.5, or 25 μg of heparin were implanted subcutaneously to induce ectopic bone formation in mice as described in Materials and Methods.  After 2, 3, or 4 weeks, the implants were removed (A) and examined by soft X-ray analysis (B).  BMD of each implant was determined by DEXA (C).  Samples were analyzed by ANOVA.  *: P < 0.05.  Sections of the implants at 4 weeks were stained with hematoxylin-and-eosin (D).

Figure 8.  Possible mechanism of effects of heparin on BMP activities.  
Left panel: In the absence of heparin, BMPs disappear quickly from culture media due to degradation. Noggin is also induced by a negative feedback loop to suppress BMP activity.  Right panel: In contrast, in the presence of sulfated polysaccharides, BMPs are maintained in culture media for longer periods of time and protected BMPs and present these ligands to receptors even in the presence of noggin.  Finally, intracellular signaling of BMPs is continuously activated in the presence of the sulfated polysaccharides.
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Heparin potentiates the in vivo ectopic bone formation induced by bone morphogenetic protein-2
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