Dual effects of heparin on BMP-2-induced osteogenic activity in MC3T3-E1 cells

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Abstract:

Heparin displays several types of biological activities by binding to various extracellular molecules, including pivotal roles in bone metabolism. We have previously reported that heparin competitively inhibits the binding activity of bone morphogenic protein-2 (BMP-2) to BMP and the BMP receptor (BMPR) and suppresses BMP-2 osteogenic activity. In the present study, we examined whether heparin affects osteoblast differentiation induced by BMP-2 at various time points *in vitro*. We found that 72 h of treatment with heparin inhibited alkaline phosphatase (ALP) activity. However, 144 h of treatment enhanced the ALP activity in BMP-2-stimulated MC3T3-E1 cells. Although heparin decreased the phosphorylation of Smad1/5/8 after 0.5 h of culture, prolonged periods of culture with heparin enhanced the Smad phosphorylation. In addition, 72 h of treatment with heparin enhanced the mRNA expression of runx2 and osterix in BMP-2-stimulated MC3T3-E1 cells. Furthermore, the mRNA expression of BMP antagonists and inhibitory Smads induced by BMP-2 was preferentially blocked by heparin at the 24 and 48 h time points. These findings indicate biphasic effects of heparin on BMP-2 activity and suggest that heparin has complex effects on the BMP-2 osteogenic bioactivities. Prolonged culture with heparin stimulated BMP-2-induced osteogenic activity *via* down-regulation of BMP-2 antagonists and inhibitory Smads.

Key words:

osteoblast, heparin, BMP-2, Smad, Runx2, osterix

Abbreviations: ALP – alkaline phosphatase, BMP – bone morphogenic protein, BMPR – BMP receptor, ECM – extracellular matrix, GAG – glycosaminoglycan

Introduction

The extracellular matrix (ECM) provides structural strength to tissues and helps to maintain the shape of organs. Proteoglycans, which are characterized by a core protein with at least one glycosaminoglycan (GAG) chain attached, commonly mediate the interactions of ECM components with extracellular molecules, including growth factors, adhesion molecules, and cytokines. Recently, the potential roles of GAGs in various biological processes [27, 30], including angiogenesis [26], viral invasion [29], tumor growth [34], and bone metabolism [1, 3, 28], have been reported.

Well-known endogenous GAGs include heparin, heparan sulfate, keratan sulfate, chondroitin sulfate and hyaluronic acid. GAG structures are based on a disaccharide repeat. Four classes of GAGs exist and

are each distinguished by a particular repeating disaccharide. Among them, heparin is based on a repeat disaccharide of iduronic acid-(β1-4)-*N*-acetylglucosamine-(α1-4).

Bone morphogenic proteins (BMPs) were originally identified as unique proteins in demineralized bone matrix that induce ectopic bone formation upon implantation into muscular tissues [33]. BMPs were later shown to regulate the differentiation and function of cells that are involved in bone and cartilage formation and degradation, including osteoblasts, chondrocytes, and osteoclasts [4].

Signaling through BMPs is initiated by binding to the specific transmembrane receptors, type I and type II serine/threonine kinase receptors [37]. Type I receptors are activated by ligand bound-type II receptors and then phosphorylate downstream molecules in the cytoplasm. Further, Smad 1/5/8 transcription factors are substrates that are phosphorylated by the BMP receptor (BMPR) in the cytoplasm and accumulate in the nucleus within 1 h after BMP stimulation [35]. Phosphorylated Smads directly regulate the expression of primary target genes by binding to their promoter or enhancer elements together with Smad 4 and other transcription factors [12].

Recently, we have found that heparin inhibits BMP-2 osteogenic bioactivity by binding to both BMP-2 and BMPR. However, the effects of GAGs, including heparin, on BMP activity have not been fully examined. For example, heparan sulfate/heparin chains have been found to bind to BMP-4 and restrict the expression pattern of BMP-4 in Xenopus embryos [22]. Heparan sulfate also binds to noggin, a secreted polypeptide that inhibits the function of BMP, resulting in modification of BMP-4 activity [23], while heparan sulfate chains bind to BMP-7 and the heparan sulfate/BMP-7 interaction is required for BMP-7 signaling [11]. In addition, heparan sulfate and heparin inhibit BMP-2 osteogenic activity by sequestering BMP-2 on the cell surface and mediating the internalization of BMP-2 [14]. In contrast, some studies have reported that heparin enhances the biological activities of BMP-2 by protecting BMP-2 from degradation and inhibition by BMP antagonists [32, 38]. Thus, the mechanism by which heparin regulates bone metabolism induced by BMP-2 remains unclear. We hypothesized that heparin can act as either a negative or positive modulator of BMP activity depending on its action time. Because we have already reported that heparin suppresses BMP-2-induced osteogenic

activity [16], we examined the effects of heparin on osteoblast differentiation induced by BMP-2 for prolonged periods of time in the present study.

Materials and Methods

Reagents

Porcine intestinal mucosal heparin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human BMP-2 was kindly supplied by Astellas Pharmaceutical Inc. (Tokyo, Japan). The anti-phospho Smad 1/5/8 polyclonal antibody, anti-phospho-p38 MAPK polyclonal antibody, and anti-p38 MAPK polyclonal antibody were obtained from Cell Signaling Technology, Inc. (Beverly, CA, USA). The anti-Smad 1/5/8/9 polyclonal antibody was purchased from Abcam (Cambridge, UK).

Cell culture

MC3T3-E1 cells, an osteoblastic cell line established from mouse calvaria, were cultured in α-minimum essential medium (α-MEM; Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS; Gibco), penicillin G (100 U/ml), and streptomycin (100 μg/ml). The cells were maintained at 37°C in an atmosphere containing 5% CO_2.

Alkaline phosphatase (ALP) activity

Quantitative analysis of ALP activity was performed biochemically using the Bessey-Lowry enzymological method [2]. Cells were distributed in 24-well plates at a density of 1×10^5/well and incubated for 24 h. The growth medium was changed, and the cells were cultured with or without BMP-2 (100 ng/ml) and heparin (100 μg/ml). After an additional 48–144 h of incubation, the cells were washed twice with Hank's balanced salt solution (HBSS) and solubilized with HBSS containing 0.2% Nonidet P-40. The ALP activity of the lysate was determined using *p*-nitrophenylphosphate (pNPP; Wako, Osaka, Japan). After a 30-min incubation at 37°C, the absorbance of pNPP was measured at 405 nm using a Multiscan JX microplate reader (Thermo Fisher Scientific, Rockford, IL, USA). The ALP activity was normalized for protein concentration using the DC protein assay kit (Bio-

Rad, Hercules, CA, USA) measured by spectrophotometry at 630 nm. The specific activity of alkaline phosphatase was calculated as μM/μg protein.

RT-PCR analysis

Gene expression levels were determined using a reverse transcription-polymerase chain reaction (RT-PCR) method. Total RNA was extracted using a Total RNA Extraction Miniprep System (Viogene Co, Sunnyvale, CA, USA) according to the manufacturer's instructions, and the reverse transcript was subjected to PCR. Oligonucleotide primers were designed to amplify cDNA fragments encoding Runx2 (381 bp) and osterix (497 bp). The following primers were used: runx2 forward; 5'-CCAGATGGGACTGTGGTTACC-3' and reverse; 5'-ACTTGGTGCAGAGTTCAGGG-3', osterix forward; 5'-CTGGGGAAAGGAGGCACAAAGAAG-3' and reverse; 5'-GGGTTAAGGGGAGCAAAGTCAGAT-3', and GAPDH forward; 5'-ACCACAGTCCATGCCATC AC-3' and reverse; 5'-TCCACCACCCTGTTGCTGTA-3'.

Real-time RT-PCR analysis

In some experiments, the extracted total RNA was reverse-transcribed and subjected to real-time RT-PCR. For real-time RT-PCR, the PCR products were detected by the FAST SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The following primer sequences were used: β-actin forward; 5'-CTGAACCCTAAGGCCAACCGTG-3' and reverse; 5'-GGCATACAGGGACAGCACAGCC-3', noggin forward; 5'-CTGGTGGACCTCATCGAACA-3' and reverse; 5'-CTCGTTCAGATCCTTCTCCTTAGG-3', follistatin forward; 5'-GAAAACCTACCGCAACGAATG-3' and reverse; 5'-TCCGGCTGCTCTTTGCAT-3', smad 6 forward; 5'-GGGTGTCTCTAGCATCGTTTCG-3' and reverse; 5'-CCGCGACCGCTCAACTC-3', and smad 7 forward; 5'-CAGCACTGCCAAGCATGG T-3' and reverse 5'-ACCGAAACGCTGATCCAAAG-5'. Thermal cycling and fluorescence detection were performed using a StepOne™ Real-Time PCR System (Applied Biosystems). The real-time RT-PCR efficiency (E) was calculated according to the equation provided by Rasmussen [25] (E = 10[–1/slope]) for β-actin and various target genes. The slope was determined from the graph of ng of cDNA substrate (x-axis) *versus* the cycle number at the crossing point (CP) (y-axis). The CP is the PCR cycle number that represents the CP in SYBR Green fluorescence intensity above the automatic noise-based threshold. The fold increase in copy numbers of mRNA was calculated as a relative ratio of the target gene to β-actin, following the mathematical model introduced by Pfaffl [24].

$$\text{Fold increase} = \frac{(E_{TARGET})^{CP\ TARGET\ (MEAN\ control-MEAN\ subject)}}{(E_{\beta\text{-}ACTIN})^{CP\ \beta\text{-}ACTIN\ (MEAN\ control-MEAN\ subject)}}$$

Western blot analysis

Cells were distributed in 6-well plates at a density of 8×10^5/well and incubated for 24 h. The growth medium was changed, and the cells were cultured with or without BMP-2 (100 ng/ml) and heparin (100 μg/ml). After an additional 0.5–48 h of incubation, the cells were washed with phosphate buffered saline (PBS) and lysed in lysis buffer (75 mM Tris-HCl containing 2% SDS and 10% glycerol, pH 6.8). The protein contents were measured using a DC protein assay kit. The samples were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). Non-specific binding sites were blocked by immersing the membranes in 10% skim milk in PBS for 60 min at room temperature, after which the membranes were washed 4 times with PBS and incubated with the diluted primary antibody overnight at 4°C. Anti-phospho-Smad 1/5/8, anti-Smad1, anti-phospho-p38, and anti-p38 antibodies and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG secondary antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) were used in this experiment. After washing the membranes, ECL reagent (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for chemiluminescence detection with Hyperfilm-ECL (Amersham Pharmacia Biotech).

Statistical analysis

Statistical analyses for ALP activity were conducted with statistics software (JMP8.0.2, SAS Institute Inc., Cary, NC, USA). The results were expressed as the mean ± SD. One-way analysis of variance was employed to analyze the manner in which the distribution of each continuous variable differed across the groups. The Tukey-Kramer HSD (honestly significant difference) test was utilized to test differences with respect to the group means.

In real-time RT-PCR analyses, statistical significance was determined using Student's *t*-test. A p value of less than 0.05 was considered significant.

Fig. 1. Heparin has biphasic effects on ALP activity induced by BMP-2 in osteoblasts. MC3T3-E1 cells (2×10^5 cells/well) were stimulated with BMP-2 (100 ng/ml) in the presence or absence of various concentrations of heparin for 48–144 h. The specific activity of ALP was determined as described in the Materials and Methods. Values are expressed as fold increases relative to untreated controls. The data are expressed as the mean ± SD of triplicate cultures. The experiment was performed three times with similar results obtained in each experiment. * p < 0.0001 as measured by the Tukey-Kramer HSD test
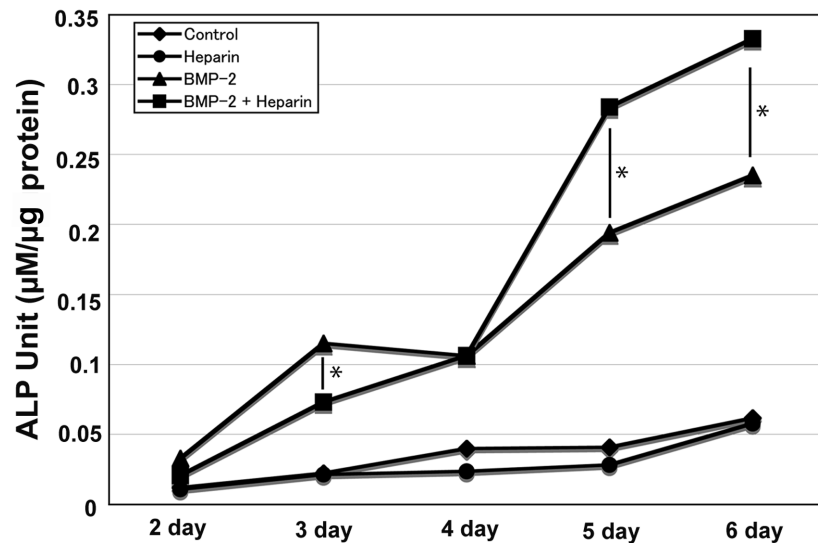
Results

Heparin enhances osteoblast differentiation induced by BMP-2

To determine the effect of heparin on osteoblast differentiation induced by BMP-2, we assessed ALP activity, a typical marker of osteoblast differentiation. ALP activity and mineralization are well known to be dramatically enhanced when MC3T3-E1 cells are cultured with BMP-2. Heparin inhibited the ALP activity induced by BMP-2 after culturing for 72 h. However, heparin remarkably enhanced the ALP activity induced by BMP-2 after culturing for 120–144 h (Fig. 1).

Heparin sustains the BMP-2-mediated signaling activity

Next, we examined the levels of Smad 1/5/8 phosphorylation for prolonged periods of time. BMPs activate identical amino acid sequences at the C-terminal domain of R-Smads. The phosphorylation of Smad 1/5/8 was noticeable after 30 min of BMP-2 treatment, which continued for up to 6 h and then gradually decreased until 48 h. As we have previously reported [16], heparin (100 μg/ml) inhibited the levels of phosphorylation of Smad 1/5/8 induced by BMP-2 (100 ng/ml) at the time points of 30 min and 1 h. However, when the cells were incubated with both BMP-2 and heparin for

Fig. 2. Heparin has biphasic effects on BMP-2-mediated Smad-1/5/8 phosphorylation. MC3T3-E1 cells (4×10^5 cells/well) were stimulated with BMP-2 (100 ng/ml) in the presence or absence of heparin (100 μg/ml) for the indicated time periods, then whole lysates were subjected to immunoblotting analyses
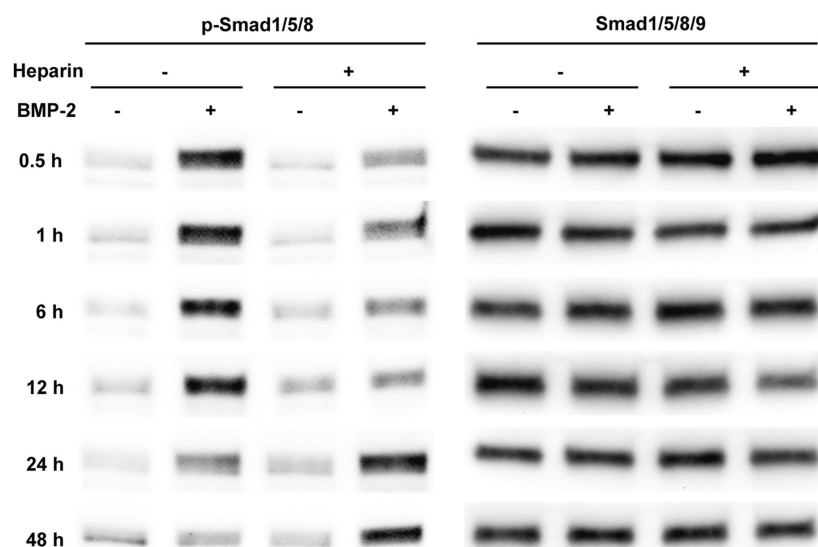
longer periods (24 and 48 h), the level of Smad 1/5/8 phosphorylation was higher than that in cells treated with BMP-2 alone (Fig. 2).

BMP receptors are well-known to determine the intensity of BMP signals *via* Smad 1 C-terminal phosphorylations, and the duration of the activated phospho-Smad signal is known to be regulated by sequential Smad linker region phosphorylation at conserved MAPK and GSK sites [8]. To elucidate the role of the p38 MAPK pathway in the regulation of BMP-2 responses, the expression of phospho-p38 MAPK was detected by Western blot analysis. However, the phosphorylation of p38 MAPK did not change when the cells were cultured with BMP-2 or heparin (data not shown).

To exclude the role of heparin in osteogenesis induced by BMP-2 for prolonged periods of time, we assessed the expression levels of genes related to osteoblast differentiation, such as runx2 and osterix, by RT-PCR. In 72-h cultures, the expression levels of

runx2 and osterix mRNA were not affected by the treatment with BMP-2 (100 ng/ml). In contrast, when the cells were incubated with both BMP-2 and heparin (100 µg/ml), the mRNA expression of these genes was remarkably enhanced (Fig. 3 A, B).

Heparin inhibits the BMP-2-induced mRNA expression of BMP-2 antagonists and inhibitory Smads

To examine the mechanisms involved in the enhancement of BMP-2-induced osteogenesis by heparin for prolonged periods of time, we assessed the expression levels of BMP-2 antagonist genes, such as noggin and follistatin, by real-time RT-PCR. In 24- and 48-h cultures, stimulation with BMP-2 (100 ng/ml) enhanced the expression levels of noggin and follistatin mRNA. In contrast, the mRNA expression of these genes was remarkably suppressed when the cells were cultured with both BMP-2 and heparin (100 µg/ml) (Fig. 4 A, B).

Finally, we assessed the expression levels of smad 6 and smad 7, which are known as inhibitory Smads, by real-time RT-PCR. In 48-h cultures, the stimulation of BMP-2 (100 ng/ml) enhanced the expression levels of smad 6 and smad 7 mRNA. However, the mRNA expression of these genes was suppressed below basal control levels when the cells were cultured with both BMP-2 and heparin (100 µg/ml) (Fig. 5 A, B).
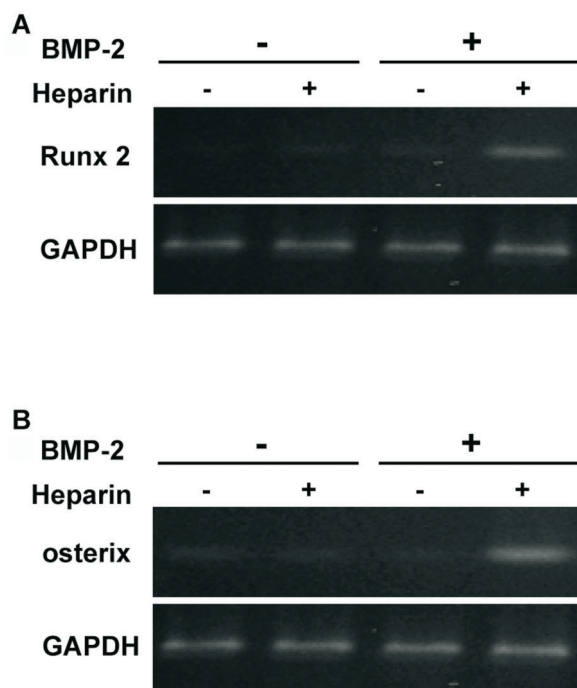

Fig. 3. Heparin enhances the gene expression of runx2 and osterix in MC3T3-E1 cells. MC3T3-E1 cells (4 × 10^5 cells/well) were incubated with BMP-2 (100 ng/ml) in the presence or absence of heparin (100 µg/ml) for 72 h, then total RNA from each cell culture was reverse-transcribed with random primers. PCR amplification was performed using primers specific for (A) Runx2, (B) osterix, and GAPDH. The PCR products were resolved on 2 % agarose gels and stained with ethidium bromide

Discussion

Long-term administration of heparin is well-known to be associated with an increased risk of developing osteoporosis [15, 36]. Heparin has also been reported to have a tendency to increase the formation of osteoclasts at lower concentrations, whereas it tends to decrease the numbers of osteoclasts in rat bone marrow cell cultures at high concentrations [7]. Recent reports have indicated that GAGs, including heparin, heparan sulfate, keratan sulfate, dermatan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, and hyaluronic acid, mediate BMP activity [14, 17, 19] and that sulfation is required for BMP activity-mediated processes [20, 21]. However, the effects of heparin (which is the most sulfated GAG) on osteogenic activity have not been fully elucidated. Takada and Zhao have indicated that heparin enhances the biological activities of
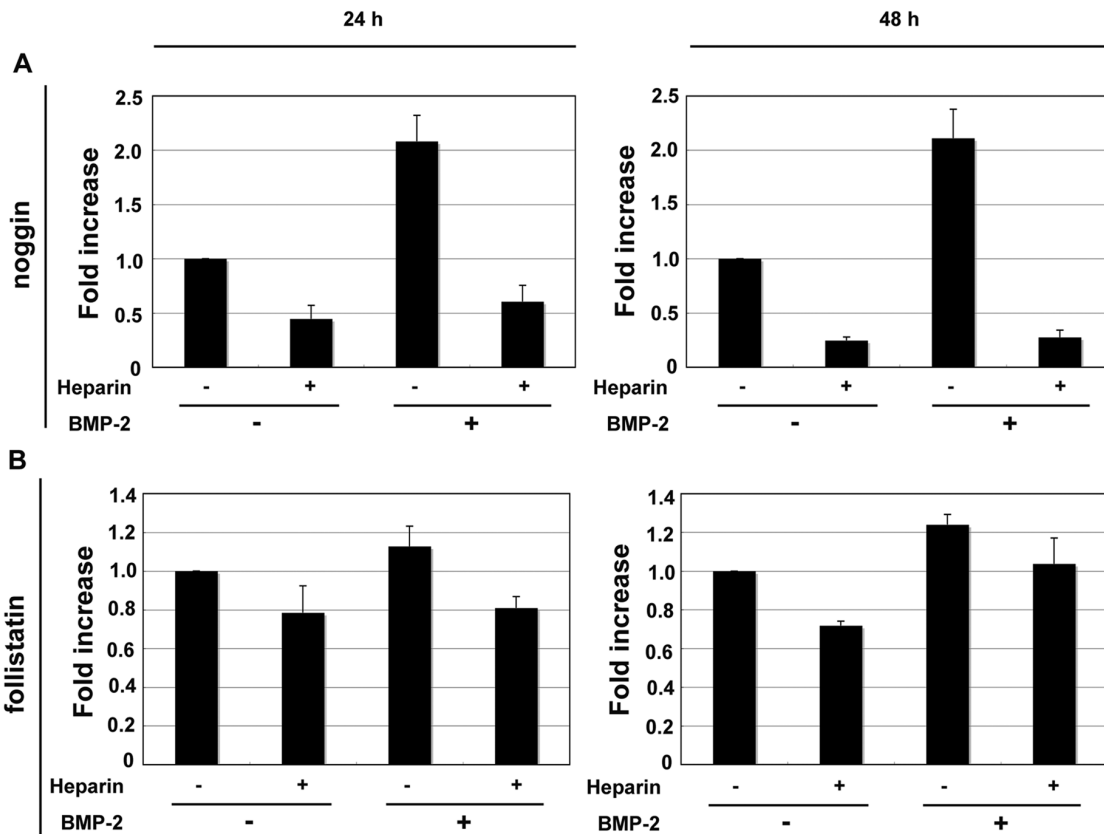
Fig. 4. Heparin suppresses the gene expression of noggin and follistatin in MC3T3-E1 cells. MC3T3-E1 cells (4×10^5 cells/well) were cultured with BMP-2 (100 ng/ml) in the presence or absence of heparin (100 µg/ml) for 24 or 48 h. Total RNA was isolated, reverse-transcribed into cDNA and PCR-amplified using SYBR green. The PCR amplification was performed using primers specific for (**A**) noggin, (**B**) follistatin, and β-actin. The fold changes in noggin and follistatin mRNA copy number values represent the average SD of data derived from triplicate cultures. * p < 0.05, ** p < 0.01, respectively, as measured by Student's *t*-test
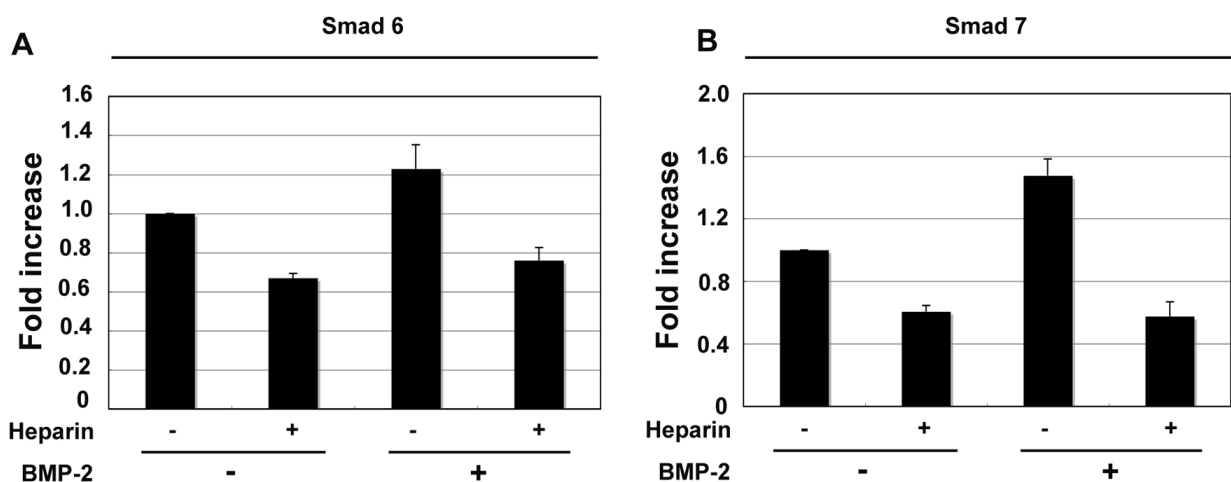

Fig. 5. Heparin supresses the gene expression of Smad 6 and Smad 7 in MC3T3-E1 cells. MC3T3-E1 cells (4×10^5 cells/well) were cultured with BMP-2 (100 ng/ml) in the presence or absence of heparin (100 µg/ml) for 48 h. Total RNA was isolated, reverse transcribed into cDNA and PCR-amplified using SYBR green. The PCR amplification was performed using primers specific for (**A**) Smad 6, (**B**) Smad 7, and β-actin. The fold changes in Smad 6 and Smad 7 mRNA copy number values represent the average ± SD of data derived from triplicate cultures. * p < 0.05, ** p < 0.05, respectively, as measured by Student's *t*-test
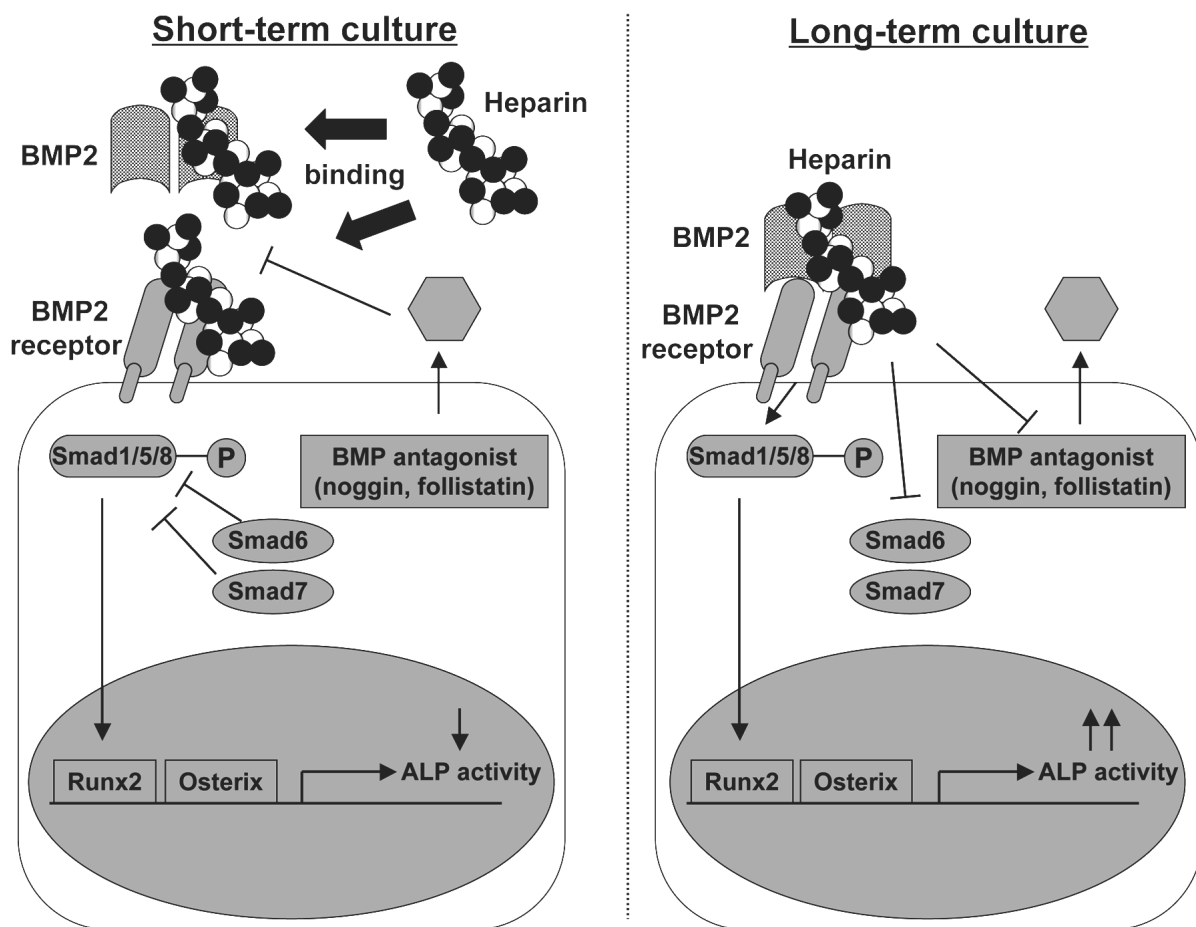
Fig. 6. Schematic of the molecular mechanism of the role of heparin in BMP-2-induced osteogenesis. In this model, short-term treatment with heparin inhibits BMP-2 osteogenic activity *via* competitive inhibition of the binding of BMP-2 to the BMP receptor. In contrast, heparin negatively regulates the negative feedback loop and enhances BMP-2- induced osteogenic activity in long- term cultures

BMP-2 by protecting BMP-2 from degradation and inhibition by BMP antagonists [32, 38]. In addition, Miyazaki has indicated that heparin alone enhances osteoblast growth, differentiation, and mineralization [20].

We have previously reported that heparin inhibits BMP-2 osteogenic bioactivities, such as ALP activity, by binding to both BMP-2 and BMPR; this binding ability of heparin also inhibits BMP-2-induced Smad 1/5/8 phosphorylation and decreases the expression levels of Runx2 and osterix genes within 12 h [16]. In the present study, we found that heparin enhanced the BMP-2 osteogenic bioactivity (Fig. 1), the phosphorylated levels of Smad 1/5/8 (Fig. 2), and the expression levels of genes related to osteoblast differentiation (Fig. 3) after a longer culture period. These data represent the first report of contrasting time-dependent effects of heparin in mediating BMP-2 activity, although previous reports have noted discrepancies between cell types, culture conditions, and heparin concentrations [6, 7, 18]. Interestingly, our results clearly indicate that time was the major factor for the discrepancy in BMP-2-mediated osteogenesis.

Previous studies have reported that bioactive BMPs remain in the extracellular space in the presence of heparin for a longer period of time and that active ligands are protected from suppression by antagonist [38]. Furthermore, our present study suggests that heparin negatively regulates the expression of BMP antagonists and inhibitory Smads that are induced by BMP signaling as part of the negative feedback loop to suppress excess signaling (Fig. 6).

We examined the mechanism by which heparin enhanced the BMP-2-mediated bioactivity for prolonged periods of time. BMP signaling is well-known to be determined by the binding of BMPs and their re-

ceptors. However, soluble BMP antagonists such as noggin and follistatin are known to directly bind to BMPs and prevent functional receptor/ligand interactions [5]. Furthermore, Smad 6 and Smad 7 have also been shown to bind to BMP receptors and inhibit BMP signaling [10, 31]. As shown in Figures 4 and 5, the mRNA expression of noggin, follistatin, Smad 6 and Smad 7 by BMP-2 was preferentially blocked by heparin in prolonged culture time periods. Although measurement of the heparin-induced protein expression of BMP-2 antagonists or inhibitory Smads is still needed, these results suggest that heparin up-regulates the BMP-2-induced osteogenic activity through the contributions of BMP-2 antagonists and inhibitory Smads. Jeon et al. have reported that heparin enhances BMP-2-induced ALP activity in rat calvarial osteoblasts using heparin-conjugated poly (L-lactide-co-glycolide) (PLGA) nanospheres (HCPNs) suspended in a fibrin gel culture system [13]. Furthermore, HCPNs have been reported to stimulate bone formation and calcium deposition *in vivo*. Taken together, these data suggest that heparin in osteoblasts may be partially dependent on this potentiation of BMP-2 activity. Heparin has been found to influence multiple pathways, including Wnt and Nodal [9]. Further experiments are needed to clarify the role of heparin in the regulation of BMP-2 both *in vivo* and *in vitro*.

In conclusion, we found that heparin inhibited BMP-2 osteogenic bioactivity in 72-h cultures and enhanced the activity in 144-h cultures. These results suggest that heparin sustains BMP-2 osteogenic activity and indicate the crucial role of heparin in bone tissue under both physiological and pathological conditions. Therefore, one might expect that the appropriate timing of heparin administration will promote bone healing mediated by BMP-2.

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