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Mechanism involved in enhancement of osteoblast differentiation by hyaluronic acid

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ABSTRACT

Objectives: Bone morphogenetic protein-2 (BMP-2) is expected to be utilized to fill bone defects and promote healing of fractures. However, it is unable to generate an adequate clinical response for use in bone regeneration. Recently, it was reported that glycosaminoglycans, including heparin, heparan sulfate, keratan sulfate, dermatan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, and hyaluronic acid (HA), regulate BMP-2 activity, though the mechanism by which HA regulates osteogenic activities has not been fully elucidated. The aim of this study was to investigate the effects of HA on osteoblast differentiation induced by BMP-2.

Materials and methods: Monolayer cultures of osteoblastic lineage MG63 cells were incubated with BMP-2 and HA for various time periods. To determine osteoblastic differentiation, alkaline phosphatase (ALP) activity in the cell lysates was quantified. Phosphorylation of Smad 1/5/8, p38, and ERK proteins was determined by Western blot analysis. To elucidate the nuclear translocation of phosphorylated Smad 1/5/8, stimulated cells were subjected to immunofluorescence microscopy. To further elucidate the role of HA in enhancement of BMP-2-induced Smad signaling, mRNA expressions of the BMP-2 receptor antagonists noggin and follistatin were detected using real-time RT-PCR.

Results: BMP-2-induced ALP activation, Smad 1/5/8 phosphorylation, and nuclear translocation were up-regulated when MG63 cells were cultured with both BMP-2 and HA. Western blot analysis revealed that phosphorylation of ERK protein was diminished by HA. Furthermore, the mRNA expressions of noggin and follistatin induced by BMP-2 were preferentially blocked by HA.

Conclusions: These results indicate that HA enhanced BMP-2 induces osteoblastic differentiation in MG63 cells via down-regulation of BMP-2 antagonists and ERK phosphorylation.

1. Introduction

Hyaluronic acid (HA) is a long polysaccharide consisting of repeating disaccharide units of *N*-acetylglucosamine and D-glucuronic acid, and a major component of extracellular matrix (ECM) proteins in mammalian tissues [1]. Together with its structural role in the matrix, HA regulates diverse cellular responses including proliferation, differentiation, motility, adhesion, and gene expression [2]. A variety of cell types function to shield against potentially harmful extracellular molecules or pathogenesis [3–9]. In bone, HA and its receptor CD44 interact to restrict osteoblast-mediated osteoclast genesis, which may play a role in inter-osteocyte and osteocyte–osteoclast communication [10,11]. Furthermore, those interactions might also provide stop signals for inducing bone resorption [12] and osteoclastogenesis [13,14].

Bone morphogenetic proteins (BMPs), members of the transforming growth factor beta (TGF-β) superfamily, were originally identified as unique proteins in demineralised bone matrix and also shown to induce ectopic bone formation when implanted into muscular tissues [15]. It is well known that BMPs regulate the differentiation and function of cells involved in bone and cartilage formation and deformation, including osteoblasts, chondrocytes, and osteoclasts [16].

Abbreviations: BMP-2, bone morphogenetic proteins-2; HA, hyaluronic acid; ALP, alkaline phosphatase; ECM, extracellular matrix; TGF-β, transforming growth factor beta; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; HBSS, Hank's balanced salt solution; FCS, fetal calf serum; real-time RT-PCR, real-time reverse transcription polymerase chain reaction; PBS, phosphate-buffered saline.

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BMP signaling is initiated by binding to type I and type II receptors, which are specific trans-membrane receptors [17]. Type I receptors are activated by ligand bound-type II receptors and then phosphorylate downstream molecules in the cytoplasm. Furthermore, Smad 1/5/8 transcription factors are phosphorylated by the BMP receptor (BMPR) in the cytoplasm as substrates and accumulate in the nucleus within 1 h after BMP stimulation [18]. 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 [19].

Recently, we found that heparin inhibits BMP-2 osteogenic bioactivity by binding to both BMP-2 and BMPR [20], while HA was reported to facilitate TGF-β1-mediated fibroblast proliferation [21] and chondrocyte response to BMP-7 [22]. Although HA is a member of the glycosaminoglycan family, such as heparan sulfate, keratan sulfate, dermatan sulfate, chondroitin-4-sulfate, and chondroitin-6-sulfate, the mechanism by which HA regulates osteogenic activities has not been fully elucidated. In the present study, we examined the effects of HA on osteoblast differentiation induced by BMP-2 *in vitro* and found that it enhanced osteogenesis.

2. Materials and methods

2.1. Reagents

High molecular weight HA (2000 kDa) and recombinant human BMP-2 were kindly supplied by Seikagaku Corp. (Tokyo, Japan) and Astellas Pharmaceutical Inc. (Tokyo, Japan), respectively. Anti-phospho-Smad 1/5/8 polyclonal, anti-p38 mitogen-activated protein kinase (MAPK) polyclonal, anti-phospho-p38 MAPK polyclonal, anti-extracellular signal-regulated kinase (ERK) monoclonal, and anti-phospho-ERK polyclonal antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-Smad 1/5/8 polyclonal antibody was obtained from Abcam plc (Cambridge, MA, USA). Anti-β-actin monoclonal antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2.2. Cell culture

The human osteoblastic cell line MG63 cells were maintained in α-minimum essential medium (α-MEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Equitech-Bio Inc., Kerrville, TX, USA), 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37 °C in an atmosphere of 5% CO_2.

2.3. Alkaline phosphatase (ALP) activity

MG63 cells were seeded at a density of 1×10^5/well in a 24-well plate, then ALP activity was evaluated, as described below. Cells were stimulated with BMP-2 (50 ng/ml) and HA (100 μg/ml) for 96 h, washed twice with Hank's balanced salt solution (HBSS), and solubilized with HBSS containing 0.2% Nonidet P-40. ALP activity of the lysate was determined using *p*-nitrophenylphosphate (*p*NPP; Wako, Osaka, Japan) using the Lowry method. After a 30-min incubation at 37 °C, absorbance of *p*NPP at 405 nm was measured using a Multiskan JX microplate reader (Thermo Fisher Scientific, Rockford, IL, USA). Protein contents were determined using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). The specific activity of ALP was calculated as μM/μg protein.

2.4. Western blot analysis

MG63 cells (4×10^5 cells/well) were cultured with BMP-2 (50 ng/ml) in the presence of HA (100 μg/ml) in 6-well plates for various time periods. Adherent cells were washed twice with phosphate-buffered saline (PBS; pH 7.2) and lysed in cell lysis buffer (75 mM Tris–HCl containing 2% SDS and 10% glycerol, pH 6.8). Samples were then 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, then the membrane was washed six times with PBS, followed by incubation with the diluted primary antibody at 4 °C. Anti-Smad 1/5/8, anti-phospho-Smad 1/5/8, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-ERK, anti-phospho-ERK, and anti-β-actin were used as the primary antibodies, while horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were used as secondary antibodies (Santa Cruz Biotechnology Inc.) in this experiment. After washing the membranes, chemiluminescence was produced using ECL reagent (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and detected with Hyperfilm-ECL (GE Healthcare Bio-Sciences Corp.). Gel images were subjected to densitometric analysis using Image Lab™ software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

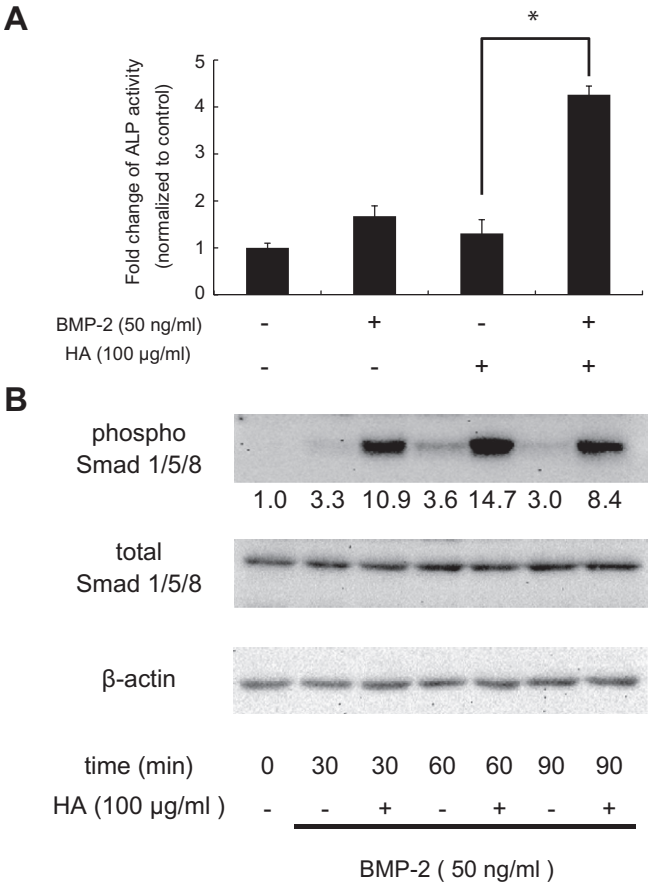

Fig. 1. (A) HA enhances ALP activity induced by BMP-2 in MG63 cells. MG63 cells (4×10^5 cells/well) were stimulated with BMP-2 (50 ng/ml) in the presence or absence of HA (100 μg/ml) for 4 days. The specific activity of ALP (μM/μg protein) was determined as described in Section 2. Values are expressed as fold increases relative to the untreated controls. Data are expressed as the mean ± SD of triplicate cultures. The experiment was performed three times, with similar results obtained in each. $^*p < 0.05$, as measured using an unpaired Student's *t*-test. (B) HA enhances BMP-2-induced Smad 1/5/8 phosphorylation. MG63 cells (4×10^5 cells/well) were stimulated with BMP-2 (50 ng/ml) in the presence or absence of HA (100 μg/ml) for the indicated time periods, then whole lysates were analyzed by SDS–PAGE and Western blotting analyses. Gel images were subjected to densitometric analysis using Image Lab™ software. The relative band intensity (RBI) of the control was set to 1.