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The effect of timing in the administration of hepatocyte growth factor to modulate BMP-2-induced osteoblast differentiation

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ABSTRACT

Development of bone morphogenetic protein (BMP) signaling modulators may provide useful therapeutic options for the treatment of large bony defects in clinical settings. Controversy remains over whether hepatocyte growth factor (HGF) is a positive or negative modulator of BMP-induced osteogenesis. This study analyzed osteogenic properties of HGF, particularly during BMP-2-induced bone formation. Using a mouse model of ectopic bone formation, HGF-impregnated gelatin sponges displayed significantly reduced bone formation induced by BMP-2, both radiologically and histologically. Abrogation of endogenous HGF production by knockdown of HGF mRNA resulted in upregulation of BMP-2-induced ALP activity for C2C12 myoblasts in vitro. In contrast, addition of exogenous HGF inhibited BMP-2-induced ALP activity and osteocalcin production by mouse embryonic fibroblasts (MEFs) through HGF–c-Met interactions. Inhibition of ALP activity by HGF was rescued by U0126, a MEK1/2 inhibitor, indicating that HGF suppresses the BMP-2-Smad axis via activation of ERK1/2. Importantly, treatment with HGF prior to administration of BMP-2 induced cellular proliferation of MEFs and did not influence subsequent osteoblast differentiation induced by BMP-2. The effects of HGF may differ according to the differentiation stage of mesenchymal stem cells, which would explain the inconsistencies seen in osteogenic properties of HGF in previous reports. The timing of HGF treatment is critical and should be carefully determined for successful induction of bone formation by BMPs.

1. Introduction

The bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF)-β superfamily, and play a central role in bone formation by inducing the differentiation of mesenchymal cells into osteoblasts [1]. Recombinant human BMPs (rhBMPs) such as BMP-2, -4, and -7 have demonstrated feasibility in the treatment of critical-sized bone defects, fractures and posterior segmental spinal fusion in animal models [2–4]. However, a high dose of rhBMPs is inevitably required to achieve clinically successful results in the setting of orthopedic surgery in humans [5,6]. The development of positive modulators of BMPs to augment BMP activities may thus be of practical significance. To date, numerous growth factors such as fibroblast growth factor (FGF)-2 [7–9], FGF-4 [10], vascular endothelial growth factor (VEGF) [11,12], and TGF-β1 [8] have been reported to act synergistically with BMPs in certain cell culture systems and animal models.

Hepatocyte growth factor (HGF) was first recognized as a highly potent hepatocyte mitogen [13,14], and is now accepted as a multifunctional cytokine that regulates proliferation, differentiation, migration, morphogenesis, and survival of a wide variety of cells [15–18]. From the perspective of osteology, HGF is a positive regulator for bone metabolism and is considered a potential candidate for a positive modulator of BMPs, since HGF and its receptor, c-Met, are reportedly expressed in both osteoblasts and osteoclasts [19,20]. However, few details are known about the roles of HGF in bone remodeling and repair. In clinical settings, HGF reportedly exerts beneficial effects not only on bone tissue-engineering, but also at the bone–implant interface of orthopedic prostheses. HGF has recently been trialed in combination with hydroxyapatite (HA) materials, based on angiogenic properties and stimulatory effects on osteoblast differentiation [21,22]. Another mechanism by which HGF favors bone formation is reportedly mediated through increased mRNA expression of BMP receptors and responsiveness of BMPs [23]. However, inhibitory effects of

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HGF on osteoblast differentiation have been proposed to play important roles in the pathogenesis of both localized osteolysis and diffuse osteopenia in multiple myeloma [24]. Indeed, whether HGF promotes positive effects on BMP-mediated osteogenesis in vivo remains a matter of debate, particularly in the field of bone tissue-engineering. The present study examined the effects of HGF on bone formation both in vivo and in vitro, and clarified reasons why previous studies have reported inconsistent results regarding the osteogenic properties of HGF.

2. Materials and methods

2.1. Recombinant proteins and antibodies

Recombinant human BMP-2 was donated by Astellas Pharma (Tokyo, Japan). Concentration of BMP-2 was adjusted using buffer solution (5 mM glutamic acid, 2.5% glycine, 0.5% sucrose, and 0.01% Tween 80) at pH 4.5. Recombinant mouse HGF was purchased from R&D Systems (Minneapolis, MN, USA). The concentration of HGF was adjusted using Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen, Carlsbad, CA, USA). Anti-phospho-ERK1/2 MAPK polyclonal antibody and anti-total-ERK1/2 MAPK polyclonal antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP) was purchased from Invitrogen.

2.2. Ectopic bone formation in muscle of mouse hind paws

Eight-week-old male C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). All experiments were performed according to the protocol approved by the Laboratory Animal Care and Use Committee of Keio University. Under general anesthesia with xylazine and pentobarbital, gelatin sponges (Gelfoam®, 5 mm in diameter, 10 mm in thickness; Pharmacia & Upjohn, Kalamazoo, Michigan, USA) were impregnated with 2 μg of BMP-2 and various amount of HGF (0, 2, 20 or 200 ng, or 2 μg), followed by implantation into hamstring muscles of mouse hind paws as described previously [11]. At 2 weeks post-implantation, mice were euthanized by cervical dislocation, and hind paws were dissected and subjected to radiological and histological analyses. Radiographs were taken using a soft X-ray instrument (CMB-2; Softex, Tokyo, Japan) for the assessment of ectopic bone formation in the transplanted site. Radiographic images were captured and the area of ectopic bone formation was measured using a planimetric method with Image J software (NIH, Bethesda, MD, USA). For histological analysis, samples were fixed in 10% formalin and embedded in paraffin. Two neighboring 5-μm-thick serial paraffin sections were prepared in each specimen, one stained with hematoxylin and eosin (HE), and the other with von Kossa stain to observe bone mineralization.

2.3. RNA isolation and real time RT-PCR

The implanted gelatin sponge containing 2 μg of BMP-2 was harvested with adjacent tissues at 0, 1, 3, 7, and 14 days post-implantation to determine changes in gene expression of *HGF* and *c-Met*. Total RNA was isolated using Trizol (Invitrogen). First-strand cDNA was prepared using PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan) according to the instructions from the manufacturer. Real-time RT-PCR was performed with a Thermal Cycler Dice Real Time System using SYBR Premix Ex Tag™ (Takara Bio). Duplicate reactions were performed for each sample, and relative mRNA expression levels were determined by comparison with *β-actin* mRNA expression. Gene-specific forward and reverse primers were as follows: 5′-CCAGCTGGTCTATGGTCCTGAAG-3′ and 3′-CCCAGCCGTAAATACTGCAAGTG-5′ for *HGF*, 5′-CGGGTGCCAAGCTACCAGTAA-3′ and 3′-TGTGTTCACGTCGGGATAAGGA-5′ for *c-Met*, and 5′-CTGAACCCTAAGGCCAACCGTG-3′ and 3′-GGCATACAGGGACAGCACAGCC-5′ for *β-actin*.

2.4. Cell cultures

The C2C12 mouse myoblast cell line was purchased from the American Type Culture Collection (ATCC). C2C12 cells were maintained in DMEM (Invitrogen) containing 15% fetal bovine serum (FBS) (Invitrogen) and antibiotics (100 units/ml penicillin, and 100 μg/ml streptomycin). Mouse embryonic fibroblasts (MEFs) taken from embryonic day (E)13.5 embryos were immortalized by introducing a vector carrying the Large-T antigen, as described previously [25]. MEFs were maintained in DMEM containing 10% FBS and antibiotics. C2C12 cells or MEFs were seeded in 96-well tissue culture plates at a density of 1×10^4 cells/well. On achieving confluence, cells were cultured in DMEM containing 2.5% FBS and antibiotics with or without BMP-2 (200 ng/ml), HGF (25 ng/ml), Met kinase inhibitor (SU11274, 2.5 μM; Calbiochem, Darmstadt, Germany) and MEK1/2 inhibitor (U0126, 10 μM; Cell Signaling Technology) for 72 h. In some experiments, cells were cultured in DMEM containing 2.5% FBS with or without HGF (25 ng/ml) for 24 h after achieving 70% confluence. MEFs were then reseeded in 96-well tissue culture plates and after achieving confluence, cells were cultured in DMEM containing 2.5% FBS with or without BMP-2 (200 ng/ml) for 72 h. Thereafter, cells were subjected to ALP assay.

2.5. Alkaline phosphatase assay

Cells were washed with DPBS, lysed in Mammalian Protein Extraction Reagent (M-PER; Pierce Biotechnology, Rockford, IL, USA) and incubated with ALP buffer (100 mM Tris–HCl (pH 9.5), 100 mM NaCl and 20 mM $MgCl_2$) in the presence of *p*-nitrophenylphosphate (Invitrogen) as a substrate for 30 min at room temperature. ALP activity in cell lysate was then assayed using a microplate reader (DS Phama Biomedical, Osaka, Japan) at 405 nm. Protein content was determined using a Bio-Rad Protein Assay kit (BioRad Laboratories, Hercules, CA, USA) according to the instructions of the manufacturer. For ALP staining, cells were fixed for 10 min with 3.7% formaldehyde at room temperature, and incubated with Nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) tablets (Roche Diagnostics, Tokyo, Japan) dissolved in water for 30 min at room temperature.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Concentrations of HGF in culture media were determined using a mouse HGF ELISA kit (Institute of Immunology, Tokyo, Japan). Similarly, concentrations of osteocalcin in culture media were determined using a mouse osteocalcin ELISA kit (DRG International, NJ, USA).

2.7. Transfection of HGF-targeted small interfering RNA

Synthesized Stealth™ RNAi against *HGF* (5′-UUAAUUGCACAAUACUCCCAAGUGG-3′ and 3′-CCACUUGGGAGUAUUGUGCAAUUAA-5′) and Stealth™ RNAi Negative Control Duplexes were purchased from Invitrogen. C2C12 cells cultured to about 50% confluence in 96-well tissue culture plates were transfected with 20 nmol/l of Stealth™ RNAi with lipofectamine 2000 (Invitrogen) according to the instructions from the manufacturer. Transfection mix was removed 3 h later and cells were grown for another 21 h in DMEM containing 15% FBS. The medium was then changed to DMEM containing 2.5% FBS and cells were cultured with BMP-2 (200 ng/ml) for 72 h.

2.8. Western blot

MEFs were cultured in 6-well plates in DMEM containing 10% FBS. On achieving confluence, cells were cultured in DMEM containing 2.5% FBS with or without BMP-2 (200 ng/ml) and HGF (25 ng/ml) for 1 h. Cell lysates were extracted by M-PER (Pierce Biotechnology) with phosphatase and protease inhibitors. Cell lysates mixed with loading buffer [tris–Glycine SDS Sample Buffer (Invitrogen) with 5% 2-mercaptoethanol (2-ME)] were incubated at 99 °C for 5 min, loaded onto 10% polyacrylamide gels, and electrophoresed in SDS–PAGE. Proteins were transferred to PVDF membranes (Nihon Millipore, Yonezawa, Japan) and blotted with anti-phospho- and anti-total-ERK1/2 MAPK antibodies followed by HRP-conjugated goat anti-rabbit IgG. Proteins were visualized using ECL Western Blotting Detection Reagents (GE Healthcare, Uppsala, Sweden).

2.9. Proliferation assay

Proliferation of MEFs in monolayer culture was analyzed using the WST method with the cell counting kit-8 (CCK-8; Dojindo, Tokyo, Japan). MEFs were cultured in 96-well plates in DMEM containing 10% FBS. On achieving 70% confluence, medium was changed to DMEM containing 2.5% FBS with or without HGF (25 ng/ml) and cultured for 24 h. The CCK-8 solution (10 μl) was then added to each well and incubated at 37 °C for 1 h. Absorbance at 450 nm was determined using a microplate reader. DMEM containing 10% CCK-8 was used as a control.

2.10. Statistical analysis

All data are provided as mean ± standard deviation (SD). Statistical analyses were performed using Student's *t*-test (for paired samples) or one-way ANOVA (for multiple samples), followed by post hoc testing using Bonferroni's method (Stat View-J 5.0 statistical software; SAS Institute). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of BMP-2 on HGF expression during intramuscular bone formation

Implantation of gelatin sponges containing BMP-2 in hamstring muscles of mouse hind paws consistently induced ectopic bone formation at 2 weeks post-implantation as assessed by radiography. Histological analysis revealed increased cartilage formation at 1 week post-implantation, followed by calcification of the cartilage with abundant osteoblasts around the surface at 2 weeks