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COMP-Ang1, a chimeric form of Angiopoietin 1, enhances BMP2-induced osteoblast differentiation and bone formation

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ABSTRACT

Introduction: Angiogenesis is closely associated with bone formation, especially endochondral ossification. Angiopoietin 1 (Ang1) is a specific growth factor functioning to generate a stable and matured vasculature through the Tie2 receptor/PI3K/AKT pathway. Recently cartilage oligomeric matrix protein (COMP)-Ang1, an Ang1 variant which is more potent than native Ang1 in phosphorylating Tie2 receptor and AKT, was developed. This study was designed to examine the effects of angiogenic COMP-Ang1 on BMP2-induced osteoblast differentiation and bone formation.

Methods: Expression of endogenous Ang-1 and its binding receptor Tie 2 mRNA was examined in osteoblast-like cells and primary mouse calvarial cells by RT-PCR analysis, and was also monitored during osteoblast differentiation induced by BMP-2 and/or ascorbic acid and β-glycerophosphate. Effects of COMP-Ang-1 on osteoblast differentiation and mineralization were evaluated by alkaline phosphatase (ALP) activity and osteocalcin (OC) production, and Alizarin red stain. For a molecular mechanism, Western blot and OG2 and 6xOSE promoter assays were done. For *in vivo* evaluation, adenoviral (Ad) vectors containing COMP-Ang-1 or BMP-2 gene were administered into thigh muscle of mice, and after 2 weeks bone formation was analyzed by micro-computed tomography and histology. Angiogenic event of COMP-Ang1 was confirmed by immunofluorescence analysis with anti-CD31 antibody.

Results: Expression of Tie2 receptor was significantly increased in the course of osteoblast differentiation. Treatment or overexpression of COMP-Ang1 enhanced BMP2-induced ALP activity, OC production, and mineral deposition in a dose-dependent manner. In addition, COMP-Ang1 synergistically increased OG2 and 6xOSE promoter activities of BMP2, and sustained p38, Smad and AKT phosphorylation of BMP2. Notably, *in vivo* intramuscular injection of COMP-Ang1 dose-dependently enhanced BMP2-induced ectopic bone formation with increases in CD31 reactivity.

Conclusions: These results suggest that COMP-Ang1 synergistically enhanced osteoblast differentiation and bone formation through potentiating BMP2 signaling pathways and angiogenesis. Combination of BMP2 and COMP-Ang1 should be clinically useful for therapeutic application to fracture and destructive bone diseases.

Introduction

Bone formation requires the concerted activity of several different pathways. Vascular endothelial growth factor (VEGF) is an important factor as a coupling factor in endochondral bone formation [1]. VEGF and BMPs play an important role in the cellular communication during angiogenesis and osteogenesis [2,3]. However, clinical strategy with use of VEGF has some limitations due to edema, inflammation, and vascular permeability [4–6]. Ang1, along with VEGF, is a critical factor in the regulation of vessel formation in both physiological and pathological context [7]. Moreover, overexpression or gene transfer of Ang1 protects the vasculature against vascular leakage [8]. However, because of aggregation and insolubility due to its unique protein structural characteristics, recombinant Ang1 protein has been limited in clinical uses. Recently recombinant COMP-Ang1 protein, a chimeric form of Ang1 containing a minimal coiled-coil domain of cartilage oligomeric matrix protein enough for oligomerization, was synthesized. COMP-Ang1 has potent and stable activity in vascular formation and also overcomes problems of aggregation and insolubility of Ang1 over time [9]. Thus, COMP-Ang1 is considered as an alternative to native Ang1 for therapeutic application. COMP-Ang1 binds to and phosphorylates a

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receptor tyrosine kinase exhibiting an Ig and epidermal growth factor homology domain 2 (Tie2) [10,11] results in receptor dimerization, which leads to activation of AKT signaling [9]. Moreover, COMP-Ang1 treatment has been shown to promote improved wound healing, which is correlated with increased angiogenesis, and an angiogenic effect with non-leaky neovascularization [12].

BMP2 promotes commitment of pluripotent mesenchymal cells to the osteoblast lineage by producing signals that stimulate specific transcriptional programs required for bone formation [13,14]. BMP2 binds to type I and II BMP receptors, and both the Smad and the mitogen-activated protein kinase (MAPK) signaling machinery relay the BMP signal from the cell surface to the nucleus to regulate target gene expression [15,16]. In addition, BMP-2 signaling involves in PI3 kinase/AKT pathway, which results in osteoblast differentiation [17].

Recent study showed that osteoblast-specific Ang1 overexpression results in increased bone mass *in vivo* and that angiogenesis induced by Ang1 expressed in osteoblasts is coupled with osteogenesis [18]. However, effects of soluble COMP-Ang1 on osteoblast differentiation and bone formation have not been elucidated. In this study, we demonstrated that COMP-Ang1 enhanced BMP-2 induced osteoblast differentiation and bone formation through potentiating BMP2 signaling pathways and induction of angiogenesis. Our results provide important implications for the formulation of more effective therapeutic strategies to improve bone formation.

Materials and methods

Recombinant proteins

Recombinant human BMP2 (rhBMP2) was purchased from R&D Systems (Minneapolis, MN). Recombinant Ang1 and COMP-Ang1 was purchased from Alexis Biochemicals (Farmingdale, NY).

Plasmids, transfection and luciferase assay

The reporter constructs, −1.3 kb OG2-Luc and six-copy osteoblast specific element (6xOSE)-Luc, and adenoviral (Ad) vector expressing BMP2 (Ad-BMP2) were kindly provided by Dr. Renny Franceschi (University of Michigan School of Dentistry, Ann Arbor, MI). Ad-COMP-Ang1 was previously described [19]. C2C12 and MC3T3-E1 cells were transfected with the indicated amounts of expression plasmids and the pCMV β-galactosidase (β-gal) expression vector, using FuGENE 6 (Roche Applied Science, Indianapolis, IN). Total amount of DNAs in each transfection was adjusted by adding appropriate amounts of pcDNA3. Approximately 40–48 h after transfection, cells lysed and then assayed with the Dual Luciferase Reporter Assay System (Promega, Madison, WI). The luciferase activity was normalized by β-gal activity.

Animals

C57BL/6J mice (male, 6-week-old) were purchased from Daehan Biolink (Taejun, Korea), and assigned randomly to each experimental group. All animal studies were carried out under the guidelines of the Chonnam National University Animal Care and Use Committee.

Preparation of primary calvarial cells

Calvariae were isolated from 10-day-old neonatal mice, and digested with 0.1% collagenase (Roche) at 37 °C for 30 min. The calvariae were sequentially digested four times. The last fraction was collected and used as primary osteoblasts.

Cell culture and adenovirus transduction

C2C12 and C3H10T1/2 were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen) in humidified air containing 5% CO_2 at 37 °C. MC3T3-E1 and primary osteoblasts were maintained in α-MEM (Invitrogen). For *in vitro* adenovirus transduction, cells were treated with indicated viruses at the designated multiplicity of infection (MOI) under serum free medium for 4 h. Subsequently an equivalent volume of media containing 4% FBS was added in the culture, and the cells were incubated for an additional 24 h before osteogenic medium treatment containing with 50 μg/ml ascorbic acid (AA) and 5 mM β-glycerophosphate (β-GP) in the presence of BMP2 (200 ng/ml). Unless indicated otherwise, osteogenic culture medium was replaced every other day.

RNA preparation and semi-quantitative RT-PCR

Total cellular RNA was prepared by TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized with the random primer and reverse transcriptase (Invitrogen) from extracted total RNA. Each reaction consisted of initial denaturation at 94 °C for 1 min followed by three-step cycling: denaturation at 94 °C for 30 s, annealing at a temperature optimized for each primer pair for 30 s, and extension at 72 °C for 30 s. After the requisite number of cycles (28-30 cycles), the reactions underwent a final extension

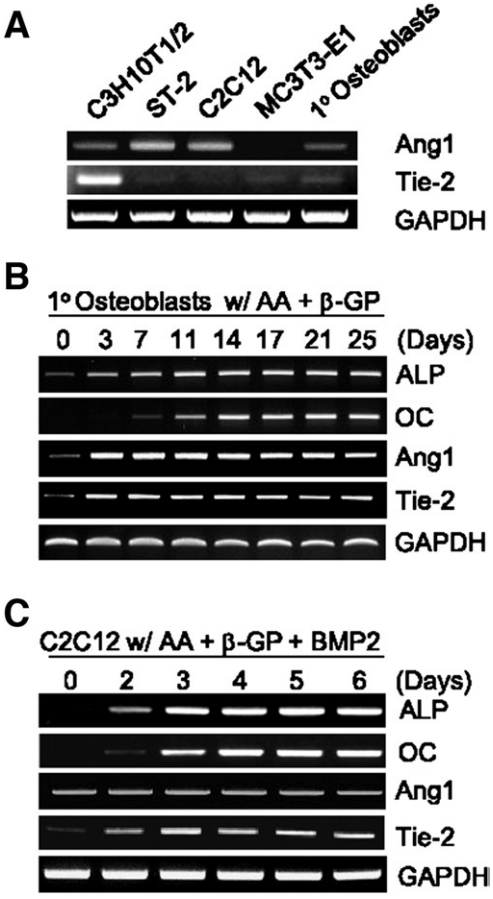

Fig. 1. Expression of Ang1 and receptor Tie2 during osteoblast differentiation. (A) Endogenous expression of Ang1 and Tie2 in various osteoblastic progenitor cells. Total RNAs were isolated from the 3-day cultured cells and used for RT-PCR. (B) Mouse primary osteoblasts were maintained for 25 days in α-MEM containing AA (50 μg/ml) and β-GP (5 mM). At the designated time points, cells were harvested for total RNA isolation, and RT-PCR was performed with the indicated primers. (C) C2C12 cells were cultured for 6 days with rhBMP2 (200 ng/ml) in the presence of AA (50 μg/ml) and β-GP (5 mM). At the designated time points, cells were harvested for total RNA isolation, and RT-PCR was performed with the indicated primers. GAPDH was used to confirm equal levels of target cDNA in the samples.