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Bone morphogenetic protein-3b (BMP-3b) inhibits osteoblast differentiation via Smad2/3 pathway by counteracting Smad1/5/8 signaling

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ABSTRACT

Despite the involvement of BMP-3b (also called GDF-10) in osteogenesis, embryogenesis and adipogenesis, the functional receptors and intracellular signaling of BMP-3b have yet to be elucidated. In the present study, we investigated the cellular mechanism of BMP-3b in osteoblast differentiation using mouse myoblastic C2C12 cells. BMP-3b stimulated activin/TGF-β-responsive promoter activities. The stimulatory actions of BMP-3b on activin/TGF-β-responsive activities were suppressed by co-treatment with BMP-2. BMP-responsive promoter activities stimulated by BMP-2 were significantly inhibited by treatment with BMP-3b. BMP-3b suppressed the expression of osteoblastic markers including Runx2, osteocalcin and type-1 collagen induced by BMP-2, -4, -6 and -7. BMP-2-induced Smad1/5/8 phosphorylation and mRNA levels of the BMP target gene Id-1 were suppressed by co-treatment with BMP-3b, although BMP-3b failed to activate Smad1/5/8 signaling. Of interest, the BMP-3b suppression of BMP-2-induced Id-1 expression was not observed in cells overexpressing Smad4 molecules. On the other hand, BMP-3b directly activated Smad2/3 phosphorylation and activin/TGF-β target gene PAI-1 mRNA expression, while BMP-2 suppressed BMP-3b-induced Smad2/3 signal activation. BMP-2 inhibition of BMP-3b-induced PAI-1 expression was also reversed by overexpression of Smad4. Analysis using inhibitors for BMP–Smad1/5/8 pathways revealed that these BMP-3b effects were mediated via receptors other than ALK-2, -3 and -6. Furthermore, results of inhibitory studies using extracellular domains for BMP receptor constructs showed that the activity of BMP-3b was functionally facilitated by a combination of ALK-4 and ActRIIA. Collectively, BMP-3b plays an inhibitory role in the process of osteoblast differentiation, in which BMP-3b and BMP-2 are mutually antagonistic possibly by competing with the availability of Smad4.

1. Introduction

Transforming growth factor (TGF)-β superfamily members play critical roles in a variety of biological processes including development, differentiation, immune responses, cell growth arrest, and tissue regeneration and maintenance (Miyazono et al., 2010). Major members of the superfamily include TGF-βs, activins/inhibins, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), nodal, and anti-Müllerian hormone (AMH). The dimeric ligands bind to a heterotetrameric complex of two sets of type-I receptors including activin receptor-like kinase (ALK)-1 to -7 and type-II receptors including activin type-II receptors (ActRII and ActRIIB), BMP type-II receptor (BMPRII), TGFβ type-II receptor (Tβ RII) and AMH type-II receptor (AMHRII) (Liu et al., 1995; Miyazono et al., 2010). Following the ligand binding to the corresponding receptors, phosphorylated type-I receptors activate downstream signaling molecules, Smads. The pathway-restricted Smads, either Smad1/5/8 or Smad2/3, are phosphorylated by type-I receptors, and then they interact with a common-mediator molecule, Smad4, to form a hetero-oligomeric complex with Smad1/5/8 or Smad2/3, leading to induction of specific gene transcription (Miyazono et al., 2001; Shimasaki et al., 2004).

Among the BMP ligands, BMP-3 and BMP-3b (also called GDF-10 Cunningham et al., 1995; Hino et al., 1996; Takao et al., 1996)

Abbreviations: ALK, activin receptor-like kinase; ActRII, activin type-II receptor; AMH, anti-Müllerian hormone; AMHRII, AMH type-II receptor; BMP, bone morphogenetic protein; BMPR, BMP receptor; BMPRII, BMP type-II receptor; Collagen1, type-1 collagen; ECD, extracellular domain; GDF, growth differentiation factor; Runx2, runt-related transcription factor 2; TGF, transforming growth factor; TβRII, TGFβ type-II receptor.

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are structurally different members of the BMP subfamily (Hino et al., 2004). BMP-3 co-purified with BMP-2 (Wozney et al., 1988) and osteogenin (Luyten et al., 1989) were found to be identical on the basis of peptide sequences in preparations of bovine osteogenic proteins. BMP-3b was originally identified as GDF-10 by screening for TGF-β family molecules using degenerative PCR from the mouse brain and lung (Cunningham et al., 1995), and it was also isolated from rat and human femurs (Hino et al., 1996; Takao et al., 1996). Although the mature region of BMP-3b and BMP-3 shares approximately 80% amino-acid sequence identity, the pro-regions of BMP-3b and BMP-3 share only 30–35% similarity (Hino et al., 2004).

Several studies have shown the expression of BMP-3b and BMP-3 in bones and other tissues such as tissues of the lung, brain, muscle, gonads and intestine (Vukicevic et al., 1994; Jaatinen et al., 1996; Takahashi and Ikeda, 1996; Thomadakis et al., 1999; Zhao et al., 1999; Erickson and Shimasaki, 2003; Hino et al., 2004). Both BMP-3b and BMP-3 are highly expressed in osteoblasts. However, the regulation of their expression is distinct. BMP-3b transcription is correlated with osteoblastic differentiation, while BMP-3 expression is inversely related to this process (Hino et al., 1999). Although some studies have shown that BMP-3 functions as an inhibitor of osteogenic BMPs, there are inconsistencies in the data, and the BMP-3 signaling pathway is still unclear (Daluiski et al., 2001; Hino et al., 2004; Gamer et al., 2005; Allendorph et al., 2007; Pearsall et al., 2008; Gamer et al., 2009). In other tissues such as brain, aorta, lungs and embryonic tissues, the expression patterns of BMP-3b and BMP-3 are diverged, suggesting that the two molecules exert distinct physiological functions. In addition, BMP-3b and BMP-3 have different roles in embryogenesis, in which BMP-3b is essential for head formation and acts as a dorsalizing factor (Hino et al., 2003). A recent study has further revealed that BMP-3b is highly expressed in adipocytes and inhibits adipogenesis (Hino et al., 2011). However, the specific receptors and cellular signaling for BMP-3b have yet to be clarified.

Osteoblast differentiation is a complex process regulated by various autocrine/paracrine factors. BMPs play pivotal regulatory roles in mesoderm induction and dorso-ventral patterning of developing limb buds and are known to promote differentiation of mesenchymal stem cells into chondrocytes and osteoblasts as well as differentiation of osteoprogenitor cells into osteoblasts (Reddi, 1997; Lieberman et al., 2002). Once matrix synthesis begins in cultured osteoblast cells, the cells differentiate and osteoblastic markers, including alkaline phosphatase (ALP), type-1 collagen and osteocalcin, are activated. Osteoblasts then embed in the extracellular matrix consisting of collagen fibrils, and the matrix is mineralized and extended in collagen fibrils. However, in the process of osteoblast differentiation, functional roles of BMP-3b, the intracellular signaling pathway for BMP-3b and its interaction with other osteogenic factors including BMP-2, -4, -6 and -7 have yet to be determined.

A subclone of the mouse myoblastic cell line C2C12 has been widely used as a model to examine the early stages of osteoblast differentiation during bone formation in muscular tissues (Mukai et al., 2007; Yamashita et al., 2008; Matsumoto et al., 2010; Takano et al., 2012). Treatment of C2C12 cells with TGF-β superfamily ligands leads to ligand-dependent differentiation, in which BMP-2, -4, -6 and -7 inhibit myoblast differentiation of C2C12 cells and promote osteoblastic cell differentiation through Smad1/5/8 signaling pathway (Katagiri et al., 1994; Ebisawa et al., 1999). In the present study, we investigated the cellular mechanisms by which BMP-3b interact in the early process of osteoblast differentiation regulated by BMPs including BMP-2, -4, -6 and -7 with focus on the interaction between BMP–Smad signaling and the possible functional receptors for BMP-3b.

2. Materials and methods

2.1. Reagents and supplies

Dulbecco's Modified Eagle's Medium (DMEM), penicillin–streptomycin solution, dimethylsulfoxide (DMSO), and recombinant human activin A were purchased from Sigma–Aldrich Co., Ltd., (St. Louis, MO). Recombinant human BMP-2, -4, -6 and -7 and extracellular domains (ECDs) that lack transmembrane and intracellular domains of human ALK-2, -3, -4, ActRII and BMPRII (Moore et al., 2003; Inagaki et al., 2006) were purchased from R&D Systems, Inc., (Minneapolis, MN). Human recombinant TGF-β1 was purchased from PeproTech (London, UK). BMP receptor signaling inhibitors, LDN193189 and dorsomorphin, were from Stemgent (San Diego, CA) and Calbiochem (San Diego, CA), respectively. Plasmids of 3TP–Luc and $(CAGA)^9$–Luc were kindly provided by Dr. Shunichi Shimasaki (University of California San Diego, CA), and BRE–Luc, Id-1–Luc and the expression plasmid for Smad4 (pcDEF3-Flag(N)-Smad4) were kindly provided by Drs. Tetsuro Watabe and Kohei Miyazono (Tokyo University, Japan),

2.2. Cell culture and morphological examination

The mouse myoblast cell line C2C12 was obtained from American Type Culture Collection (Manassas, VA). C2C12 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and penicillin–streptomycin solution at 37 °C under a humid atmosphere of 95% air/5% CO_2. Changes in cell morphology were monitored using an inverted microscope.

2.3. Recombinant rat BMP-3b production

CHO cells stably expressing BMP-3b generated by the dihydrofolate reductase-coupled method were used to prepare the recombinant BMP-3b protein (Takao et al., 1996; Hino et al., 2011). Parental wild-type CHO cells were used as controls. The CHO CM was collected and filtered through a 0.45-mm Milliex Filter Unit (Millipore, Billerica, MA, USA). Recombinant BMP-3b in the conditioned medium inhibited adipocyte differentiation and the effect was abolished by an anti-BMP-3b antibody (Hino et al., 2011). Concentration of recombinant mature protein of BMP-3b in the conditioned medium was estimated by the intensities of Western immunoblots using a BMP-3b-specific antibody.

2.4. RNA extraction, RT-PCR, and quantitative real-time PCR analysis

To prepare total cellular RNA, C2C12 cells were cultured in a 12-well plate (1×10^5 viable cells/well) and treated with indicated concentrations of BMP-3b, BMP-2, -4, -6 and -7, and activin A or TGF-β1 in serum-free DMEM for 24 or 48 h. In the indicated experiments, 500 ng of an expression plasmid encoding wild-type Smad4 DNA or an empty vector was transfected using FuGENE6® (Roche Molecular Biochemicals, Indianapolis, IN) for 24 h, and then cells were treated with BMP-3b and BMP-2 in serum-free conditions. After 24-h culture, the medium was removed, and total cellular RNA was extracted using TRIzol® (Invitrogen Corp., Carlsbad, CA), quantified by measuring absorbance at 260 nm, and stored at −80 °C until assay. The extracted RNA (1.0 μg) was subjected to an RT reaction using the First-Strand cDNA synthesis system® (Invitrogen Corp.) with random hexamer (50 ng/μl), reverse transcriptase (200 U), and deoxynucleotide triphosphate (2.5 mM) at 42 °C for 55 min and at 70 °C for 10 min. Subsequently, hot-start PCR was performed using $MgCl_2$ (50 mM), deoxynucleotide triphosphate (2.5 mM), and 1.5 U of *Taq* DNA polymerase (Invitrogen Corp.) under the conditions we previously reported (Mukai et al.,