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Bone morphogenetic protein signaling in articular chondrocyte differentiation

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Abstract

Articular chondrocytes progressively undergo dedifferentiation into a spindle-shaped mesenchymal cellular phenotype in monolayers. Chondrocyte dedifferentiation is stimulated by retinoic acid. On the other hand, bone morphogenic proteins (BMPs) stimulate differentiation of chondrocytes. We examined the mechanism of effects of BMP in chondrocyte differentiation with use of a recombinant adenovirus vector system. Constitutively active forms of BMP type I receptors (BMPR-IA and BMPR-IB) and those of activin receptor-like kinase (ALK)-1 and ALK-2 maintained differentiation of chondrocytes in the presence of retinoic acid. The BMP receptor-regulated signaling substrates, Smad1/5, weakly induced chondrocyte differentiation; the effects of Smad1/5 were enhanced by BMP-7 treatment. Inhibitory Smad, Smad6, blocked increase of expression of chondrocyte markers by BMP-7 in a dose-dependent manner. SB202190, a p38 mitogen-activated protein kinase inhibitor, inhibited this effect of BMP-7; however, since SB202190 suppressed phosphorylation of Smad1/5, this may be due to blockade of BMP receptor activation. These results together strongly suggest that induction of chondrocyte differentiation by BMP-7 is regulated by Smad pathways.
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Keywords: BMP-7; Chondrocytic differentiation; Retinoic acid; Smad

Initiation and maintenance of chondrocyte differentiation in articular cartilage is critical for joint function. The vitamin A derivative retinoic acid is a well-characterized molecule that induces dedifferentiation of chondrocytes in cell culture and inhibits chondrogenesis in vivo [1,2]. Retinoic acid-induced dedifferentiation of articular chondrocytes mimics the loss of chondrocyte phenotype and associated down-regulation of type II collagen during osteoarthritis. On the other hand, bone morphogenetic proteins (BMPs) initiate bone- and cartilage-differentiation in rats [3]. In addition, BMPs promote and maintain articular chondrocyte phenotype in cell culture [4–6].

Members of the transforming growth factor-β (TGF-β)/BMP superfamily transduce their signals via two types of serine/threonine kinase receptors, type I and type II, both of which are required for signal transduction [7,8]. Seven types of type I receptors, termed activin receptor-like kinase (ALK) 1–7, have been identified in mammals and have similar structures [9,10]. Among them, three type I receptors, BMP type IA receptor (BMPR-IA or ALK-3), BMPR-IB (ALK-6), and ALK-2, mediate BMP signaling [10–12]. In addition, ALK-1 mediates BMP-like signals, although it binds to TGF-β in endothelial cells [13].

Upon ligand-binding, type I receptors are phosphorylated by type II receptors, resulting in activation of Smad proteins. Mutations of Thr-204 in ALK-5 and corresponding threonine or glutamine residues in ALK-1, ALK-2, ALK-3, ALK-4, ALK-6, and ALK-7 to

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acidic amino acids such as aspartic acid and glutamic acid lead to constitutive activation of type I receptor kinases [14].

Smads are the major intracellular signal transducers of TGF-β/BMP superfamily proteins [8,15–17]. Eight different Smads have been identified in mammals and are classified into three subgroups. Receptor-regulated Smads (R-Smads) are phosphorylated at SSXS motifs at their C-termini by type I receptors. Smad1, Smad5, and Smad8 transduce BMP signals. Phosphorylated R-Smads form heteromers with common-partner Smad (Co-Smad), Smad4, and translocate into the nucleus where they bind directly or indirectly via other DNA binding proteins to specific DNA sequences for activation of gene transcription. Inhibitory Smads, Smad6 and Smad7, inhibit phosphorylation of R-Smads by competing with R-Smads for binding to activated type I receptors. In addition, various MAP kinases, including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 MAP kinase, have been reported to be activated by TGF-β/BMP superfamily proteins in certain types of cells [18–22].

The involvement of Smad pathways in chondrogenesis was previously examined using ATDC5 cells; although Smad6 and Smad7 inhibited chondrogenesis, the roles of Smad1 and Smad5 in chondrocyte differentiation were unclear [23]. In this study, we examined the ability of BMP-7 to block the effect of retinoic acid and maintain chondrocyte phenotype in bovine articular chondrocytes. We also studied the roles of type I receptors, Smad pathways, and MAP kinase pathways in maintenance of the chondrocyte phenotype.

Materials and methods

Cell culture. Bovine articular cartilage was minced into 1–2 mm^3 pieces. After washing with medium (DMEM/F12 containing 50 μg/ml ascorbic acid 2-phosphate, 100 μg/ml sodium pyruvate, 100 U/ml penicillin, and 1 U/ml Fungizone), they were digested in medium with 0.2% collagenase P (Roche Molecular Biochemicals) overnight at 37 °C. The cells were filtered through a 70-μm Cell Strainer, washed, and cultured in monolayers in the same medium containing 1% FBS. The mouse muscle myoblast cell line C2C12 was obtained from American Type Culture Collection (Rockville) and cultured in DMEM containing 15% FBS and 100 U/ml penicillin.

Reagents. A mitogen-activated protein kinase kinase (MEK) inhibitor (PD98059) (New England Biolabs) and a p38 MAP kinase inhibitor (SB202190) (Calbiochem) were dissolved in dimethyl sulfoxide (DMSO) before use. All-*trans* retinoic acid (Sigma) was dissolved in absolute ethanol. The final concentrations of DMSO and ethanol added to the cells were 0.5%.

Recombinant adenovirus constructions. Constructions of recombinant adenovirus were described previously [23]. Briefly, each cDNA was subcloned into the pAxCAwt cassette cosmid at the *Swa*I site. The cosmid DNA and adenovirus DNA-terminal protein complex of Ad5-dlX were cotransfected into E1 transcomplemental cell line 293 cells. The recombinant adenoviruses were generated through homologous recombination in 293 cells and digested by restriction endonucleases to confirm the insertion of cDNAs. Recombinant adenoviruses were grown in 293 cells to high titers and purified. Infection was performed at a multiplicity of infection (m.o.i.) of less than 1×10^3 (pfu/cell).

Determination of chondrocytic differentiation. Chondrocytic differentiation of cells was examined by staining of sulfated glycosaminoglycans using Alcian blue as described [24]. Six days after adenovirus infection, cells were fixed with 10% formalin for 10 min and stained by 0.5% Alcian blue 8GX (Sigma) in HCl, pH 1.0, for 1 h. To quantify Alcian blue intensity, cells were washed with distilled water and incubated with 4 M guanidine–HCl, 50 mM Tris–HCl, and 0.1% Chaps, pH 7.4, for 2 h at room temperature. Optical density of the extracted dye was evaluated at 595 nm in a microplate reader (Bio-Rad).

cDNA probes. Bovine type II collagen and 18S ribosomal cDNA were generated by reverse-transcription (RT)-PCR. The products of RT-PCR were subcloned into TOPO TA cloning vector using the TOPO TA Cloning System (Invitrogen).

Northern blot analysis. Six days after adenovirus infection, total RNAs were isolated from bovine chondrocytes with an RNeasy Mini Kit (Qiagen) and electrophoresed on 1% agarose gel. Then, RNAs were transferred to Hybond-N^+ membrane (Amersham Pharmacia). After prehybridization, the membranes were hybridized with appropriate ^{32}P-labeled DNA probes at 45 °C overnight using ExpressHyb Hybridization Solution (Clontech). Washing of the membranes was performed with 2× SSC, 0.1% SDS and 0.5× SSC, and 0.1% SDS at 55 °C. The membranes were exposed to Storage Phosphor Screen (Molecular Dynamics), and scanned by Molecular Dynamics fluorescence scanning systems.

Immunoblotting. Cells were lysed in lysis buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 1% Triton X-100. The lysates were subjected to SDS–gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membrane, and after blocking with 5% skim milk in TBS-T (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, and 0.05% Tween 20) were immunoblotted with anti-phospho-Smad1/5/8 antibody (Cell Signaling). Phospho-Smad1/5/8 bands were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Results and discussion

Inhibition of retinoic acid-induced dedifferentiation by BMP-7

In the present investigation, we used primary bovine articular chondrocytes, which were dedifferentiated by retinoic acid. To investigate chondrogenesis, we examined Alcian blue staining of sulfated glycosaminoglycans, because this type of cartilage extracellular matrix is a hallmark of chondrogenic differentiation [4]. Examination of intensities of Alcian blue staining showed that 10 nM all-*trans* retinoic acid induced dedifferentiation of cells, whereas BMP-7 counteracted the effect of retinoic acid in a dose-dependent manner (Fig. 1A).

Type II collagen is another marker of chondrogenesis [25]. Northern blot analysis of expression of type II collagen mRNA revealed that retinoic acid decreased type II collagen expression and that BMP-7 counteracted this effect of retinoic acid in a dose-dependent manner (Fig. 1B). These results suggest that BMP-7 is involved in differentiation of chondrocytes.

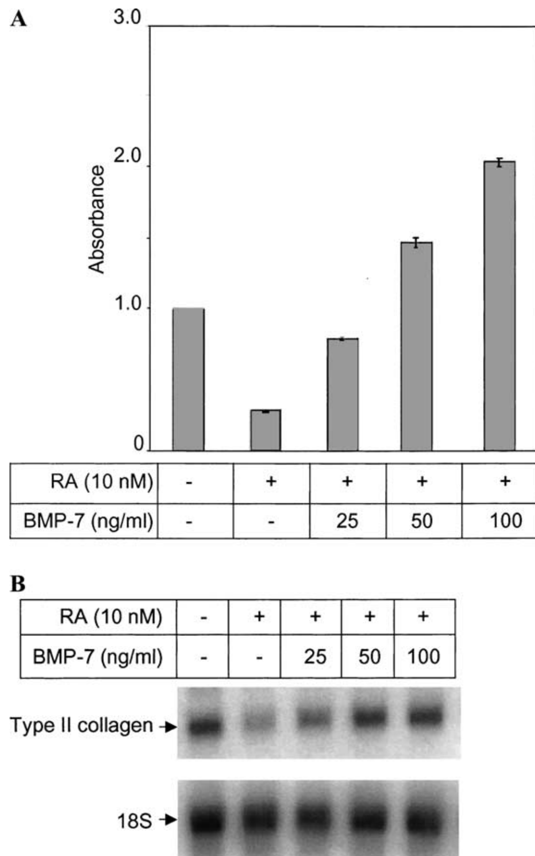

Fig. 1. Maintenance of chondrocyte phenotype by BMP-7 in bovine articular chondrocytes. Bovine articular chondrocytes were stimulated by retinoic acid and BMP-7 for 6 days. (A) Intensity of Alcian blue staining. (B) Northern blot analysis of type II collagen mRNA expression in bovine articular chondrocytes. 18S ribosome was used as a loading control. RA, retinoic acid.

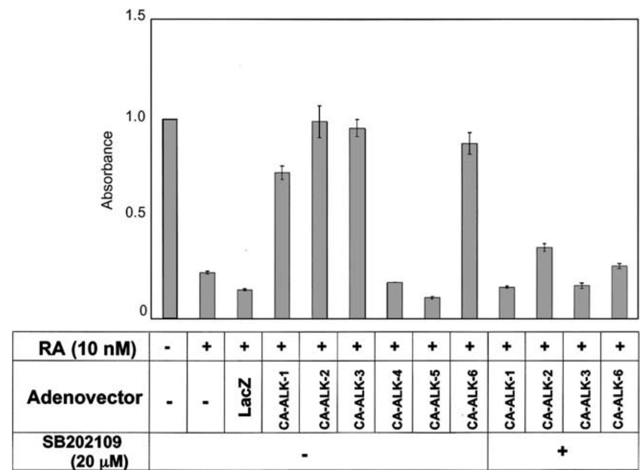

Fig. 2. Induction of chondrocytic differentiation by constitutively active forms of type I receptors. Cells were evaluated for intensity of Alcian blue staining. Bovine articular chondrocytes were infected with adenovirus vectors carrying constitutively type I receptors at m.o.i. of 900 (ALK-1, -2, -3) or 1000 (ALK-4, -5, -6). Adenovirus carrying β-galactosidase (m.o.i. of 1000) was used as a control. SB202190 is a p38 MAP kinase inhibitor (see Fig. 5).

Chondrocyte differentiation maintained by BMP type I receptors

We next examined whether type I receptors for the members of the TGF-β/BMP superfamily modulate dedifferentiation of chondrocytes induced by retinoic acid. To obtain a high transfection efficiency, we used a recombinant adenovirus system as previously described [23]. Constitutively active forms of type I receptors were infected in bovine articular chondrocytes. The intensity of Alcian blue staining revealed that of the six constitutively active type I receptors, ALK-1, ALK-2, ALK-3, and ALK-6 maintained the chondrocytic phenotype. BMPs bind to ALK-3 and ALK-6. Moreover, BMP-6, BMP-7, and probably other BMPs bind to ALK-2, which is structurally similar to ALK-1 [9,12,26]. In contrast to these receptors, ALK-4 and ALK-5, type I receptors for activin and TGF-β, respectively, did not help maintain chondrocytic phenotype (Fig. 2). It has been reported that in the chondrocyte cell line ATDC5, constitutively active forms of ALK-3 and ALK-6 but not those of ALK-1 or ALK-2 stimulated chondrogen-

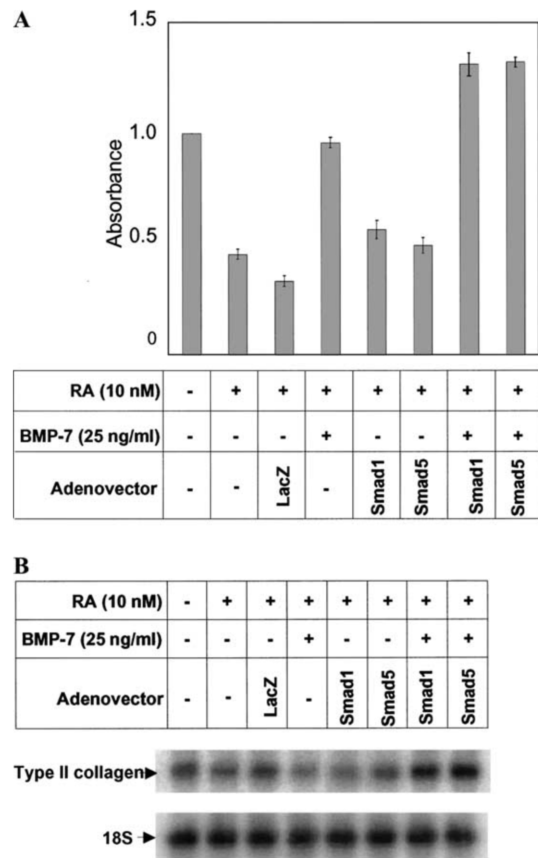

Fig. 3. Induction of chondrocytic differentiation by Smad1 and Smad5. (A) Intensity of Alcian blue staining. Bovine articular chondrocytes were infected with adenovirus vectors carrying R-Smads and β-galactosidase at m.o.i. of 1000 for 6 days. (B) Northern blot analysis of type II collagen mRNA expression. Each adenovirus vector was infected at m.o.i. of 500 for 6 days.

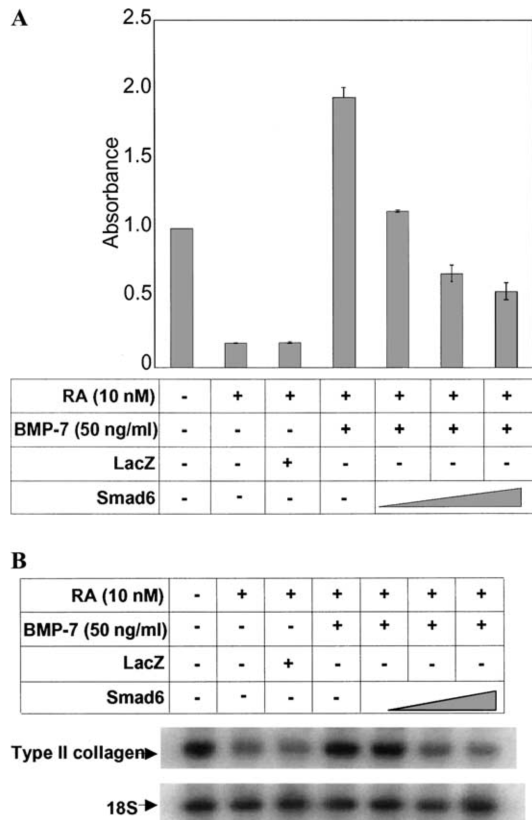

Fig. 4. Inhibition of chondrocytic differentiation by Smad6. Bovine articular chondrocytes were infected with adenovirus vector carrying Smad6. (A) Intensity of Alcian blue staining. (B) Northern blot analysis of type II collagen mRNA expression. Smad6 adenovirus vector was infected at m.o.i of 250, 500, and 1000.

esis [23]. This difference in findings is probably due to differences in cell types tested, i.e., primary chondrocytes in the present investigation and the cell line used in the earlier study.

Stimulation of chondrocyte differentiation by R-Smads

The next question is whether Smad1 or Smad5 is sufficient to induce chondrocytic differentiation in bovine articular chondrocytes. Intensity of Alcian blue staining revealed that overexpression of Smad1 or Smad5 using adenovirus vector had only a weak effect (Fig. 3A). On the other hand, when cells were stimulated with sub-threshold concentration of BMP-7 (25 ng/ml) to change the localization of Smad1 and Smad5 to the nucleus, Smad1 and Smad5 enhanced the effect of BMP-7 (Fig. 3A). Northern blot analysis of expression of type II collagen mRNA yielded results similar to those of Alcian blue staining (Fig. 3B).

Inhibition of chondrocytic differentiation by I-Smads

Smad6 and Smad7 function as inhibitory Smads [27–29]. Smad6 inhibits BMP signaling selectively, whereas Smad7 inhibits both TGF-β/activin and BMP signaling [30]. We examined whether Smad6 inhibits the effect of BMP-7 on chondrocytic differentiation of retinoic acid-treated articular chondrocytes. Alcian blue staining revealed that Smad6 inhibited chondrocytic differentiation by BMP-7 in a dose-dependent manner (Fig. 4A). Smad6 also inhibited induction of type II collagen

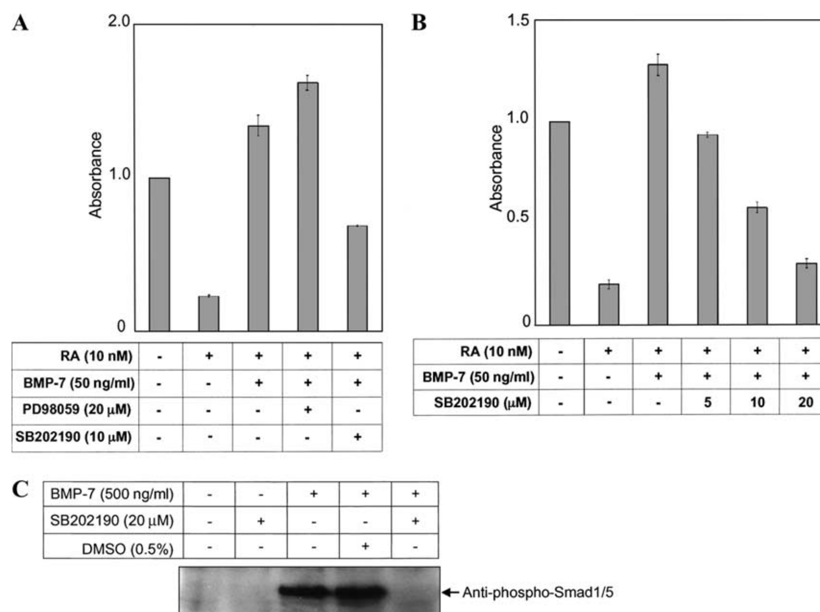

Fig. 5. Effects of SB202190 on chondrocytic differentiation by BMP-7. (A and B) Bovine articular chondrocytes were treated with retinoic acid, BMP-7, SB202190, and PD98059 for 6 days. The cells were then stained by Alcian blue and measured for intensity of staining. (C) Effect of SB202190 on Smad1/5 phosphorylation induced by BMP-7. After preincubation with SB202190, C2C12 cells were stimulated with BMP-7 for 1 h. Cell lysates were subjected to SDS–PAGE and immunoblotting using anti-phospho-Smad1/5/8 antibody. Phospho-Smad1/5, which was larger than phospho-Smad8, was detected in C2C12 cells treated with BMP-7.

mRNA expression by BMP-7 in a dose-dependent manner (Fig. 4B), indicating that Smad6 can antagonize the chondrocytic differentiation induced by BMP-7. These results suggest that the Smad pathway is necessary for induction of chondrocytic differentiation by BMP-7.

Role of p38 MAP kinase inhibitor in chondrocytic induction by BMP-7

Next, we examined whether a p38 MAP kinase inhibitor regulates chondrocytic differentiation by BMP-7. A p38 MAP kinase inhibitor, SB202190, inhibited the effect of BMP-7 in a dose-dependent manner (Figs. 5A and B). The p38 MAP kinase inhibitor also inhibited differentiation of chondrocytes by the constitutively active forms of ALK-1, ALK-2, ALK-3, and ALK-6 (Fig. 2). These findings agree with the previous report that p38 MAP kinase plays a role in chondrogenic differentiation in the ATDC5 chondrocyte cell line [31,32]. However, it was recently reported that SB202190 could inhibit not only p38 MAP kinase, but also TGF-β type I receptor kinase activity [33]. To evaluate whether SB202190 inhibits BMP type I receptor kinase, we performed immunoblotting analysis using anti-phospho-Smad1/5/8 antibody. BMP-7 induced phosphorylation of Smad1/5 and this was blocked by SB202190 (Fig. 5C). These findings thus suggest that the effects of SB202190 in chondrogenesis may be due to suppression of BMP type I receptor kinase.

A MEK1 inhibitor, PD98059, enhanced sulfated glycosaminoglycan production by BMP-7 (Fig. 5A). MEK1 functions as an activator of ERK1 and ERK2. The ERK MAP kinase pathway has been reported to negatively regulate the Smad1 pathway by induction of phosphorylation of the linker domain of Smad1 [34]. The MEK1 signaling pathway may thus be involved in regulation of the Smad pathway in articular chondrocytes, although further studies are needed to elucidate the roles of MEK1 in chondrogenesis.

In conclusion, chondrogenic differentiation requires the signaling pathway involving BMP receptors and Smads, although it is possible that other pathways are involved in chondrogenic differentiation. Future studies will explore the cross-talk between the various signaling pathways in articular chondrocytes.

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