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Noggin and Bone Morphogenetic Protein-4 Coordinately Regulate the Progression of Chondrogenic Differentiation in Mouse Clonal EC Cells, ATDC5

Hiromu Ito,* Haruhiko Akiyama,*,[1] Chohei Shigeno,† and Takashi Nakamura*

**Department of Orthopaedic Surgery, and †Calcium Laboratory, Department of Nuclear Medicine and Diagnostic Imaging, Graduate School of Medicine, Kyoto University, Sakyo, Kyoto 606-8507, Japan*

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Here we report the gene expression and regulation and the function of noggin in clonal mouse chondrogenic EC cells, ATDC5. In ATDC5 cells, the expression of Noggin mRNA increased in parallel with the progression of chondrogenic differentiation. The treatment with conditioned medium of noggin-transfected COS-7 cells decreased the levels of type II and type X collagen gene transcripts of differentiated ATDC5 cells in a dose-dependent manner, and this inhibitory action was reversed by exogenously administered BMP-4 in a dose-dependent manner. The steady-state level of noggin gene transcripts was markedly upregulated by exogenously administered BMP-4 in time- and dose-dependent manners. Furthermore, this stimulatory effect of BMP-4 was attenuated by treatment with actinomycin D, but not with cycloheximide. These results indicate that noggin and BMP-4 coordinately regulate the progression of chondrogenic differentiation in ATDC5 cells.

Key Words: **chondrogenic differentiation; Noggin; bone morphogenetic protein.**

Noggin is a dominant inhibitor of bone morphogenetic protein (BMP) activities and involved in a variety of developmental processes during embryogenesis [1–7]. Noggin is a secreted glycoprotein mimicking the actions of the Spemann organizer, which can induce the formation of neural tissue from ectoderm and dorsalization of the ventral mesoderm. Recent studies show that noggin limits BMP activity in cultured osteoblasts [8] and that noggin regulates chondrogenesis in somite [9] and limb buds [10]. The observations that the noggin gene transcripts are localized in differentiated chondrocytes and that homozygous mice carrying noggin null mutation exhibit widespread abnormalities of cartilage development [11], provide evidence that noggin regulates chondrogenic differentiation during endochondral bone formation.

Accumulating evidence shows that BMP signal plays important roles in chondrogenic differentiation in vivo and in vitro [12–18]. BMP-4 transcripts are detectable in perichondrium and chondrocytes, and BMP receptors are expressed in chondrocytes [18, 19]. Zou and coworkers [18] demonstrated that BMP receptor type IB expression precedes chondrogenesis and the signaling from this receptor is necessary for the early steps of mesenchymal condensation and cell death. Moreover, BMP receptor type IA is essential for proper progression of the cartilage differentiation program during its later stages. However, it remains unknown whether noggin modulates BMP signal during chondrogenic differentiation of differentiated chondrocytes.

We and others previously indicated that clonal mouse EC cells, ATDC5, keep track of the multistep chondrogenic differentiation processes, serving as a powerful in vitro model for the analyses of molecular mechanisms underlying chondrogenic differentiation [17, 20–23]. In ATDC5 cells, BMP-4 and BMP receptor IA are continuously expressed throughout processes of chondrogenic differentiation, and BMP signals stimulate the progression of chondrogenic differentiation of these cells (Akiyama *et al.*, submitted). In this study, we show that noggin mRNA is expressed after the onset of chondrogenesis of ATDC5 cells and that BMP-4 and noggin coordinately regulate the progression of chondrogenic differentiation of these cells. Moreover, we indicate that the level of noggin transcripts is under the regulatory control by the amplitude of BMP-4 signal.

[1] To whom correspondence should be addressed at Department of Orthopaedic Surgery, Graduate School of Medicine, Kyoto University, Sakyo, Kyoto 606-8507, Japan. Fax: 81 75 751 8409. E-mail: akiy@kuhp.kyoto-u.ac.jp.

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MATERIALS AND METHODS

Materials. Xenopus recombinant BMP-4 was a generous gift from Takeda Chemical Industries Ltd. (Osaka, Japan) [24], and cycloheximide and actinomycin D were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells and culture conditions. ATDC5 cells were cultured as previously described [21, 22]. Briefly, cells were maintained in a log growth phase in DMEM/Ham's F12 hybrid medium (Flow laboratories, Irvine, U.K.) containing 5% (V/V) FBS (GIBCO BRL, Gaithersburg, MD), 10 μg/ml human transferrin (Boehringer Mannheim Gmbh, Mannheim, Germany), and 3×10^{-8} M sodium selenite (Sigma, St. Louis, MO) (the maintenance medium) at 37°C in a humidified 5% CO_2/95% air atmosphere. Chondrogenesis and cartilage nodule formation could be induced only in a postconfluent phase when cells were cultured in the maintenance medium supplemented further with 10 μg/ml bovine insulin (Wako Pure Chemical, Osaka, Japan) (the differentiation medium). In the present study, we plated ATDC5 cells in six-multiwell plastic plates at an initial density of 6×10^4 cells/well and cultured these cells for a total of 23 days with medium replacement every other day. Cells were cultured for the initial 21 day-period in the differentiation medium in a 5% CO_2/95% air atmosphere and then cells were treated for the indicated time periods with various doses of the test substances in the absence of insulin.

RNA extraction and Northern analysis. Total RNA was prepared from the cultures as previously described [17, 21–23] and poly $(A)^+$ RNA was purified by binding to Oligotex-dT30 Super (Takara, Ohtsu, Japan) as described by the manufacturer's instructions at the indicated time points. For northern hybridization, total RNA (20 μg) or poly$(A)^+$ RNA (3 μg) was denatured, separated by 1% agarose gel electrophoresis, and transferred on Nytran membranes (Schleicher & Schuell, Dassel, Germany). Hybridization probes were prepared by the random-primer method with a BcaBEST labeling kit (Takara, Ohtsu, Japan). The following cDNA fragments were used as hybridization probes: a 0.7-Kb fragment of noggin mRNA; a 1.4-Kb EcoRI fragment of pKT1180 for α1 (II) collagen mRNA [25]; a 0.65-Kb Hind III fragment of pSAm10h for α1 (X) collagen mRNA [26]; and a 0.98-Kb fragment of mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA. After hybridization, the membranes were exposed to X-Omat films (Kodak, Rochester, NY) at −80°C with Cronex lightening plus intensifying screens (DuPont, Boston, MA). The quantitation of hybridization signals was performed by scanning densitometry with Model 301 Densitometer (Fuji X-Ray, Tokyo, Japan).

Transfection of noggin to COS-7 cells and Western analysis of its conditioned medium. A DNA fragment encoding the mouse noggin protein appended with a FLAG-tag at its C-terminus was generated by PCR using the 0.9-Kb noggin cDNA as a template and the primer sequences as follows: sense primer 5′-ACGCGGGACGAAGAGGCAGCC-3′; antisense primer 5′-CTACTTGTCATCGTCGTCCTTGTAATCGCAGGAACACTTACACTCG-3′. Antisense primer contained sequences for a FLAG-tag (underlined) and a stop codon. Amplification consisted of initial denaturation at 94°C for 5 minutes, followed by 25 reaction cycles (30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C). The PCR product was gel-purified and cloned into pCRII vector (pCRII/Nog-FLAG), and its nucleotide sequence was verified by ALFred DNA Sequencer. The insert of the pCRII/Nog-FLAG vector was excised with SpeI and XhoI and recloned into the SpeI-XhoI site of pcDNA3.1 vector. The resultant expression vector, pCMV/Nog-FLAG, or pcDNA3.1 as a control, was transiently transfected into COS-7 cells by lipofection using SuperFect Transfection Reagent (Qiagen GmbH, Hilden, Germany). Forty-eight hours later, the conditioned medium was collected, and noggin-FLAG fusion protein secreted was identified by Western blot using anti-FLAG M2 monoclonal antibody (Sigma, St. Louis, MO) according to the manufacturer's instructions.

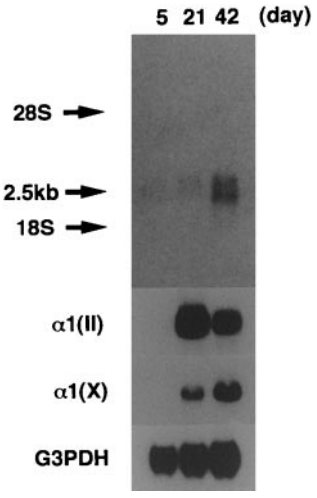

FIG. 1. Expression pattern of noggin mRNA during the processes of chondrogenic differentiation of ATDC5 cells. Undifferentiated ATDC5 cells were plated as described under Materials and Methods and cultured for a total 42 days. Poly$(A)^+$ RNA were prepared at the time points indicated and subjected to Northern analysis (3 μg of poly$(A)^+$ RNA per lane). The gene transcript levels of type II collagen and type X collagen, phenotypic markers of proliferating and hypertrophic chondrocytes, respectively, were also examined. The integrity of the RNA analyzed was confirmed by G3PDH mRNA, as shown in the bottom panel. Three independent experiments were performed and gave similar results.

RESULTS

Differentiated ATDC5 cells express noggin mRNA. We previously showed that chondrogenic differentiation of ATDC5 cells takes place in the presence of insulin and that transitions of the differentiation stages occur in a synchronous manner as evidenced by the orderly expression of cartilage phenotypic marker genes [21]. We extracted poly$(A)^+$ RNA at the time points indicated, and analyzed the level of noggin gene transcripts. We also examined the levels of type II collagen and type X collagen gene transcripts, phenotypic markers of proliferating and hypertrophic chondrocytes, respectively. The expression of noggin mRNA was observed after the expression of type II collagen mRNA was detected and increased in parallel with the progression of chondrogenic differentiation of ATDC5 cells (Fig. 1).

Treatment with noggin-FLAG fusion protein containing conditioned medium inhibits chondrogenic differentiation of differentiated ATDC5 cells. Noggin inhibits BMP activities by directly binding BMP-2 and BMP-4 [7]. Recent studies showed that BMP-4 is expressed in both perichondrium and chondrocytes and that this molecule promotes chondrogenic differentiation via BMP receptors expressed in chondrocytes [14, 18, 19]. In ATDC5 cells, BMP-4-BMP type IA receptor signal also promotes chondrogenic differentiation (Akiyama *et al.*, submitted), suggesting that noggin

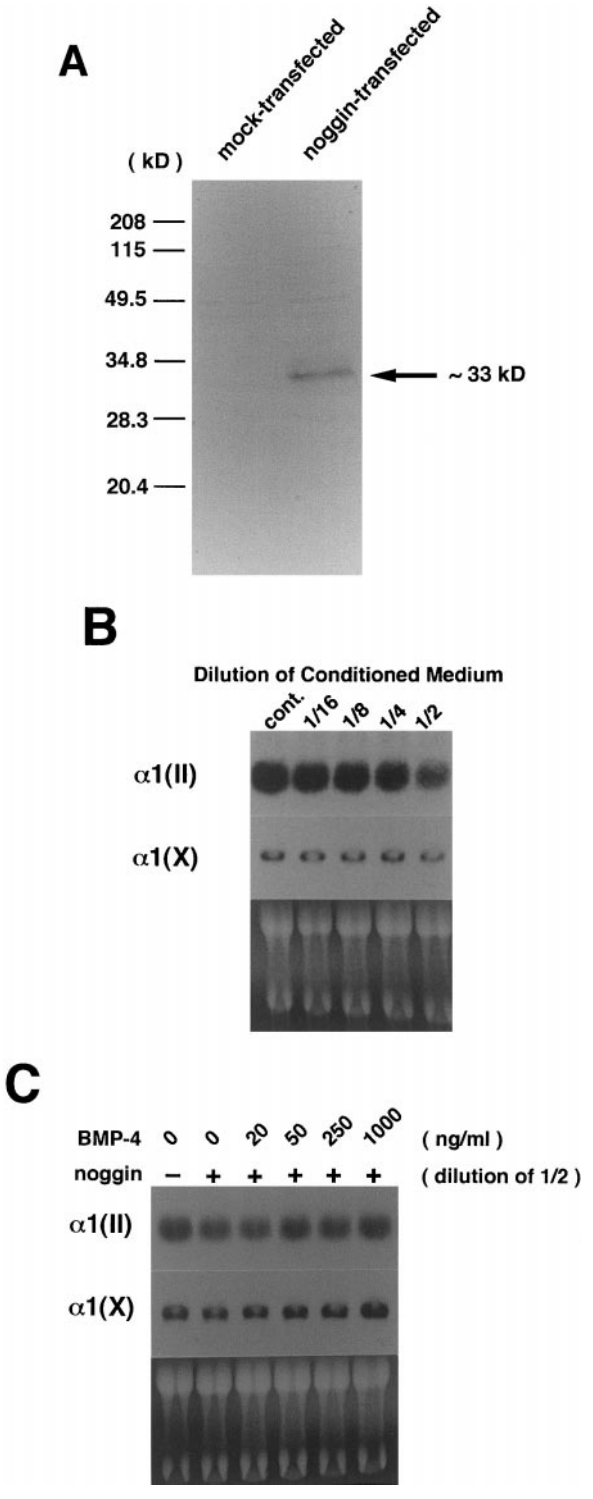

FIG. 2. (**A**) Production of noggin-FLAG fusion protein in COS-7 cells. The anti-FLAG M2 monoclonal antibody was used for detection. The protein corresponding to the mouse noggin cDNA with FLAG-tag (approx. 33 kDa) was detected in the culture supernatant from COS-7 cells transfected by lipofection with pCMV/NOG-FLAG expression vector as described under Materials and Methods. There is no detectable band in the culture supernatant from COS-7 cells transfected with pcDNA 3.1 vector alone (mock-transfected). (**B**) Effects of the treatment with noggin-containing conditioned medium (dilution of 0-1/2) and (**C**) reversal by varying doses of BMP-4 (0, 20, 50, 250 and 1000 ng/ml) of the inhibitory actions of the maximal dilution of conditioned media (1/2) on the expression of type II and type X collagen mRNAs in differentiated ATDC5 cells. Cells were cultured in 6-multiwell plastic plates as described under Materials and Methods for 21 days and were then treated for an additional 24 h with test substances described above. Total RNA were prepared and subjected to Northern analysis (20 μg of total RNA per lane). The integrity of the RNA analyzed was confirmed by ethidium bromide staining, as shown in the bottom panel. Three independent experiments were performed and gave similar results.

may modulate the progression of chondrogenic differentiation of ATDC5 cells. We transfected noggin or FLAG tagged noggin cDNA containing expression vector to COS-7 cells and production of noggin-FLAG fusion protein was confirmed by Western blot (Fig. 2A). The treatment of differentiated ATDC5 cells with conditioned medium containing noggin or noggin-FLAG fusion protein resulted in decrease of the transcript levels of type II and type X collagen genes in a dose-dependent manner (dilution of 0-1/2) (Fig. 2B). Both conditioned medium containing noggin and those containing noggin-FLAG fusion protein showed the similar effects (data not shown). Conditioned medium prepared from mock-transfected COS-7 cells did not affect their expression (dilution of 0-1/2) (data not shown). Moreover, the reduced levels of type II and type X mRNAs (to 63% and 68%, respectively) by noggin containing-conditioned media (dilution of 1/2) were rescued by exogenously administered BMP-4 in a dose-dependent manner (Fig. 2C).

BMP-4 upregulates the level of noggin gene transcripts in differentiated ATDC5 cells. Our observations suggest that noggin may regulate the progression of chondrogenic differentiation of ATDC5 cells mediated by BMP-4 signal. As shown in Fig. 3, exogenously administered BMP-4 markedly upregulated the steady-state level of noggin gene transcripts in time- and dose-dependent manners. Furthermore, pretreatment of ATDC5 cells with the transcriptional inhibitor actinomycin D attenuated the effect of BMP-4 on the induction of noggin mRNA, but not with the translational inhibitor cycloheximide, indicating that the induction of noggin mRNA by BMP-4 in differentiated ATDC5 cells does not require new protein synthesis (Fig. 4).

DISCUSSION

Chondrogenic differentiation is a programmed multistep event in the skeletal development. Undifferentiated mesenchymal cells differentiate into chondrocytes. Differentiated chondrocytes then go through the sequential processes of proliferation, maturation, hypertrophic conversion and calcification. This sequential cellular event is under the regulatory control by a

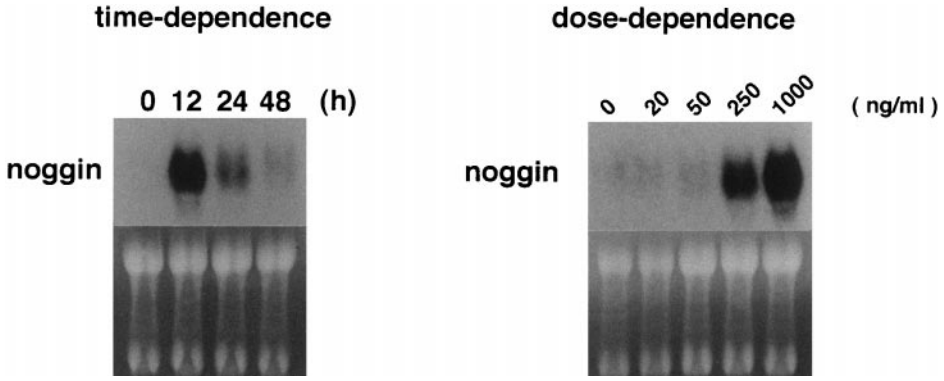

FIG. 3. Effect of exogenously administered BMP-4 on the expression of noggin mRNA in differentiated ATDC5 cells. Cells were cultured in 6-multiwell plastic plates as described under Materials and Methods for 21 days and were then exposed for the indicated time periods to 1000 ng/ml of BMP-4 and for an additional 12 h to either vehicle or BMP-4 (20, 50, 250, and 1000 ng/ml). Total RNA was prepared and subjected to Northern analysis (20 μg of total RNA per lane). The integrity of the RNA analyzed was confirmed by ethidium bromide staining, as shown in the bottom panel. Three independent experiments were performed and gave similar results.

variety of growth/differentiation molecules, including BMP family, parathyroid hormone-related protein and Indian hedgehog secreted from either chondrocytes or perichondrium in an autocrine/paracrine fashion [15, 18, 27, 28]. BMP family plays a vital role in skeletal development, and several BMP family genes are expressed at sites of bone formation with a unique spatiotemporal pattern, suggesting that the coordinated expression of these molecules is crucial for the cascade of events underlying chondrogenic differentiation [15, 18, 28, 29].

ATDC5 cells provide an *in vitro* model system in which chondrogenesis is observed in a synchronous and stepwise manner [17, 20–23]. By using this system, we showed that noggin regulates the progression of chondrogenic differentiation mediated by BMP-4. Three lines of evidence support this conclusion. (1) Noggin gene transcript was not detectable in undifferentiated ATDC5 cells, and increased in parallel with the progression of chondrogenic differentiation of these cells. (2) When differentiated ATDC5 cells were treated with the conditioned medium containing noggin protein, the steady-state levels of type II and type X collagen gene transcripts were downregulated. (3) This inhibitory effect of noggin on chondrogenic differentiation is reversed by exogenously administered BMP-4.

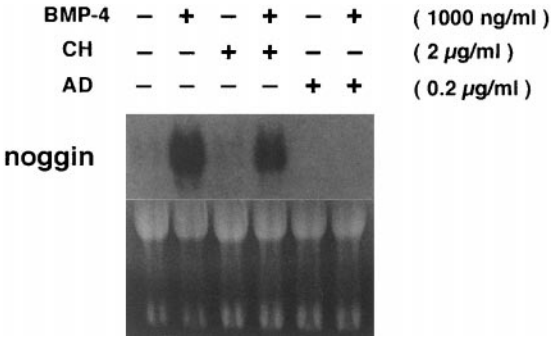

FIG. 4. Effect of actinomycin D (AD) or cycloheximide (CH) on noggin gene transcript enhancement by BMP-4. ATDC5 cells were cultured in 6-multiwell plastic plates as described under Materials and Methods for 21 days. The medium was changed and cells were cultured in the presence or absence of AD (0.2 μg/ml), CH (2 μg/ml), BMP-4 (1000 ng/ml), or combinations as indicated. Inhibitors were added 15 min prior to addition of BMP-4. RNA was extracted 12 h later and analyzed as described under Materials and Methods (20 μg of total RNA per lane). The integrity of the RNA analyzed was confirmed by ethidium bromide staining, as shown in the bottom panel. Three independent experiments were performed and gave similar results.

Our recent observations that exogenously administered BMP-2 and BMP-4 markedly downregulated the transcript levels of BMP-4 in ATDC5 cells, indicate that the stimulatory effects of BMP-4 on chondrogenic differentiation of ATDC5 cells are under the regulatory control by the negative-feedback mechanisms which maintain the amplitude of endogenous BMP-4 signal relatively constant (Akiyama *et al.*, submitted). Moreover, in the present study, we revealed that the increase of BMP-4 signal in ATDC5 cells resulted in the induction of the gene transcripts of noggin, which may antagonize the BMP-4 actions on the progression of chondrogenic differentiation of these cells. These lines of evidence favor the notion that the gross amplitude of BMP-4 signal in ATDC5 cells is maintained at a relatively constant level via modulation of both transcript levels and protein activities of BMP-4.

In summary, regulatory mechanisms underlying chondrogenic differentiation by a variety of growth/differentiation molecules are prerequisite for the proper progression of skeletal formation and development. Our observations in the present study using the chondrogenic ATDC5 cells raise the possibility that noggin may at least be involved in such functional

cooperation with BMPs during chondrogenic differentiation of chondrocytes.

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