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Stimulation of osteoblast proliferation by the cartilage-derived growth promoting factors chondromodulin-I and -II

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Abstract We previously reported the isolation of the cartilage-derived growth promoting factors chondromodulin-I (ChM-I) and chondromodulin-II (ChM-II) from fetal bovine epiphyseal cartilage. Both of these factors stimulate the growth and matrix formation of chondrocytes in vitro. In the present study, we found that ChM-I and ChM-II stimulated the proliferation of clonal mouse osteoblastic MC3T3-E1 cells as well as primary mouse osteoblasts in culture. Unlike other known growth factors, these factors did not support the proliferation of fibroblasts. Concomitantly with growth stimulation of osteoblasts, there was a reduction of alkaline phosphatase (ALP) activity in the cells, the expression of the differentiated phenotype. These results suggest that epiphyseal cartilage may play a functional role in longitudinal bone growth by production of these unique growth-promoting factors.

Key words: Chondromodulin-I; Chondromodulin-II; Osteoblast proliferation; MC3T3-E1; Alkaline phosphatase

1. Introduction

The longitudinal growth of long bones is supported by the growth of epiphyseal growth plate cartilage coupled with active bone formation (endochondral bone formation). A complex signaling network of local growth factors underlies the active proliferation of osteoblasts. Bone is known to be one of the major sources of growth factors such as insulin-like growth factor-I, fibroblast growth factor (FGF), transforming growth factor-β and bone morphogenetic protein [1–3]. These local growth factors exert profound effects on the growth and expression of the differentiated phenotype of osteoblasts [4,5] as well as chondrocytes [6–8]. Previous studies revealed that cartilage also contains these growth factors [9–11], suggesting that bone and cartilage interact with each other, exchanging growth signals in a paracrine fashion.

The growth and maturation of chondrocytes in the growth plate is believed to provide the physical scaffold for bone cells at the osteochondral junction. However, the active proliferation of osteoblasts at the osteochondral junction cannot be accounted for by only the common set of growth factors present in bone and cartilage. There is a possibility that cartilage may send a paracrine growth signal to the bone forming cells. We previously isolated the novel heparin-binding growth promoting factors chondromodulin-I (ChM-I) and -II (ChM-II) from fetal bovine epiphyseal cartilage [12,13]. These factors stimulated growth and proteoglycan synthesis in rabbit growth plate chondrocytes in culture [12,13]. In the present study, we tested our hypothesis that ChM-I and ChM-II may participate in the growth control of osteoblasts by using mouse osteoblastic MC3T3-E1 cells and primary osteoblasts in culture.

2. Materials and methods

2.1. Materials

ChM-I and ChM-II were purified from guanidinium chloride extracts of fetal bovine epiphyseal cartilage (embryos 3–6 months old) by acetone precipitation, ultrafiltrations, and successive chromatography with a Sephacryl S-200 column, a heparin Toyopearl affinity column and a YMC C_4 reverse-phase column, as previously described [12,13]. Human recombinant FGF-2 was obtained from R & D Systems (Minneapolis, MN).

2.2. Cell culture

A clonal mouse osteoblastic cell line, MC3T3-E1, was maintained in α-MEM containing 10% fetal bovine serum (FBS) at 37°C under 5% CO_2 in air and subcultured every third day [14]. In each well of a 48-microwell plate, 1.5×10^3 cells were plated with α-MEM containing 0.3% FBS and various concentrations of ChM-I or ChM-II. After incubation for 3 days, the cells were stripped off the plates with trypsin-EDTA solution (Sigma, St. Louis, MO), and their number was counted with a hemocytometer. For the determination of DNA synthesis and alkaline phosphatase (ALP) activity, the cells were grown to confluency in α-MEM containing 10% FBS. The cells were then preincubated in α-MEM containing a low concentration of serum (0.3% FBS) for 24 h to allow the cells to become quiescent, and then incubated in α-MEM containing test samples in the presence of 0.3% FBS for another 24 h ([^{3}H]thymidine incorporation) or 48 h (ALP activity). For the isolation of primary osteoblasts and fibroblasts, calvariae and lungs from 3-day-old ICR mice were cut into pieces with scissors and cultured separately in 35-mm dishes with α-MEM containing 10% FBS. After 5 days, cells migrating from the tissue fragments were subcultured. The [^{3}H]thymidine incorporation and ALP activity of these cells were measured under the culture conditions described for the MC3T3-E1 cells.

A clonal mouse chondrogenic cell line, ATDC5, was maintained in the medium consisting of a 1:1 mixture of DME and Ham's F-12 medium containing 5% FBS containing 10 μg/ml human transferrin (Boehringer Mannheim GmbH, Mannheim, Germany) and 3×10^{-8} M sodium selenite (Sigma, St. Louis, MO) at 37°C under 5% CO_2 in air, as previously described [15]. Chondrogenic differentiation of the cells was induced by the above medium supplemented with 10 μg/ml bovine insulin (Wako Pure Chemicals, Osaka, Japan) [15].

2.3. [^{3}H]Thymidine incorporation and ALP activity

The cells treated for 24 h with test samples were labeled for the last 3 h with [methyl-^{3}H]thymidine (1 μCi/well; NEN, Boston, MA). The cells were then scraped into 1 ml of cold phosphate-buffered saline (PBS) and precipitated with 10% trichloroacetic acid (final concentra-

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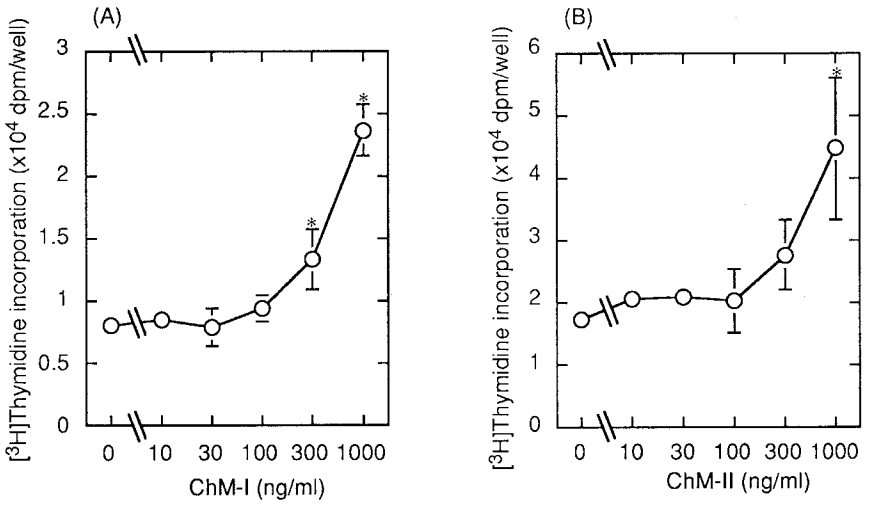

Fig. 1. Effects of ChM-I and ChM-II on [^{3}H]thymidine incorporation in the mouse osteoblastic cell line MC3T3-E1. The cells were treated with various concentrations of ChM-I (A) or ChM-II (B) for 24 h, and labeled with [^{3}H]thymidine for the last 3 h of the incubation. Values are means ± S.D. of 4 wells. $^*P < 0.05$ versus the control.

tion). The precipitates were washed with diethylether-ethanol (1:3, v/v). The radioactivity in the acid-insoluble materials was counted in a liquid scintillation counter. For the determination of ALP activity, the cells treated for 48 h with test samples were scraped into 0.05% Triton X-100 and solubilized by three cycles of freezing and thawing. The resultant supernatant was used for the assay of ALP activity by the method described previously [16], with *p*-nitrophenyl phosphate used as the substrate. Part of the supernatant was used to determine the protein content, which was measured by the method of Bradford [17]. The data are expressed as means ± S.D. Statistical analysis was carried out by one-way analysis of variance, and significance was determined by a multiple comparison procedure (Tukey-Kramer test).

2.4. RNA extraction and hybridization analysis

MC3T3-E1 (6×10^4 cells/well) and ATDC5 cells (6×10^4 cells/well) were inoculated in 6-multiwell plates. For induction of chondrogenesis, ATDC5 cells were cultured in the presence of 10 μg/ml insulin for 21 days. Total RNA was prepared from the cultures by a single-step method according to Chomczynski and Sacchi [18]. Total RNA (20 μg) was denatured with 6% formaldehyde, separated by 1% agarose electrophoresis, and transferred on Nytran membranes (Schleicher & Schuell, Germany). Hybridization was performed overnight at 42°C with an appropriate probe (10^6 cpm/ml) in solutions containing 50% formamide, 6×SSPE, as described [15]. Hybridization probes were prepared by the random-primer method with a BcaBEST Labeling kit (Takara, Otsu, Japan) using the appropriate cDNA fragments: 1.4 kb *Eco*RI fragment of pKT1180 [19] as a probe for α1(II) collagen mRNA; 1.3 kb *Eco*RI fragment of pCS1 harboring mouse ChM-I cDNA which was isolated from a mouse 17-day embryo cDNA library and subcloned into pGEM 3Zf(+) (Promega Corporation, Madison, WI) (C. Shukunami, submitted for publication). The filters were washed twice for 15 min at 55°C in 0.1×SSPE and 0.1% SDS, and were exposed to Kodak X-OMAT film at −80°C with a Cronex lightening plus intensifying screen (DuPont, Boston, MA).

3. Results and discussion

In a preliminary study, the medium conditioned by rabbit growth plate chondrocytes stimulated [^{3}H]thymidine incorporation into osteoblastic MC3T3-E1 cells (M. Kumegawa, unpublished data). This implies that growth plate chondrocytes produce some humoral factors that stimulate the proliferation

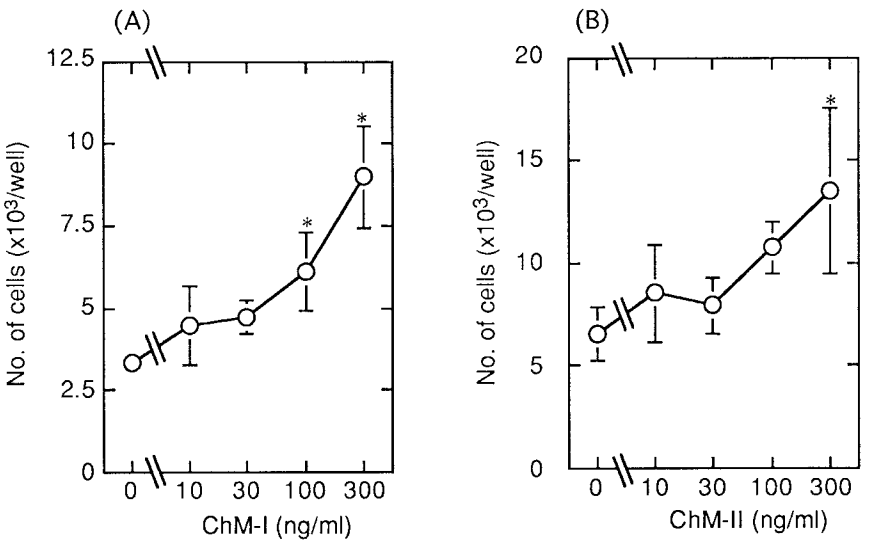

Fig. 2. Effects of ChM-I and ChM-II on the number of MC3T3-E1 cells. The cells were treated for 3 days with various concentrations of ChM-I (A) or ChM-II (B). Values are means ± S.D. of 4 wells. $^*P < 0.05$ versus the control.

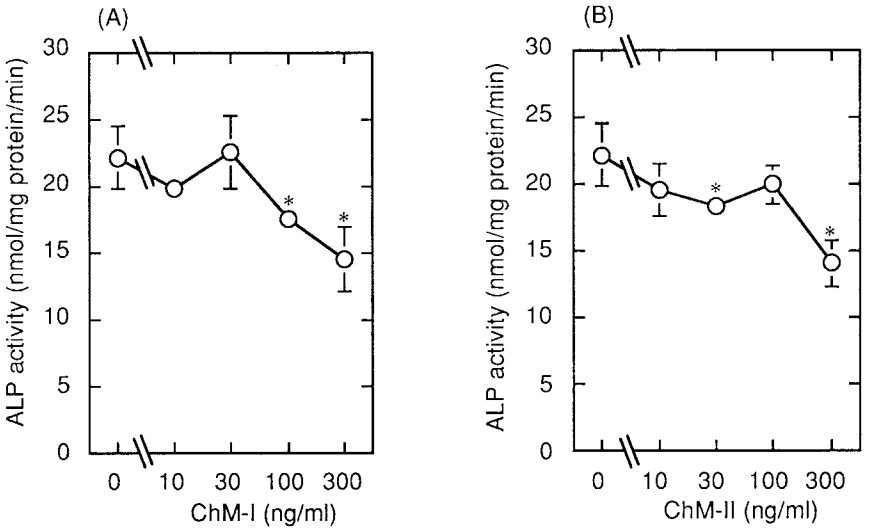

Fig. 3. Effects of ChM-I and ChM-II on the alkaline phosphatase (ALP) activity of MC3T3-E1 cells. The cells were treated with varying concentrations of ChM-I (A) or ChM-II (B) for 48 h. Values are means ± S.D. of 4 wells. $^{*}P < 0.05$ versus the control.

of osteoblasts. The discovery of the novel factors ChM-I and -II in epiphyseal cartilage suggested to us that these factors could be candidates for the cartilage-derived signaling molecules which act on osteoblasts.

To make the cells quiescent, MC3T3-E1 cells were grown to confluency and further preincubated under the low serum condition (0.3% FBS). Quiescent confluent cultures of MC3T3-E1 cells were then treated with ChM-I or ChM-II in the presence of 0.3% FBS for 24 h (Fig. 1). The [^{3}H]thymidine incorporation of the cells was stimulated in a dose-dependent manner. The stimulatory effects of ChM-I and ChM-II were detectable at 300 ng/ml, and DNA synthesis of the cells reached a 2–3-fold increase at 1 μg/ml. In accordance with these findings, ChM-I and ChM-II stimulated the proliferation of MC3T3-E1 cells (Fig. 2). The cells were plated at the density of 1.5×10^3 cells/well, and further incubated with various concentrations of ChM-I or ChM-II in the presence of 0.3% FBS. During the 3-day incubation of the sparse culture, the cell number increased by 2–4-fold without ChM-I or ChM-II in the control cultures, depending on the experiment. In the presence of ChM-I or ChM-II, the cell number increased in a dose-dependent manner, and became 2–3-fold greater than that of the control at the concentration of 300 ng/ml.

As shown in Fig. 3, we studied the effect of these ChM-I and ChM-II on the expression of the differentiated phenotype of osteoblasts, i.e. the ALP activity in MC3T3-E1 cells. The ALP activity was reduced with increasing doses of ChM-I or ChM-II. The effect was evident at the concentration of 100–300 ng/ml, at which dose ChM-I and ChM-II stimulated DNA synthesis of the cells. The expression of the differentiated phenotype is reciprocally correlated with the growth rate

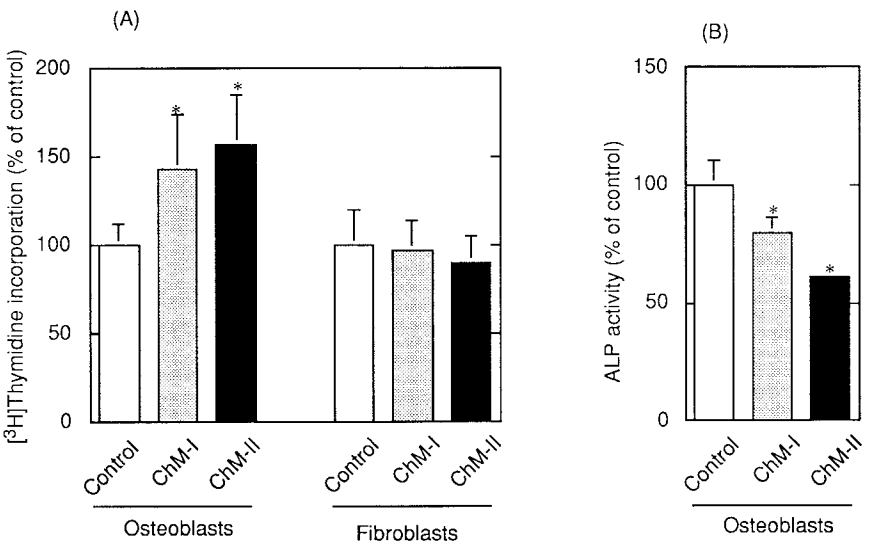

Fig. 4. Effects of ChM-I and ChM-II on [^{3}H]thymidine incorporation into primary calvarial osteoblasts and pulmonary fibroblasts, and on the ALP activity of the calvarial osteoblasts. For the assay of [^{3}H]thymidine (A) and ALP activity (B), the cells were cultured for 24 h and 48 h, respectively, with or without ChM-I or ChM-II at 300 ng/ml. The [^{3}H]thymidine incorporations in control wells of the osteoblasts and the fibroblasts were 1687 ± 214 dpm/well and 814 ± 163 dpm/well, respectively. The ALP activity in the control wells of the osteoblasts was 75.5 ± 7.8 nmol/mg protein/min. Values are means ± S.D. for 4 wells. $^{*}P < 0.05$ versus the control.

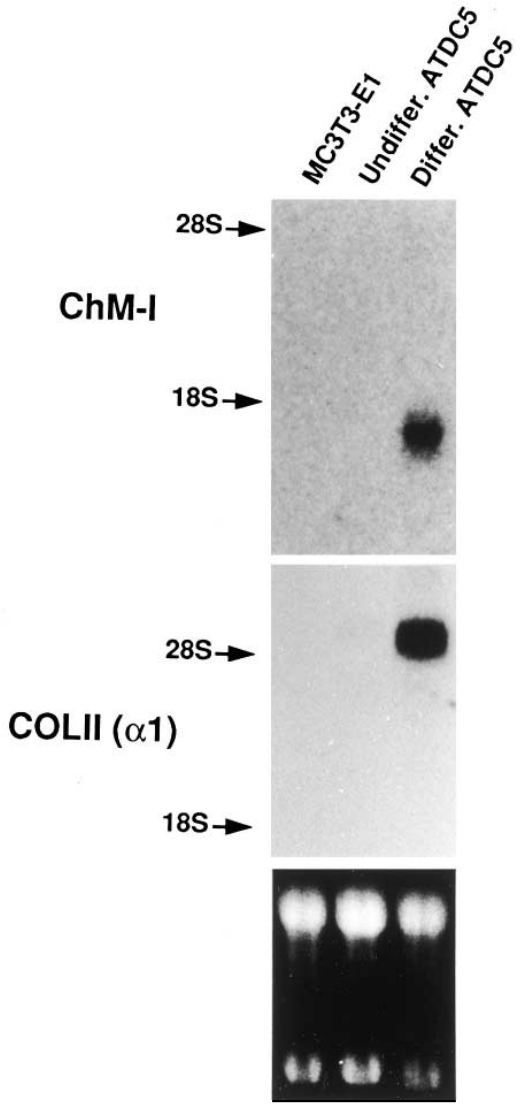

Fig. 5. Northern blot analysis of ChM-I and type II collagen mRNAs in MC3T3-E1 cells and chondrogenic ATDC5 cells. Cells were grown in 6-multiwell plates. Total RNA was isolated from MC3T3-E1 cells on day 10, from the undifferentiated ATDC5 cells on day 3, or from the differentiated ATDC5 cells on day 21 of culture. RNA (20 μg) was loaded in each lane and then hybridized with mouse ChM-I cDNA or rat type II collagen cDNA. The positions of 28S and 18S ribosomal RNAs are indicated. The lowest panel shows ethidium bromide-stained gel.

of MC3T3-E1 cells [16], as generally observed in other cell types [20]. Thus, the treatments of the cells with ChM-I or ChM-II resulted in a shift of cellular status from the maturing to the actively growing status.

The primary osteoblasts isolated from mouse calvariae showed a more mature phenotype than those of the clonal MC3T3-E1 cells, as characterized by the higher ALP activity and the lower growth rate. Fig. 4 illustrates that the above observations gained from MC3T3-E1 cell cultures can be extended to the authentic primary osteoblasts: stimulation of DNA synthesis and reduction of the ALP activity of osteoblasts by ChM-I and by ChM-II.

Among the known growth factors, FGF is known as the most potent growth stimulator of osteoblasts as well as chondrocytes [5]. As shown in Table 1, FGF-2 stimulated the [^{3}H]thymidine incorporation of MC3T3-E1 cells to a much higher level than did ChM-I or ChM-II. However, FGF stimulates the proliferation of the cells of mesenchymal origin, irrespective of cell type. Intriguingly, ChM-I and ChM-II stimulated the DNA synthesis of osteoblasts, whereas they exerted no growth stimulation in mouse lung fibroblasts (Fig. 4A). ChM-I and ChM-II additively stimulated DNA synthesis of the osteoblastic cells even in the presence of FGF-2 (Table 1). The additive nature and specificity of action suggest that ChM-I and ChM-II bring to osteoblasts and chondrocytes a growth advantage over the surrounding mesenchymal cells under the conditions that FGF is commonly available for the cells. Compared to FGF, ChM-I and ChM-II require a much higher dose to stimulate osteoblasts and chondrocytes (Figs. 1 and 2). However, an abundance of these factors in cartilage may compensate for the higher dose requirement for action in osteoblasts at the osteochondral junction [12,13].

Fibroblast growth factor exhibits pleiotropic effects depending on the target tissue. Cartilage and bone are the major source of FGF [21], although factors of the FGF family are also widely distributed in the body [22]. In contrast, expression of ChM-I mRNA was specific to cartilage. No transcript for the ChM-I gene was detected in the bony tissue by the in situ hybridization technique (Y. Hiraki, unpublished data). As shown in Fig. 5, there was no ChM-I mRNA detected in osteoblasts such as MC3T3-E1 cells by Northern blot analysis. However, the transcript was evidently expressed in the differentiated ATDC5 cells which expressed cartilage phenotype such as expression of the type II collagen gene [15], while no ChM-I mRNA was detected in the undifferentiated ATDC5 cells (Fig. 5). The tissue-specific distribution of ChM-II transcripts is not clear at this moment because molecular cloning of ChM-II is still uncompleted. However, it has been shown that there is an abundant expression of ChM-II protein in cartilage [13]. These results substantiate a possibility that these proteins may function as the paracrine growth promoting factors.

The present findings suggest that epiphyseal growth plate cartilage may be functionally involved in the active proliferation of osteoblasts at the osteochondral junction, and hence in the longitudinal growth of long bones by sending a specific

Table 1
DNA synthesis of MC3T3-E1 cells stimulated by ChM-I or ChM-II in the presence or absence of FGF-2

	[^{3}H]Thymidine incorporation (% of control)		
	None	ChM-I	ChM-II
None	100.0 ± 11.4	146.9 ± 16.8	140.5 ± 14.7
FGF-2 (0.2 ng/ml)	295.9 ± 31.6	366.2 ± 42.4	342.9 ± 51.4

ChM-I and ChM-II were used at 300 ng/ml. [^{3}H]Thymidine incorporation of cultures without any agents (control) was $(1.34 \pm 0.15) \times 10^4$ dpm/well. Data are presented as the mean ± S.D. (n=5).

growth-stimulating signal. The cell-surface receptors for ChM-I and ChM-II and the mechanism of their signal transduction remain to be elucidated.

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