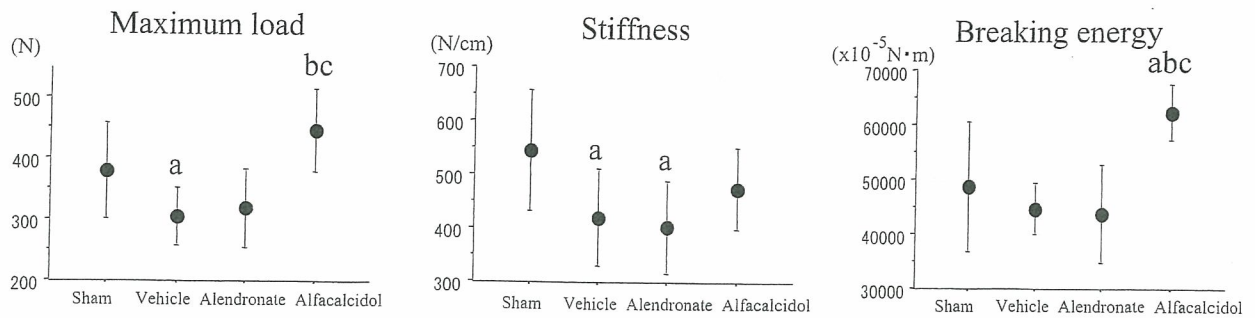


(A) Distal metaphysis of the femur



(B) Diaphysis of the femur

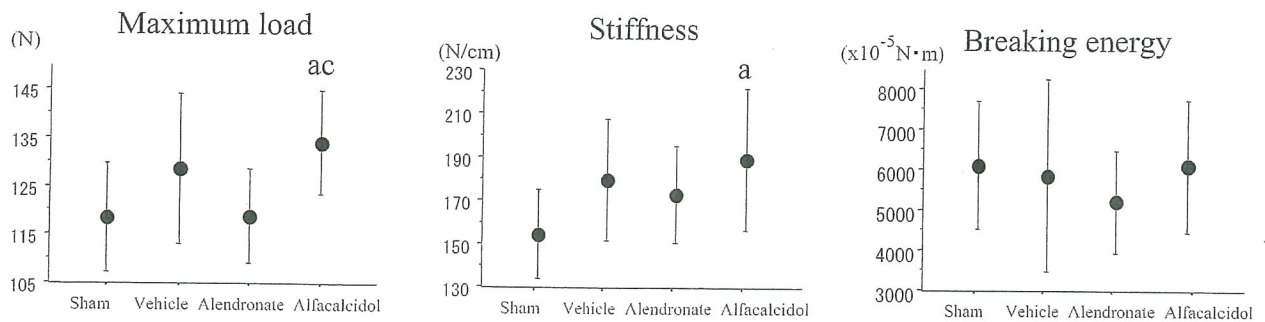


Fig 4. Mechanical properties of the femur. Data are expressed as mean \pm SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. a: significant vs Sham, b: significant vs Vehicle, c: significant vs Alendronate.

on Ct Ar. On the other hand, alfacalcidol increased both cancellous BV/TV and Ct Ar by mildly decreasing cancellous bone turnover as indicated by bone histomorphometric analysis and increasing periosteal and endocortical bone formation. The effect of alfacalcidol at the present dose on cancellous BV/TV was greater than that of alendronate, because the decrease of bone turnover by alfacalcidol was comparatively mild, and bone formation was maintained by alfacalcidol. Alfacalcidol, but not alendronate, increased the maximum load of the femoral distal metaphysis and femoral diaphysis. Thus, the present study clearly showed the differential effects of alendronate and alfacalcidol on cancellous and cortical bone mass and bone mechanical properties in ovariectomized rats.

In the present study, OVX increased bone turnover, resulting in the loss of cancellous BV/TV, and Tb N, and the deterioration of the maximum load of the femoral distal metaphysis. However, neither the loss of Tt Ar, and Ct Ar, nor the deterioration of the mechanical

properties of the femoral diaphysis was observed following OVX. The bone loss and deterioration of mechanical properties following OVX were observed primarily in regions rich in cancellous bone.

Bisphosphonates inhibit osteoclast-mediated bone resorption. Because of coupling of bone resorption and bone formation, suppression of bone resorption by bisphosphonates is followed by a reduction in bone formation. In other words, bisphosphonates decrease bone turnover. In the present study, the bisphosphonate, alendronate prevented a decrease in cancellous BV/TV in ovariectomized rats by suppressing bone turnover, without exerting any effect on Ct Ar, or maximum load of the femoral distal metaphysis and femoral diaphysis, despite suppressed endocortical bone resorption and maintained periosteal bone formation. Risedronate and pamidronate have been reported to prevent the loss of BMD and the mechanical properties of the femoral distal metaphysis, without having any effect on BMD or the mechanical properties of the femoral diaphysis in sciatic neurectomized and tail-suspended rats, respec-

tively [12, 13]. Furthermore, alendronate and risedronate have also been reported to effectively prevent immobilization (hindlimb-bandage)-induced loss of BMD and the mechanical properties of the femoral metaphysis [15]. These results suggest that bisphosphonates could prevent BMD loss in cancellous bone of hindlimb-immobilized or tail-suspended rats, but not the loss of BMD and the mechanical properties of cortical bone. Clinically, alendronate has been shown to markedly increase BMD of the lumbar spine which is rich in cancellous bone and reduce the incidence of vertebral fractures, but it does not affect metacarpal cortical BMD in postmenopausal women with osteoporosis [11, 14, 23]. All of these results demonstrate that alendronate is effective against the loss of bone mass and bone mechanical properties at skeletal sites rich in cancellous bone.

Despite inducing improvement of cancellous BV/TV, alendronate had no effect on the mechanical properties of the femoral distal metaphysis. Very few studies have clearly shown the beneficial effect of preventive treatment with alendronate alone on the mechanical properties of skeletal sites rich in cancellous bone in ovariectomized rats. Sato *et al.* [19] reported that long-term treatment with alendronate attenuated cancellous BV/TV loss, but scarcely affected the mechanical properties of the lumbar vertebrae in ovariectomized rats, because of marked suppression of bone formation. This result suggests that marked suppression of bone formation can also affect bone quality in ovariectomized rats. In the present study, marked suppression of bone formation might explain the non-significant effect of alendronate on the mechanical properties of the femoral distal metaphysis. It has been established that alendronate (10 mg/day) markedly increases lumbar and femoral neck BMD and reduces the incidence of vertebral and nonvertebral fractures in postmenopausal women with osteoporosis [2, 3, 5]. Thus, no significant effect of alendronate on the mechanical properties of the femoral distal metaphysis in the present study might possibly be attributable to the deterioration of bone quality caused by its high-dose administration.

The active vitamin D, 1α -hydroxyvitamin D₃ (alfacalcidol), is the prodrug of 1α , 25-dihydroxyvitamin D₃, which stimulates calcium absorption in the intestine, enhances calcium reabsorption in the kidney, suppresses parathyroid hormone secretion, and regu-

lates bone formation as well as bone resorption [4, 18]. An experimental study clearly showed that alfacalcidol caused a dose-dependent suppression of bone resorption and yet maintained or even stimulated bone formation, as reflected by the increases in the serum OC levels and BFR/BS at both cancellous and cortical bone sites, resulting in an increase in BMD and improvement of mechanical properties in ovariectomized rats [21]. In the present study, alfacalcidol increased cancellous BV/TV, Tb Th, and Ct Ar in ovariectomized rats by mildly decreasing bone resorption, but maintaining bone formation, increasing periosteal and endocortical bone formation, and preventing an increase of endocortical bone resorption. Alfacalcidol also increased the maximum load of the femoral distal metaphysis and femoral diaphysis. These results were consistent with those of a previous study [21]. Clinically, alfacalcidol maintains BMD of the metacarpus, which consists of cortical bone, and the lumbar spine, which is rich in cancellous bone, and decreases the incidence of vertebral fractures in patients with postmenopausal osteoporosis [8, 16]. All of these results suggest that alfacalcidol may mildly affect both cancellous and cortical bone mass and bone mechanical properties. However, alfacalcidol increased bone turnover, as evaluated by urinary DPD and serum OC levels, which reflect both cancellous and cortical bone turnover.

Clinically, randomized controlled head-to-head trials have demonstrated that alendronate (5 mg/day) is more effective than alfacalcidol (1 μ g/day) in increasing lumbar BMD and reducing the incidence of vertebral fractures in Japanese postmenopausal women with osteoporosis [14, 23]. Nevertheless, in the present study, the effect of alfacalcidol (0.5 μ g/kg) on cancellous BV/TV was greater than that of alendronate (2.5 mg/kg), and only alfacalcidol increased the maximum load of the femoral distal metaphysis, presumably because the decrease of bone turnover by alfacalcidol administration was comparatively mild as compared with that following alendronate administration, and bone formation was maintained by alfacalcidol. This discrepancy may be due to a differential response of cancellous bone to alfacalcidol between ovariectomized rats and postmenopausal women (human); the beneficial effects of alfacalcidol might be greater on rat bones than on human bones. Only alfacalcidol increased Ct Ar and

the maximum load of the femoral diaphysis. Thus, it would be of interest to investigate the comparative effect of alendronate and alfacalcidol on the incidence of nonvertebral fractures by further clinical trials.

In conclusion, the strengths of the present study included the detailed bone histomorphometric analyses of cancellous and cortical bone, the measurement of biochemical markers, and the measurement of the bone mechanical properties. The results showed that alendronate prevented the decrease in cancellous bone mass observed in ovariectomized rats, without exerting any effect on the cortical bone mass. On the other hand, alfacalcidol increased both cancellous and cortical bone mass. Alfacalcidol, but not alendronate, increased the maximum load of the bone at both sites rich in cortical and cancellous bone. Thus, the present study clearly showed the differential effects of alendronate and alfacalcidol on cancellous and cortical bone mass and bone mechanical properties in ovariectomized rats.

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Rapid communication

Organic–inorganic hybrid-nanocarrier of siRNA constructing through the self-assembly of calcium phosphate and PEG-based block anionomer

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Abstract

The development of siRNA delivery systems is a major key for practical RNA therapy that holds promise for the treatment of life-threatening human diseases, yet there still exists significant difficulties in their construction because of the various requirements including high transfection efficacy, tolerability in the biological medium, and low toxicity. Here we report the novel preparation route of organic–inorganic hybrid-nanocarriers entrapping siRNA based on the self-assembly of the block anionomer, poly(ethylene glycol)-*block*-poly(methacrylic acid), with calcium phosphate crystals. The nanocarriers have diameters in the range of several hundreds of nanometers and revealed excellent colloidal stability due to the steric stabilization effect of the PEG palisade. The biological activity of siRNA loaded in nanocarriers was assessed using 293 cells stably expressing luciferase gene, showing the remarkably high gene silencing-efficacy without the use of any adjuvant molecules such as chroloquin. Further advantage of the system is the serum tolerability, which is of a critical issue in in vivo application.

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Short interfering RNA (siRNA) that mediates gene-silencing phenomena of RNA interference (RNAi) needs a carrier system to exert biological activity, especially, for in vivo application [1]. Some lipid- and polymer-based systems are available for the in vitro application of siRNA, yet they often have drawbacks including a low stability in a serum-containing medium and significant cytotoxicity. From the standpoint of developing a safe and serum-tolerable carrier system, the calcium phosphate (CaP) co-precipitation method is of interest [2]. To control the growth of CaP, that is the determining factor for the transfection efficacy, a novel concept has recently been introduced by our group based on the self-assembly of poly(ethylene glycol)-*block*-poly(aspartic acid) block copolymers (PEG-P(Asp)) with CaP to form the narrowly distributed hybrid nanoparticles covered with PEG palisades [3].

siRNA-entrapped PEG-P(Asp)/CaP hybrid nanoparticles indeed revealed an appreciable gene silencing in a cultured cell line [4]. Nevertheless, the requirement of chroloquin, a reagent facilitating endosomal-escape, impedes the wide applicability of this method. Herein, we overcome this critical issue using the newly designed system of siRNA-entrapped CaP nanoparticles. The strategy is to incorporate the molecular units facilitating endosomal-escape directly into the block copolymer structure. In this regard, poly(methacrylic acid) (PMA) was selected as the polyanion unit in the block copolymer. PMA undergoes a conformational transition at pH 4–6, which almost corresponds to the endosomal pH, to adopt a more hydrophobic conformation as compared to neutral pH, thus expected to have an increased interaction with the endosomal membrane in order to perturb its structure to facilitate escape of the nanoparticles into the cytoplasm [5]. Here, two different compositions of the poly(ethylene glycol)-*block*-poly(methacrylic acid) (PEG-PMA) were used for the nanoparticle formation entrapping siRNA. PEG-PMA1 has a PEG segment with the molecular weight (Mw) of 7800 and the PMA segment with the Mw of

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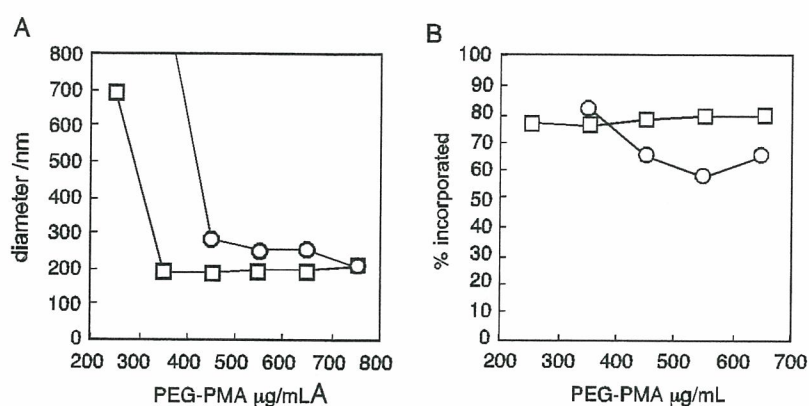


Fig. 1. Physicochemical characterization of nanoparticles composed of PEG-PMA1 (□) and PEG-PMA2 (○). (A) Size of the nanoparticles entrapping siRNA determined by DLS measurements. (B) Percentage of siRNA loaded in the nanoparticles formed at varying PEG-PMA concentrations.

2000, while the Mw of the PEG and PMA segments of PEG-PMA2 are 7500 and 15,500, respectively.

The diameters of the nanoparticles formed in the presence of the PEG-PMAs are determined by dynamic light scattering measurements [6,7]. Fig. 1A shows the change in diameters of nanoparticles with varying concentrations of PEG-PMAs at 3.0 mM phosphate. A similar trend was observed for PEG-PMA1 and PEG-PMA2, and the stable dispersion of nanoparticles was obtained over certain concentrations; 350 µg/mL for PEG-PMA1 and 450 µg/mL for PEG-PMA2. Diameters above these critical concentrations of PEG-PMA were about 180–200 and 200–280 nm for PEG-PMA1 and PEG-PMA2, respectively. It is noteworthy that at those concentrations no precipitation of CaP was observed 24 h after the mixing of Ca/siRNA and phosphate/PEG-PMA solution. The polydispersity indices of the nanoparticles were below 0.1 for PEG-PMA1, suggesting a unimodal distribution, while the values were slightly higher (0.12–0.15) for PEG-PMA2. The zeta potential of nanoparticles composed of

450 µg/mL of PEG-PMA1 or PEG-PMA2 was almost zero, -1.50 ± 0.36 and -0.439 ± 0.118 mV, respectively, indicating that the colloidal stability of the nanoparticles is caused by the PEG palisades with the steric repulsion effect. The incorporation efficacy of siRNA in the nanoparticles determined by the centrifugation method was around 80% for PEG-PMA1 and 60–80% for PEG-PMA2 [8] (Fig. 1B).

In order to investigate RNAi activity of siRNA incorporated in the nanoparticles, we have established a 293 cell line stably expressing the GL3-luciferase gene and estimated the degree of gene silencing using the siRNA targeting GL3-luciferase gene [9–11]. Fig. 2A shows the effect of the PEG-PMA concentrations on the RNAi activity of the siRNA-loaded nanoparticles, expressed as the relative inhibition activity to the control (non-silencing) siRNA. PEG-PMA1 showed no remarkable RNAi activity (■), while the PEG-PMA2 system (●) achieved a gene silencing down to 20% of the control (80% inhibition) in the polymer concentration range of 450 to 550 µg/mL. The

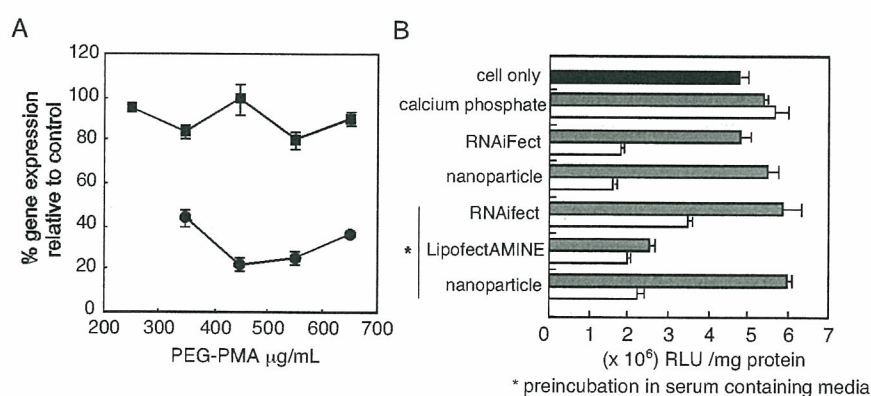


Fig. 2. Biological activity of siRNA incorporated in nanoparticles. (A) RNA interference by siRNA incorporated in nanoparticles formed at various concentrations of PEG-PMAs. GL3 luciferase gene expression in 293 cells was inhibited by nanoparticles composed of PEG-PMA1 (■) and PEG-PMA2 (●). Results are shown as the percentage to the control expression of cells treated with nanoparticles entrapping non-silencing siRNA. (B) RNA interference by siRNA using various transfection reagents. Grey bars indicate GL3 luciferase gene expressions in 293 cells treated with non-silencing control siRNA, and white bars indicate those treated with GL3 siRNA. The nanoparticle samples are composed of calcium phosphate and PEG-PMA2 formed at 450 µg/mL. For the preincubation study (*), DMEM containing 20% FCS was added to an equal amount of nanoparticle or lipoplex solutions and incubated at 37 °C for 30 min prior to the transfection. In the calcium phosphate method, the transfection was performed under the same condition as the nanoparticle except in the absence of PEG-PMA. The concentrations of siRNA and calcium ion in all the experiments were 125 nM and 6.25 mM, respectively.

reason for the presence of the maximum transfection efficacy in terms of the PEG-PMA concentration is not yet clear, however, nanoparticles with smaller diameters seem to be more efficient than the larger CaP/PEG-PMA complex or precipitate formed at the lower PEG-PMA concentration. For example, at 250 $\mu\text{g/mL}$ PEG-PMA1, at which a large precipitate was formed, no silencing was recognized. The siRNA concentration dependency was evaluated using the sample solutions prepared by dilution of the stock nanoparticle solution formed at 450 $\mu\text{g/mL}$ of PEG-PMA2 and 2.5 μM of siRNA. The significant inhibition was observed at a concentration as low as 6.7 nM (45% inhibition) and reached a plateau value around 25 nM (80% inhibition).

To clarify the advantages of this system, the transfection efficacy was further compared with other transfection reagents and the conventional calcium phosphate method (Fig. 2B). Under the experimental conditions, the hybrid nanoparticles formed at 450 $\mu\text{g/mL}$ of PEG-PMA showed transfection efficacy similar to RNAiFect. Worth noting is that the nanoparticles are more efficient in silencing than RNAiFect at the more physiologically relevant conditions: Preincubation in the DMEM containing 10% FCS was performed for 30 min prior to the transfection (*—marked conditions in Fig. 2B). Upon serum pre-incubation, the GL3 luciferase expression relative to the control increased from 36% to 58% in the case of RNAiFect, while the increase is only 29% to 35% for the hybrid nanoparticle system. Another type of lipid-based transfection reagent, lipofectAMINE, showed a non-specific effect in the serum-containing medium, which resulted in a significantly reduced luciferase activity even using the controlled non-silencing siRNA, presumably due to the toxicity. Tolerability to the serum is crucial when considering the in vivo application of the carrier system, especially, in the case of the systemic administration. Indeed, the in vivo application of a conventionally available cationic-lipid system is reported to be hampered in the serum-containing medium [12]. Furthermore, no significant activity of siRNA was observed for the calcium phosphate crystal prepared in the absence of PEG-PMA, indicating the necessity of the block copolymer for the improved transfection activity.

In summary, we demonstrated a highly efficient transfection of siRNA to cultured mammalian cells using nano-sized calcium phosphate crystals with appreciable serum tolerability. This nano-particulate siRNA carrier possessing a biocompatible PEG palisade and a pH-responsive moiety of PMA to facilitate endosomal escape may have a promising utility in RNAi research directed toward disease treatment.

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- [9] siRNA targeting GL3-luciferase gene, antisense sequence; 5'-UCGAA GUACUCAGCGUAAGdTdT-3', sense sequence; 5'-CUUACGCUGA GUACUUCGAdTdT-3', control (non-silencing) siRNA; antisense sequence; 5'-UUCUCCGAACGUGUCACGUDdT-3', sense sequence; 5'-ACGUGACACGUUCGAGAAAdTdT-3').
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Pro-inflammatory Cytokine Tumor Necrosis Factor- α Induces Bone Morphogenetic Protein-2 in Chondrocytes via mRNA Stabilization and Transcriptional Up-regulation*

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In articular chondrocytes, the inflammatory cytokine tumor necrosis factor- α (TNF- α) induces the expression of bone morphogenetic protein-2 (BMP-2), a growth factor known to be involved in the induction of cartilage and bone. A study was performed to clarify the mechanism(s) underlying the induction of BMP-2 in chondrogenic ATDC5 cells and primary cultured adult human articular chondrocytes. In ATDC5 cells, the endogenous BMP-2 expression was consistently low throughout the process of chondrogenic differentiation, and TNF- α induced BMP-2 expression only after the cells acquired the chondrogenic phenotype. The results of nuclear run-off assay and cycloheximide treatment consistently indicated that ATDC5 cells acquire the capacity to synthesize BMP-2 mRNA in the nuclei during the differentiation process. In an attempt to explain the discrepancy between the active nuclear mRNA synthesis and the observed low expression level in differentiated ATDC5 cells, the stability of BMP-2 mRNA was evaluated, and the cells were found to regulate the expression of BMP-2 at the post-transcriptional level. Human chondrocytes were confirmed to have a similar post-transcriptional regulation. The result of 3'-rapid amplification of cDNA end revealed that both human and mouse BMP-2 mRNAs contain multiple pentameric AUUUA motifs in a conserved manner in the 3'-untranslated regions, and transient transfection experiments demonstrated that TNF- α increases the stability of BMP-2 mRNA through the pentameric motifs. Further experiments revealed that TNF- α modulates mRNA stability via p38 signal transduction pathway, whereas the cytokine also augmented the expression of BMP-2 through transcriptional up-regulation via the transcriptional factor NF- κ B.

Bone morphogenetic proteins (BMPs)² are a group of secreted signaling proteins that were originally identified by

their ability to induce ectopic bone formation (1). Molecular cloning of the BMPs later revealed that the proteins belong to the transforming growth factor- β superfamily (2). The evolutionary conservation of BMPs is remarkable. For example, the amino acid sequence of *Drosophila* protein *decapentaplegic* (*dpp*) is ~75% identical to human BMP-2 and is functionally interchangeable with recombinant human protein as to its ability to ectopically induce bone formation in rodents (3).

BMPs are critical in embryonal development and postnatal growth. Among them, BMP-2 plays a vital role in fetal development. Mice lacking functional BMP-2 gene die during early embryogenesis due to malformation of the proamniotic canal and a defect in cardiac development (4). Subsequent studies have shown that the growth factor is involved in various aspects of development such as skin and hair formation, neural cell differentiation, and cartilage and bone formation (5). The role of BMP-2 in skeletal development is particularly crucial. At the early stage of embryogenesis, BMP-2 is expressed in specific areas of limb buds to form prechondrogenic condensations, and it later promotes cellular differentiation into chondrocytes (6).

Besides its roles in developmental and growth processes, BMP-2 is also expressed in postnatal animals and is often associated with various pathologies. The growth factor is expressed in the process of bone healing where it regulates cellular differentiation, proliferation, and matrix production (7, 8). Various tumors have been found to express BMP-2. Indeed, this protein is known to exert diverse effects on tumor cells, ranging from the facilitation of tumor growth to the induction of cellular apoptosis (9–11). We and other investigators have found that BMP-2 is expressed at high levels in arthritic joints by both chondrocytes and synovial cells, possibly promoting chondrocyte anabolism and osteophyte formation (12–16).

Because of its potent biological actions, the expression of BMP-2 must be strictly regulated *in vivo*. In fact, BMP-2 is expressed in a highly tissue- and stage-specific pattern during embryogenesis (17, 18), and either enhancement or inhibition

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² The abbreviations used are: BMP, bone morphogenetic protein; UTR, untranslated region; TNF, tumor necrosis factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ARE, AU-rich element; nt, nucleotide; NF- κ B,

nuclear factor- κ B; I κ B α , inhibitor of κ B α ; PDTC, pyrrolidine dithiocarbamate; MAP, mitogen-activated protein; MKK, mitogen-activated protein kinase kinase; CHX, cycloheximide; ActD, actinomycin D; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; RNAi, RNA interference; siRNA, small interfering RNA.

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of its activity is known to cause a significant disturbance in skeletal formation (19, 20). However, although the function of BMP-2 has been extensively studied, less is known about the mechanisms that regulate production of the growth factor. To date, several studies have shown that the expression of the gene is regulated at the transcriptional level. Retinoic acid induces BMP-2 expression through transcriptional activation in osteoblastic cells, possibly via retinoic acid receptor γ (21–23). NF- κ B has been shown to regulate the transcriptional activity of BMP-2 in growth plate chondrocytes during endochondral bone development (24). The transcriptional activity might be enhanced by estrogen (25) and, interestingly, by BMP-2 itself (26). Thus, transcriptional regulation is considered to play an important role in expression of the protein.

On the other hand, BMP-2 expression could also be regulated at the post-transcriptional level. Computer analyses have revealed that the proximal part of the 3'-untranslated region (3'-UTR) of BMP-2 gene is highly conserved across a wide range of species (21, 27). That fact suggests the involvement of post-transcriptional regulation for the expression of BMP-2, because 3'-UTRs of mRNA often contain sequences to regulate post-transcriptional events (28, 29). In fact, a recent report has shown that degradation of the gene transcripts could be regulated by the region (27). However, details of the regulatory mechanism are not yet known, and the biological significance of transcriptional and post-transcriptional regulation has yet to be established.

We and others recently reported that the pro-inflammatory cytokines interleukin-1 β and TNF- α induce BMP-2 expression in adult articular chondrocytes and a chondrosarcoma cell line (12, 30). Similar BMP-2 induction by those cytokines is also observed in synovial cells (14, 31) and could be a widespread event in arthritic joints. In this study, the mechanism of BMP-2 induction by TNF- α was studied in chondrogenic ATDC5 cells and primary cultured adult human articular chondrocytes. Our results indicated that both transcriptional and post-transcriptional regulation are involved in the induction of BMP-2.

EXPERIMENTAL PROCEDURES

Cell Culture—ATDC5 cells were obtained from the RIKEN cell bank (Tsukuba, Japan) and cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F-12, Invitrogen) containing 5% fetal bovine serum (fetal bovine serum, Invitrogen), 50 units/ml penicillin, 50 μ g/ml streptomycin, 10 μ g/ml human transferrin (Roche Molecular Biochemicals, Indianapolis, IN), and 3×10^{-8} M sodium selenite (Sigma) (32). To induce chondrogenic differentiation, bovine insulin (Sigma) was added to the media at a concentration of 10 μ g/ml. Human chondrocytes were obtained from 29 osteoarthritic knee joints in 28 patients at the time of joint replacement surgery. The material collection was performed under the approval of institutional review boards, and informed consent in writing was obtained from all patients. Articular chondrocytes were liberated from cartilage tissue by sequential enzymic digestion of 0.5% Pronase (Calbiochem) and 0.025% collagenase P (Roche Diagnostics, Basel, Switzerland) (33). Isolated cells were plated onto 6- or 12-well plates at a density of 2×10^5 cells/cm² and cultured in DMEM/F-12

media containing 10% fetal bovine serum, penicillin, streptomycin, and 25 μ g/ml ascorbic acid (Sigma). For cartilage explants, full thickness articular cartilage was aseptically obtained from metacarpophalangeal joints of adult bovine animals and punched out into discs 3 mm in diameter. The explants were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum, penicillin, streptomycin, and 25 μ g/ml ascorbic acid.

For the inhibitors used in the study, cycloheximide, actinomycin D, pyrrolidine dithiocarbamate, ionomycin, and wortmannin were purchased from Sigma, U0126 and SP600125 were from Biomol (Plymouth Meeting, PA), and SB202190, SB202474, SB203580, PD98059, and GF109203X were obtained from Calbiochem. Recombinant mouse and human TNF- α were purchased from Chemicon International (Temecula, CA); recombinant mouse noggin was from R&D Systems (Minneapolis, MN).

Real-time Quantitative PCR Analysis—Total RNA was extracted from the cells using the RNeasy kit (Qiagen, Valencia, CA) with DNaseI (Qiagen) treatment, and 1 μ g of total RNA was employed to synthesize cDNA using avian myeloblastosis virus reverse transcriptase (Roche Diagnostics). The cDNA was then used for real-time quantitative PCR on a LightCycler (Roche Diagnostics). For mouse BMP-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, respective pairs of gene-specific hybridization probes labeled with fluorescein (Flu) and LightCycler-Red640 (LC640) dyes, respectively, were used to monitor the amount of PCR product. The primer sequences were 5'-TGCACCAAGATGAACACAG-3' and 5'-GCTGTTTGTGTTTGGCTTG-3' for BMP-2, and 5'-TGAA-CGGGAAGCTCACTGG-3' and 5'-TCCACCACCCTGTTG-CGTGA-3' for GAPDH. The probe sequences were 5'-TCGT-TTGTGGAGCGGATGTCCTTT/Flu/-3' and 5'-/LC640/C-ATCATGTCCAAAAGTCACTAGCAATGGC-3' for BMP-2, and 5'-CTGAGGACCAGTGTCTCTCTCCGCA/Flu/-3' and 5'-/LC640/TTCAACAGCAACTCCCACTCTTCCACC-3' for GAPDH (Nihon Gene Research Laboratory, Sendai, Japan). PCR of human BMP-2, RelA, I κ B α , and GAPDH genes was performed using the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics), and the amount of PCR product was monitored by the intensity of fluorescence from the dye bound to the product. The primer sequences were 5'-CCCCG-GGGTATCACGCCTTT-3' and 5'-GCGACACCCACAACC-CTCCA-3' for BMP-2, 5'-CAGAGTTACCTACCAGGGCT-ATTC-3' and 5'-TCTGACTCTGTGTCATAGCTCTCC-3' for RelA, 5'-ACGAGCTTGTAGGAAAGGACTG-3' and 5'-GCTGCTCTTCTATAGGAACTTGGGA-3' for I κ B α , and 5'-CAGGGACTCCCCAGCAGT-3' and 5'-GGCATTGCCCTC-AACGACCA-3' for GAPDH.

The PCR protocol was the same for all genes, *i.e.* 95 °C for 10 min to activate *Taq* polymerase, then 40 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 6 s. When SYBR green dye was used to monitor PCR, melting curves were routinely recorded to verify the singularity of the PCR product. In each sample, the level of cDNA was normalized by the expression of GAPDH.

Nuclear Run-off Assay—ATDC5 cells were seeded to 225-cm² flasks at a density of 2×10^3 /cm² and incubated under the aforementioned conditions. Soon after reaching confluency or

following 15 days of culture in the insulin-containing media, the cells were incubated for another 48 h in the presence or absence of 20 ng/ml of recombinant mouse TNF- α . The cells were then harvested, lysed in 4 ml of the Nonidet P-40 buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40). The nuclei thus obtained were resuspended in glycerol storage buffer (40% glycerol, 50 mM Tris, pH 8.3, 5 mM MgCl₂, and 0.1 mM EDTA) and immediately stored in liquid nitrogen until use.

RNA transcripts were labeled by incubation of the nuclei in a reaction buffer (5 mM Tris, pH 8.0, 2.5 mM MgCl₂, 150 mM KCl, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, and 1 mM dithiothreitol) containing 100 μ Ci of [α -³²P]UTP (10 mCi/ml, GE Healthcare, Piscataway, NJ) for 30 min at 30 °C. Nuclei from 1–3 \times 10⁸ cells were used for each transcript reaction. The reaction mixture was treated with DNase I and proteinase K, and RNA was isolated by phenol-chloroform extraction and ethanol precipitation. pCR2.1 vectors (Invitrogen) containing cDNAs for the entire mouse BMP-2 3'-UTR, partial mouse GAPDH, and full-length mouse β -actin were prepared respectively, together with an empty vector. Five micrograms of each plasmid was linearized by HindIII digestion, denatured with 0.2 M NaOH, blotted onto nylon membranes (Hybond-N, GE Healthcare), and immobilized by UV-cross-linking. The membrane was pre-hybridized with ULTRAhyb (Ambion, Austin, TX), and hybridization was then allowed to proceed at 42 °C for 14–16 h. The membranes were washed twice in 2 \times SSC at 65 °C, treated with RNase A, and washed again in 2 \times SSC at 37 °C. The analysis was performed by phosphorimaging using a Typhoon 9410 (GE Healthcare) with ImageQuant software (GE Healthcare).

3'-Rapid Amplification of cDNA Ends—The 3'-end of mouse BMP-2 gene was determined using a GeneRacer kit (Invitrogen) following the manufacturer's protocol. In brief, total RNA was extracted from bone tissues of C57BL/6 mice, and cDNA was synthesized using an oligo(dT)-adapter primer provided by the kit. The first PCR was performed using 1 μ g of the synthesized cDNA as a template with mGSP1 (5'-AGAAAACGTCTCGCCACCCTCC-3') and 3' primer for the adaptor. The PCR product containing the 3'-end of human BMP-2 mRNA was obtained by using a nested PCR using the first PCR product as a template, and mGSP2 (5'-TACATTTGCCTGACACGCA-GCA-3') and the 3'-nested primer provided by the kit as primers. The PCR product was subcloned into pCR2.1 vector and subjected to DNA sequencing. The 3'-rapid amplification of cDNA ends of human BMP-2 mRNA was performed in the same manner, using total RNA from primary cultured chondrocytes to generate cDNA, and an external primer hGSP1 (5'-TGGCACGTCCGGGTTACCATGTTTCATTA-3') and an internal primer hGSP2 (5'-TGAAGCCCTTACAGGCCAAA-GGACCACA-3') for the first and nested PCR, respectively. DNA sequences were determined by using the Long-Read Tower System (GE Healthcare). Three clones were sequenced for each gene.

Plasmid Constructions—Luciferase reporter expression constructs harboring mouse BMP-2 3'-UTR at the 3'-end of the luciferase coding region were prepared in pcDNA3.1/Zeo(+) expression vector (Invitrogen). The luciferase cDNA was generated from pGL3-Basic vector (Promega) by PCR and

cloned into the KpnI and EcoRI sites of pcDNA3.1/Zeo(+). cDNA for the entire 3'-UTR of mouse BMP-2 was generated by PCR and subcloned into pCR2.1 vector. Using the vector as a template, specific regions of the 3'-UTR were amplified by PCR and ligated into pcDNA3.1/Zeo(+) between the EcoRI and XbaI sites. Thus, either the entire or a specific region of mouse BMP-2 3'-UTR was placed between the luciferase gene and the BGH polyadenylation site of the vector. All constructs were analyzed by restriction mapping and DNA sequencing.

Transient Transfection Assays—Transient transfection experiments were performed in differentiated ATDC5 cells using each of the prepared luciferase vectors and a phRL-TK vector (Promega). The cells were plated on 12-well plates, and differentiation was induced as previously described. DNA transfection was carried out using the SuperFect transfection reagent (Qiagen). For each well, 0.5 μ g of the luciferase vector and 1.5 μ g of phRL-TK vector were mixed with 4 μ l of SuperFect reagent and 75 μ l of DMEM/F-12 medium, and the mixture was incubated for 10 min at room temperature. The reagent mixture was combined with 400 μ l of the complete culture medium, which was transferred to the washed cell monolayer in each well. After incubation for 14–18 h, the medium containing transfection reagent was replaced with the complete medium. For some experiments, recombinant mouse TNF- α was added to the medium at a concentration of 10 ng/ml. The transfected cells were cultured for 48 h and then lysed in 100 μ l of reporter lysis buffer (Promega). Luciferase activity was assayed on a luminometer (JNR AB-2100, Atto, Tokyo, Japan) using a Dual-Glo luciferase assay system (Promega). All transfection experiments were repeated at least three times in duplicate.

Preparation of Adenovirus Vector and Gene Transduction—The recombinant adenovirus vector carrying constitutively active MAP kinase kinase 6 (MKK6) gene under the control of chicken β -actin promoter and cytomegalovirus IE enhancer was generated by the DNA-terminal protein complex method (34). The adenovirus carrying β -galactosidase was obtained from BD Biosciences (San Jose, CA). Titers of the adenovirus were determined by an Adeno-X Rapid Titer kit (BD Biosciences) following the manufacturer's protocol. The titer was represented by the multiplicity of infection.

To infect the adenovirus vectors in cultured human chondrocytes, the culture media in each well of 6- or 12-well plates was replaced by 1.0 or 0.5 ml of serum-free DMEM/F-12 medium containing the adenoviruses at the indicated multiplicity of infection. The cells were incubated for 2 h under normal culture conditions, after which 5 times the volume of the complete medium was added to each well.

Western Blotting—Approximately 1 \times 10⁶ cells were lysed in 0.5 ml of radioimmune precipitation assay buffer containing proteinase inhibitors (0.5 ml of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, and a mixture of proteinase inhibitors (Complete Mini, Roche Diagnostics)) for 10 min on ice with occasional mixing. The lysate was clarified by centrifugation at 12,000 \times g for 20 min at 4 °C. Twenty micrograms of protein was subjected to SDS-PAGE with 8–16% polyacrylamide sepa-

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rating gel in the reducing condition and electronically transferred onto a nitrocellulose membrane (Bio-Rad). After blocking, the membrane was incubated with either anti-p38 MAP kinase or anti-phospho-p38 MAP kinase (Thr-180/Thy-182) antibody (Cell Signaling Technology, Beverly, MA) and then with the secondary antibody conjugated with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive protein was finally visualized using a SuperSignal West Pico chemiluminescent substrate (Pierce).

RNAi Experiments—The expression of p65 subunit of NF- κ B or RelA and I κ B α was suppressed by RNAi in primary cultured human chondrocytes. All siRNAs were purchased from Qiagen, and two siRNAs with distinctive sequences were used for each gene. The sense strand sequences of the RNA duplexes were as follows: RelA#1, 5'-GGACAUAUGAGACCUUCAAdTdT-3'; RelA#2, 5'-GAUUGAGGAGAAACGUAAAAdTdT-3'; I κ B α #1, 5'-GGGUGUACUUUAUCCACATdT-3'; I κ B α #2, 5'-GGGCCAGCUGACACUAGAAAdTdT-3'; and control siRNA, 5'-UUCUCCGAACGUGUCACGUDTdT-3'.

The siRNAs were delivered into human chondrocytes by electroporation following cell isolation from cartilage matrix. Electroporation was performed using a Nucleofector[®] device and a Human Chondrocyte Nucleofector kit (Amaxa, Cologne, Germany) following the manufacturer's instructions. In brief, 1×10^6 cells were suspended in 100 μ l of the electroporation buffer provided by the kit, along with 1.5 μ l of 68 μ M stock solution of siRNA. Thus, the final concentration of siRNA in the buffer was \sim 100 nM. The buffer was then transferred to a supplied cuvette, and electroporation was performed using the protocol recommended by the manufacturer. After electroporation, the cells were immediately transferred onto each well of 12-well plates and cultured in DMEM/F-12 containing 20% fetal bovine serum. Next day, the media were replaced to the aforementioned regular culture media for human chondrocytes. The viability of cells was \sim 60% with the procedure.

Incorporation of [³⁵S]Sulfate into Cartilage Explants—For this experiment, 20–40 cartilage explants were prepared from normal bovine articular cartilage and equally divided into 4 groups. The first group was cultured in the aforementioned culture medium for 6 days, while changing the medium every 2 days. The second group was first cultured in the same medium for 4 days, and then for 2 days in a medium containing recombinant mouse noggin (1 μ g/ml). The third and fourth groups were initially cultured in a medium containing 2.5 ng/ml recombinant human TNF- α for 4 days. The third group was then cultured in the regular culture medium for 2 days, whereas the fourth group was cultured in a medium containing 1 μ g/ml recombinant noggin. For all four groups, newly synthesized sulfated proteoglycan was radiolabeled for the last 8 h of culture with 10 μ Ci/ml [³⁵S]sulfate (GE Healthcare). The explants were then recovered, extensively rinsed with ice-cold phosphate-buffered saline, and subjected to papain digestion. The digestion was performed using 50 μ g/ml papain (Sigma) in 500 μ l of digestion buffer (0.2 M sodium acetate, pH 6.0) at 60 °C overnight. The digest was then centrifuged, and 20 μ l of supernatant was used to measure the radioactivity. DNA content was also determined using a PicoGreen[®] double-stranded DNA quanti-

tation kit (Invitrogen), and the radioactivity was normalized by the amount of DNA.

Immunohistochemistry—Immunohistochemistry of cartilage explants was performed following a previously described method (12) with some modifications. In brief, 6- μ m-thick cryosections were prepared from the explants, fixed in acetone, and digested with 1.0% hyaluronidase (Sigma) for antigen retrieval. To detect the presence of BMP-2, anti-human BMP-2 goat polyclonal antisera (Santa Cruz Biotechnology) were used at the concentration of 1:100, which was visualized with the avidine-linked peroxidase system (goat ABC staining system, Santa Cruz Biotechnology) coupled with 3-amino-9-ethylcarbazole substrate (DakoCytomation, Carpinteria, CA). The sections were observed under a light microscope without nuclei staining, to facilitate direct comparison of the staining intensities among the sections.

Statistical Analyses—For statistical analyses, data were compared using one-way factorial analysis of variance, and when necessary, Fisher's PLSD was used as a post-hoc test. Statistical significance was set at $p < 0.05$.

RESULTS

TNF- α and Cycloheximide Induced BMP-2 Expression in Differentiated, Not Undifferentiated, ATDC5 Cells—As a preliminary experiment, the time course of chondrogenic differentiation of ATDC5 cells was determined by evaluating the expression of type II procollagen and aggrecan as well as type X procollagen from Day 0 until Day 25, every 5 days after addition of insulin to the media. Under our experimental conditions, the expression of type II procollagen and aggrecan began to increase at Day 10 and rose continuously up to Day 25 (data not shown). Meanwhile, the expression level of type X procollagen was very low from Day 0 to Day 15 and then was up-regulated at Day 20 and beyond (data not shown), indicating the occurrence of a hypertrophic change in the cells. Based on these observations, the induction of BMP-2 by TNF- α in ATDC5 cells was investigated between Days 0 and 15 in the following experiments.

Without TNF- α stimulation, the expression level of BMP-2 was consistently low in ATDC5 cells from Day 0 to Day 15 (Fig. 1, A–D). The response to TNF- α varied according to the stage of chondrogenic differentiation. At Days 0 and 5 when the cells were still undifferentiated, TNF- α did not induce the expression of BMP-2. With the onset of chondrogenic differentiation, a weak induction was observed at Day 10 (Fig. 1C), and it became obvious at Day 15 as the differentiation progressed (Fig. 1D). In Day-15 cells, the induction was dose-dependent, and its maximum was observed with 100 ng/ml TNF- α . The cells cultured for 15 days without insulin showed a weaker response to TNF- α , presumably due to the lack of chondrogenic differentiation (Fig. 1E).

Next, the effect of a protein synthesis inhibitor, cycloheximide (CHX), was studied at different stages of differentiation (Fig. 1F). In Day-0 cells, the expression of BMP-2 was not affected by CHX. In Day-10 cells, CHX induced a moderate expression of BMP-2, and in Day-15 cells the induction was obvious. In various genes, CHX is known to induce mRNA expression by disrupting the linkage between mRNA transla-

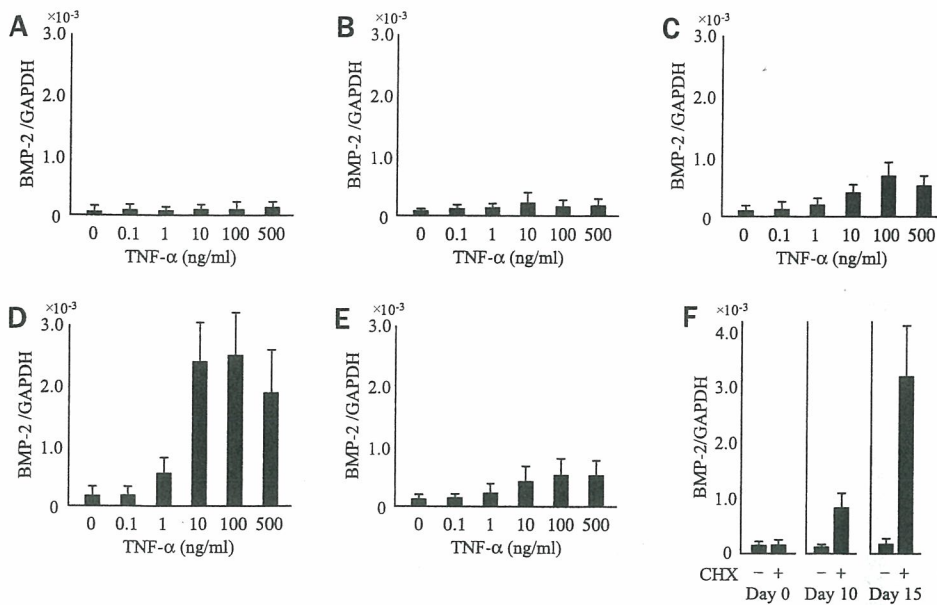


FIGURE 1. Induction of BMP-2 in ATDC5 cells by TNF- α and cycloheximide. A–E, effect of TNF- α on BMP-2 expression was evaluated on ATDC5 cells at various stages of chondrogenic differentiation. Before (A) and after culture for 5 (B), 10 (C), or 15 days (D) in insulin-containing media, ATDC5 cells were treated with graded doses of TNF- α for 48 h, and expression of BMP-2 mRNA was evaluated. In parallel, cells maintained for 15 days in insulin-free media were treated with TNF- α , and expression of BMP-2 was evaluated (E). F, ATDC5 cells cultured in insulin-containing media for 10, 15 days were treated with 2.5 μ g/ml CHX for 24 h, and BMP-2 mRNA expression was evaluated. For these experiments, expression of BMP-2 mRNA was evaluated by real-time PCR together with GAPDH expression; results are shown in relative ratios against GAPDH. Data are mean \pm S.D. of three to five experiments.

tion and mRNA degradation (35). Therefore, our result suggested the possibility that BMP-2 mRNA might have been synthesized at a certain level in differentiated ATDC5 cells, even though the observed expression level was very low.

BMP-2 Expression Is Regulated at Both Transcriptional and Post-transcriptional Levels in ATDC5 Cells—To determine the transcriptional activity of BMP-2 gene, a nuclear run-off assay was performed on differentiated and undifferentiated ATDC5 cells with or without TNF- α stimulation (Fig. 2, A and B). The result revealed that the expression of BMP-2 was suppressed at the transcriptional level in undifferentiated cells but that the gene transcripts were being synthesized at a substantial level in the nuclei of differentiated cells. In the differentiated ATDC5 cells, TNF- α treatment enhanced mRNA synthesis, whereas the cytokine had virtually no effect on it in undifferentiated cells.

Thus, results of the CHX treatment and nuclear run-off assay both indicated that, in ATDC5 cells, BMP-2 mRNA is synthesized at a certain level in the nuclei of cells once they acquired the chondrogenic phenotype. However, the observed level of BMP-2 expression was very low in the differentiated ATDC5 cells as long as they were not treated with TNF- α (Fig. 1, A–D). The apparent contradiction between the results strongly suggested the involvement of a post-transcriptional mechanism in the regulation of BMP-2 expression.

To examine this hypothesis, we evaluated the stability of BMP-2 mRNA in differentiated ATDC5 cells in the presence or absence of TNF- α . Because the basal expression level of BMP-2 was too low to evaluate the degradation rate, the cells were initially treated with TNF- α for 48 h to induce certain levels of BMP-2 expression, after which the degradation rate of mRNA

was evaluated using an RNA synthesis inhibitor, actinomycin D (ActD). The result of this experiment revealed that the degradation rate of BMP-2 mRNA was indeed reduced by TNF- α (Fig. 2C). In the presence of TNF- α , the half-life of BMP-2 mRNA was extended from 137 to 257 min, an increase of \sim 88%. Thus, the involvement of a post-transcriptional mechanism in the induction of BMP-2 by TNF- α was indicated.

TNF- α Induces BMP-2 in Human Articular Chondrocytes by Increasing mRNA Stability—In the next experiment, the involvement of post-transcriptional mechanism(s) in the induction of BMP-2 was examined in primary cultured adult human articular chondrocytes. Before examining that hypothesis, the induction of BMP-2 in human chondrocytes was evaluated under our experimental conditions. The endogenous expression level of BMP-2 in human chondrocytes was considerably higher than that in dif-

ferentiated ATDC5 cells, and, in accordance with our previous observation (12), TNF- α up-regulated BMP-2 expression \sim 10-fold (Fig. 3A). The induction was dose-dependent, and similar to that in ATDC5 cells, the maximum induction was observed at 100 ng/ml.

The experiment with CHX was repeated with human chondrocytes, and a strong induction of BMP-2 was again observed, suggesting the presence of a similar post-transcriptional regulation in human cells (Fig. 3B). The effect of CHX was observed at and above a concentration of 2.5 μ g/ml, and 10 μ g/ml CHX seemed sufficient to obtain the maximum induction.

Next, the stability of BMP-2 mRNA in human chondrocytes was evaluated in the presence or absence of TNF- α (Fig. 3C). Some cells were treated with TNF- α for 48 h, and the mRNA stability was then evaluated in the presence of TNF- α . The results showed that TNF- α also stabilizes BMP-2 mRNA in human articular chondrocytes. The mRNA half-life was extended \sim 2.6 times from 72 to 185 min by TNF- α . Interestingly, an extended TNF- α treatment for 48 h resulted in further mRNA stabilization, and the half-life was prolonged to 278 min, which was 390% of that in the untreated cells.

Human and Mouse BMP-2 mRNA Contain AU-rich Elements in 3'-UTRs—To determine the 3'-UTR sequence, 3'-rapid amplification of cDNA ends was performed on human and mouse BMP-2 mRNA. The results revealed that the 3'-UTR of human BMP-2 had a length of 3736 nt, whereas that of mouse 3'-UTR was 1170 nt (Fig. 4). Mouse 3'-UTR shows an 82% sequence identity with human 3'-UTR. Although both 3'-UTRs are longer than the average lengths of the respective species (29), they are both encoded by the third or the last exon of the gene together with the last part of the translated region.

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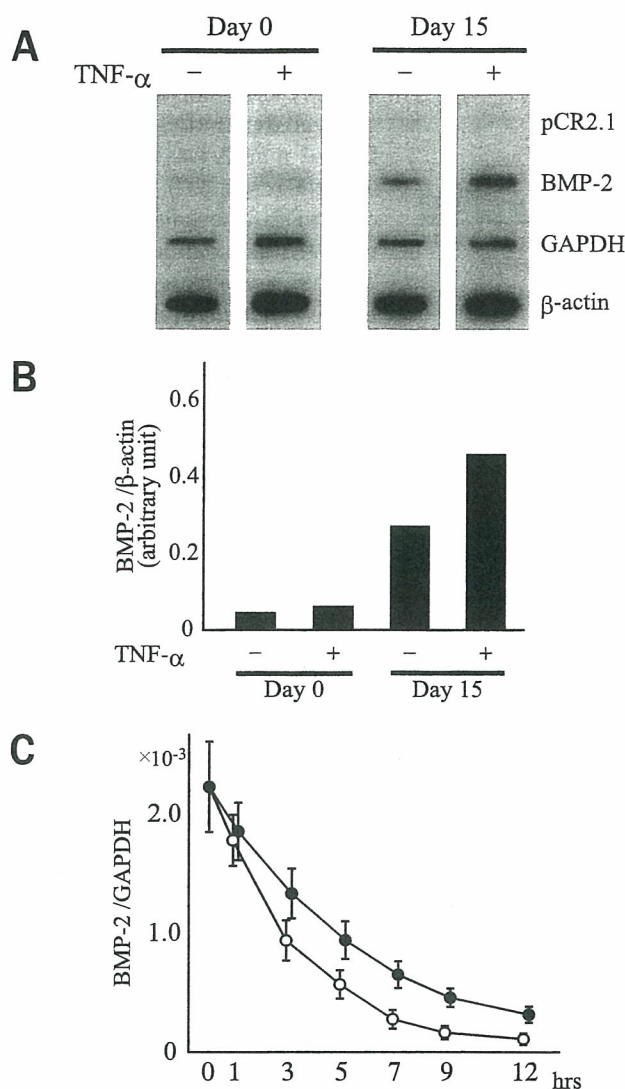


FIGURE 2. Synthesis of BMP-2 mRNA in nuclei, and stability of mRNA in cytoplasm in the presence or absence of TNF- α . *A*, nuclear run-offs from differentiated and undifferentiated ATDC5 cells. Immediately after reaching confluency (Day 0) or after culture in insulin-containing media for 15 days (Day 15), ATDC5 cells were treated with or without 20 ng/ml TNF- α for 48 h, and nuclei were obtained. RNA synthesis was allowed to proceed in nuclei in the presence of [α - 32 P]UTP, and amounts of synthesized BMP-2 gene transcripts were evaluated by slot blot analysis together with those for GAPDH, β -actin, and pCR2.1 vector without insert (for background). The experiment was repeated twice with consistent results. *B*, signal ratios between BMP-2 3'-UTR and β -actin were quantified after subtracting background. *C*, ATDC5 cells were maintained in insulin-containing media for 15 days, and stimulated with 100 ng/ml TNF- α for 48 h to induce BMP-2 expression. Degradation of BMP-2 mRNA was then evaluated by addition of ActD (1 μ M), with (open circles) or without (filled circles) withdrawal of TNF- α from media. At each time point, remaining amounts of BMP-2 mRNA were evaluated by real-time PCR together with GAPDH mRNA; results are shown in relative ratios against GAPDH. Data are mean \pm S.D. of three experiments.

For various genes, the stability of mRNA is regulated by cis-acting elements in 3'-UTR (28, 29). Among them, the adenosine/uridine-rich element (ARE) is a well characterized sequence that has the function of modulating mRNA degradation in response to various extracellular stimuli. The element often contains repeats of pentameric AUUUA motifs. The result of sequencing revealed that both human and mouse BMP-2 3'-UTRs contain multiple copies of the pentameric motif. Human 3'-UTR contains 22 motifs throughout the

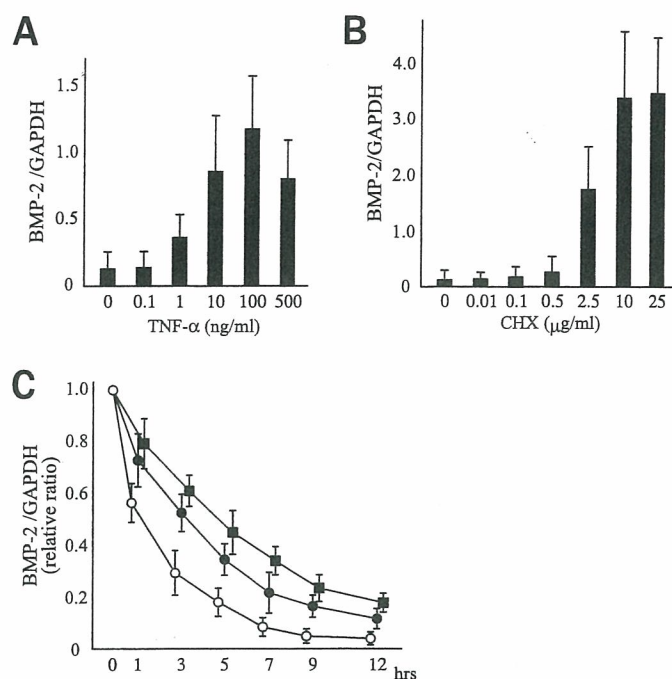


FIGURE 3. Involvement of post-transcriptional regulation in expression of BMP-2 in human chondrocytes. *A*, human articular chondrocytes were maintained by monolayer culture and treated with graded concentrations of TNF- α . Induction of BMP-2 was evaluated 48 h later. *B*, chondrocytes were treated with graded doses of CHX for 24 h, and BMP-2 expression was evaluated. *C*, decay of BMP-2 mRNA was evaluated in presence (filled circles) or absence (open circles) of TNF- α , using ActD (1 μ M). Decay of mRNA was also evaluated in cells treated with TNF- α for 48 h prior to addition of ActD and in the continuous presence of the cytokine (filled squares). For these experiments, expression of BMP-2 was evaluated by real-time PCR together with GAPDH expression. In *A* and *B*, results are shown as ratios against GAPDH. In *C*, RNA ratios at respective time points were normalized by the ratio at beginning of the evaluation (i.e. time 0) in each experiment to facilitate direct comparison. Data are mean \pm S.D. of three to five experiments.

region, and mouse has 8 in the proximal third of the region (Fig. 4). The alignment of the 8 motifs in the mouse gene is well conserved in the human gene, suggesting their functional significance.

Induction of BMP-2 by TNF- α Is Mediated by the AU-rich Element in 3'-UTR—The functional significance of the ARE in BMP-2 3'-UTR was investigated by generating luciferase reporter constructs harboring either the entire or various parts of mouse BMP-2 3'-UTR at the 3'-end of the luciferase coding region (Fig. 5A).

To analyze the function of the region, that 3'-UTR was divided into four parts according to the distribution of pentameric AUUUA motifs. The first part, designated part A, involves the first 189 nt with two pentamers. The next 137 nt containing four pentamers was designated part B. Part C contained the following 114 nt with two pentamers, and the remaining 730 nt without motifs was designated part D. These parts were inserted, alone or in combination, after the stop codon of the luciferase coding region.

The constructs were transiently transfected to the differentiated ATDC5 cells, and the luciferase activity was measured (Fig. 5B). The results revealed that the addition of the entire 3'-UTR reduced luciferase activity by \sim 75%. When parts A, B, or D were inserted alone, a significant reduction was observed only with part B, whereas the reduction with parts A or D was

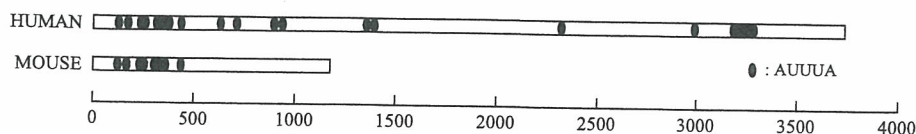


FIGURE 4. Distribution of AUUUA motifs in human and mouse BMP-2 3'-UTRs. Relative lengths and distributions of AUUUA motifs (filled ellipses) in human and mouse BMP-2 3'-UTRs are shown.

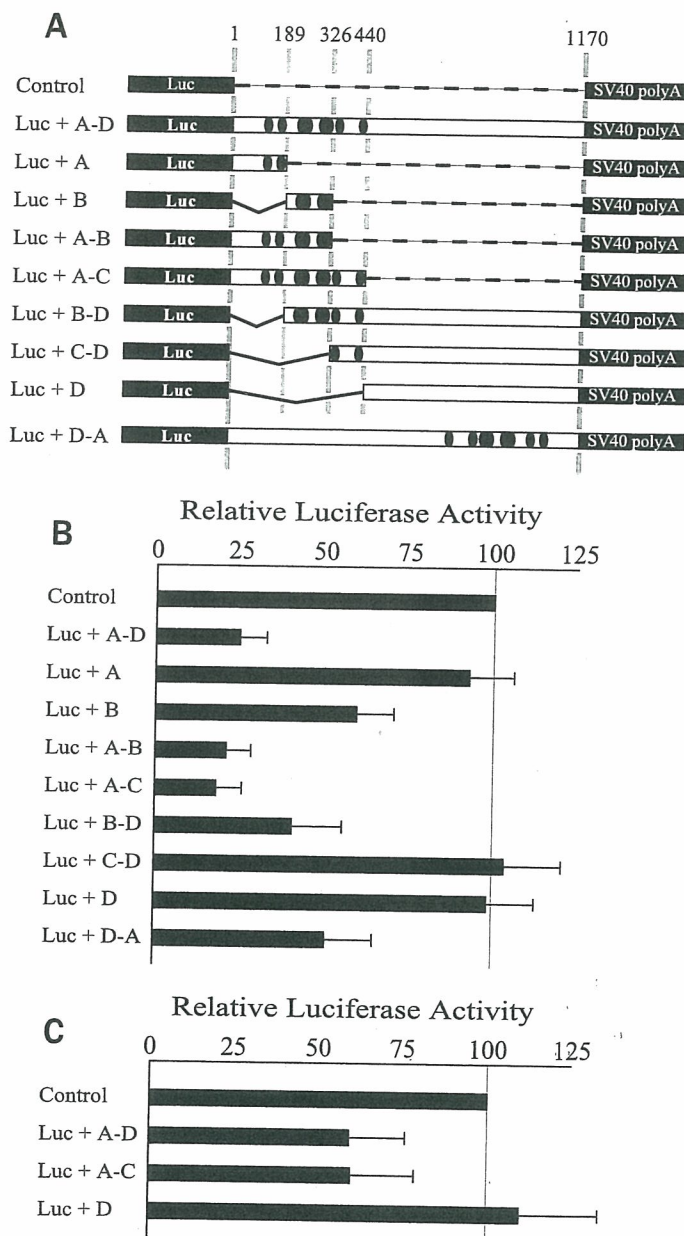


FIGURE 5. Chimeric constructs used for transient transfection experiments and their luciferase activity. A, chimeric constructs used for transient transfection experiments. Constructs have all or a part of mouse BMP-2 3'-UTR (open bars) at the 3'-end of luciferase coding sequence (Luc), followed by SV40 polyadenylation signal (SV40 polyA). The chimeric region was under the control of SV40 promoter and enhancer. Solid ellipses indicate locations of pentameric AUUUA motifs, and numbers at the top denote positions of nucleotide in 3'-UTR. B, luciferase activity of constructs. ATDC5 cells maintained for 15 days in insulin-containing media were transiently transfected with one of the above constructs, and luciferase activity was measured 48 h later. C, effect of TNF- α on luciferase activity. For some constructs, luciferase activity was measured after treating cells with 10 ng/ml TNF- α for 48 h following transfection. Luciferase assays were repeated three to six times, and activity of *Firefly* luciferase was normalized by that of *Renilla* luciferase. Data are expressed as a percentage of control (mean \pm S.D.).

not apparent. The reduction of luciferase activity with part B was augmented by the addition of part A or parts C and D, and the strongest reduction was observed when parts A through C were inserted together.

Interestingly, the addition of the reversed 3'-UTR sequence also suppressed luciferase activity, although the effect was less obvious than that of the regular orientation.

For some constructs, luciferase activity was also evaluated in the presence of TNF- α (Fig. 5C). For the two constructs that strongly reduced luciferase activity in the previous experiment, the suppressed activity was partly recovered by TNF- α , whereas the cytokine did not affect luciferase activity with the insert containing no AUUUA motifs. Thus, our results suggest the possibility that the expression of BMP-2 could have been suppressed in the chondrocytes by the ARE in 3'-UTR and that the induction of BMP-2 by TNF- α could have been the result of a release from the ARE-mediated suppression.

p38 Signaling Pathway Is Involved in Stabilization of BMP-2 mRNA by TNF- α in Human Chondrocytes—Next, experiments were performed to determine the signal transduction pathway(s) involved in the stabilization of BMP-2 mRNA by TNF- α . It is known that TNF- α stabilizes mRNA of various genes through the signal pathway involving p38 MAP kinase (36). For some other genes, the stability of mRNA is regulated by signal pathways involving extracellular signal-regulated kinase-1/2 (ERK-1/2) (37, 38), c-Jun N-terminal kinase (JNK) (39, 40), protein kinase C (38, 41), or phosphatidylinositol 3-kinase (42). mRNA turnover can be regulated by the intracellular calcium concentration (43). Thus, seven specific inhibitors for these signals were examined to determine whether they inhibited the induction of BMP-2 by TNF- α (Fig. 6A). Among them, SB203580, an inhibitor for p38 pathway, was found to strongly suppress the BMP-2 induction; it also suppressed the expression of BMP-2 in untreated chondrocytes by \sim 50%, suggesting that p38 signaling could be important in maintaining the endogenous expression of the protein. On the other hand, wortmannin and ionomycin, which inhibit phosphatidylinositol 3-kinase activity and cause calcium ion influx, respectively, induced the expression of BMP-2 in chondrocytes, although the induction by TNF- α was not enhanced but rather suppressed by these inhibitors. The inhibitors for ERK-1/2, JNK, or protein kinase C showed no significant effect on either the endogenous expression or induction levels, indicating that these pathways might not be involved in the regulation of BMP-2 expression in chondrocytes.

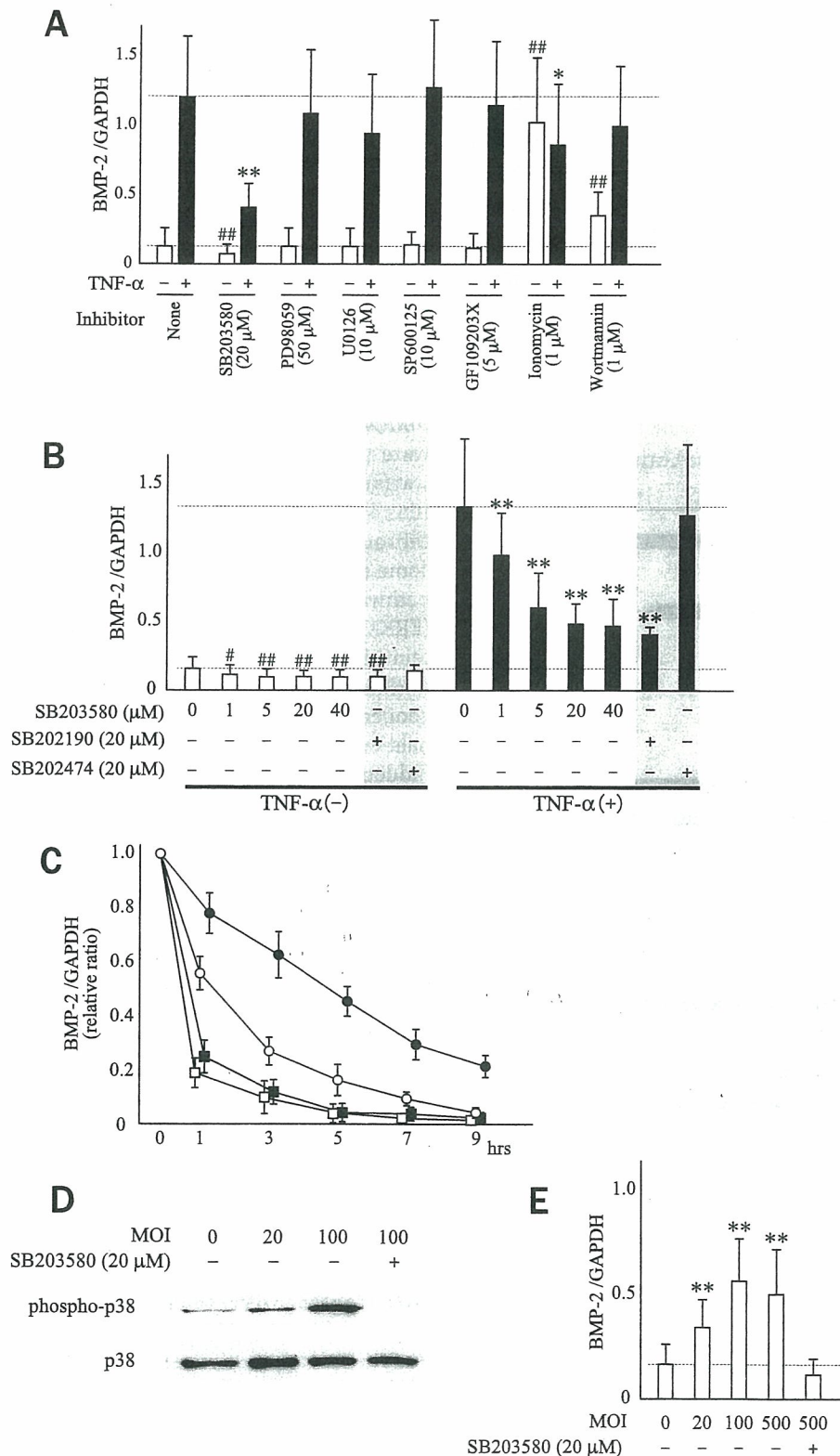
Because SB203580 showed significant suppression of BMP-2 expression, the dose response to the inhibitor was evaluated in the untreated and TNF- α -stimulated chondrocytes together with the effect of SB202190, another specific inhibitor for the p38 pathway (Fig. 6B). The result showed that the endogenous expression and induction of BMP-2 was inhibited by SB203580 in a similar dose-dependent manner. The endogenous expression and induction were both reduced by \sim 25% with 1 μ M SB203580, and the inhibitory effects were almost maximal at the concentration of 5 μ M.

Induction Mechanisms of BMP-2 by TNF- α in Chondrocytes

The expression of BMP-2 was inhibited by SB202190 to an extent similar to SB203580 in both untreated and TNF- α stimulated chondrocytes, confirming the significance of p38 signaling in the maintenance of endogenous expression and induction of the protein by TNF- α .

We then evaluated the effect of SB203580 on the stability of BMP-2 mRNA in the TNF- α -treated chondrocytes and

untreated cells (Fig. 6C). Even without TNF- α stimulation, the inhibition of the p38 pathway resulted in the facilitation of mRNA degradation in chondrocytes; the half-life of BMP-2 mRNA declined from 70 to 42 min, a reduction of ~40%. The decrease in mRNA stability was considered to account for the suppression of endogenous BMP-2 expression by the inhibitor. Furthermore, although the mRNA stability was significantly



increased in the TNF- α -treated cells, the inhibitor reduced the stability below the level of that in untreated cells, completely abrogating the cytokine's effect. The mRNA half-life was 282 min in the TNF- α -treated cells, which was reduced to 53 min by the addition of SB203580.

The significance of p38 MAP kinase in the regulation of BMP-2 expression was confirmed by use of the adenovirus carrying constitutively active MKK6, a kinase that directly phosphorylates p38 MAP kinase. Infection with the adenovirus induced the expression of BMP-2 mRNA along with the phosphorylation of p38 (Fig. 6, *D* and *E*), but that induction was completely inhibited by SB203580, together with p38 phosphorylation. Thus, the results of these experiments showed that BMP-2 expression in primary cultured adult human chondrocytes is regulated by a post-transcriptional mechanism predominantly modulated by the p38 signal pathway.

Coordination of NF- κ B and p38 Signal Pathway in BMP-2 Induction by TNF- α —Since it has been reported that the expression of BMP-2 is transcriptionally regulated by NF- κ B in epiphyseal chondrocytes (24), experiments were performed to determine the role of NF- κ B in the induction of BMP-2 by TNF- α . In the first experiment, nuclear translocation of NF- κ B was inhibited by pyrrolidine dithiocarbamate (PDTC), and the levels of endogenous expression and induction by TNF- α were evaluated (Fig. 7A). Unlike SB203580, PDTC did not change the endogenous level of BMP-2 expression in human chondrocytes, suggesting that the transcriptional factor may not be responsible for the maintenance of BMP-2 expression in adult human articular chondrocytes. This finding differed from a previously reported result with epiphyseal chondrocytes showing that the inhibition of NF- κ B strongly reduced the expression of BMP-2 in the growth plates (24). On the other hand, the inhibitor suppressed the induction of BMP-2 by TNF- α by ~50%. The suppressive effect seemed to reach a plateau at a concentration of 100 μ M, because no further suppression was observed with a higher concentration of PDTC. In contrast, when SB203580 was used together with PDTC, the induction was completely abrogated, and the expression of BMP-2 declined below the level of endogenous expression. Although PDTC partly suppressed BMP-2 induction, the inhibitor did not change the stability of BMP-2 mRNA in TNF- α -treated chondrocytes (Fig. 7B), suggesting the possibility that the

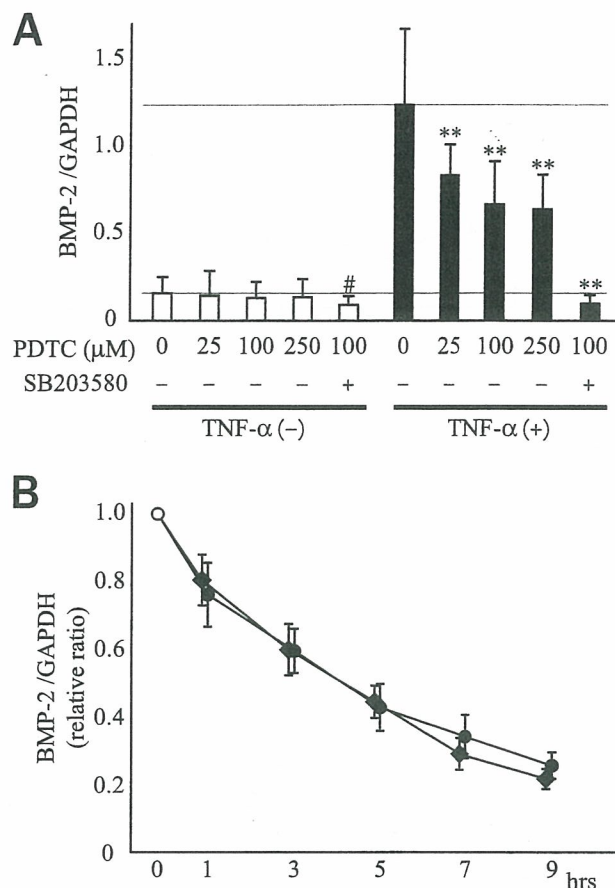


FIGURE 7. Involvement of NF- κ B in induction of BMP-2. A, effect of PDTC on endogenous expression and induction of BMP-2 by TNF- α was evaluated in primary cultured human articular chondrocytes. In presence or absence of TNF- α , PDTC was given to chondrocytes at various concentrations. For some cells, SB203580 (20 μ M) was given together with PDTC. Forty-eight hours later, expression of BMP-2 was evaluated by real-time PCR and represented by ratios against GAPDH. #, $p < 0.05$ against untreated cells, and **, $p < 0.01$ against cells treated with TNF- α alone. B, stability of BMP-2 mRNA was evaluated using ActD (1 μ M) in chondrocytes treated with TNF- α for 48 h with (filled diamonds) or without (filled circles) PDTC (100 μ M). At each time point, expression of BMP-2 was determined by real-time PCR together with GAPDH, and their RNA ratio was obtained. To facilitate direct comparison, ratios at respective time points were normalized by the ratio at beginning of the evaluation (i.e. Time 0) under each experimental condition. For these experiments, TNF- α was used at a concentration of 100 ng/ml. Data are shown by mean \pm S.D. or mean \pm S.D. of three to five experiments.

induction was suppressed not through a change in post-transcriptional regulation, but rather through the decrease in transcriptional activity.

FIGURE 6. Involvement of p38 MAP kinase in the induction of BMP-2 by TNF- α . A, human articular chondrocytes were maintained by monolayer culture, and seven specific inhibitors of signal transduction pathways were examined at indicated concentrations to determine whether they exerted significant effects on endogenous expression and induction of BMP-2 by TNF- α . Effects on endogenous expression were evaluated 48 h after addition of inhibitors. To assess effects on induction, inhibitors were given 30 min prior to addition of TNF- α to media, and induction levels were determined 48 h later. Expression of BMP-2 was represented by ratios against GAPDH. #, $p < 0.05$; **, $p < 0.01$ against control cells; *, $p < 0.05$; ***, $p < 0.01$ against control cells treated with TNF- α alone. B, chondrocytes were cultured with graded doses of SB203580 in the presence (solid bars) or absence (open bars) of TNF- α stimulation, and the expression of BMP-2 was evaluated 48 h later. In parallel, the effect of SB202190 on the expression of BMP-2 was evaluated together with that of SB202474, a negative control for the p38 inhibitors (bars in shaded areas). Expression of BMP-2 was represented by ratios against GAPDH. #, $p < 0.05$, and ***, $p < 0.01$ against untreated cells; **, $p < 0.01$ against cells treated with TNF- α alone. C, chondrocytes were cultured for 48 h in the presence (open squares) or absence (open circles) of SB203580, and the stability of BMP-2 mRNA was evaluated using ActD (1 μ M). Evaluation was also performed on cells treated with TNF- α for 48 h with (filled squares) or without (filled circles) SB203580. To facilitate direct comparison, RNA ratios at respective time points were normalized by the ratio at beginning of the evaluation (i.e. Time 0) under each experimental condition. D and E, chondrocytes were infected with the adenovirus carrying constitutively active MKK6 at indicated titers, and 72 h later, the amount of phosphorylated p38 and expression of BMP-2 mRNA were evaluated together with those of total p38 protein and GAPDH, respectively. SB203580 was given to some cells together with the adenovirus. In E, BMP-2 expression was represented by ratios against GAPDH. **, $p < 0.01$ against untreated cells. For these experiments, expression of BMP-2 and GAPDH was quantitatively evaluated by real-time PCR. TNF- α was used at the concentration of 100 ng/ml, and media containing inhibitors were replaced every 24 h to preclude possible degradation of the inhibitors. Data are shown by mean \pm S.D. or mean \pm S.D. of three or four experiments.

Induction Mechanisms of BMP-2 by TNF- α in Chondrocytes

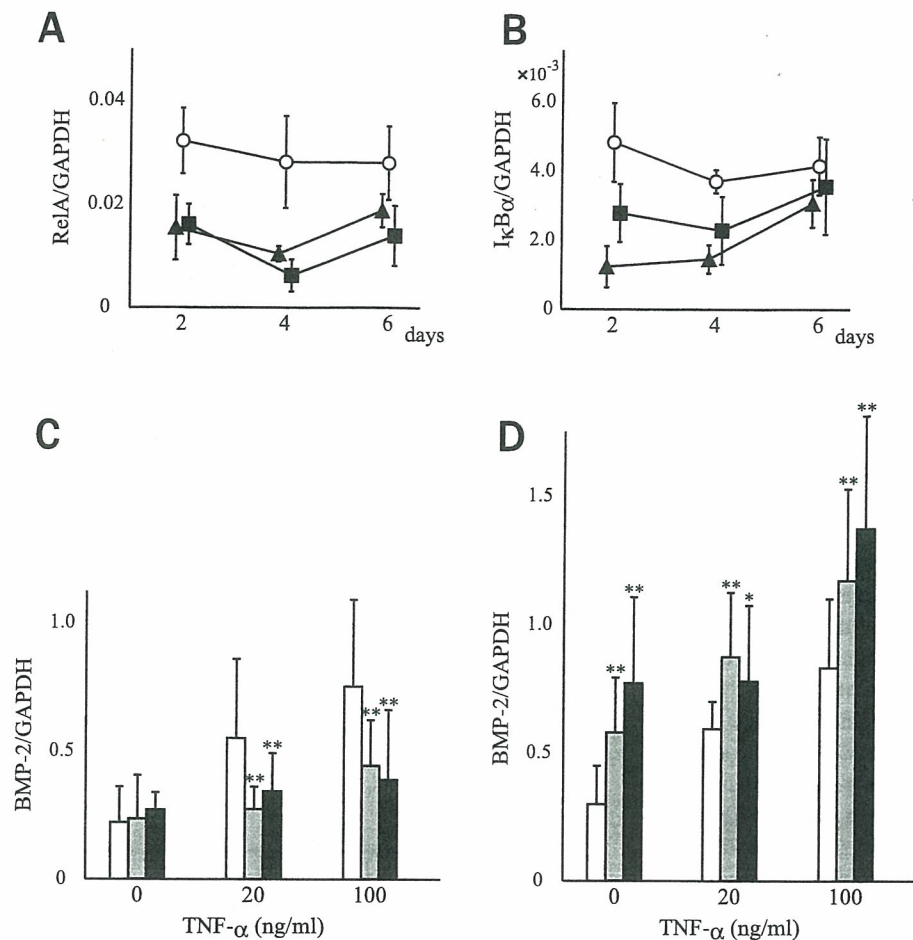


FIGURE 8. Influence of reduced RelA and I κ B α expression on the induction of BMP-2 by TNF- α . *A* and *B*, siRNAs for RelA (*A*) and I κ B α (*B*) were delivered into primary cultured human chondrocytes by electroporation, and the expression levels of respective genes were monitored every 2 days until Day 6 by real-time PCR. For each gene, two siRNAs with respective target sequences were used (filled squares and triangles), and the results are shown by ratios against GAPDH together with that of the control cells given a control siRNA (open circles). *C* and *D*, the cells to which the siRNAs for RelA (*C*) and I κ B α (*D*) were delivered were treated with 20 or 100 ng/ml of TNF- α for 48 h, and the induction of BMP-2 was evaluated by real-time PCR. Considering the time course of gene suppression, the cells were treated with TNF- α from Day 2 to Day 4. Open bars represent the result of control cells, and light and dark shaded bars show the results of respective siRNAs used for the gene. The results are shown by ratios against GAPDH. *, $p < 0.05$ and **, $p < 0.01$ against control cells in respective treatment groups. Data are mean \pm S.D. or mean \pm S.D. of three to five experiments.

Induction of BMP-2 by TNF- α Was Significantly Modulated by the Suppression of RelA and I κ B α Expression—The involvement of NF- κ B in the induction of BMP-2 was further confirmed by RNAi experiments. In primary cultured human articular chondrocytes, the expression of RelA and I κ B α was effectively reduced by RNAi. The result of real-time PCR demonstrated that the expression of RelA gene was suppressed from Day 2 to Day 6 after the delivery of siRNAs into the cells (Fig. 8*A*). The strongest inhibition was observed at Day 4 for both siRNAs used for the gene, when the expression levels were reduced to 22 and 37%, respectively, of that of the control. For I κ B α , the suppression was strongest at Day 2 and Day 4 for respective siRNAs, when the expression was 25 and 44% of the control (Fig. 8*B*). Next, the induction of BMP-2 by TNF- α was evaluated in the cells given the siRNAs. The introduction of siRNA by electroporation considerably reduced the induction of BMP-2 by TNF- α (Fig. 8, *C* and *D*). Nonetheless, it was noticed that the gene silencing of RelA significantly inhibited the induction of BMP-2 (Fig. 8*C*). Meanwhile, the reduction of

I κ B α expression caused a \sim 2-fold increase of endogenous BMP-2 expression (Fig. 8*D*). The response to TNF- α was preserved in those cells, although the magnitude of BMP-2 induction was not augmented by the suppression of I κ B α .

Noggin Showed Stronger Inhibitory Effects on Chondrocyte Anabolism after TNF- α Treatment—To evaluate the biological significance of BMP-2 induced by TNF- α , cartilage explants were cultured in the presence or absence of the cytokine, and the inhibitory effect of noggin on the synthetic activity of chondrocytes in those explants was investigated. In this experiment, the concentration of TNF- α was set at a relatively low level based on our previous experience (12). First, the induction of BMP-2 by TNF- α was confirmed by immunostaining. Compared with the untreated controls (Fig. 9*A*), staining for BMP-2 was much stronger in the TNF- α -treated explants (Fig. 9*B*). In those explants, the staining was observed both inside and around the chondrocytes, which was consistent with our previous observation with human cartilage explants (12). Next, the synthesis of sulfated proteoglycan in the explants was evaluated by the incorporation of [35 S] sulfate. In the control explants without TNF- α treatment, the incorporation of [35 S] sulfate into the explants was not significantly influenced by the

addition of noggin to the culture media (Fig. 9*C*). On the other hand, noggin showed a strong inhibitory effect on the explants treated with TNF- α . Although the low dose of cytokine did not cause significant suppression of the synthetic activity in the explants, noggin reduced the incorporation of [35 S] sulfate by $>50\%$. Thus, in the TNF- α -treated explants, BMP-2 induced by TNF- α was considered to play a significant anabolic role on the synthetic activity of chondrocytes, likely counteracting the suppressive effects of TNF- α .

DISCUSSION

In the current work, the presence of a complex regulation for BMP-2 expression was initially suggested in ATDC5 cells by the experiment using CHX. The expression of BMP-2 was induced by CHX in differentiated but not undifferentiated ATDC5 cells, whereas the basal level of BMP-2 expression was consistently low throughout the differentiation process. Because CHX is known to induce gene expression through the inhibition of RNA degradation (35), that observation suggested the possibil-

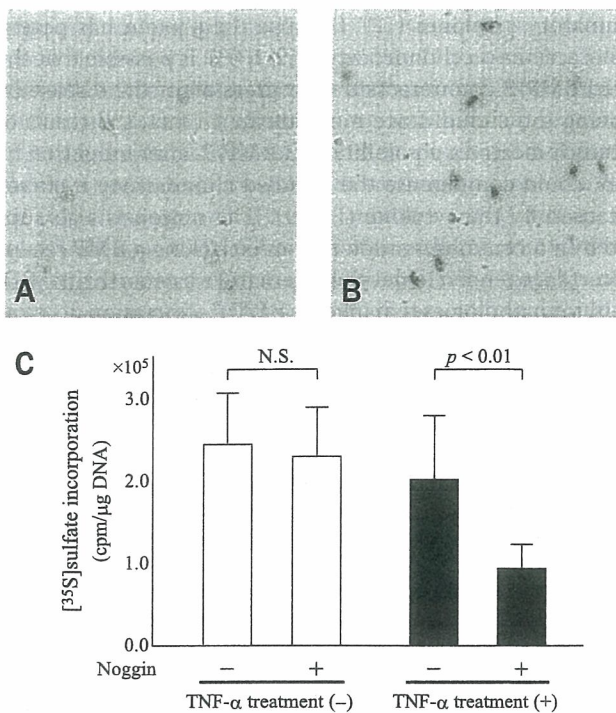


FIGURE 9. Expression of BMP-2 and effect of noggin on sulfate incorporation in cartilage explants with and without TNF- α treatment. A and B, cartilage explants were cultured in the presence or absence of TNF- α (2.5 ng/ml) for 4 days, and the expression of BMP-2 was detected by immunostaining. Representative photomicrographs of the control explants without TNF- α treatment (A) and those treated with TNF- α (B) are shown. Magnification, $\times 200$. C, the explants with and without TNF- α treatment were further cultured for 2 days in the presence or absence of recombinant mouse noggin (1 μ g/ml), and the synthetic activity of chondrocytes was evaluated by the incorporation of [³⁵S]sulfate into the explants. The experiments were repeated four times using cartilage from two animals. *Open bars* represent the results of the explants without TNF- α treatment, and *solid bars* represent for those with TNF- α treatment. The results are shown as cpm normalized by DNA contents. Data are mean \pm S.D.

ity that BMP-2 mRNA was synthesized at a certain level in differentiated cells, even without TNF- α stimulation. Accordingly, the results of nuclear run-off experiments revealed that differentiated ATDC5 cells were in fact synthesizing BMP-2 mRNA at the nuclei, whereas such synthesis was barely detectable in undifferentiated cells. The discrepancy between the active mRNA synthesis in the nuclei and the low level of expression observed in differentiated cells can be explained by the involvement of post-transcriptional control in the regulation of BMP-2 expression. On the other hand, the results of nuclear run-off experiments also revealed that the transcriptional activity of BMP-2 mRNA was increased by TNF- α . Taking all these results into account, the regulation of BMP-2 in ATDC5 cells may be understood as follows. In undifferentiated cells, expression is suppressed at the transcriptional level. The cells acquire the ability to synthesize BMP-2 mRNA with the progression of chondrogenic differentiation, but the overall expression is still suppressed by a post-transcriptional regulatory mechanism that facilitates mRNA degradation. TNF- α induces the expression of BMP-2 in differentiated cells through the transcriptional up-regulation and stabilization of mRNA. The regulatory mechanisms were then investigated in primary cultured human chondrocytes, and the results of those experiments indicated

that human chondrocytes most likely have a similar regulatory system for the control of BMP-2 expression.

In eukaryotic cells, the level of gene expression is strictly regulated at both transcriptional and post-transcriptional levels. Modulation of the mRNA decay rate is a strategy widely used by cells to adjust the intensity of expression (44). The decay of mRNA is often mediated by the specific *cis*-acting sequences in 3'-UTR, represented by an AU-rich element or ARE (29, 45). The ARE often contains multiple copies of pentameric AUUUA motifs. Such motifs have been found in many unstable and inducible genes such as cytokines and oncogenes, where the elements control the degeneration of mRNA in response to a variety of intra- and extracellular signals, enabling a rapid adjustment of RNA levels (46). In the present study, the result of 3'-rapid amplification of cDNA ends revealed that both human and mouse BMP-2 mRNA contain multiple AUUUA motifs in the 3'-UTRs. Mouse 3'-UTR contains 8 motifs within a proximal 320-nt AU-rich stretch, and their alignment is highly conserved in the human gene. In various genes whose expression is regulated at the post-transcriptional level, the nucleotide sequences of AREs are often evolutionarily conserved (47–50). The result of this study indicated that BMP-2 could be one such gene. Because BMP-2 protein is highly conserved during the evolutionary process, it is reasonable to assume that the regulatory mechanism is conserved as well. In fact, a sequence comparison has revealed that a 265-nucleotide region in the BMP-2 3'-UTR is 73% conserved over a span of 450 million years of evolution from fish to mammals (27). Interestingly, with all its functional and sequential similarity to BMP-2, the BMP-4 gene lacks an equivalent conserved region in the 3'-UTR. The possible absence of post-transcriptional regulation may account for the difference in the expression patterns between the two BMPs during embryogenesis (5, 17, 18).

Although AREs regulate the decay of mRNA in many genes, the presence of ARE in 3'-UTR does not necessarily indicate that the element is actually functional (45). Furthermore, the significance of AREs may vary depending on the cell types (51) or states of cellular differentiation (21, 27, 45, 52), possibly due to the change in trans-acting regulators (45). Thus, the function of ARE needs to be evaluated within the biological context in which the gene is expressed. In this study, we therefore examined the function of BMP-2 3'-UTR in differentiated ATDC5 cells, in which the addition of 3'-UTR to the luciferase gene did indeed reduce the enzyme activity. That reduction was related to the number of inserted AUUUA motifs, rather than to the specific sequence in the region. Thus, out of eight pentameric motifs, the first or last two pentamers alone did not change the luciferase activity significantly, whereas the middle four within 86 bases suppressed it by over 40%. The addition of two motifs, either proximal or distal, was enough to obtain suppression equal to the entire 3'-UTR.

AREs are often divided into three classes, with the ARE of BMP-2 falling into class II, in which the region is characterized by multiple copies of clustered AUUUA motifs (53, 54). The other members of this class are mostly cytokines and enzymes such as interleukin-3 (40), TNF- α (55), and cyclooxygenase-2 (51, 56, 57). In these genes, the effect of mRNA stabilization by

the pentameric sequence has often been related to the number of motifs in the AREs (40, 57). Our current observations are in good agreement with those previous results.

It is worth noting that a few previous studies reported contradictory results on whether or not the addition of 3'-UTR increased the stability of BMP-2 mRNA (21, 27). Considering that AREs primarily destabilize rather than stabilize mRNA (29, 36, 44, 45), it is possible that the previous observations might not reflect the actual function of the 3'-UTR *in vivo*. Because an embryonic carcinoma cell line was used in those studies, the inconsistency might stem from differences in the cell types and/or stages of cellular differentiation. Because the reduced luciferase activity by the addition of 3'-UTR was significantly recovered by TNF- α , we think the current result reasonably reflects the biological function of the region.

Based on the results of our current study, it seems very likely that the p38 signal pathway mediates the stabilization of BMP-2 mRNA by TNF- α . In genes containing AREs, the stability of mRNA is regulated by proteins that bind to the region or ARE-binding proteins. The function of such proteins is often modulated by the p38 signal pathway. For example, the ARE-binding proteins HuR, AUF1, and tristetraprolin are all known to stabilize or destabilize mRNA in response to p38 signaling (42, 58–60). Although the protein that binds to BMP-2 ARE has not been determined, it is likely that mRNA stability is regulated by one such protein. AREs regulated by the p38 pathway have a common feature in that they contain several closely adjacent AUUUA motifs (36). That feature is indeed shared with the BMP-2 gene.

Besides the post-transcriptional regulation, the modulation of transcriptional activity seemed to play another important role in the induction of BMP-2 by TNF- α . In differentiated ATDC5 cells, TNF- α increased the transcriptional rate of BMP-2, whereas in human cells, the induction of BMP-2 was partly suppressed by an NF- κ B inhibitor without changing the stability of mRNA. The involvement of NF- κ B in the induction of BMP-2 was further suggested by the result of RNAi experiment. In the present study, the reduction of RelA expression by RNAi strongly suppressed the induction of BMP-2 by TNF- α . In the meantime, because neither addition of PDTC nor suppression of RelA changed the level of endogenous BMP-2 expression, it is assumed that the transcriptional factor may not play a significant role in the maintenance of BMP-2 in the untreated chondrocytes. This speculation is consistent with the result that the suppression of I κ B α by RNAi caused significant elevation of endogenous BMP-2 expression. The evidence that the expression of BMP-2 is transcriptionally regulated by NF- κ B has been shown in a previous study in the growth plate chondrocytes (24), and our current results indicated that the transcriptional regulation could be involved in the induction of BMP-2 by TNF- α in articular chondrocytes, together with the mRNA stabilization mechanism.

The induction of BMP-2 in chondrocytes could be a critical factor in several pathologies, *e.g.* osteoarthritis. In osteoarthritis joints, the anabolic activity of the chondrocytes is highly up-regulated, which likely retards disease progression (61). In osteoarthritis cartilage, the expression of BMP-2 is significantly increased (12, 15), possibly through the induction by the pro-

inflammatory cytokines (12). Because the protein has potent anabolic actions on chondrocytes (62, 63), it is possible that the induced BMP-2 counteracts the progression of the disease by enhancing the chondrocyte metabolism. In fact, the result of this study indicated a possibility that BMP-2, after induction by TNF- α , could compensate the reduced chondrocyte metabolism caused by the cytokine (Fig. 9). The notion is also supported by a recent observation that mice lacking a BMP receptor in cartilage tended to develop premature osteoarthritis (64).

Thus, for pathologies that involve BMP-2 expression, its control could be a key to regulating the disease process. The results of this study will provide useful clues in developing new strategies to treat various diseases involving chondrocytes.

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