

ORIGINAL ARTICLE

Yukio Nakamura · Shigeyuki Wakitani · Naoto Saito
Kunio Takaoka

Expression profiles of BMP-related molecules induced by BMP-2 or -4 in muscle-derived primary culture cells

Received: October 7, 2004 / Accepted: April 19, 2005

Abstract The formation of ectopic bone in muscle following the implantation of decalcified bone matrix led to the search and eventual discovery of bone morphogenetic proteins (BMPs) in bone matrix. The precise sequence of molecular events that underpin the cellular transformation of undifferentiated mesenchymal cells into bone has not been established, and is the subject of this study. Northern and Western blot analyses were used to examine changes in gene expression of cells treated with BMP-2 or -4. The molecules, which included BMP receptors (BMPRs), Noggin (a BMP-specific antagonist), osteocalcin (OC), Smad-4, and MyoD, were examined at messenger RNA (mRNA) and protein levels. The changes in expression of these molecules were followed in mouse muscle-derived primary culture cells, and osteoblastic or nonosteoblastic embryonic cell lines. We show the early up-regulation of BMPR-1A, -2, Noggin, OC, and Smad-4 in muscle-derived primary culture cells in a dose-dependent manner in response to BMP-2 or -4. MyoD expression was not detected after BMP stimulation. The differential expression of these positive and negative regulators of BMP signaling points to a potential regulatory mechanism for bone induction in mesenchymal cells.

Key words BMP signaling · Feedback · Mesenchymal cells · Muscle · Up-regulation

Introduction

Bone morphogenetic proteins (BMPs) are multifunctional cytokines belonging to the transforming growth factor- β

(TGF- β) superfamily. Among the BMP family, BMP-2, -4, and -7 (osteogenic protein-1) have been recognized as potent bone inducers [1–3], and BMP-2 and -4 also play critical roles in early embryogenesis and skeletal development [4]. Signaling by BMPs requires binding of the BMP (BMP-2, -4, and -7) molecules to the BMP receptors (BMPRs), which consist of two different types of serine-threonine kinase receptors, known as BMP type 1 receptors (1A and 1B) and BMP type 2 receptor [5]. These receptors then phosphorylate intracellular proteins such as the Smad-1 or -5 to effect intracellular signaling and physiological responses [6–9]. Therefore, BMPR expression is a prerequisite for the biological action of the BMPs [1–5]. BMP action may also be modulated by a group of BMP-binding proteins outside the responding cells. Noggin is a representative molecule with such a function, and is assumed to contribute to the negative regulation of BMP action or bone formation under physiological conditions [6–9].

Changes in expression of those molecules involved in BMP signaling are critical to understanding the mechanism of BMP-induced osteogenic differentiation and feedback mechanisms following treatment with BMPs. We previously examined an ectopic bone-forming model in mice [10], and found that BMPR-1A, -2, and Noggin were induced by BMP-2 in muscle tissues during the early phase of the reaction. To confirm this mechanism in *in vitro* systems, we used muscle-derived primary culture cells, and osteoblastic or non-osteoblastic embryonic cell lines were used as controls.

Materials and methods

Recombinant human BMP-2 (rhBMP-2) and mouse BMP-4 (mBMP-4)

rhBMP-2 was produced by the Genetics Institute (Cambridge, MA, USA) using DNA recombination techniques, and donated to us through Yamanouchi Pharmaceutical (Tokyo, Japan), as described elsewhere [11].

Y. Nakamura (✉) · S. Wakitani · N. Saito
Department of Orthopaedic Surgery, Shinshu University School of
Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan
Tel. +81-263-37-2659; Fax +81-263-35-8844

K. Takaoka
Department of Orthopaedic Surgery, Osaka City University
Hospital, Osaka, Japan

Conditioned media of mBMP-4-transfected Chinese hamster ovary (CHO) cells (BMP-CHO) were the source of mBMP-4. Details of the BMP-CHO cells have been described previously [12,13]. In mBMP-4-conditioned media, the alkaline phosphatase activity of the 10% conditioned media corresponds to approximately 70 ng/ml rhBMP-2 [13]. The BMP-CHO cells transfected with mBMP-4 cDNA or mock vector (for a control) were propagated at a density of 1×10^6 cells/100-mm plastic dish (Falcon no. 3003; Becton Dickinson Labware, Tokyo, Japan), and were then cultured in 10 ml Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) with 10% fetal calf serum (FCS; Sigma Chemical, St. Louis, MO, USA) at 37°C for 5 days. The conditioned media were collected after 5 days and stored at 4°C.

Cell culture

Muscle-derived primary culture cells were prepared from the thigh muscles of newborn ddy mice (Nippon SLC, Shizuoka, Japan), as described previously [14], and cultured on a 100-mm plastic dish in DMEM containing 10% (vol/vol:v/v) heat-inactivated FCS and penicillin-streptomycin (PSM) antibiotic mixture (Invitrogen). A murine osteoblastic cell line, MC3T3-E1, and murine embryonic fibroblast-like cell line, NIH3T3, were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and cultured on a 100-mm plastic dish in α -minimal essential medium (Gibco-BRL) and DMEM, respectively, containing 10% (v/v) heat-inactivated FCS.

Experimental protocols

To examine the effects of rhBMP-2 and mBMP-4 on the expression of BMPRs, Noggin, OC, Smad-4, and MyoD in muscle-derived primary culture cells, and MC3T3-E1 and NIH3T3 cells, the culture media were replaced with fresh media containing rhBMP-2 or mBMP-4 at various concentrations (0, 10, 100, 500, 1000, or 1500 ng/ml for rhBMP-2, and 0%, 10%, 20%, 40%, 60%, or 80% conditioned media for mBMP-4). The cells were cultured at 37°C in a humidified 5% CO₂ incubator for a period of 6 days, with a change of media on day 3. Cells cultivated for 0, 12, 24, 48, 72, 96, 120, or 144 h with each medium containing rhBMP-2 (0, 10, 100, 500, 1000, or 1500 ng/ml) or mBMP-4 (0%, 10%, 20%, 40%, 60%, or 80%) were collected and processed for Northern blot analysis. Cells cultivated for 24, 48, 72, or 96 h with each medium containing rhBMP-2 (1000 or 1500 ng/ml) or mBMP-4 (60% or 80%) were collected and processed for Western blot analysis, as described below.

RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from primary culture cells derived from embryonic mouse thigh muscle and MC3T3-E1 and NIH3T3 cells was extracted using Isogen (Nippon Gene, Tokyo, Japan)

according to the manufacturer's instructions. After treating with RNase-free deoxyribonucleases II (Gibco-BRL), complementary DNA (cDNA) was synthesized using an RNA polymerase chain reaction (PCR) kit (Takara Shuzo, Ohtsu, Japan) according to the manufacturer's instructions. The reaction time was 30 min at 42°C. Aliquots of the cDNA pool obtained were subjected to PCR and amplified in a 20 μ l reaction mixture using *Taq* polymerase (Takara Shuzo). Amplifications were performed in a Program Temp Control System (PC800; ASTEC, Fukuoka, Japan) for 30 cycles after an initial denaturation step at 94°C for 3 min, denaturation at 94°C for 30 s, annealing for 30 s at 60°C, and extension at 72°C for 90 s, with a final extension at 72°C for 10 min. Reaction products were electrophoresed in a 1.5% agarose gel, and the amplified DNA fragments were visualized by ethidium bromide staining under UV light. PCR products were subcloned and sequenced using a DNA sequencing kit (Applied Biosystems, Warrington, UK). The primers of Noggin, Smad-4, OC, and MyoD for PCR were set as described previously [10,13,15]. The primers of BMPRs for PCR were set as follows: BMPR-1A, 5'-CTCATGTTCAAGGGCAG-3' (5' sense) and 5'-CCCCTGCTTGAGATACTC-3' (3' antisense; 346–362 and 850–833, respectively); BMPR-1B, 5'-ATGTGGG CACCAAGAAG-3' and 5'-CTGCTCCAGCCCAATGC T-3' (215–231 and 681–664, respectively); BMPR-2, 5'-GTGCCCTGGCTGCTATGG-3' and 5'-TGCCGCCTC CATCATGTT-3' (47–64 and 592–575, respectively). Nucleotide sequences of the cDNA fragments were checked and found to be identical to mouse BMPRs (BMPR-1A, NM009758; BMPR-1B, NM007560; BMPR-2, NM007561). The specificity of these cDNAs was confirmed by sequencing using an autosequence analyzer (ABI Prism 310 Genetic Analyzer; Perkin-Elmer Japan, Tokyo, Japan).

Northern blot analysis

Twenty micrograms of total RNA were separated by electrophoresis on a 1.0% agarose-formaldehyde gel and blotted onto Hybond-N⁺ membrane (Amersham Intl., Piscataway, NJ, USA) for Northern blotting. Filters were hybridized overnight with random-primed [³²P]-labeled mouse BMPRs, Noggin, OC, Smad-4, and MyoD cDNA fragment probes at 65°C for 3 h in hybridization buffer (50 mM Tris-HCl (pH 7.5), 1 mg/ml denatured salmon sperm DNA, 1% SDS, 1 M NaCl, 10 mM EDTA, 0.2% Ficol 400, 0.2% polyvinylpyrrolidone, and 0.2% bovine serum albumin) and washed three times with 0.1 \times SSC and NaDodSO₄ for 1 h at 68°C. The signals were detected by a BioImaging Analyzer BAS-1500 (Fuji Photo Film, Tokyo, Japan). For reprobing, each hybridized probe was removed by boiling the membrane in 0.5% SDS, and then sequentially hybridized with the respective target probes.

Western blotting

Muscle-derived primary culture cells, MC3T3-E1 cells, NIH3T3 cells, and mouse skeletal muscle tissue (as a posi-

tive control) were homogenized and dissolved in 0.5 ml sample buffer (0.05M Tris-HCl (pH 6.8), 2% SDS, 6% β -mercaptoethanol, and 10% glycerol) and centrifuged at 12000g for 5 min at 4°C. The supernatant was used as the sample, and the protein content of each sample was measured by UV assay at an OD of 280 nm. Anti-mouse Noggin antibody (R&D Systems, Minneapolis, MN, USA) was used at 1 μ g/ml. Polyclonal goat antibodies for BMPRs (Santa Cruz, San Diego, CA, USA) were also used at a dilution of 1 μ g/ml. Aliquots of protein solution (5 μ l) were adjusted to 1 μ g/ μ l, mixed with 1% BPB (1 μ l), and then boiled for 2 min and loaded onto each lane of SDS (10%–20%) acrylamide gradient gels (35 mA, low voltage, 90 min). After running the gels, BMPR-1A, -1B, -2, and Noggin proteins in mouse embryo muscle-derived cells, and MC3T3-E1 and NIH3T3 cells, and mouse skeletal muscle tissue were stained with Coomassie brilliant blue (Sigma Chemical). The protein bands were then transferred to polyvinylidene difluoride membrane (Immunobilon-P Transfermembrane, Millipore, Bedford, MA, USA) according to the manufacturer's instructions. After treatment with Blocking Reagent (Nippon Roche, Tokyo, Japan) for 1 h at room temperature, the membranes were washed with PBS for 5 min, and then incubated for 1 h with primary antibody (BMPRs, 1:200; Noggin, 1:100). After two 5-min washes with PBS, the membranes were incubated with peroxidase-conjugated rabbit anti-goat antibody (1:50; Histofine, Nichirei, Tokyo, Japan) for 1 h. After two further 5-min washes with PBS, the immunoblot was developed using an ImmunoStar Kit for Rabbit (Wako Pure Chemical Industries, Tokyo, Japan) to detect biotin and chemiluminescence.

Results

Expression level of messenger RNA (mRNA)

The increase in transcription of BMPR-1A, -2, Noggin, OC, and Smad-4 appeared to be dose-dependent. The expression pattern of these molecules in muscle-derived primary culture cells after 24 h stimulation by rh-BMP-2 or mBMP-4 is shown in Fig. 1. When we performed Northern blotting on all cell sources using 0, 10, 100, 500, 1000, and 1500 ng/ml doses of rhBMP-2, or 0%, 10%, 20%, 40%, 60%, and 80% doses of mBMP-4, the gene expression levels of these molecules were the similar at the following doses: rhBMP-2 (1000 ng/ml), rhBMP-2 (1500 ng/ml), or mBMP-4 (60%) and mBMP-4 (80%). Therefore, the expression of BMPR-1A, -2, Noggin, OC, and Smad-4 appeared to reach a plateau at the 1000 ng/ml dose of rhBMP-2 and 60% mBMP-4. The mRNA expression of these molecules was readily detected at the 10 ng/ml dose of rhBMP-2 and 10% mBMP-4 (Figs. 1 and 2).

The expression levels of BMPR-2 and Noggin mRNA were sharply elevated on day 1, and then decreased gradually in the muscle-derived primary culture cells at all concentrations. Representative expression patterns using rhBMP-2 (100 ng/ml) or mBMP-4 (20%) are shown in Figs.

3A and 4A. BMPR-1A transcription was also elevated on day 1, but at lower levels when compared with BMPR-2 at all concentrations (Figs. 3 and 4).

Similar patterns for BMPR-1A, -2, and Noggin were observed in MC3T3-E1 and NIH3T3 cell lines, but to a much lower degree than that seen in the muscle-derived primary culture cells at all concentrations. The typical expression figures using rhBMP-2 (1000 ng/ml) or mBMP-4 (60%) in MC3T3-E1 and NIH3T3 cell lines are shown in Figs. 5–8. BMPR-1B expression was not detected by Northern blotting before or after BMP stimulation in any cell sources examined, or in OC later on day 4. The Smad-4 mRNA level gradually increased, and reached a plateau from day 2 (Figs. 5–8).

MyoD mRNA expression was detected without BMP-2 or -4 exposure throughout the experimental period. A typical figure in which muscle-derived primary culture cells were stimulated by BMP-2 or -4 after 24 h is shown in Fig. 9A or 9B.

Protein expression levels

Western blotting on the muscle-derived primary culture cells using either 1000 or 1500 ng/ml doses of rhBMP-2, or 60% or 80% of mBMP-4, revealed that the protein expression levels of BMPR-1A, -2, and Noggin were the same at the 1000 ng/ml and 1500 ng/ml doses of rhBMP-2, and at 60% or 80% concentrations of mBMP-4. Therefore, rhBMP-2 (1000 ng/ml) or mBMP-4 (60%) was used for all subsequent studies of protein expression levels in the muscle-derived primary culture cells (Fig. 10). We did not perform Western blotting on MC3T3-E1 and NIH3T3 cell lines because of the weak expression revealed by Northern blotting.

The translational expression levels of BMPR-1A, -2, and Noggin were enhanced on day 2 and then decreased gradually in the muscle-derived primary culture cells. BMPR-1B expression was not detectable by Western blotting before or after BMP stimulation in the muscle-derived primary culture cell (Fig. 10).

Discussion

This study showed increased transcription and translation of BMPR-1A, -2, and Noggin and increased transcription of OC and Smad-4 in response to rhBMP-2 or mBMP-4 in muscle-derived primary culture cells. Clearly, the muscle-derived primary culture cells are capable of responding to changes in the external concentrations of the bone growth factors. Induction of BMPR-1A and -2 following exposure to BMPs points to the activation of a receptor-mediated pathway to effect intracellular signaling by these molecules. Although the reason for the predominant induction of BMPR-2 among BMPRs is unknown at present, it is possible that an increased number of BMPR-2 molecules with a high affinity for BMP might allow greater capture of this

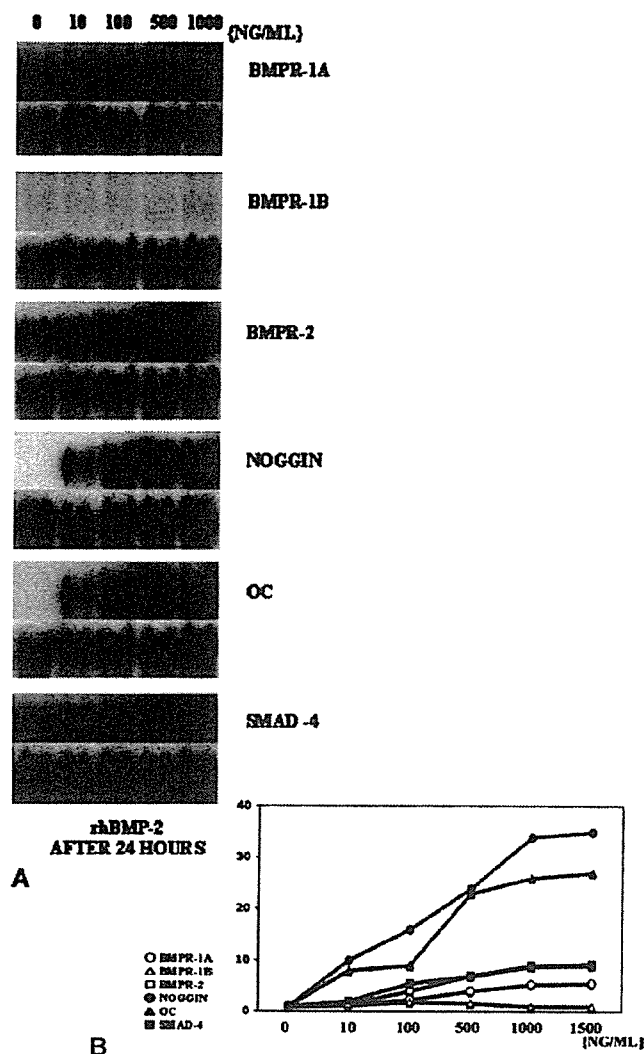


Fig. 1. Gene expression level of BMPR-1A, -1B, -2, Noggin, OC, and Smad-4 after 24-h stimulation of rhBMP-2 (0, 10, 100, 500, 1000ng/ml) in muscle-derived primary culture cells by Northern blot analysis (**A**) and quantitation of the data of Northern blot analysis by Densitometry (**B**). G3PDH mRNA levels (the bottoms of all lanes are G3PDH) obtained by Northern blotting were used for normalization (**A**). The score on hour 0 (just after BMP stimulation) was used as a standard (**B**). BMPR-1A, -2, Noggin, OC, and Smad-4 were up-regulated dose-dependently. No increase in BMPR-1B expression was observed during the course of the study. The expression levels of these molecules were almost the same using 1000ng/ml and 1500ng/ml rhBMP-2 (1500ng/ml rhBMP-2 data not shown in Fig. 1A)

ligand and subsequent activation of BMPR-1A for enhanced transduction of the BMP signal into cells.

We have observed that expression of BMPR-1A and -2 is significantly increased during the early phase of ectopic bone formation following the implantation of rhBMP-2 into the back muscles of adult mice [10]. Based on these data from in vivo and in vitro studies, the activation of BMPR-1A after BMPR-2 might be a key event following BMP stimulation of muscle tissue. BMPR-1A, -2, and Noggin were induced in MC3T3-E1 and NIH3T3 cell lines, but to a

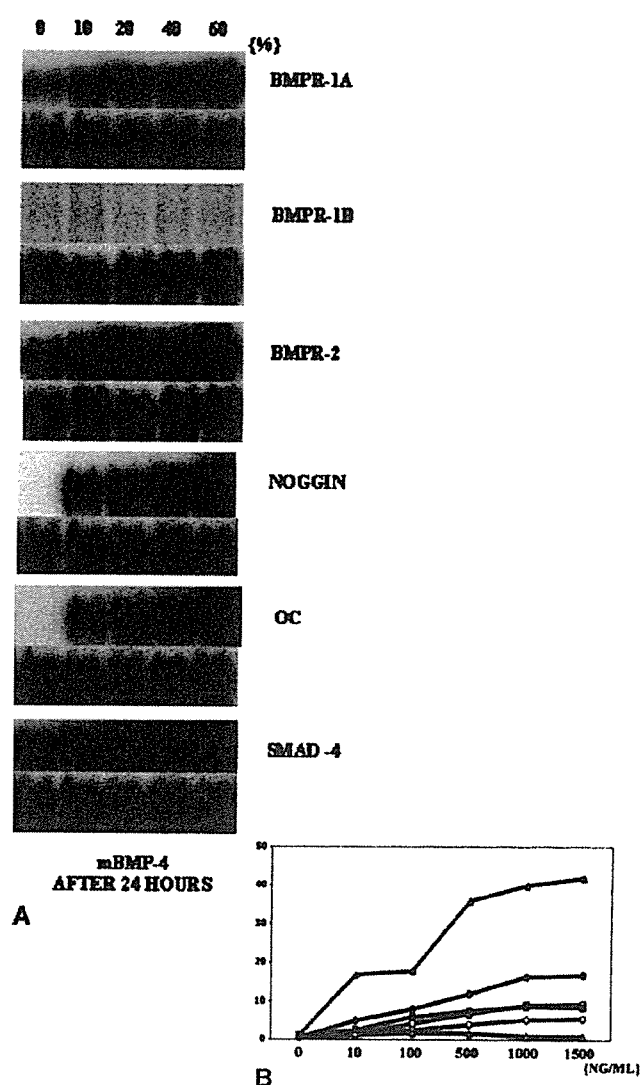


Fig. 2. Gene expression level of BMPR-1A, -1B, -2, Noggin, OC, and Smad-4 after 24-h stimulation of mBMP-4 [0%, 10%, 20%, 40%, and 60% (v/v) conditioned media] in muscle-derived primary culture cells by Northern blot analysis (**A**) and quantitation of the data of Northern blot analysis by Densitometry (**B**). G3PDH mRNA levels (the bottoms of all lanes are G3PDH) obtained by Northern blotting were used for normalization (**A**). The score on hour 0 (just after BMP stimulation) was used as a standard (**B**). The gene expression pattern of the molecules after stimulation of mBMP-4 was similar to that observed after stimulation of rhBMP-2. The expression levels of these molecules were almost the same using 60% and 80% mBMP-4. (80% mBMP-4 data not shown in Fig. 2A)

much lesser degree than that seen in the muscle-derived primary culture cells used in this study.

A specific role of BMPR-1B in skeletal development has been proposed based on the abnormal interphalangeal joint formation in an animal with a null mutation in this receptor. However, the expression of BMPR-1B appeared to be limited in the muscle-derived primary culture cells and the osteoblastic or nonosteoblastic embryonic cell lines, even after exposure to BMPs [16–19]. The lack of expression of BMPR-1B was in accordance with results in a previous

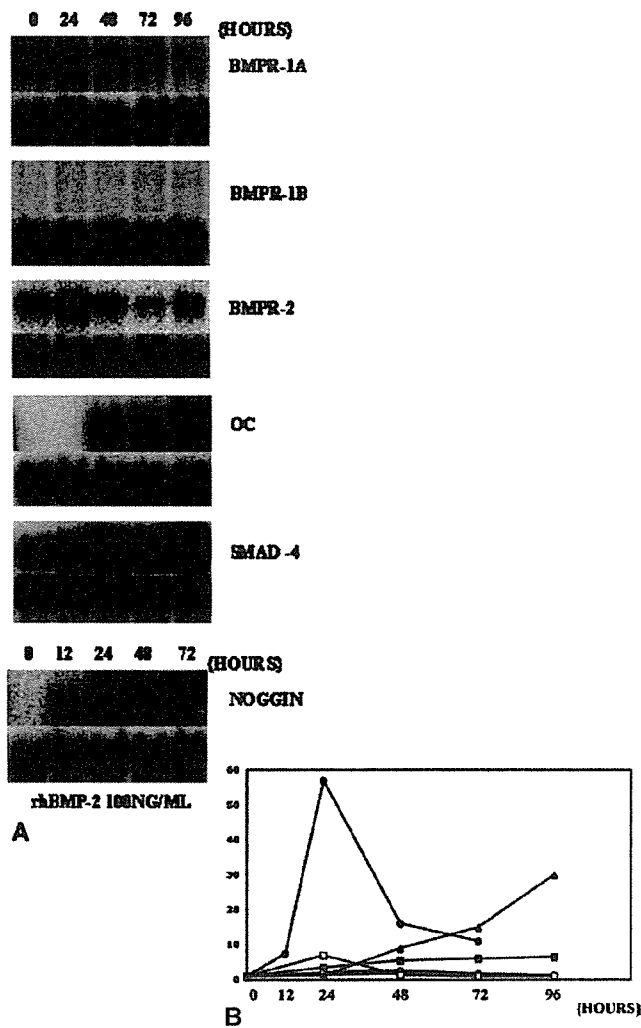


Fig. 3. Gene expression of BMPR-1A, -1B, -2, OC, and Smad-4 for 0, 24, 48, 72, and 96 h and Noggin for 0, 12, 24, 48, and 72 h after 100 ng/ml rhBMP-2 stimulation in muscle-derived primary culture cells by Northern blot analysis (A) and quantitation of the data of Northern blot analysis by Densitometry (B). G3PDH mRNA levels (the bottoms of all lanes are G3PDH) obtained by Northern blotting were used for normalization (A). The score on hour 0 (just after BMP stimulation) was used as a standard (B). After rhBMP-2 stimulation, OC was up-regulated time-dependently. Noggin level peaked at 24h. Expression of BMPR-1A and -2 was increased moderately after 24h, then gradually decreased thereafter. Smad-4 was gradually and weakly up-regulated after stimulation. BMPR-1B was not increased during the experimental period

report using the pluripotent C2C12 cell line, and another study that revealed predominant expression of BMPR-1B in brain and not skeleton [20].

The induction of Noggin gene expression in cells of the osteoblastic lineage following exposure to rhBMP-2, and in fetal rat limb explants by BMP-7, has been reported [21,22]. In this study, Noggin gene expression was also confirmed in muscle-derived primary culture cells, an osteoblastic cell line (MC3T3-E1), and a nonosteoblastic, embryonic fibroblast-like cell line (NIH3T3) [16,23,24]. As Noggin is a representative antagonist of BMP action, the expression of

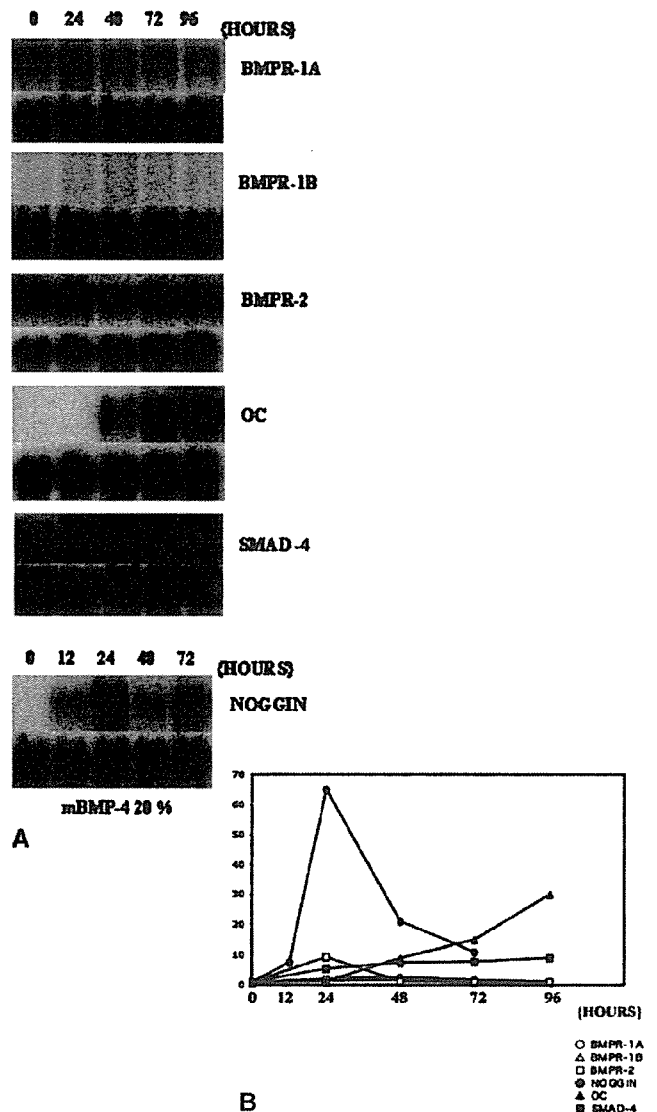


Fig. 4. Gene expression of BMPR-1A, -1B, -2, OC, and Smad-4 for 0, 24, 48, 72, and 96 h and Noggin for 0, 12, 24, 48, and 72 h after 20% mBMP-4 stimulation in muscle-derived primary culture cells by Northern blot analysis (A) and quantitation of the data of Northern blot analysis by Densitometry (B). G3PDH mRNA levels (the bottoms of all lanes are G3PDH) obtained by Northern blotting were used for normalization (A). The score on hour 0 (just after BMP stimulation) was used as a standard (B). The gene expression pattern of the molecules after stimulation of mBMP-4 (20%) was similar to that seen after stimulation of 100ng/ml rhBMP-2

Noggin might act as a negative regulator of the BMP-induced cellular reactions, and consequently reduce the susceptibility of the cells to BMPs.

Three classes of Smads, termed receptor-activated Smads (R-Smads), common Smads (Co-Smads), and inhibitory Smads (I-Smads), have been identified in mammals. Smads1, 5, and 8 are R-Smads that primarily mediate BMP signaling from the receptors to the nucleus [16,25]. Therefore, the up-regulation of Smad-4, which is a representative BMP signaling Co-Smad, in a time- or dose-

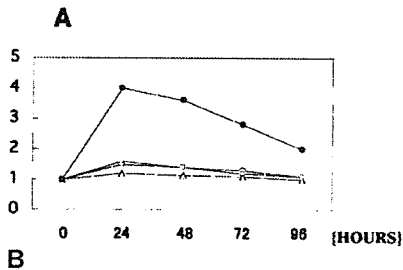
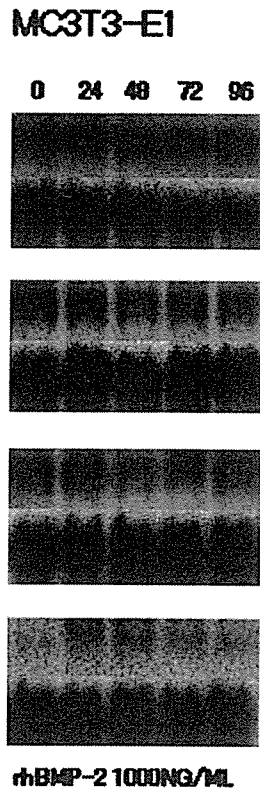


Fig. 5. Gene expression of BMPR-1A, -1B, -2, and Noggin for 0, 24, 48, 72, and 96 h after 1000 ng/ml rhBMP-2 stimulation in MC3T3-E1 cell line by Northern blot analysis (A) and quantitation of the data of Northern blot analysis by Densitometry (B). G3PDH mRNA levels (the bottoms of all lanes are G3PDH) obtained by Northern blotting were used for normalization (A). The score on hour 0 (just after BMP stimulation) was used as a standard (B). BMPR-1A and -2 were weakly induced after rhBMP-2 stimulation, peaked at 24h, then decreased gradually. Noggin was also moderately induced after stimulation showed maximal expression at 24h, then decreased thereafter. BMPR-1B was not induced during the course of the reaction

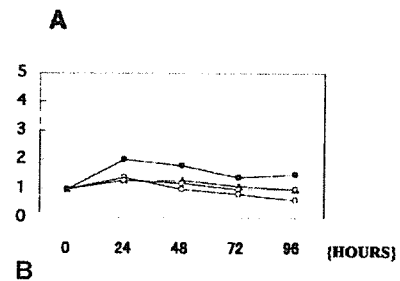
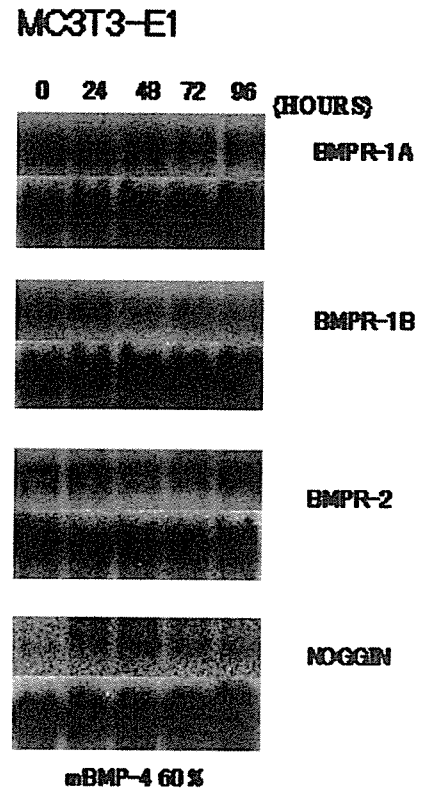


Fig. 6. Gene expression of BMPR-1A, -1B, -2, and Noggin for 0, 24, 48, 72, and 96 h after mBMP-4 (20%) stimulation in MC3T3-E1 cell line by Northern blot analysis (A) and quantitation of the data of Northern blot analysis by Densitometry (B). G3PDH mRNA levels (the bottoms of all lanes are G3PDH) obtained by Northern blotting were used for normalization (A). The score on hour 0 (just after BMP stimulation) was used as a standard (B). The gene expression pattern of the molecules after stimulation of mBMP-4 (20%) was similar to that seen after stimulation of 1000 ng/ml rhBMP-2, but the expression levels with mBMP-4 (20%) were smaller than those with 1000 ng/ml rhBMP-2

dependent manner suggests that BMP signaling in muscle tissue is regulated in a coordinated manner. OC is a well-characterized osteoblast differentiation marker, and MyoD is also a good marker for myoblastic differentiation [26]. Although the expression of MyoD was not detected in this study, the expression of OC was enhanced on day 2 after BMP-2 or -4 stimulation. These results indicate that BMP-induced osteogenic differentiation in muscle tissue might occur through a BMP/Smad signaling pathway, and

muscle-derived primary culture cells might lose the muscle phenotype after BMP exposure.

The expression profiles were much more prominent for primary undifferentiated mesenchymal cells derived from muscle than for MC3T3-E1 or NIH3T3 cells in this study. Muscle-derived primary culture cells include a large population of undifferentiated mesenchymal cells, as described elsewhere [14]. Clearly, undifferentiated mesenchymal cells in muscle tissue are highly responsive to BMPs, based on

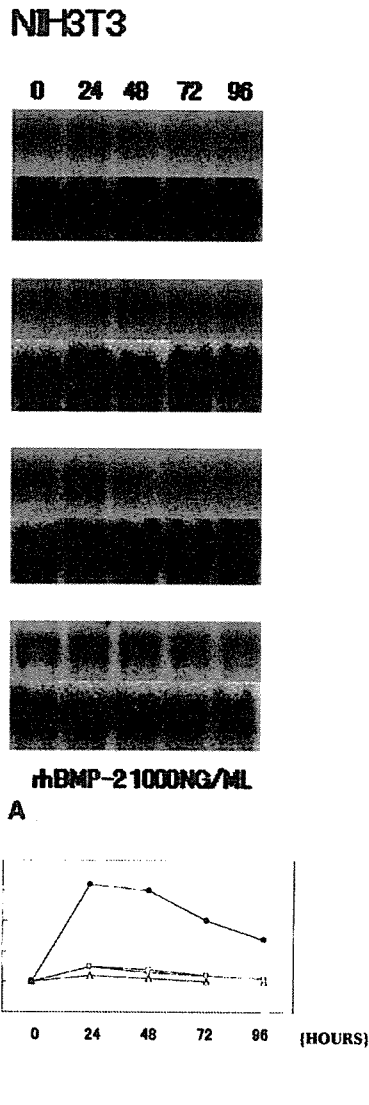


Fig. 7. Gene expression of BMPR-1A, -1B, -2, and Noggin for 0, 24, 48, 72, and 96 h after 1000ng/ml rhBMP-2 stimulation in NIH3T3 cell line by Northern blot analysis (**A**) and quantitation of the data of Northern blot analysis by Densitometry (**B**). G3PDH mRNA levels (the bottoms of all lanes are G3PDH) obtained by Northern blotting were used for normalization (**A**). The score on hour 0 (just after BMP stimulation) was used as a standard (**B**). BMPR-1A and -2 were weakly induced after rhBMP-2 stimulation, peaked at 24h, then decreased gradually. Noggin was moderately induced after stimulation showed maximal expression at 24h, then decreased thereafter

the changes in gene and protein expression levels observed in this study. The proliferation and differentiation of osteoblasts from osteoprogenitor cells in murine bone marrow cultures induced by BMP-2 or -4 have been reported [27,28]. However, there have been few reports using muscle-derived primary culture cells with BMPs. In this study, the expression of BMP-related molecules was examined using undifferentiated mesenchymal cells derived from mouse muscle tissue.

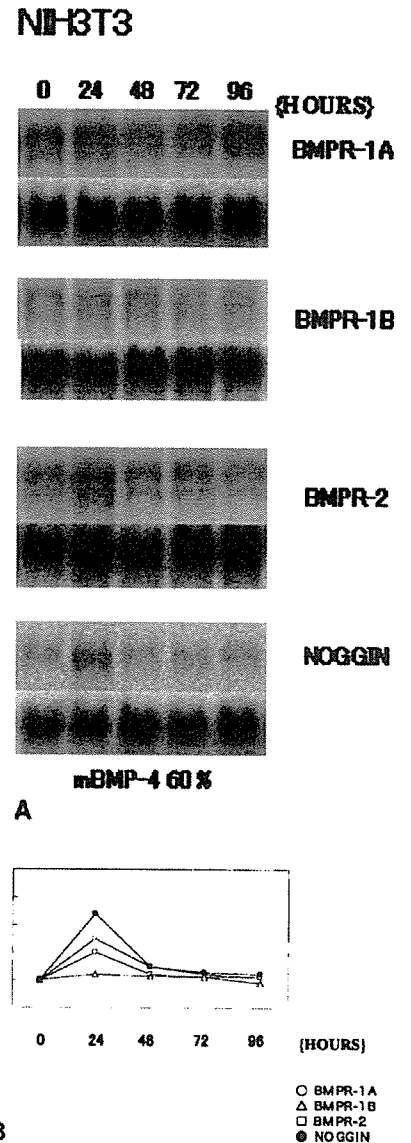


Fig. 8. Gene expression of BMPR-1A, -1B, -2, and Noggin for 0, 24, 48, 72, and 96 h after mBMP-4 (20%) stimulation in NIH3T3 cell line by Northern blot analysis (**A**) and quantitation of the data of Northern blot analysis by Densitometry (**B**). G3PDH mRNA levels (the bottoms of all lanes are G3PDH) obtained by Northern blotting were used for normalization (**A**). The score on hour 0 (just after BMP stimulation) was used as a standard (**B**). BMPR-1A and -2 were weakly induced after rhBMP-2 stimulation, peaked at 24h, then decreased gradually. Noggin was moderately induced after stimulation showed maximal expression at 24h, then decreased thereafter. BMPR-1B was not induced in all experimental stages. In NIH3T3 cells, the expression pattern was similar to that observed in the MC3T3-E1 culture experiments. Expression levels were greater in NIH3T3 cells than in MC3T3-E1 cells

The majority of undifferentiated mesenchymal cells in muscle-derived primary culture cells showed a fibroblastic appearance. These cells are considered to be heterogenous, and contain some kinds of precursor cells such as bone, cartilage, and muscle. They differentiate into each phenotype when they are placed in each differentiation condition.

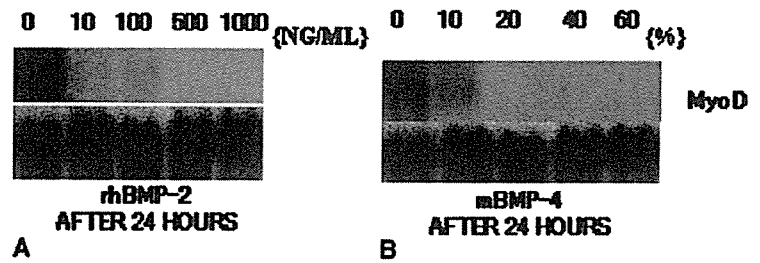


Fig. 9. The expression of MyoD in muscle-derived primary culture cells by Northern blot analyses. G3PDH mRNA levels obtained by Northern blotting were used for normalization. The expression of

MyoD mRNA was not detected after BMP-2 or -4 exposure, and the expression was detected only at 0 and 24h, and not after 24h BMP stimulation

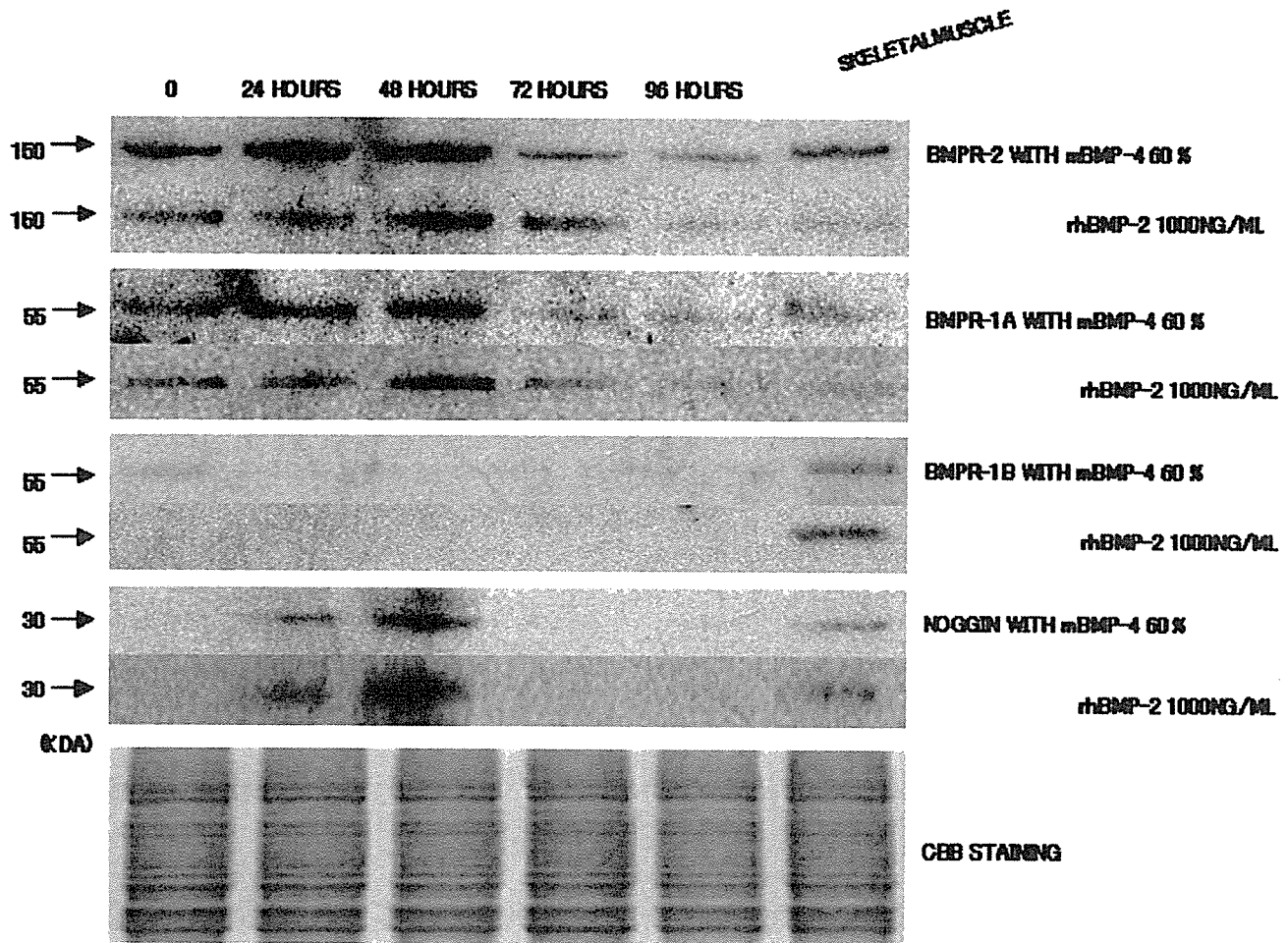


Fig. 10. Western blot analysis of BMPR-1A, -1B, -2, and Noggin after 60% mBMP-4 or 1000ng/ml rhBMP-2 stimulation in muscle-derived primary culture cells. Equivalent loading and integrity of protein were confirmed by Coomassie brilliant blue staining on the gel (*lower panel*). Mouse skeletal muscle proteins were used as positive controls. BMPR-1A and -2 were detected at 0h, induced at 24h, peaked at 48h, and then

gradually decreased in both 60% mBMP-4 and 1000ng/ml rhBMP-2 stimulation groups. Expression was greater for BMPR-2 than for BMPR-1A. BMPR-1B was not detectable during any stages in either treatment group. Noggin was not detected at 0h, was up-regulated at 24h, peaked at 48h, and decreased thereafter

In our study, BMPs stimulated them to upregulate the expressions of a bone marker (OC) and cartilage markers (type II collagen and aggrecan, data not shown), but not the muscle marker examined previously. However, it is unclear

whether bone and cartilage phenotypes were induced by BMPs in separate cells or in a single cell.

To further understand the potential autoregulatory mechanism in response to BMP, further gene expression

studies will be necessary. Ultimately, this knowledge may provide new approaches to the regulation of local and systemic bone formation.

References

- Aspenberg P, Turek T (1996) BMP-2 for intramuscular bone induction: effect in squirrel monkeys is dependent on implantation site. *Acta Orthop Scand* 67:3-6
- Itoh H, Ebara S, Kamimura M, Tateiwa Y, Kinoshita T, Yuzawa Y, Takaoka K (1999) Experimental spinal fusion with use of recombinant human bone morphogenetic protein. 2. *Spine* 24:1402-405
- Takaoka K, Yoshikawa H, Hashimoto J, Ono K, Matsui M, Nakazato H (1994) Transfilter bone induction by Chinese hamster ovary (CHO) cells transfected by DNA encoding bone morphogenetic protein-4. *Clin Orthop* 300:269-273
- Kaplan FS, Shore EM (1999) Illustrative disorders of ectopic skeletal morphogenesis: a childhood parallax for studies in gravitational and space biology. *Gravit Space Biol Bull* 12:27-38
- Miyazono K, Ichijo H, Heldin CH (1993) Transforming growth factor-beta: latent forms, binding proteins and receptors. *Growth Factors* 8:11-22
- Fujii M, Takeda K, Imamura T, Aoki H, Sampath TK, et al. (1999) Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. *Mol Biol Cell* 10:3801-3813
- Ishidou Y, Kitajima I, Obama H, Maruyama I, Murata F, et al. (1995) Enhanced expression of type I receptors for bone morphogenetic proteins during bone formation. *J Bone Miner Res* 10:1651-1659
- Onishi T, Ishidou Y, Nagamine T, Yone K, Imamura T, et al. (1998) Distinct and overlapping patterns of localization of bone morphogenetic protein (BMP) family members and a BMP type II receptor during fracture healing in rats. *Bone* 22:605-612
- Kaneko H, Arakawa T, Mano H, Kaneda T, Ogasawara A, et al. (2000) Direct stimulation of osteoclastic bone resorption by bone morphogenetic protein (BMP)-2 and expression of BMP receptors in mature osteoclasts. *Bone* 27:479-486
- Nakamura Y, Wakitani S, Nakayama J, Wakabayashi S, Horiuchi H, Takaoka K (2003) Temporal and spatial expression profiles of BMP receptors and Noggin during BMP-2-induced ectopic bone formation. *J Bone Miner Res* 18:1854-1862
- Horiuchi H, Saito N, Kinoshita T, Wakabayashi S, Tsutsumimoto T, Takaoka K (2001) Enhancement of bone morphogenetic protein-2-induced new bone formation in mice by the phosphodiesterase inhibitor pentoxifylline. *Bone* 28:290-294
- Takaoka K, Yoshikawa H, Hashimoto J, Ono K, Matsui M, Nakazato H (1994) Transfilter bone induction by Chinese hamster ovary (CHO) cells transfected by DNA encoding bone morphogenetic protein-4. *Clin Orthop* 300:269-273
- Tsutsumimoto T, Wakabayashi S, Kinoshita T, Horiuchi H, Takaoka K (2002) A phosphodiesterase inhibitor, pentoxifylline, enhances the bone morphogenetic protein-4 (BMP-4)-dependent differentiation of osteoprogenitor cells. *Bone* 31:396-401
- Yaffe D (1973) Rat skeletal muscle cells. In: Kruse PF, Patterson MK (eds) *Tissue Culture*, vol 16. Academic Press, New York, p 106-114
- Yoshimura Y, Nomura S, Kawasaki S, Tsutsumimoto T, Shimizu T, Takaoka K (2001) Colocalization of Noggin and bone morphogenetic protein-4 during fracture healing. *J Bone Miner Res* 16:876-884
- Kamiya N, Jikko A, Kimata K, Damsky C, Shimizu K, Watanabe H (2002) Establishment of a novel chondrocytic cell line N1511 derived from p53-null mice. *J Bone Miner Res* 17:1832-1842
- Jiao K, Zhou Y, Hogan BL (2002) Identification of mZnf8, a mouse Kruppel-like transcriptional repressor, as a novel nuclear interaction partner of Smad1. *Mol Cell Biol* 22:7633-7644
- Yamaguchi A, Ishizuya T, Kitou N, Wada Y, Katagiri T, et al. (1996) Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6. *Biochem Biophys Res Commun* 220:366-371
- Zhao M, Harris SE, Horn D, Geng Z, Nishimura R, Mundy GR, Chen D (2002) Bone morphogenetic protein receptor signaling is necessary for normal murine postnatal bone formation. *J Cell Biol* 157:1049-1060
- Wlodarski KH, Reddi AH (1986) Importance of skeletal muscle environment for ectopic bone induction in mice. *Folia Biol (Krakow)* 34:425-434
- Chen D, Ji X, Harris MA, Feng JQ, Karsenty G, et al. (1998) Differential roles for bone morphogenetic protein (BMP) receptor type 1B and 1A in differentiation and specification of mesenchymal precursor cells to osteoblast and adipocyte lineages. *J Cell Biol* 142:295-305
- Akiyama S, Katagiri T, Namiki M, Yamaji N, Yamamoto N, et al. (1997) Constitutively active BMP type I receptors transduce BMP-2 signals without the ligand in C2C12 myoblasts. *Exp Cell Res* 235:362-369
- Gazzerro E, Gangji V, Canalis E (1998) Bone morphogenetic proteins induce the expression of Noggin, which limits their activity in cultured rat osteoblasts. *J Clin Invest* 15:2106-2114
- Nifuji A, Noda M (1999) Coordinated expression of Noggin and bone morphogenetic proteins (BMPs) during early skeletogenesis and induction of Noggin expression by BMP-7. *J Bone Miner Res* 14:2057-2066
- Attisano L, Tuen Lee-Hoeflich S (2001) The Smads Genome. *Biol* 2:Reviews3010
- Yamamoto N, Akiyama S, Katagiri T, Namiki M, Kurosawa T, Suda T (1997) Smad1 and smad5 act downstream of intracellular signalings of BMP-2 that inhibits myogenic differentiation and induces osteoblast differentiation in C2C12 myoblasts. *Biochem Biophys Res Commun* 238:574-580
- Zou H, Wieser R, Massague J, Niswander L (1997) Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev* 11:2191-2203
- Abe E, Yamamoto M, Taguchi Y, Lecka-Czernik B, O'Brien CA, et al. (2000) Essential requirement of BMPs-2/4 for both osteoblast and osteoclast formation in murine bone marrow cultures from adult mice: antagonism by Noggin. *J Bone Miner Res* 15:663-673

Use of Local Electroporation Enhances Methotrexate Effects With Minimum Dose in Adjuvant-Induced Arthritis

Masahiro Tada, Kentaro Inui, Tatsuya Koike, and Kunio Takaoka

Objective. To investigate the effects of electrical pulses on the ability of methotrexate (MTX) to attenuate inflammation and subsequent joint destruction in rats with adjuvant-induced arthritis (AIA).

Methods. Rats in the experimental group received an intraperitoneal injection of MTX (0.125 mg/kg body weight), followed 30 minutes later by application of direct electrical pulses (50V, 8 Hz) to their left hind paws with an electroporation apparatus (M+/E+ group; n = 8). The procedure was repeated twice weekly for 3 weeks. Three control groups received the following treatments, respectively: MTX without electrical treatment (M+/E- group; n = 9), electrical treatment but no MTX (M-/E+ group; n = 10), or no electrical treatment and no MTX (M-/E- group; n = 9). Progression of AIA was monitored by joint swelling and radiologic and histologic changes in the ankle joint.

Results. Three weeks after injection of the adjuvant, and at the height of the arthritic reaction, the swelling and radiologic and histologic changes in the left hind paws in the M+/E+ rats were significantly reduced, as compared with changes observed in the control groups.

Conclusion. These results demonstrate that application of electrical pulses in combination with use of systemic low-dose MTX can ameliorate local arthritic reactions. This response probably occurs because electrical stimulation promotes transient passage of MTX through pores in the cell membranes, with a resultant

local increase in the concentration of the drug within the cells. These results point to a potential use of electrochemotherapy to increase the efficacy of MTX or other drugs in an arthritic joint that is refractory to treatment, without increasing the dose of the drug.

Although new biologic agents (1) can ameliorate inflammatory reactions and consequently protect the joints of patients with rheumatoid disease from progressive damage (2), methotrexate (MTX) remains one of the most effective and widely used disease-modifying antirheumatic drugs (DMARDs) (3). However, chronic inflammation often persists in isolated joints even after effective systemic MTX treatment, presumably as a result of an inadequate concentration of MTX in the joint that is refractory to treatment. In patients with persistent inflammation, synovectomy is often indicated for symptomatic relief, although data on the long-term clinical effectiveness of this approach are limited (4). Another option is an additional dose of MTX, but this increases the risk of adverse events. Because MTX has weak cell permeability, and the pharmacologic effects of this drug depend upon its intracellular concentration, any method for increasing intracellular MTX levels in the joint may be effective in attenuating the inflammatory response.

Electroporation has been used to facilitate the transport of nonpermeable molecules into cells. Transient cell membrane pores, generated electrically, allow nonpermeable molecules, including genes and drugs, to enter into the cells (5). Electroporation systems are now available for clinical use to deliver anticancer drugs into malignant solid tumor cells (6–8) as electrochemotherapy. Encouraging clinical results have been reported for the treatment of malignancies, in terms of efficacy, safety, and cost (9). This suggests that electroporation may be useful for the local treatment of rheumatoid arthritis (RA) that is refractory to conventional therapy.

We used electroporation to enhance the effect of

Supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant-in-aid 15591594).

Masahiro Tada, MD, Kentaro Inui, MD, PhD, Tatsuya Koike, MD, PhD, Kunio Takaoka, MD, PhD: Osaka City University, Graduate School of Medicine, Osaka, Japan.

Address correspondence and reprint requests to Masahiro Tada, MD, Department of Orthopaedic Surgery, Osaka City University, Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan. E-mail: m-tada@med.osaka-cu.ac.jp.

Submitted for publication May 11, 2004; accepted in revised form October 21, 2004.

low-dose MTX treatment on the progression to severe arthritis and associated joint destruction in a rat model of adjuvant-induced arthritis (AIA) (10–12).

MATERIALS AND METHODS

Animals. Inbred 7-week-old male Lewis rats were purchased from Charles River Japan (Kanagawa, Japan) and housed with free access to standard laboratory chow and water, under 12-hour dark/light cycles in conditioned air.

Induction of arthritis. The adjuvant mixture was prepared by mixing dried heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI) in paraffin oil (Wako, Tokyo, Japan) at a concentration of 5 mg/ml. To induce systemic arthritis, 0.2 ml of the preparation was injected into the tail bases of 8-week-old rats that had received anesthesia via ethyl ether inhalation.

Pulsed electrical stimulation for electroporation. For electrical stimulation to generate transient pores in cell membranes at the target tissue site, we used an electroporation apparatus (CUY-21; Gene System, Osaka, Japan). Direct-current electrical pulses (8 Hz, 75 msec pulse duration, 50 volts/cm electrode distance) of 1-second duration were delivered 6 times during a single procedure. Each of the six 1-second pulses was applied by 2 parallel stainless steel electrodes that were moved between each pulse through 60° in a plane perpendicular to the long axis of the left hind paws, 30 minutes after an intraperitoneal injection of MTX or saline. We used electrode paste (Gelaid; Nihon Kodan, Tokyo, Japan) to prevent skin burns.

Experimental protocol. The animals were assigned to an experimental group or to 1 of 3 control groups, as follows: MTX injection with electroporation (M+/E+ [experimental] group; n = 8), MTX without electroporation (M+/E- group; n = 9), electroporation with saline (M-/E+ group; n = 10), or no treatment (M-/E- group; n = 9).

MTX was provided by Wyeth-Pharmaceutical (Tokyo, Japan). The dose of MTX was set to 0.125 mg/kg body weight, based on preliminary experimental data indicating that no significant systemic antiarthritic changes were recognized at this dose. The drug was administered intraperitoneally twice weekly for 3 weeks, and the animals were killed by asphyxia in carbon dioxide (for radiologic and histologic examination).

These experimental protocols were in accordance with institutional regulations for animal care and were approved by the Institutional Committee for Animal Care of Osaka City University.

Gross inspection and radiologic evaluation. Twice weekly, the animals were weighed using an electronic balance, and hind paw thickness was measured with digital calipers. Three weeks after the adjuvant was injected, the animals were killed using CO₂ asphyxiation, and both hind limbs were harvested and fixed by perfusing cold 4% paraformaldehyde through the left ventricle, followed by immersion in cold 4% paraformaldehyde solution. Soft x-ray images of the hind paws were obtained with a soft x-ray apparatus (DCS-600EX; Aloka, Tokyo, Japan) using settings of 45 kV, 4 mA, and 30 seconds of exposure time. Destructive changes in hind paw bones seen on radiographs were evaluated by criteria previously described by Clark et al (13), with some modifications. Briefly, radiographic

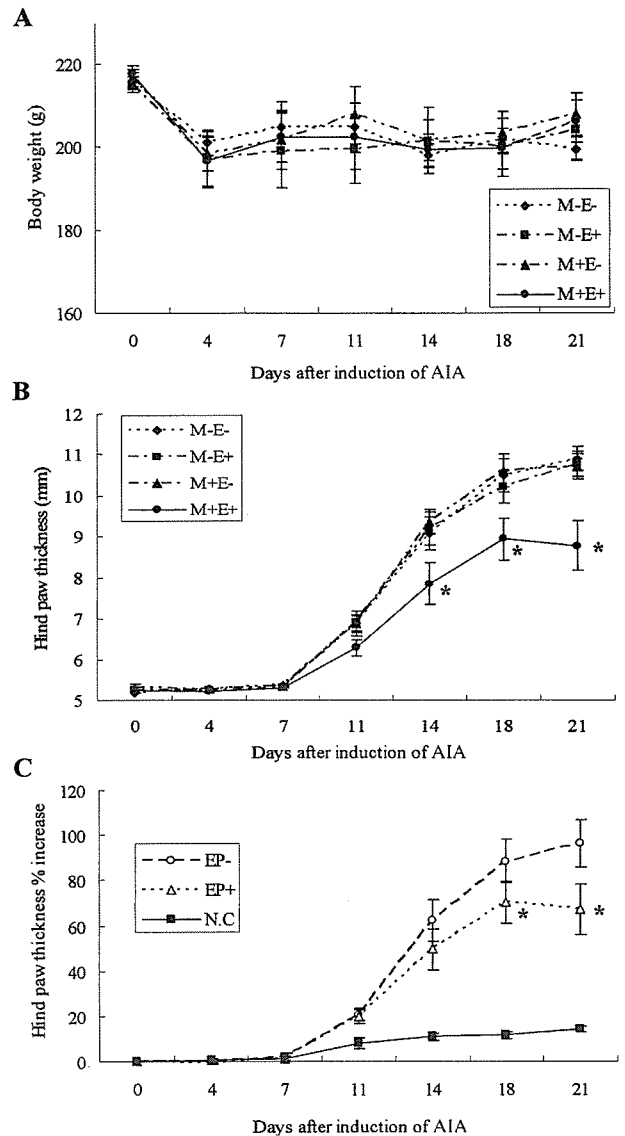


Figure 1. Effects of electrochemotherapy with methotrexate (MTX) on body weight and paw swelling in rats with adjuvant-induced arthritis (AIA). **A**, Weight loss was observed in all groups on day 4. There was no significant weight difference between the 4 groups throughout the entire study period. **B**, Left hind paw thickness, as measured by digital calipers, was maximal on day 21 in the M-/E- (no treatment; n = 9), M-/E+ (electroporation with saline; n = 10), and M+/E- (MTX without electroporation; n = 9) groups. The thickness of the left hind paw treated with electrical pulses after administration of MTX, 0.125 mg/kg/week (M+/E+; n = 8) was significantly decreased when compared with the other groups. * = $P < 0.05$ versus the M-/E-, M-/E+, and M+/E- groups. **C**, Effects of electrical pulses on paw swelling in the M+/E+ group. Electrical pulses were applied to the left hind paw only (electrically treated [EP+]) (n = 8), not the right paw (not electrically treated [EP-]) (n = 8). Application of electrical pulses after administration of low-dose MTX significantly inhibited hind paw swelling on days 18 and 21, as assessed by paw thickness and when compared with EP- paws. NC = negative control (non-adjuvant-injected model) (n = 5). * = $P < 0.05$ versus EP-. Bars show the mean \pm SEM.

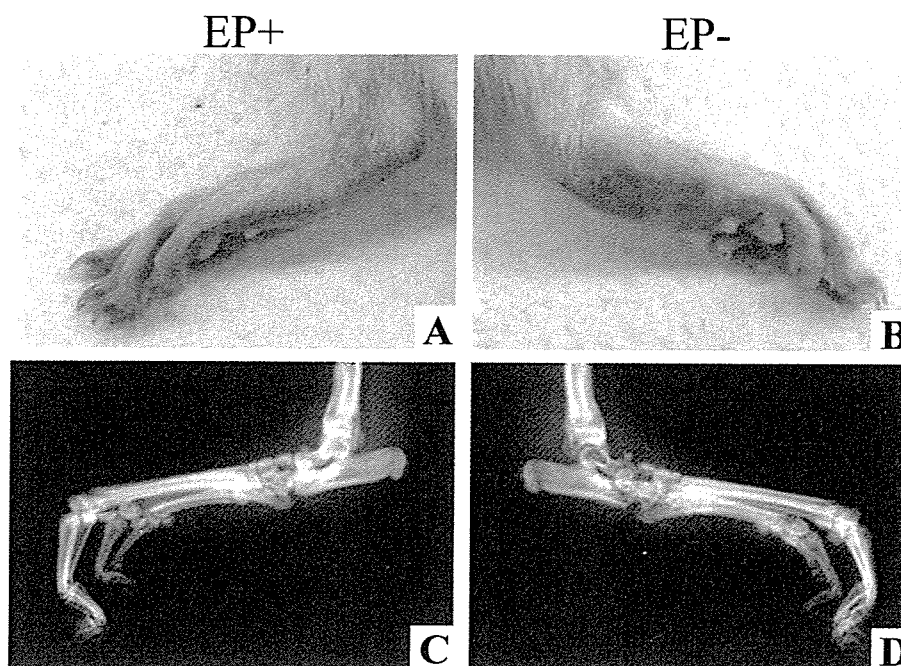


Figure 2. Gross appearance and radiographs of the hind paws of the same rat in the M+/E+ group on day 21. Following administration of MTX (0.125 mg/kg/week), electrical pulses were applied to the left hind paw only (EP+) (A and C). Note the obvious difference in the degree of swelling and joint damage between the left paw (EP+) and right paw (EP-) in gross appearance (A and B), as well as on soft x-ray (C and D). See Figure 1 for definitions.

changes in terms of radiodensity, subchondral bone erosion, periosteal reaction, and cartilage space were evaluated under blinded conditions by 2 rheumatologists (KI and TK) and graded on a 0–3 scale (where 0 = normal and 3 = severely damaged).

Histologic sections. Both hind paws were harvested from the animals for histopathologic examination. After the removal of skin, bones in the hind paws were decalcified in a neutral buffered 14% solution of EDTA/10% formalin, dehydrated in a graded ethanol series, embedded in paraffin, sectioned sagittally into 4- μ m sections, and stained with hematoxylin and eosin or toluidine blue. Pathologic changes were evaluated by 2 observers according to a previously reported rating system (14), as follows: grade 0 = normal synovium, cartilage, and bone; grade 1 = hypertrophic synovium with cellular infiltration without pathologic change in bone and cartilage; grade 2 = pannus formation and cartilage erosion in addition to the hypertrophic synovium; grade 3 = additional severe erosion of cartilage and subchondral bone; grade 4 = loss of joint integrity and ankylosis.

In order to identify and count osteoclastic cells, sections were stained for tartrate-resistant acid phosphate (TRAP) using a staining kit (Sigma-Aldrich, St. Louis, MO). TRAP-positive multinucleated cells were counted in 11 selected fields (8 fields in the distal tibia and 3 fields in the talus), all at 100 \times magnification.

Statistical analysis. Body weight and hind paw thickness were evaluated by repeated analysis of variance and Fisher's protected least significant difference test. Pairwise comparisons were made using Wilcoxon's signed rank tests

among groups. All statistical analyses were carried out using StatView software version 5.0 (SAS Institute, Cary, NC). *P* values less than or equal to 0.05 were considered significant.

RESULTS

Effects of electrochemotherapy on progression of AIA. No significant difference in body weight was noted between the 4 groups during the course of this experiment (Figure 1A), indicating that low-dose MTX, with or without electroporation, had little effect on the systemic physical condition of the rats with AIA.

The thickness of the hind paws in all rats was

Table 1. Radiologic and histologic scores and osteoclast numbers in rat AIA, 21 days after injection of adjuvant*

Group	Radiologic score (n = 8)	Histologic score (n = 8)	Osteoclast number (n = 5)
Right hind paw, EP-negative	3.8 \pm 4.5	2.5 \pm 1.2	77.6 \pm 10.2
Left hind paw, EP-positive [†]	1.8 \pm 2.2	1.3 \pm 0.5	22.0 \pm 2.4

* Values are the mean \pm SD. AIA = adjuvant-induced arthritis; EP = electroporation.

[†] For all comparisons, *P* < 0.05 versus EP-negative.

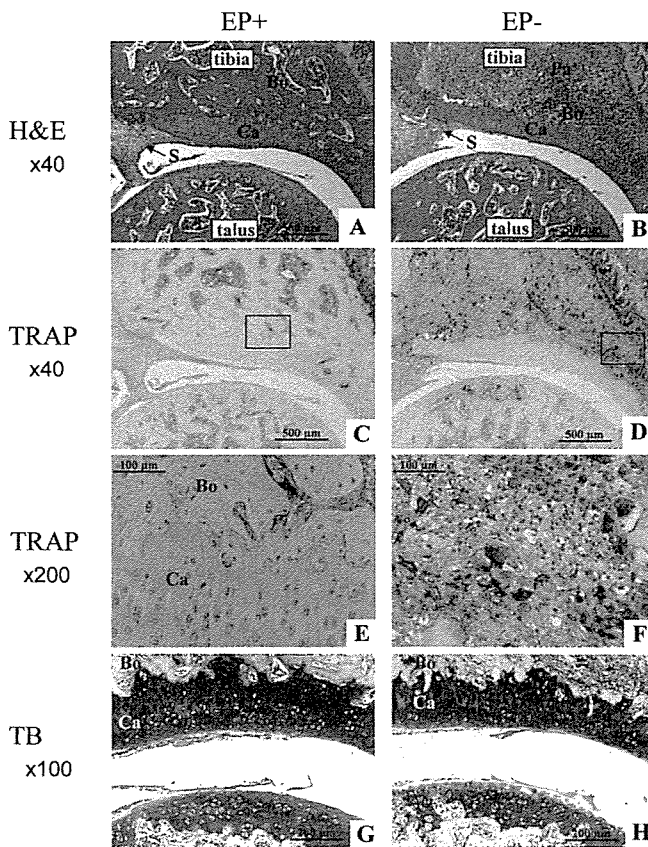


Figure 3. Histologic analysis of the ankle joints of the same rat in the M+/E+ group on day 21. **A** and **B**, Staining with hematoxylin and eosin (H&E). **C**, **D**, **E**, and **F**, Staining with tartrate-resistant acid phosphate (TRAP). **G** and **H**, Staining with toluidine blue (TB). The electroporation procedure was applied to the left ankle joint only (EP+) (**A**, **C**, **E**, and **G**). No inflammatory synovial tissue erosion into subchondral bone was observed with application of electroporation (**A**) compared with MTX only (**B**). Inflamed synovium infiltrated with lymphocytes was found to contain abundant osteoclastic multinucleated cells on TRAP staining (**D** and **F**). However, there was no difference in metachromasia of articular cartilage in the left and right hind paws. **E**, Higher-magnification view of the boxed area in **C**. **F**, Higher-magnification view of the boxed area in **D**. Bo = subchondral bone; Ca = cartilage; Pa = pannus; S = synovial tissue (see Figure 1 for other definitions).

significantly and consistently increased from day 11 until the end of the experiment. However, in the M+/E+ group, swelling of the left hind paw was significantly suppressed on days 14, 18, and 21 (Figure 1B) when compared with the 3 control groups (M+/E-, M-/E+, and M-/E-). The gross appearance of the hind paws is shown in Figures 2A and B. Thus, application of electrical pulses appeared to prevent the hind paw joints from progressing to advanced AIA. The degree of swelling differed significantly between the left (electrically treated [EP+]) and right (EP-) paws of the same rat in the M+/E+ group (Figure 1C).

cally treated [EP+]) and right (EP-) paws of the same rat in the M+/E+ group (Figure 1C).

Radiologic evaluation of bones and joints. Radiologic analysis revealed that the hind paw joints were severely damaged in the M-/E-, M-/E+, and M+/E- groups at 21 days after injection of the adjuvant. Therefore, at a dose of 0.125 mg/kg body weight, MTX did not prevent the joint damage (Figure 2D) or local swelling (Figure 2B) caused by progression of arthritis. In contrast, the radiologic damage score was significantly lower in the electrically treated left (EP+) hind paws in the M+/E+ group (Figures 2A and C and Table 1).

Histologic analyses. In the M+/E+ group, the histologic scores were significantly lower in the left hind paws (EP+) than in the right hind paws (EP-) (Figures 3A and B and Table 1). Inflamed synovial tissues with abundant lymphocytes were observed to erode into subchondral bone (Figure 3B). In sections of these joints, the population of TRAP-positive multinucleated osteoclastic cells was significantly lower in the bones of the left hind paw (EP+) than in those of the right hind paw (EP-) (Figures 3C, D, E, and F and Table 1). Toluidine blue staining revealed no degenerative changes of cartilage tissue, including irregularity of articular surface, disorganization of tidemark, and alternation of metachromasia, in either hind paw (Figures 3G and H).

DISCUSSION

These results indicate positive effects of pulsed electrical stimulation for attenuating arthritis by enhancing the antiarthritic effect of MTX. We believe that this is attributable to micropores created by the electrical pulses in the cytoplasmic membranes of cells in the synovium or other inflamed cells. The subsequent passive influx of MTX into the cells would attenuate the inflammatory responses that led to the AIA, although this study did not provide direct evidence of MTX influx. In this preliminary study, we could not identify the cells targeted by electrochemotherapy, and MTX-negative synovial cells, inflammatory cells, or both, may be targets for the drug.

The effects of electrical fields on living cells have been investigated since the 1960s, and high-voltage electrical pulses have been reported to generate transient and reversible pores in cell membranes. This phenomenon has been termed electroporation and is currently used to transfer genes or drugs into cells (6). Electrochemotherapy involves electroporation with drugs, and this methodology is used for the treatment of malignant tumors (5-9). The use of electrochemotherapy to introduce anticancer drugs into malignant tumors has been reported, e.g., bleomycin

for melanoma, basal cell carcinoma, Kaposi's sarcoma, squamous cell carcinoma (6), or chondrosarcoma (15). However, electrochemotherapy with MTX for the treatment of RA has not been reported, although the less-permeable character of MTX and its use as a DMARD in RA would make it an ideal candidate for this approach. Because the effect of pulsed electrical stimulation is expected only at the local site, this method might be applicable for an isolated joint with arthritis that is refractory to systemic chemotherapy or in the early stages of RA involving a limited number of joints without significant joint-destructive changes.

Clinical application of this therapy should not affect normal tissues. Using TUNEL staining, we did not observe any difference in the number of apoptotic cells between the M+/E+ and M+/E- groups (data not shown). We also confirmed in the pilot study that electrical pulses, used under the same conditions as those used in this experiment, did not influence the normal tissues of inbred 9-week-old male Lewis rats. In this pilot study, no inflammatory reactions were observed on histologic examination of the area treated with the electrical pulses, suggesting that electroporation under these conditions did not cause any damage to normal tissue, including cartilage, bone, muscle, and blood vessels (results not shown). However, the clinical application of electrochemotherapy requires further study, including the dose of MTX and the parameters of the electrical pulses.

This experimental study is limited in 2 key areas. First, electrochemotherapy was not applied to joints with established arthritis, and the effect of electrochemotherapy was estimated based on the progression of arthritis. This differs from the clinical situation, in which, as indicated previously (10,11), the inflammatory phase in this AIA model is self-limiting. Therefore, the efficacy of electrochemotherapy for the treatment of established chronic arthritis is difficult to determine in this model. Second, optimization of the application of pulsed electrical current may not be sufficient to obtain maximum delivery of MTX into cells and to achieve maximal antiinflammatory effect in RA. The conditions that enable the efficacy of electrical stimulation in electrochemotherapy may be quite different from the condi-

tions used in the clinical treatment of malignancies that were reference sources for the present study. The potential value of electrochemotherapy for the treatment of RA has been illustrated by these studies, and further work is required to optimize electrochemotherapy to control disease in joints with RA refractory to treatment.

REFERENCES

1. Maini R, St Clair EW, Breedveld F, Furst D, Kalden J, Weisman M, et al, and the ATTRACT Study Group. Infliximab (chimeric anti-tumour necrosis factor α monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. *Lancet* 1999;354:1932-9.
2. Lipsky PE, van der Heijde DM, St Clair EW, Furst DE, Breedveld FC, Kalden JR, et al, and the Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. Infliximab and methotrexate in the treatment of rheumatoid arthritis. *N Engl J Med* 2000;343:1594-602.
3. Furst DE, Kremer JM. Methotrexate in rheumatoid arthritis. *Arthritis Rheum* 1988;31:305-14.
4. Multicenter evaluation of synovectomy in the treatment of rheumatoid arthritis: report of results at the end of three years. *Arthritis Rheum* 1977;20:765-71.
5. Heller R, Gilbert R, Jaroszeski MJ. Clinical applications of electrochemotherapy. *Adv Drug Deliv Rev* 1999;35:119-29.
6. Mir LM, Orlowski S. Mechanisms of electrochemotherapy. *Adv Drug Deliv Rev* 1999;35:107-18.
7. Hyacinthe M, Jaroszeski MJ, Dang VV, Coppola D, Karl RC, Gilbert RA, et al. Electrically enhanced drug delivery for the treatment of soft tissue sarcoma. *Cancer* 1999;85:409-17.
8. Horiuchi A, Nikaido T, Mitsushita J, Toki T, Konishi I, Fujii S. Enhancement of antitumor effect of bleomycin by low-voltage in vivo electroporation: a study of human uterine leiomyosarcomas in nude mice. *Int J Cancer* 2000;88:640-4.
9. Gothelf A, Mir LM, Gehl J. Electrochemotherapy: results of cancer treatment using enhanced delivery of bleomycin by electroporation. *Cancer Treat Rev* 2003;29:371-87.
10. Welles WL, Silkworth J, Oronsky AL, Kerwar SS, Galivan J. Studies on the effect of low dose methotrexate on rat adjuvant arthritis. *J Rheumatol* 1985;12:904-6.
11. Kawai S, Nagai K, Nishida S, Sakyo K, Murai E, Mizushima Y. Low-dose pulse methotrexate inhibits articular destruction of adjuvant arthritis in rats. *J Pharm Pharmacol* 1997;49:213-5.
12. Morgan SL, Baggott JE, Bernreuter WK, Gay RE, Arani R, Alarcon GS. MTX affects inflammation and tissue destruction differently in the rat AA model. *J Rheumatol* 2001;28:1476-81.
13. Clark RL, Cuttino JT Jr, Anderle SK, Cromartie WJ, Schwab JH. Radiologic analysis of arthritis in rats after systemic injection of streptococcal cell walls. *Arthritis Rheum* 1979;22:25-35.
14. Shiozawa S, Shimizu K, Tanaka K, Hino K. Studies on the contribution of c-fos/AP-1 to arthritic joint destruction. *J Clin Invest* 1997;99:1210-6.
15. Shimizu T, Nikaido T, Gomyo H, Yoshimura Y, Horiuchi A, Isobe K, et al. Electrochemotherapy for digital chondrosarcoma. *J Orthop Sci* 2003;8:248-51.

Use of Bone Morphogenetic Protein 2 and Diffusion Chambers to Engineer Cartilage Tissue for the Repair of Defects in Articular Cartilage

Masashi Nawata,¹ Shigeyuki Wakitani,¹ Hiroyuki Nakaya,² Akira Tanigami,³ Toyokazu Seki,³ Yukio Nakamura,¹ Naoto Saito,¹ Kenji Sano,¹ Eiko Hidaka,¹ and Kunio Takaoka⁴

Objective. To examine the ability of cartilage-like tissue, generated ectopically in a diffusion chamber using recombinant human bone morphogenetic protein 2 (rHuBMP-2), to repair cartilage defects in rats.

Methods. Muscle-derived mesenchymal cells were prepared by dissecting thigh muscles of 19-day postcoital rat embryos. Cells were propagated in vitro in monolayer culture for 10 days and packed within diffusion chambers (10⁶/chamber) together with type I collagen (CI) and 0, 1, or 10 μ g rHuBMP-2, and implanted into abdominal subfascial pockets of adult rats. Tissue pellets were harvested from the diffusion chambers at 2 days to 6 weeks after implantation, and examined by histology, by reverse transcription–polymerase chain reaction (PCR) for aggrecan, CII, CIX, CX, and CXI, MyoD1, and core binding factor a1/runt-related gene 2, and by real-time PCR for CII. Tissue pellets generated in the chamber 5 weeks after implantation were transplanted into a full-thickness cartilage defect made in the patellar groove of the same strain of adult rat.

Results. In the presence of 10 μ g rHuBMP-2, muscle-derived mesenchymal cells expressed CII messenger RNA at 4 days after transplantation, and a

mature cartilage mass was formed 5 weeks after transplantation in the diffusion chamber. Cartilage was not formed in the presence of 1 μ g rHuBMP-2 or in the absence of rHuBMP-2. Defects receiving cartilage engineered with 10 μ g rHuBMP-2 were repaired and restored to normal morphologic condition within 6 months after transplantation.

Conclusion. This method of tissue engineering for repair of articular defects may preclude the need to harvest cartilage tissue prior to mosaic arthroplasty or autologous chondrocyte implantation. Further studies in large animals will be necessary to validate this technique for application in clinical practice.

Regeneration of articular cartilage is a challenging subject for research on joint surgery (1), and several methods have been devised and attempted in clinical practice to repair focal defects in articular cartilage, especially in young patients (2–5). Currently, mosaic arthroplasty (6), a procedure in which pieces of autogeneic chondro-osseous mass are procured from peripheral parts of the joint surface and transplanted into the focal cartilage defects, is often used with success in the knee joint (7). However, a number of limitations persist, and these include the limited source of the autogeneic osteochondral tissue mass and the potential risk of progression to osteoarthritis due to the injury caused by procurement of graft tissue from the normal joint surface. In addition, the functional durability of the repaired cartilage and the limited application of the approach to small joints are further areas of concern.

Recently, technologies have been developed in order to fabricate tissues for the repair of skeletal defects. The transplantation of chondrocytes of auto- or allogeneic origin has been demonstrated in both experimental (8–11) and clinical (12) situations. In these

Supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology (16390436).

¹Masashi Nawata, MD, Shigeyuki Wakitani, MD, PhD, Yukio Nakamura, MD, Naoto Saito, MD, PhD, Kenji Sano, PhD, Eiko Hidaka, PhD: Shinshu University School of Medicine, Matsumoto, Japan; ²Hiroyuki Nakaya, MD: Osaka University Medical School, Osaka, Japan; ³Akira Tanigami, MD, PhD, Toyokazu Seki, PhD: Otsuka Pharmaceutical Company, Tokushima, Japan; ⁴Kunio Takaoka, MD, PhD: Osaka City University Medical School, Osaka, Japan.

Address correspondence and reprint requests to Shigeyuki Wakitani, MD, PhD, Department of Orthopaedic Surgery, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. E-mail: wakitani@hsp.md.shinshu-u.ac.jp.

Submitted for publication May 6, 2004; accepted in revised form September 14, 2004.

cases, cells are dissociated from pieces of articular cartilage, propagated (or left unpropagated) on dishes in *ex vivo* conditions to expand the cell population, and then transplanted with or without scaffolding carrier materials into the cartilage defect of the recipient. Although these methods can repair cartilage defects, some difficulties persist. Allogeneic transplantation has the inherent risks of disease transmission and rejection; autologous transplantation causes damage to the donor site.

In an effort to address the limitations of existing approaches, we attempted to generate cartilage tissue by inducing the differentiation of muscle-derived cells into the chondrocytic lineage in an *in vivo* environment with recombinant human bone morphogenetic protein 2 (rHuBMP-2). Articular defects in rat joints that received the induced cartilage-like tissue were repaired and restored to normal condition. The present report provides evidence to support this approach for the successful treatment of articular cartilage defects.

MATERIALS AND METHODS

Preparation of muscle-derived mesenchymal cells and diffusion chambers. Mesenchymal cells were obtained from the thigh muscles of 19-day, postcoital, F344 rat embryos (purchased from Japan SLC, Hamamatsu, Japan). The muscle tissues were minced with scissors and digested in 0.25% trypsin with 1 mM EDTA- Na_4 (Invitrogen, Carlsbad, CA). The dissociated cells were propagated on plastic culture dishes (10 cm in diameter) in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (volume/volume) fetal calf serum (Invitrogen) and antibiotics (mixture of 5 mg/ml penicillin G, 5 mg/ml streptomycin, 10 mg/ml neomycin; Invitrogen) and passaged under routine culture conditions for 10 days. At the end of this period, the cells were detached from the dishes with 0.25% trypsin with 1 mM EDTA- Na_4 and packed within diffusion chambers (10^6 cells/chamber).

In order to construct a diffusion chamber for cell transplantation, a diffusion chamber kit (Millipore, Billerica, MA), consisting of a plastic ring (14 mm in outer diameter and 10 mm in inner diameter), a membrane filter (comprising a mixture of cellulose acetate and cellulose nitrate [0.45 μm in pore size]), and adhesive sealant, was utilized. The inner diameter of the ring was reduced to 5 mm by inserting another plastic ring. Only one side of the larger plastic ring was initially sealed with a membrane filter and adhesive sealant. For the next step, 40 μl of 0.3% (weight/weight) pig type I collagen (Cellmatrix LA; Nitta Gelatin, Osaka, Japan) and 0, 1, or 10 μg of rHuBMP-2 (Yamanouchi Pharmaceutical, Tokyo, Japan) were introduced into the diffusion chamber. The chamber was then freeze-dried and sterilized with ethylene oxide gas.

After these processes were completed, 10^6 cells suspended in 40 μl of serum-free culture medium containing 0.3% (w/w) pig type I collagen (Cellmatrix I-A; Nitta Gelatin) were introduced into the diffusion chamber, and another open side

of the chamber was sealed with a filter and adhesive sealant. Sixty-two chambers (42 for histologic examination, 8 for reverse transcription-polymerase chain reaction [RT-PCR] analysis, and 12 for real-time PCR analysis) with 10 μg of rHuBMP-2 (group B10), 10 chambers (all for histologic examination) with 1 μg of rHuBMP-2 (group B1), and 46 chambers (26 for histologic examination, 8 for RT-PCR analysis, and 12 for real-time PCR analysis) without rHuBMP-2 (group B0) were prepared for analysis and implantation.

Transplantation of the diffusion chamber into the abdominal pocket of rats. Immediately after loading the cells into the diffusion chambers, each chamber was surgically inserted into a pocket in the abdominal muscles of 8-week-old F344 rats under diethyl ether anesthesia. After surgery, the rats were housed in cages and were given free access to standard chalk-like food and water. At 2, 4, 6, 8, 14, 21, 28, 35, and 42 days after implantation, the animals were killed in due order and the diffusion chambers were harvested (Table 1) for histologic examination. For RT-PCR analysis, 2 chambers were harvested at 2-, 4-, 7-, and 14-day intervals after implantation. For real-time PCR analysis, 2 chambers were harvested at 2-, 4-, 6-, 14-, 28-, and 42-day intervals after implantation.

Harvested tissue pellets within the chambers were inspected for vascular invasion caused by seal failure or breakage of the filter membranes. When vascular invasion was noted, the tissue was excluded from the transplantation into the cartilage defect and from PCR analysis. The tissue pellets for histologic examination were radiographed and fixed in 20% neutral buffered formalin solution, prior to processing for histologic examination. Some parts of the tissue pellet from the 5-week-old sample were used for transplantation into the rat-knee defect. Tissue pellets for RT-PCR or real-time PCR were frozen in liquid nitrogen immediately after harvesting.

Transplantation of tissue pellets from diffusion chambers into osteochondral defects of rats. Some portions of the tissue pellet removed from the diffusion chambers at 5 weeks after implantation were transplanted into cartilage defects generated on the patellar grooves of the knee joints of 7 (4 from group B10, 3 from group B0) mature, same-strain rats (a quarter tissue pellet/animal). The transplantation procedure was performed with the rats under anesthesia, using an intramuscular injection of a mixture of ketamin (100 mg/ml, 0.6

Table 1. Cartilage formation in diffusion chamber*

	rHuBMP-2			Area of cartilage tissue in cross-section
	0 μg	1 μg	10 μg	
2 days	0/2	-	0/2	-
4 days	0/2	-	0/2	-
6 days	0/2	-	0/2	-
8 days	0/2	-	0/2	-
14 days	0/2	-	0/2	-
21 days	0/4	-	4/6	1/4
28 days	0/4	0/4	9/10	1/3
35 days	0/4	0/6	9/10	Almost all
42 days	0/4	-	6/6	Almost all

* Except where indicated otherwise, values are the number of samples with cartilage formation/number of experiments. rHuBMP-2 = recombinant human bone morphogenetic protein 2.

ml/kg body weight; Sankyo, Tokyo, Japan) and xylazine (20 mg/ml, 0.3 ml/kg body weight; Bayel, Osaka, Japan). Pellets were transplanted into the left knees, and defects made on the right knees did not receive the implants.

In order to generate an osteochondral defect on the patellar groove of the distal femur of the rats, a longitudinal skin incision was made in the midline of the knee and the patellar groove was exposed by medial parapatellar arthrotomy and lateral dislocation of the patella. The osteochondral defect was made by drilling in 2 mm in depth and 2 mm in diameter, vertically to the patellar groove. The tissue pellet was detached from the inner surface of the membrane filters of the diffusion chamber and press-fitted into the defect. The knee joint was then closed with sutures. After surgery, the rats were fed in cages and killed at 24 weeks after surgery. The knee joints were excised and processed for histologic examination.

Histologic examination. Diffusion chambers and distal femurs with an articular cartilage defect were removed from the animals at 24 weeks after implantation and fixed in 20% buffered formalin. The harvested chambers were radiographed with a soft x-ray apparatus (Sofron, Tokyo, Japan) and visualized on radiographic films (Fuji Photo Film, Tokyo, Japan). The harvested chambers with calcified tissue and the distal ends of femurs with articular defects were decalcified in 4% EDTA solution, and then dehydrated with a gradient ethanol series, embedded in paraffin, sectioned in 5- μ m thickness, and stained with hematoxylin and eosin or toluidine blue. Results of the histologic examination were evaluated using the scoring system described by Wakitani et al (13) for histologic grading of a cartilage defect (Wakitani's score; a lower score indicates improvement).

RT-PCR analysis. In order to detect changes in the expression of cartilage matrix-specific molecules in cells from the harvested diffusion chambers, RT-PCR analyses for aggrecan, types II, IX, X, and XI collagens, MyoD1, and core binding factor a1 (Cbfa1)/runt-related gene 2 (Runx2) were performed with the tissue pellets from the B10 and B0 groups. Frozen tissue pellets were ground down to powder with liquid nitrogen in a mortar on dry ice, and total messenger RNA (mRNA) was extracted from the tissue using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. After treating samples with RNase-free deoxyribonuclease I (Takara Bio, Otsu, Japan), 500 ng of total mRNA from each sample was reverse transcribed using SuperScript II (Invitrogen). The reaction time was 60 minutes at 42°C. Thereafter, 1 μ l of each reaction product was amplified in a 15- μ l PCR mixture containing 0.5 units TaKaRa EX Taq (Takara Bio) and 10 pmoles of each primer to detect mRNA specific to each molecule.

Amplifications were performed in a Program Temp Control System (DNA Engine PTC-200; MJ Research, Waltham, MA) for 35 cycles after an initial denaturation step at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, annealing for 30 seconds at 60°C, and extension at 72°C for 30 seconds, with a final extension at 72°C for 3 minutes. The PCR products (10 μ l) were electrophoresed in a 3% agarose gel and detected by ethidium bromide staining. The nucleotide sequences of the primers for each of these genes are as follows: for AGC1, 5'-TCCAAACCAACCCGACAAT-3' (forward) and 5'-TTCTGCCAAAGGGTTCTG-3' (reverse); for Col2A1, 5'-GCTCGAGGAGACTGGTG-3' (forward)

and 5'-ACCTGGGGGACCATCAGA-3' (reverse); for Col9A1, 5'-GGTCCTCCGGGGAAGCCT-3' (forward) and 5'-CCAACCTCTCCCGGGGT-3' (reverse); for Col10A1, 5'-CGAGGTCTGTGTGGCCCTAC-3' (forward) and 5'-CCTGGGTCTCTGTCCGCT-3' (reverse); for Col11A1, 5'-ATTGCCACCAGTCAACTGCT-3' (forward) and 5'-TTGGACTGTGCCTCCGTC-3' (reverse); for MyoD1, 5'-ACTACAGCGGCGACTCAGAC-3' (forward) and 5'-GTG-GAGATGCGCTCCACTAT-3' (reverse); and for Cbfa1/Runx2, 5'-TGCTTCATTGCGCTCACAAAC-3' (forward) and 5'-TAGAAGTTGTGCCCTCTGTTG-3' (reverse).

Real-time quantitative RT-PCR. Quantitative RT-PCR assay for type II collagen was carried out with the use of gene-specific expression-labeled fluorescent probes and sets of specific primers in an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). On the basis of the published sequence of rat type II collagen, specific primer pair and probe sets were designed with the aid of Primer Express software, version 2.0 (Applied Biosystems). The sequences of the primers were 5'-AGGCGCTTCTGTAAACCA-3' (forward) and 5'-GACCAGTTGCACCTTGAGGAC-3' (reverse), and the probe was 5'-TTCCCGGAGCCAAAGGATCTGCTG-3'. We used 6-carboxyfluorescein for type II collagen as the 5' fluorescent reporter for the probe, while we added 6-carboxy-tetramethylrhodamine (Tamura Pharmaceutical, Osaka, Japan) to the 3' end as a quencher.

Standard curves were constructed with the use of dilutions of accurately determined pCR2.1 plasmid vector (Invitrogen) containing complementary DNA (cDNA) products of type II collagen. A relative standard curve representing 10-fold dilutions of a rat type II collagen cDNA ranging from 2×10 to 2×10^5 copies/ μ l was used for linear regression analysis of the samples. PCR was carried out in 50 μ l of reaction mixture containing 3 μ l of the RT reaction, 1 \times Universal Master Mixture (Applied Biosystems), 500 nM of each primer, and 200 nM of the Taqman probe purchased from Applied Biosystems.

To compensate for the differences in cell number and/or RNA recovery, the copy number of type II collagen mRNA was determined relative to 18S ribosomal RNA (rRNA) (Applied Biosystems), which was also analyzed quantitatively. Thus, a partial cDNA of 18S rRNA was amplified from rat bone and cartilage samples using a specific primer set for 18S rRNA, and then subcloned into pCR2.1 (Invitrogen). Ten-fold dilutions of the resultant vector, pCR2.1-18S rRNA, ranging from 2×10 to 2×10^5 copies/ μ l, were used to construct a relative standard curve for 18S rRNA. The PCR mixture was basically the same as that for type II collagen, except for 200 nM of an 18S rRNA-specific Taqman probe set carrying a 5'-VIC reporter label and 3'-TAMURA quencher group, and 500 nM of the specific primer for 18S rRNA that was purchased from Applied Biosystems. These samples were placed in the ABI PRISM 7700 Sequence Analyzer and preheated at 95°C for 10 minutes, then amplified for 50 cycles of 95°C for 15 seconds, followed by 60°C for 1 minute. These experimental protocols were in compliance with the guidelines established by the Institutional Committee for Animal Care and Experiments of Shinshu University.

Statistical analysis. The histologic score was statistically analyzed using the SPSS software package (SPSS Japan, Tokyo, Japan). The Kruskal-Wallis H test followed by the

Mann-Whitney U test was used to determine differences between the groups.

RESULTS

Cartilage induction in diffusion chambers by rHuBMP-2. The tissue mass harvested from group B10 chambers (those receiving 10 μg rHuBMP-2) had a gelatinous appearance, with no histologic features characteristic of cartilage until 2 weeks after implantation. At 3 and 4 weeks after implantation, the tissue had a pale, opaque gelatinous appearance and revealed some cartilaginous characteristics along the inner surface of the filter membranes of the chamber on histologic examination (Figures 1A–H).

At 5 and 6 weeks postimplantation (Figures 1I–P), the cells of group B10 formed an elastic tissue mass with opaque appearance and no evidence of calcification on radiography (Figure 2B). Histologic examination of the opaque tissue mass in the chambers indicated normal features of cartilage, with round chondrocytic cells enclosed in a metachromatic matrix, as revealed by toluidine blue staining (Figures 1L and P).

Small amounts of osseous tissue were found on the outer or host-side surfaces of the membrane filter of those samples. In one chamber with an accidental “hole” on the membrane filter, containing 5-week postimplantation tissue of group B10, the tissue became a hard mass with a reddish appearance; on radiography, the tissue was highly calcified (Figure 2C) and showed a normal histologic appearance of bone with hematopoietic marrow (Figure 2A). In contrast, the tissue of groups B0 (Figure 1) and B1 (chambers without rHuBMP-2 or with 1 μg rHuBMP-2, respectively) showed a gelatinous appearance with no histologic evidence of cartilage formation throughout the experimental period.

PCR findings. PCR analysis of the tissue in the diffusion chambers revealed a consistent expression of types X and XI collagen (Figure 3). Expression of type X collagen gradually increased in group B10. The expression of type II collagen was detected at low levels 2 days after implantation in group B10 (Figure 3). After 4 days, the expression of type II collagen was clearly detected in group B10. The expression of Cbfa1/Runx2 was clearly detected after 96

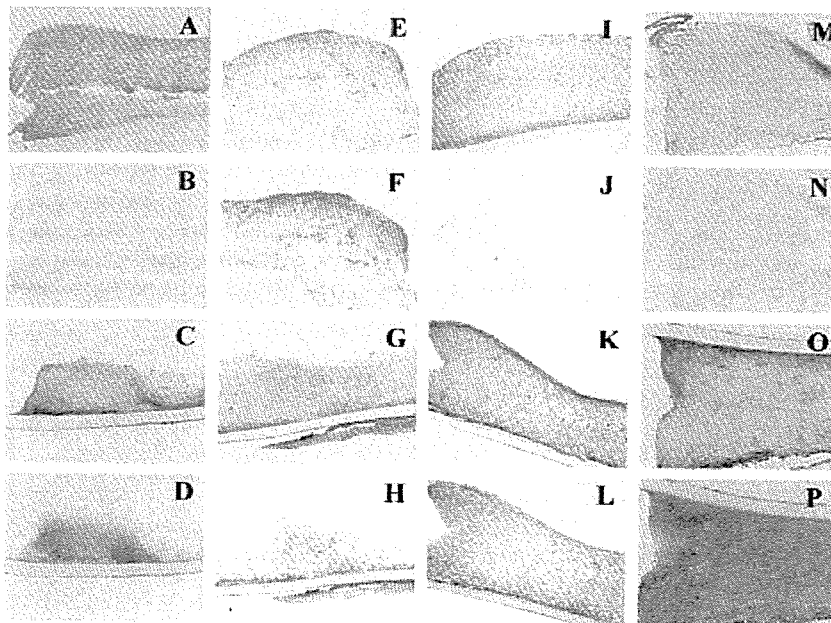


Figure 1. Cartilage formation in the diffusion chamber. Tissue pellets in diffusion chambers were examined at 3 weeks (A–D), 4 weeks (E–H), 5 weeks (I–L), and 6 weeks (M–P) postimplantation, in group B0 (without recombinant human bone morphogenetic protein 2 [rHuBMP-2]) (A, B, E, F, I, J, M, and N) compared with group B10 (with 10 μg rHuBMP-2) (C, D, G, H, K, L, O, and P). (Stained with hematoxylin and eosin in A, C, E, G, I, K, M, and O, with toluidine blue in B, D, F, H, J, L, N, and P; original magnification $\times 40$.)

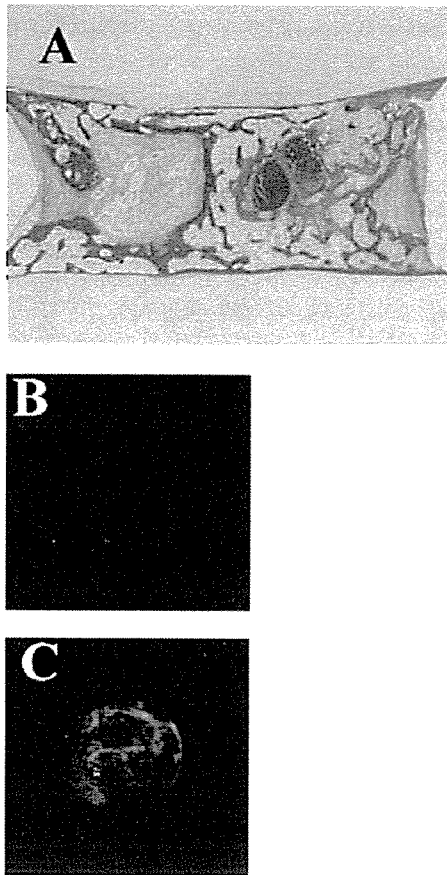


Figure 2. Histologic and radiologic evaluations of engineered cartilage tissue. For the tissue pellet in the diffusion chamber with an accidental hole on the filter (at 5 weeks posttransplantation; obtained from group B10), the normal histologic appearance of bone is clearly visible (stained with hematoxylin and eosin; original magnification $\times 20$) (A), and the soft radiographic view shows bone trabeculae (C). Another soft radiographic view of group B10 tissue (same sample as in Figures 1K and L) shows no calcification (B).

hours in group B10 only (Figure 3). The expression of MyoD1 was not detected in either group at any time point.

Real-time PCR revealed that the expression of type II collagen increased markedly at 4 days after implantation (Figure 4). A high level of aggrecan was seen in group B10 after 2 days. Type IX collagen was weakly expressed in group B10 after 4 days, but increased significantly after 1 week. Low expression levels of aggrecan and type II collagen were detected in all groups at later time points in the study.

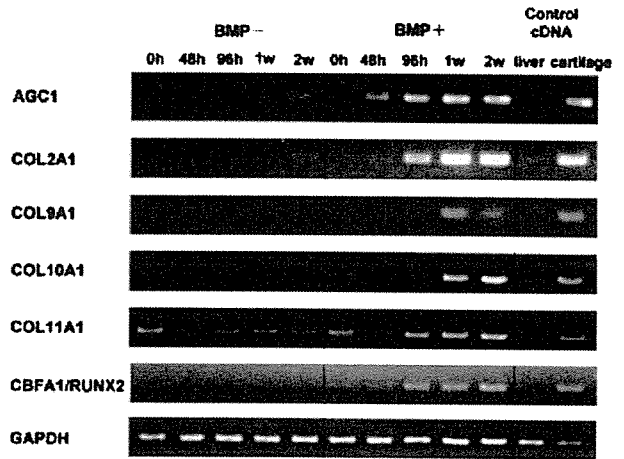


Figure 3. Reverse transcription-polymerase chain reaction analysis. Expression of types X and XI collagen (COL10A1 and COL11A1, respectively) was detected consistently in both groups (with 10 μ g recombinant human bone morphogenetic protein 2 [BMP+; group B10] and without [BMP-]) throughout the experimental period. Expression of type IX collagen (COL9A1) was detected after 96 hours, indicating that effective cartilage matrix synthesis begins 3 or 4 days after implantation. Expression of type II collagen (COL2A1) was detected at low levels after 2 days in group B10 only, and after 4 days, it became more prominent. The expression of core binding factor $\alpha 1$ / runt-related gene 2 (CBFA1/RUNX2) was clearly detected after 96 hours in group B10 only. AGC1 = aggrecan.

Repair of cartilage defects by transplantation of the engineered cartilage. The osteochondral defects that received the cartilaginous tissue mass, which was generated for 5 weeks in diffusion chambers containing tissue from group B10, were restored to a normal appearance at 24 weeks after transplantation. Upon examination, the site of the defects had a smooth surface and no

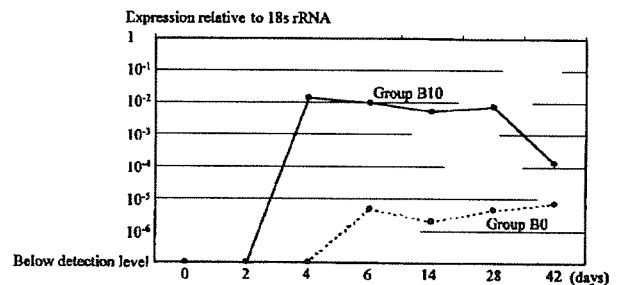


Figure 4. Real-time polymerase chain reaction analysis for type II collagen mRNA. After 4 days, expression of type II collagen mRNA was markedly increased in group B10 (with 10 μ g recombinant human bone morphogenetic protein 2 [rHuBMP-2]). Group B0 = without rHuBMP-2; rRNA = ribosomal RNA.

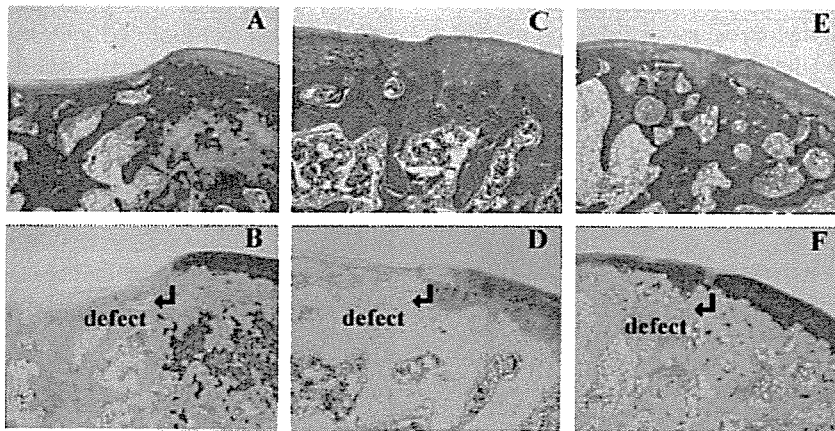


Figure 5. Osteochondral defects of a rat knee repaired with tissue pellets generated in diffusion chambers 24 weeks after transplantation. A and B, Defect with no implant. C and D, Defect implanted with tissue pellet generated in the chamber of group B0 (without recombinant human bone morphogenetic protein 2 [rHuBMP-2]). E and F, Defect implanted with tissue pellet generated in the chamber of group B10 (10 μ g rHuBMP-2). (Stained with hematoxylin and eosin in A, C, and E, with toluidine blue in B, D, and F; original magnification \times 40.)

obvious border with the surrounding normal articular cartilage (Figures 5E and F). The defects were filled with a layer of cartilage exhibiting subchondral cancellous bone connecting to the original subchondral bone. Although the architecture of the repaired articular cartilage was similar to that of normal cartilage with regard to cell arrangement, differences were noted. A tidemark was visible at the base of the cartilage layer adjacent to the subchondral bone, and the thickness of the regenerated cartilage was slightly less than that of the neighboring normal articular cartilage.

In contrast, the defects transplanted with tissue mass from group B0 were partially repaired, with a depressed surface visible at the defect site (Figures 5C and D). Histologic assessment of the defects that received either the tissue from group B0 or no implant revealed a small amount of fibrocartilage, with slightly positive metachromatic staining at the periphery of the defects and dominant fibrous tissue in the defect space.

Upon histologic evaluation of the knee cartilage after repair, the average histologic score (Wakitani's score) was 4.25 for group B10, 11.67 for group B0, and 14.00 for the defect-only group. The score for group B10 was significantly better than that for group B0 ($P = 0.032$) and the defect-only group ($P = 0.002$).

DISCUSSION

The experimental data presented herein indicate the capacity of rHuBMP-2 to induce the differentiation of young muscle-derived mesenchymal cells into chondrocytes within diffusion chambers in *in vivo* conditions. The resultant heterotopic cartilage formation represents a significant volume of induced tissue mass derived from these cells.

In order to induce the cartilage tissue, the diffusion chamber system was essential. When vascular invasion into the chamber occurred as a result of membrane seal failure, new bone with hematopoietic marrow was seen in the chambers harvested at 5 weeks after transplantation. Budenz and Bernard have reported similar findings (14). This bone was likely formed through the process of endochondral ossification, as deduced from classic reports describing the actions of BMP (15) and from comparison with the process of direct ossification (16,17). In the process of BMP-induced endochondral bone formation, cartilage is formed in the early phase of the bone-forming process. The cartilage tissue is then absorbed by invading vascular connective tissue and replaced by newly formed bone, as seen in embryonic osteogenesis (18) and in callus in fracture repair (19). During the process of ectopic bone formation elicited by