

ORIGINAL ARTICLE

Hiroshi Ohta · Shigeyuki Wakitani · Keiji Tensho
Hiroshi Horiuchi · Shinji Wakabayashi · Naoto Saito
Yukio Nakamura · Kazutoshi Nozaki · Yuuki Imai
Kunio Takaoka

The effects of heat on the biological activity of recombinant human bone morphogenetic protein-2

Received: January 17, 2005 / Accepted: May 25, 2005

Abstract This study was designed to investigate effects of heat on the bone-inducing activity of recombinant human bone morphogenetic protein (rhBMP)-2. rhBMP-2 samples were heated at 50, 70, 90, or 100°C for 15 min, or 1, 2, 4, or 8 h, or autoclaved at 120°C for 15 min. The bone-inducing activity of the rhBMP-2 before and after heating was assayed in *in vivo* and *in vitro* systems. For the *in vivo* assay, 5 µg rhBMP-2 samples were impregnated into porous collagen disks (6 mm in diameter, 1 mm thickness), freeze dried, and implanted into the back muscles of ddY mice. Three weeks later, the implant was harvested from the host and examined for ectopic new bone tissue by radiography. The new bone mass was quantified by single-energy X-ray absorptiometry. The *in vitro* activity of the rhBMP-2 was assayed by adding the BMP sample at a concentration of 100 ng/ml to cultures of MC3T3-E1 cells. After 48 h, the alkaline phosphatase activity was measured. After heating at 50° or 70°C, no significant reduction in bone-inducing activity was noted in either *in vivo* or *in vitro* assay systems unless the protein was exposed to sustained heat at 70°C for 8 h, based on *in vitro* assay data. However, heating above 90°C and for longer periods led to a decrease in the biological activity of the rhBMP-2 in a time- and temperature-dependent manner. rhBMP-2 was rendered inactive when exposed to temperatures at or in excess of 120°C.

Key words Heat stability · Bone induction · Molecular structure

H. Ohta · S. Wakitani (✉) · K. Tensho · H. Horiuchi · S. Wakabayashi · N. Saito · Y. Nakamura
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. Nozaki
Applied Pharmacology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical, Tsukuba, Japan

Y. Imai · K. Takaoka
Department of Orthopaedic Surgery, Osaka City University Graduate School of Medicine, Osaka, Japan

Introduction

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor- β superfamily [1] and possess unique biologic activity that leads to new bone formation [2–4]. BMP-2 is a member of the BMP family, and the human form (hBMP) has been successfully synthesized by the use of DNA recombination technology (rhBMP) for commercial purposes. This protein is now being evaluated as a bone-graft substitute for the treatment of nonunion fractures, bone defect repairs, and spinal fusions [5–11]. In order to use rhBMPs clinically, it is necessary to understand how the molecular structure or bone-inducing activity of BMPs may be modified during transportation, storage, and intraoperative handling. In particular, the stabilization of BMP-retaining devices, or the use of BMP-2 in combination with heat-generating bone cement, are two instances where the stability of the protein would be challenged. Several authors have described the heat resistance of natural crude BMP extracted from the rat, rabbit, and human [12–16], but there has been little work done to look at the effects of heat on the stability and biological activity of rhBMP-2. This study describes how the biological activity and molecular structure of rhBMP respond to varying degrees of heat using *in vivo* and *in vitro* assay systems.

Materials and methods

Heat treatment of rhBMP-2

rhBMP-2 was provided by Yamanouchi Pharmaceutical (Tokyo, Japan) in a buffered solution (1 mg rhBMP-2/ml). This was diluted 3-fold (15 µl saline containing 5 µg rhBMP-2) and heated at 50, 70, 90, or 100°C for 15 min, or 1, 2, 4, or 8 h using a heat block (Dry Thermo Unit, Taitec, Saitama, Japan), or autoclaved at 120°C for 15 min. The biological activity of these heat-treated rhBMP-2 samples was then evaluated in *in vitro* and *in vivo* experiments to examine

changes in the biological activities of the rhBMP-2. As a control, rhBMP-2 that had not been exposed to heat was used in the assays.

Cell culture

A mouse osteoblastic cell line MC3T3-E1 was obtained from the RIKEN Cell Bank (Tsukuba Science City, Japan). These cells were seeded at a cell density of 3×10^5 cells/100-mm plastic dish and cultured with α -minimal essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco) at 37°C in an incubator with 95% humidified air and 5% CO₂.

Induction of alkaline phosphatase (ALP) by rhBMP-2

The biological activity of the rhBMP-2 was assayed based on the observation that BMP is capable of inducing expression of ALP in a dose-dependent manner. ALP activity is an early marker of osteoblastic differentiation [17]. The rhBMP-2 samples, heated as described above, were added to the MC3T3-E1 cells at a final concentration of 100 ng/ml. After 48 h, the osteoblastic cells were washed twice with phosphate-buffered saline, scraped from each well into 0.3 ml 0.5% NP-40 containing 1 mM MgCl₂ and 10 mM Tris (pH 7.5), and sonicated twice for 20 s with a sonicator (model W-220; Wakenyaku, Kyoto, Japan). The cell lysates were then centrifuged for 10 min at 3000 r.p.m., and the supernatants were used for the enzyme assay. ALP activity was assayed using the method of Kind-King [18] and a test kit (Alkaline Phosphatase K, Wako Pure Chemical Industries, Osaka, Japan) with phenylphosphate as a substrate.

The enzyme activity was expressed in King-Armstrong (K-A) units normalized to the protein content of the sample. The protein content was determined with a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL, USA) using bovine serum albumin as the standard. Each heat-treated rhBMP sample was measured in three separate dishes, and the ALP activity from each group was expressed as mean \pm SD.

Preparation of BMP-containing collagen disks

In order to evaluate the influence of heating on the bone-inducing activities of the rhBMP-2, a classical *in vivo* experimental system of ectopic bone induction in mice was utilized. In this system, BMP was combined with collagen and implanted into muscle, whereupon an ectopic ossicle was elicited if the BMP was biologically active. In order to deliver the BMP, commercially available porous collagen sheets made from bovine Achilles tendon (Helistat Integra Life Sciences, Plainsboro, NJ, USA) were cut into round strips (6 mm in diameter, 1 mm thick), soaked in the sample solutions containing 5 μ g of either the heated or nonheated control rhBMP-2, freeze-dried, and stocked at -20°C until implantation into mice.

Assay for the bone-inducing activities of heat-treated rhBMP-2

One hundred and seventy-six male ddY mice, 5 weeks of age, were purchased from Nippon SLC Co. (Shizuoka, Japan). The mice were housed in cages with free access to food and water for 1 week before the start of the experiment. Prior to surgery for implantation of the collagen/rhBMP-2 composite collagen disks, the mice were anesthetized with diethylether. The BMP-retaining pellets were implanted into the left dorsal muscle pouches (one pellet per animal). Twenty-two groups of mice (8 mice in each group) were implanted with collagen disks containing 5 μ g rhBMP-2 that had never been heated (control), or heated at 50, 70, 90, or 100°C for 15 min, or for 1, 2, 4, or 8 h, or autoclaved at 120°C for 15 min. The mice were fed for 3 weeks and then killed. The implants were harvested and examined for ectopic new bone formation *in situ* by radiographic and histological methods. X-rays of the harvested tissues were taken with a soft X-ray apparatus (Sofron Co., Tokyo, Japan). In order to quantify the ectopically induced bony mass, the bone mineral content (BMC, mg) of each ossicle was measured by single-energy X-ray absorptiometry using a bone mineral analyzer (DCS-600; Aloka Tokyo, Japan). For the histology, the harvested tissue mass from each group was fixed in neutralized 10% formalin, defatted in chloroform, decalcified with 10% ethylenediamine tetraacetic acid, and embedded in paraffin wax. Sections of 5 μ m thickness were cut, stained with hematoxylin-eosin, and observed under a light microscope.

All procedures for the animal experiments were carried out in compliance with the guidelines of the Institutional Animal Care Committee of Shinshu University.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

In order to examine the effects of heat on the rhBMP-2 molecules, heated or nonheated samples of the protein were electrophoresed on a SDS-PAGE slab gel. Five micrograms of nonheated rhBMP-2 or protein heated at 90°C on a heat block or at 120°C in an autoclave for 2 h were dissolved into 20 μ l sample buffer solution (0.5 μ M, pH 6.8 Tris-HCl buffer solution/0.2% SDS/10% glycerol/0.01% bromophenol blue) with or without 100 mM dithiothreitol (DTT, a disulfide-bond reducing agent) and boiled for 5 min. Each sample was run on a SDS gel at 40 mA for 60 min. The gel was then stained with Coomassie brilliant blue and destained in 10% acetic acid solution.

Statistical analysis

Quantitative data were expressed as the mean \pm SD. Differences between control and experimental groups were considered to be statistically significant at $P < 0.05$ using the Kruskal-Wallis *H*-test with Bonferroni correction.

Fig. 1. The influence of heat treatment on rhBMP-2-induced alkaline phosphatase (ALP) activity of MC3T3-E1 cells. Data are means \pm SD of three culture wells. A significant difference from the control is indicated as * ($P < 0.05$)

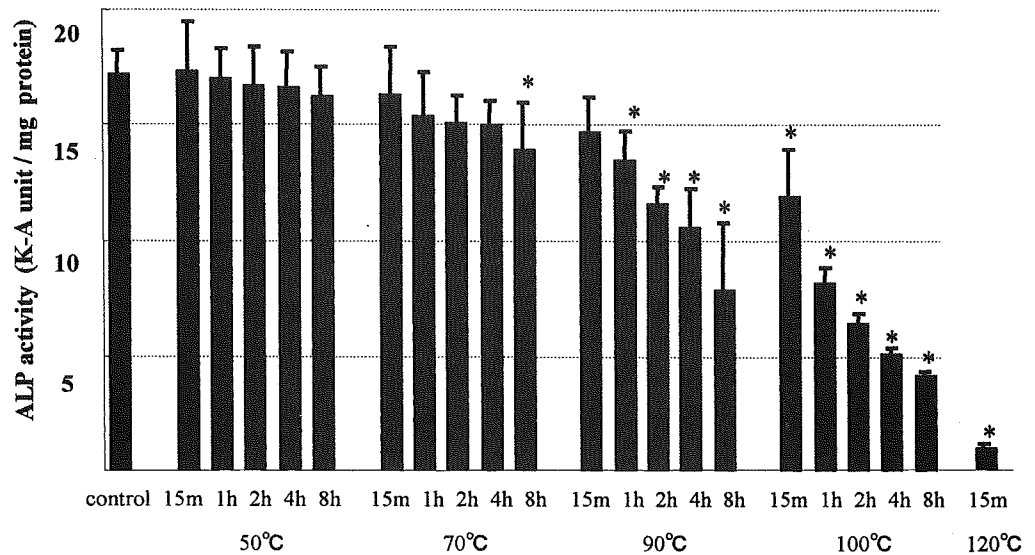
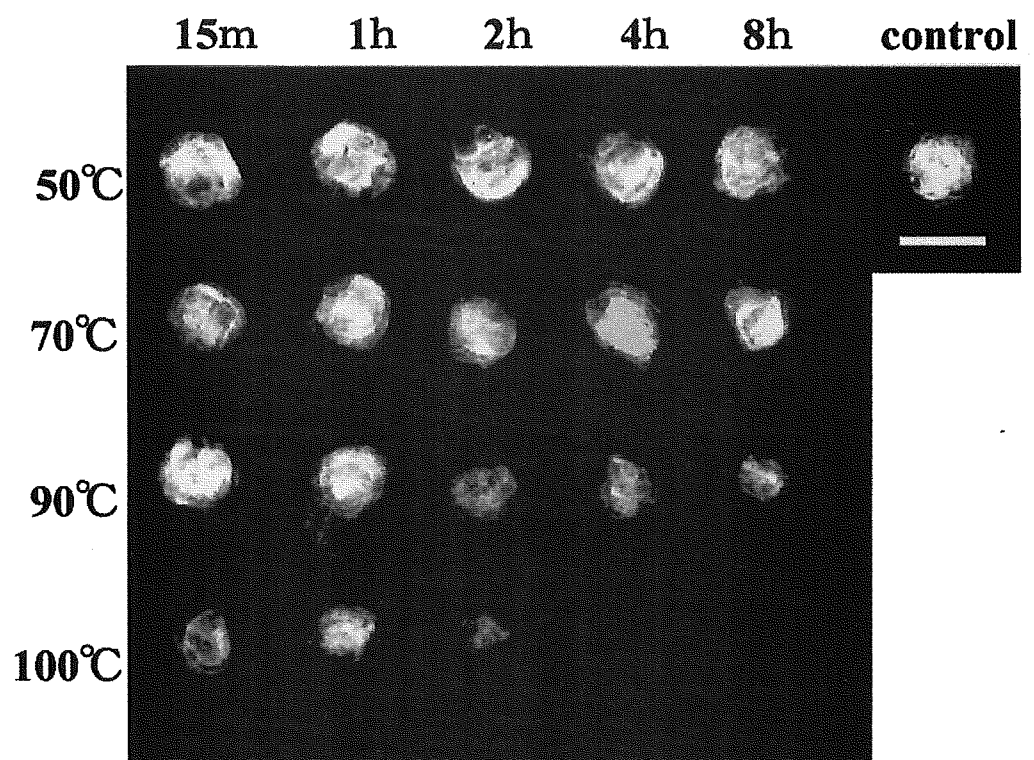


Fig. 2. Soft X-ray photograph of an ossicle formed at 3 weeks after implantation. A typical implant from each group is shown. Scale bar = 6 mm. There is no difference in the trabecular pattern within ossicles from each group. However, a reduction in size was observed over 90°C in a time-dependent manner. In the groups heated at 100°C for 4 h, at 100°C for 8 h, and at 120°C for 15 min, there was no firm mass around the implant location. Thus, we decided not to collect tissues from these areas for further analysis (softex and bone mineral content)



Results

Effects on ALP expression by heated rhBMP-2

Figure 1 shows the effects of the heat treatment on the ALP-inducing activity of rhBMP-2 in MC3T3-E1 cells. ALP-inducing activity was preserved at 50°C even after the rhBMP-2 was heated at this temperature for 8 h. A notable reduction in the ALP-inducing activity of rhBMP-2 was seen using protein samples heated at 70°C for 8 h. At 90°C, a significant reduction of activity was noted by heating for 1 h or more in a time-dependent manner. By heating

at 100°C, the time-dependent reduction was profound. The ALP-inducing activity was reduced to less than 1 unit/mg protein when the rhBMP-2 was heated at 120°C for 15 min.

Change in the bone-inducing activity of rhBMP-2 by heating

Figure 2 shows representative ossicles elicited in mice within a 3-week period by implanting collagen disks impregnated with 5 μ g rhBMP-2 heated at 50, 70, 90, or 100°C for 15 min, or for 1, 2, 4, or 8 h. Ossicle size tended to become

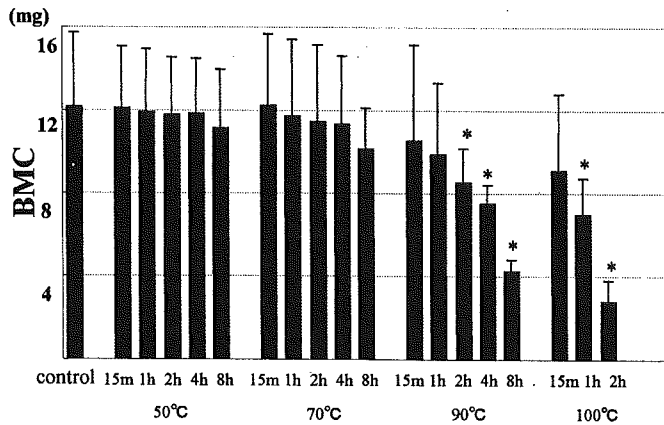


Fig. 3. The bone mineral content (BMC) of the tissues harvested at 3 weeks after surgery. Data are means \pm SD of 8 samples. A significant difference from the control is indicated as * ($P < 0.05$). There was no significant difference between the BMC of the 50°C and 70°C groups and the control group. However, the mean BMCs of groups heated at 90°C for 2, 4, or 8 h and the one heated at 100°C for 1, or 2 h ($P < 0.01$) were significantly lower than that found in the control group. In the groups heated at 100°C for 4 h, at 100°C for 8 h, and at 120°C for 15 min, we found no evidence of new ectopic bone formation, as described in the legend to Fig. 2

small when the rhBMP-2 was treated at 90°C for 2 h or more. After treatment for more than 4 h at 100°C or for 15 min at 120°C, the bone-inducing activity of rhBMP-2 was lost.

Figure 3 shows the mean BMC values of the ossicles from each of the groups. The mean (\pm SD) BMC of the control group was 12.2 ± 3.56 mg. In the group treated at 50°C for 8 h, the mean BMC was 11.2 ± 2.81 mg, which was not significantly lower than that of the controls. In the group with protein heated at 70°C, the mean BMC in the group treated at 70°C for 15 min was 12.2 ± 3.45 mg. The BMC in the 70°C group showed a downward trend, but no statistically significant difference was observed when compared with the control group. Similarly, in the group using protein heated at 90°C, the BMC of the harvested ossicles decreased with an extension of the heating time. The BMC of ossicles from the groups treated at 90°C for 15 min or 90°C for 1 h were not significantly different from those of the control group. However, the mean BMC values of the groups treated at 90°C for 2 h (8.48 ± 1.68 mg), at 90°C for 4 h (6.98 ± 1.06 mg), or at 90°C for 8 h (4.26 ± 0.53 mg) were significantly reduced when compared with those of the control group ($P < 0.01$). Again, in the group treated at 100°C for 15 min, the BMC was less than that of the controls, although there was no significant difference between the values. The mean BMC of the groups treated at 100°C for 1 h (6.96 ± 1.71 mg) or at 100°C for 2 h (2.80 ± 0.99 mg) were significantly lower than those from the control group ($P < 0.01$).

Histological analysis of the ossicles from all groups revealed normal bone histology with hematopoietic marrow and bony trabeculae (Fig. 4).

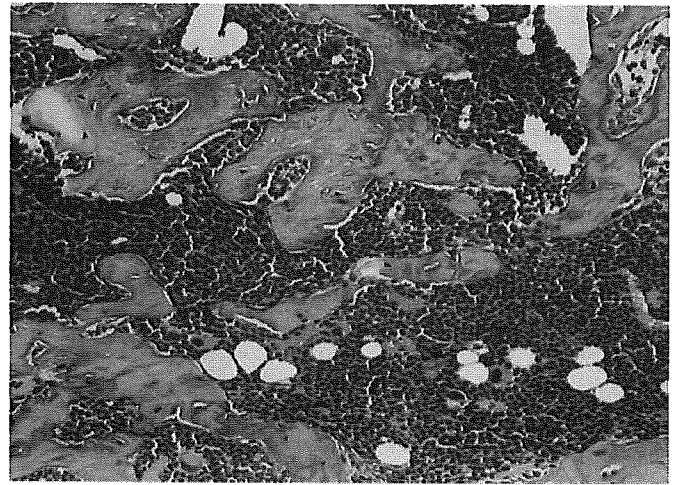


Fig. 4. Photomicrograph of the tissue harvested at 3 weeks after implantation (heated to 50°C for 8 h) (H&E, $\times 200$). Normal bone histology with hematopoietic marrow and bony trabeculae was observed

Changes in the SDS-PAGE profile of rhBMP-2 by heating

The SDS-PAGE profiles of rhBMP-2 before and after heating at 90 or 120°C are shown in Fig. 5. The original rhBMP-2 showed three dark bands at around 30-k Daltons (kD) and two light bands below 20kD without DTT (lane 1). With DTT, the 30-kD bands became lighter and the lower molecular-sized bands became darker (lane 4). By heating at 90°C for 2 h, in the absence of DTT the 30-kD bands became a little indistinct and two lower sized bands disappeared (lane 2), and with DTT the 30-kD bands became lighter and the lower bands became darker (lane 5). By heating at 120°C for 2 h, in the absence of DTT the 30-kD bands remained although the three bands became indistinct (lane 3), and with DTT both of the 30-kD bands and the lower bands were lost (lane 6).

Discussion

BMPs (BMP-2, BMP-7) with potent bone-inducing activity have been successfully produced and developed for clinical use through DNA recombinant technology. Examples of clinical applications include the repair of damaged bone, the reconstruction of bone defects resulting from trauma, and resection of bone tumors. In terms of a successful clinical outcome, the bone-inducing activity of rhBMP-2 must be able to withstand the conditions associated with transportation and storage. In addition, rhBMP has to survive sterilization and the high temperatures encountered when implants are used in close contact with curing bone cement. Previous studies have qualitatively described the stable character of natural BMP based on the ability of crude insoluble BMP-retaining decalcified bone matrix to induce ectopic new bone. Nakanishi et al. [12] reported that rabbit bone-derived BMP extracts elicited ectopic bone after

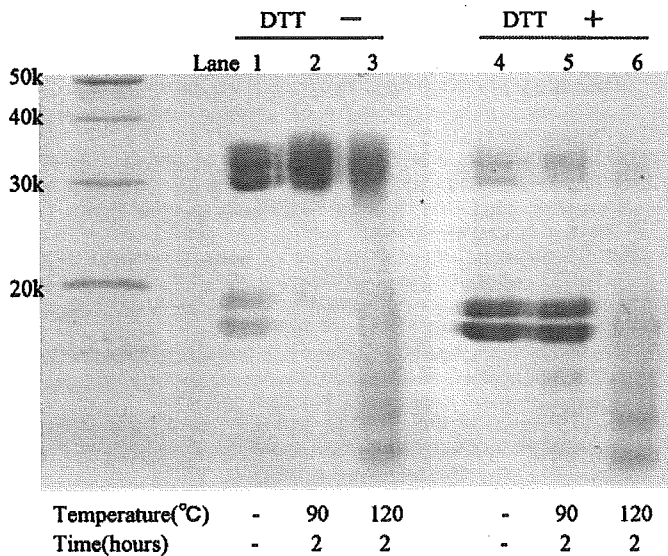


Fig. 5. SDS-PAGE profiles of rhBMP-2 before (*lane 1*) and after (*lane 2*) heating at 90°C and at 120°C (*lane 3*) for 2h. *Lanes 4, 5, and 6* show the respective samples under reducing conditions in the presence of dithiothreitol (DTT, a disulfide-bond reducing agent). The original rhBMP-2 showed three dark bands at around 30k Daltons (kD) and two light bands below 20kD without DTT (*lane 1*). With DTT, bands at 30kD became lighter and the lower bands became darker (*lane 4*), which indicates that the dimers were reduced to monomers. By heating at 90°C for 2h in the absence of DTT, the major 30-kD bands became a little indistinct, and the two lower-sized bands disappeared (*lane 2*). With DTT, bands at 30kD became lighter and the lower bands became darker (*lane 5*), indicating that the bands at 30kD without DTT maintained a dimeric structure. By heating at 120°C for 2h, the original size of rhBMP-2 was maintained, although the three distinct bands were lost (*lane 3*). With DTT, both of the original 30-kD bands and those lower molecular-sized bands were lost (*lane 6*), indicating complete denaturation of the original configuration of the molecules

heating at 70°C for 10min. However, the bone-inducing activity was lost when the sample was heated at over 100°C for 20min. Some reports have also described similar results using crude BMP extracts as the bone-inducer in rodent models [13,14]. One report demonstrated that BMP activity was retained in a rabbit model after heating the matrix protein at 170°C for 10min or at 140°C for 30min [15].

In this study, the bone mineral content of the rhBMP-2-induced ossicles was used to evaluate the bone-inducing activity of the heat-treated rhBMP-2 in an *in vivo* system. In addition, the ability of rhBMP-2 to induce ALP activity in osteoblastic cells in culture was used as a model to confirm the results from the *in vivo* experiments. The ALP-inducing activity of the rhBMP-2 was little affected by heat treatment up to 70°C for 8h or up to 90°C for 1h, but then decreased gradually with increasing temperature and time. The ALP-inducing activity was eventually lost after heating the rhBMP-2 at 120°C, a treatment that denatured the molecular structure of the rhBMP-2 as shown on a SDS-PAGE profile. These results were in accordance with the *in vivo* results, and suggest that rhBMP-2 is largely stable in nature. The use of autopolymerising polymethylmethacrylate (PMMA)-based bone cement is the most common method

chosen for prosthetic component fixation in total joint replacement surgery. During the polymerization of PMMA, high peak temperatures are reached. The temperature peak ranges from 48°C to 105°C at the bone-cement interface, and from 80°C to 124°C in the cement. The exposure time over 50°C is reported to be between 30 and 400s [19]. Thus, the data from the present study suggest that contact with curing bone cement would not affect the bone-inducing activity of rhBMP-2. Sterilizing by autoclave (120°C) would effectively inactivate the biological action of this protein, but sterilizing by ethylene oxide gas (60°C for 3h or 40°C for 6h) would not, as reported previously [20].

The heat-stable character of BMP may be derived from its molecular structure. It is known that the bioactive BMP-2 molecule consists of a homodimer which is essential for its biological activity. The homodimer consists of two 114 amino acid monomers with 7 cysteine residues that form three intramolecular disulfide bonds, with one intermolecular disulfide bond forming a cysteine knot [21]. The heat-resistant nature of the BMP might be explained by the cysteine-knot formation, which is known to contribute to the structural stability of the protein.

The biological activity of the rhBMP was reduced following heat denaturation at 90°C for 2h as evaluated in an *in vivo* assay system, and for 1h in an *in vitro* assay system. At these higher temperatures, the reduction in rhBMP-2 activity was temperature- and time-dependent. In order to visualize the heat-dependent changes in molecular structure, changes in the SDS-PAGE gel profile after heating were observed. As previously described, the rhBMP-2 molecule has a molecular size of around 16kD with one N-glycosylation site, and it forms a homodimer which has biological activity [6-8,22]. Therefore, the three bands at 30kD in the control lane on the SDS-PAGE with no disulfide bond reduction indicated dimeric BMP-2 molecules with 2, 1, or no sugar chains, respectively (see Fig. 5). Further, the two faint bands below 20kD were monomers with or without glycosylation because of their similarity to the molecular sizes of the DTT-reduced rhBMP-2 monomer. By heating at 90°C for 2h, in the absence of DTT the dimer bands became a little indistinct and the monomer bands were lost, which indicated complete deformation of the rhBMP-2 monomer. With DTT, the dimer bands became lighter and the monomer bands became darker. These changes most likely reflect partial deformation of the original three-dimensional configuration of the rhBMP-2 dimers, which corresponds with the partial loss of bone inducing activity. A higher temperature (120°C for 2h) made the dimer bands more indistinct and eventually degraded the structure of the monomers, as seen on DTT-reduced SDS-PAGE, with the resultant loss of biological activity.

In conclusion, the biological activity and molecular structure of rhBMP-2 were shown to be fairly resistant to heat treatment. In considering the potential clinical uses of rhBMP-2, the stable character of the protein suggests that it would not need to be cooled during transportation or storage. In addition, it was found that contact with heated material such as curing bone cement would not affect the

bone-inducing activity of the rhBMP-2. However, the auto-claving of rhBMP-2 causes this protein to lose its biological activity based on the results of the present study.

References

- Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, Wozney JM (1990) Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone. *Proc Natl Acad Sci USA* 87:9843-9847
- Urist MR (1965) Bone formation by autoinduction. *Science* 150:893-899
- Urist MR, Iwata H (1973) Preservation and biodegradation of the morphogenetic property of bone matrix. *J Theor Biol* 38:155-167
- Reddi AH, Anderson WA (1976) Collagenous bone matrix-induced endochondral ossification hemopoiesis. *J Cell Biol* 69:557-572
- Sampath TK, Muthukumaran N, Reddi AH (1987) Isolation of osteogenin, an extracellular matrix-associated, bone-inductive protein, by heparin affinity chromatography. *Proc Natl Acad Sci USA* 84:7109-7113
- Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: molecular clones and activities. *Science* 242:1528-1534
- Wang EA, Rosen V, Cordes P, Hewick RM, Kriz MJ, Luxenberg DP, Sibley BS, Wozney JM (1988) Purification and characterization of other distinct bone-inducing factors. *Proc Natl Acad Sci USA* 85:9484-9488
- Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, Israel DI, Hewick RM, Kerns KM, LaPan P, Luxenberg DH, McQuid D, Moutsatsos IK, Nove J, Wozney JM (1990) Recombinant human bone morphogenetic protein induces bone formation. *Proc Natl Acad Sci USA* 87:2220-2224
- Wozney JM (1992) The bone morphogenetic protein family and osteogenesis. *Mol Reprod Dev* 32:160-167
- Takaoka K, Yoshikawa H, Hashimoto J, Miyamoto S, Masuhara K, Nakahara H, Matsui M, Ono K (1993) Purification and characterization of a bone-inducing protein from a murine osteosarcoma (Dunn type). *Clin Orthop* 292:329-336
- Takaoka K, Yoshikawa H, Hashimoto J, Masuhara K, Miyamoto S, Suzuki S, Ono K, Matsui M, Oikawa S, Tsuruoka N, Tawaragi Y, Inuzuka C, Katayama T, Sugiyama M, Tsujimoto M, Nakanishi T, Nakazato H (1993) Gene cloning and expression of a bone morphogenetic protein derived from a murine osteosarcoma. *Clin Orthop* 294:344-352
- Nakanishi K, Sato K, Sato T, Takahashi M, Fukaya N, Miura T (1992) Preservation of bone morphogenetic protein in heat-treated bone (in Japanese). *Nippon Seikeigeka Gakkai Zasshi (J Jpn Orthop Assoc)* 66:949-955
- Ito T, Sakano S, Sato K, Sugiura H, Iwata H, Murata Y, Seo H (1995) Sensitivity of osteoinductive activity of demineralized and defatted rat femur to temperature and duration of heating. *Clin Orthop* 316:267-275
- Inokuchi T, Ninomiya H, Hironaka R, Yoshida S, Araki M, Sano K (1991) Studies on heat treatment for immediate reimplantation of resected bone. *J Cranio-Max-Fac Surg* 19:31-39
- Sato T, Iwata H, Takahashi M, Miura T (1993) Heat tolerance of activity toward ectopic bone formation by rabbit bone matrix protein. *Ann Chir Gynaecol Suppl* 207:37-40
- Izawa H, Hachiya Y, Kawai T, Muramatsu K, Narita Y, Ban N, Yoshizawa H (2001) The effect of heat-treated human bone morphogenetic protein on clinical implantation. *Clin Orthop* 390:252-258
- Dunlop LL, Hall BK (1995) Relationships between cellular condensation, preosteoblast formation and epithelial-mesenchymal interactions in initiation of osteogenesis. *Int J Dev Biol* 39:357-371
- Kind PRN, King EJ (1954) Estimation of plasma phosphatase by determination of hydrolyzed phenol with amino-antipyrine. *J Clin Pathol* 7:322-326
- Borzacchiello A, Ambrosio L, Nicolais L, Ronca D, Guida G (2001). The temperature at the bone-cement interface: modeling and in vitro analysis. In: Pipino F (ed) *Bone Cement and Cemented Fixation of Implants*. Zenit, Rome, p 135-140
- Nawata M, Wakitani S, Nakaya H, Tanigami A, Seki T, Nakamura Y, Saito N, Sano K, Hidaka E, Takaoka K (2005). Use of bone morphogenetic protein-2 and diffusion chambers to engineer cartilage tissue for the repair of defects in articular cartilage. *Arthritis Rheum* 52:155-163
- Scheuffer C, Sebald W, Hulsmeyer M (1999) Crystal structure of human bone morphogenetic protein-2 at 2.7-Å resolution. *J Mol Biol* 287:103-115
- Wozney JM (1989) Bone morphogenetic proteins. *Prog Growth Factor Res* 1:267-280

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 30s, annealing for 30s at 60°C, and extension at 72°C for 90s, 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'-CCCCTGCTGAGATACTC-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'-TGCCGCTC 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% Ficoll 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 (0.05 M 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 Transmembrane, 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

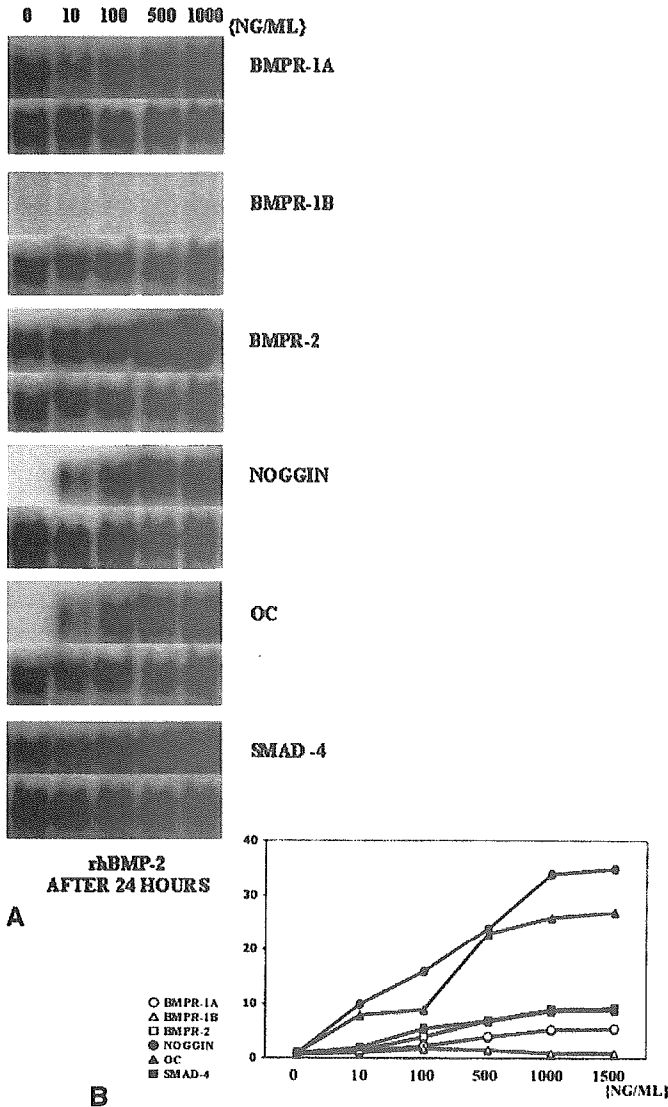


Fig. 1. Gene expression level of BMPR-1A, -1B, -2, Noggin, OC, and Smad-4 after 24-h stimulation of rhBMP-2 (0, 10, 100, 500, 1000 ng/ml) in muscle-derived primary culture cells by Northern blot analysis (A) and quantitation of the data of Northern blot analysis by Densitometry (B). G3PDH mRNA levels (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 1000 ng/ml and 1500 ng/ml rhBMP-2 (1500 ng/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 BMP-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

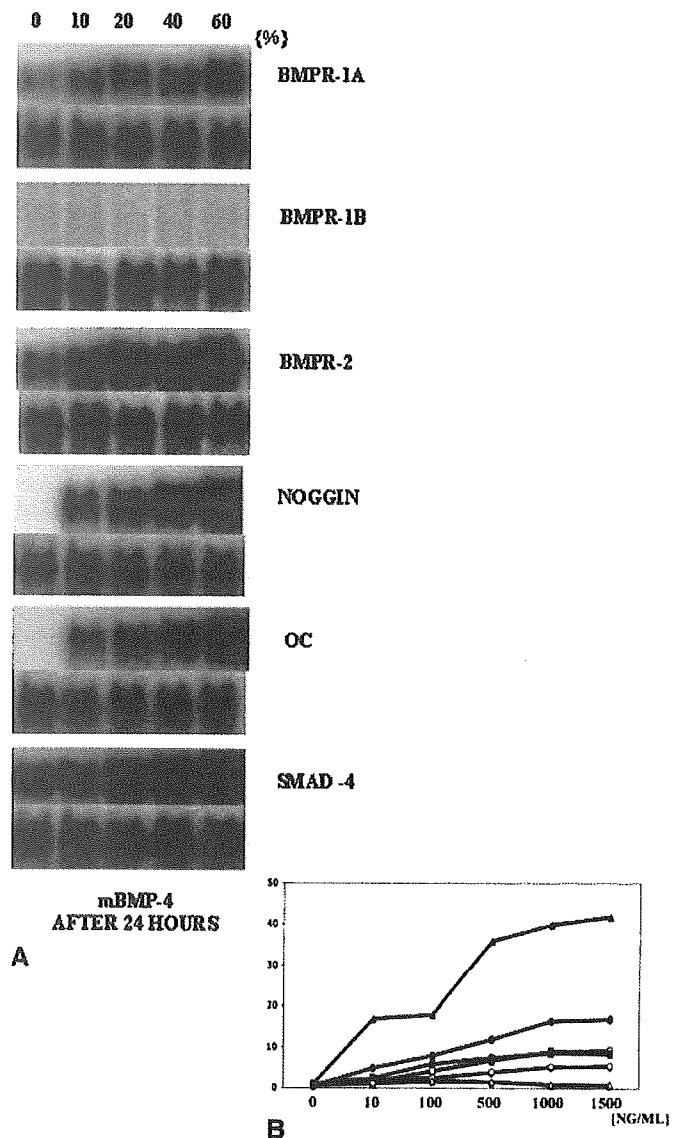


Fig. 2. Gene expression level of BMPR-1A, -1B, -2, Noggin, OC, and Smad-4 after 24-h stimulation of mBMP-4 [0%, 10%, 20%, 40%, and 60% (v/v) conditioned media] in muscle-derived primary culture cells by Northern blot analysis (A) and quantitation of the data of Northern blot analysis by Densitometry (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

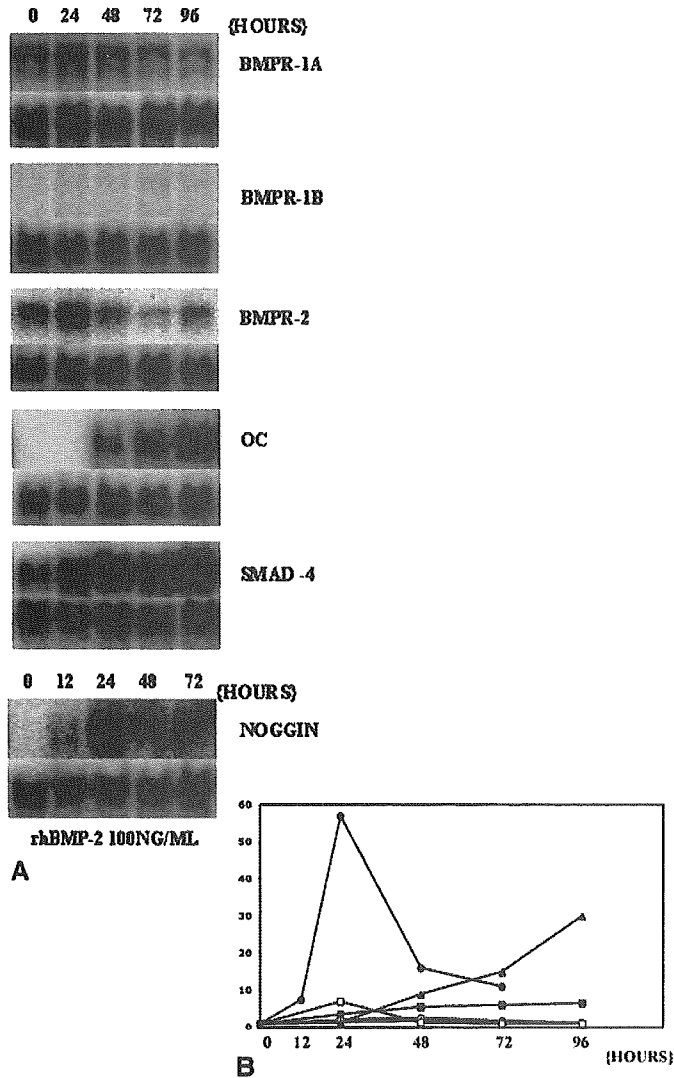


Fig. 3. Gene expression of BMPR-1A, -1B, -2, OC, and Smad-4 for 0, 24, 48, 72, and 96h and Noggin for 0, 12, 24, 48, and 72h after 100 ng/ml rhBMP-2 stimulation in muscle-derived primary culture cells by Northern blot analysis (A) and quantitation of the data of Northern blot analysis by Densitometry (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

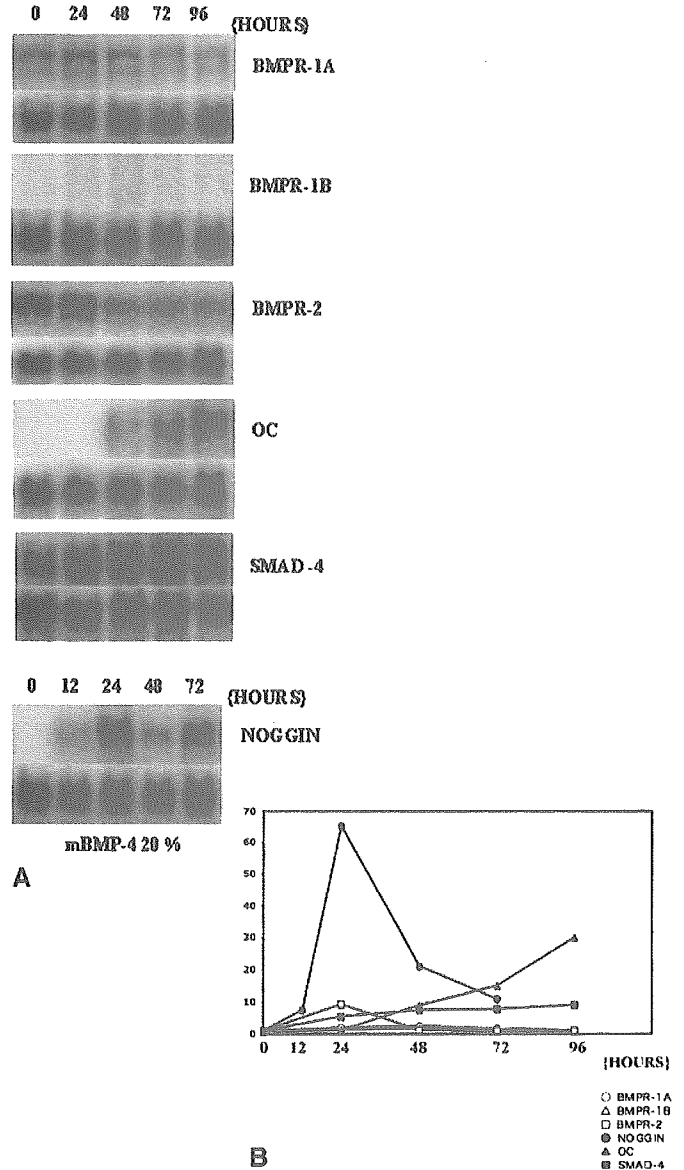


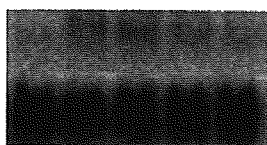
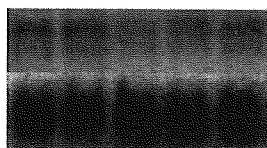
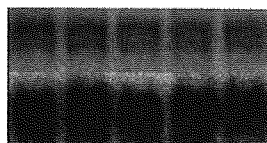
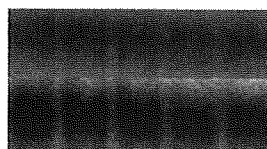
Fig. 4. Gene expression of BMPR-1A, -1B, -2, OC, and Smad-4 for 0, 24, 48, 72, and 96h and Noggin for 0, 12, 24, 48, and 72h after 20% mBMP-4 stimulation in muscle-derived primary culture cells by Northern blot analysis (A) and quantitation of the data of Northern blot analysis by Densitometry (B). G3PDH 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-

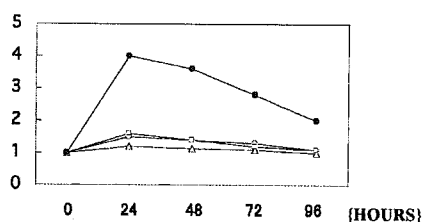
MC3T3-E1

0 24 48 72 96



rhBMP-2 1000NG/ML

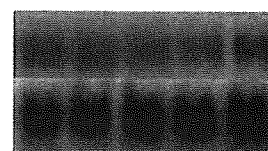
A



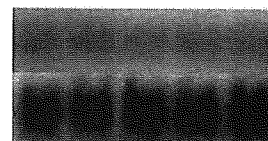
B

MC3T3-E1

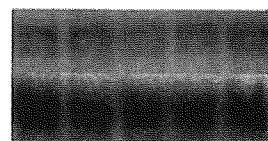
0 24 48 72 96 (HOURS)



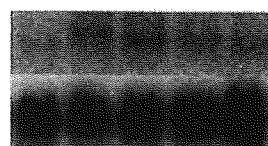
BMPR-1A



BMPR-1B



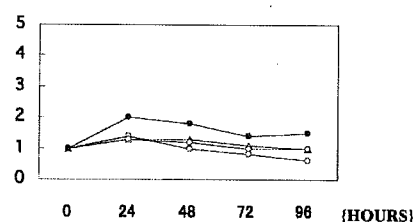
BMPR-2



NOGGIN

mBMP-4 60%

A



B

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 24 h, then decreased gradually. Noggin was also moderately induced after stimulation showed maximal expression at 24 h, 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

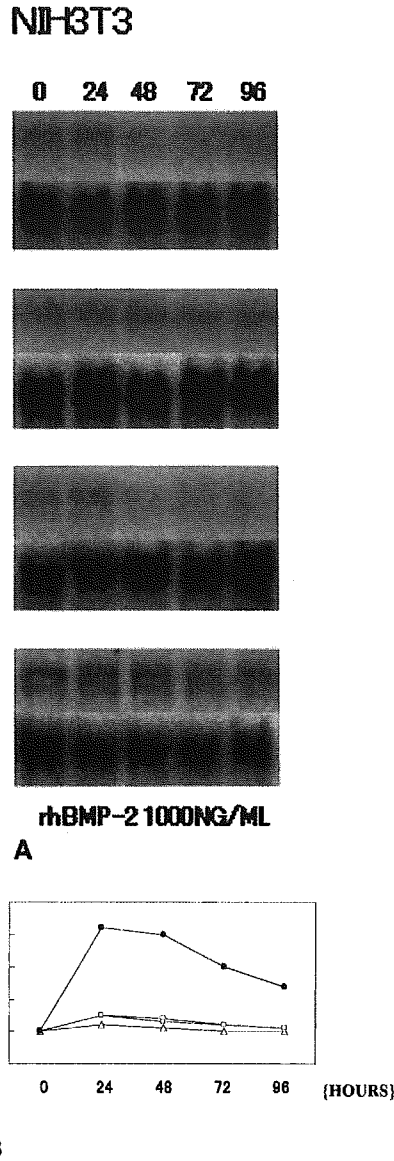


Fig. 7. Gene expression of BMPR-1A, -1B, -2, and Noggin for 0, 24, 48, 72, and 96 h after 1000 ng/ml rhBMP-2 stimulation in NIH3T3 cell line by Northern blot analysis (A) and quantitation of the data of Northern blot analysis by Densitometry (B). G3PDH mRNA levels (the bottoms of all lanes are G3PDH) 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 24 h, then decreased gradually. Noggin was moderately induced after stimulation showed maximal expression at 24 h, 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.

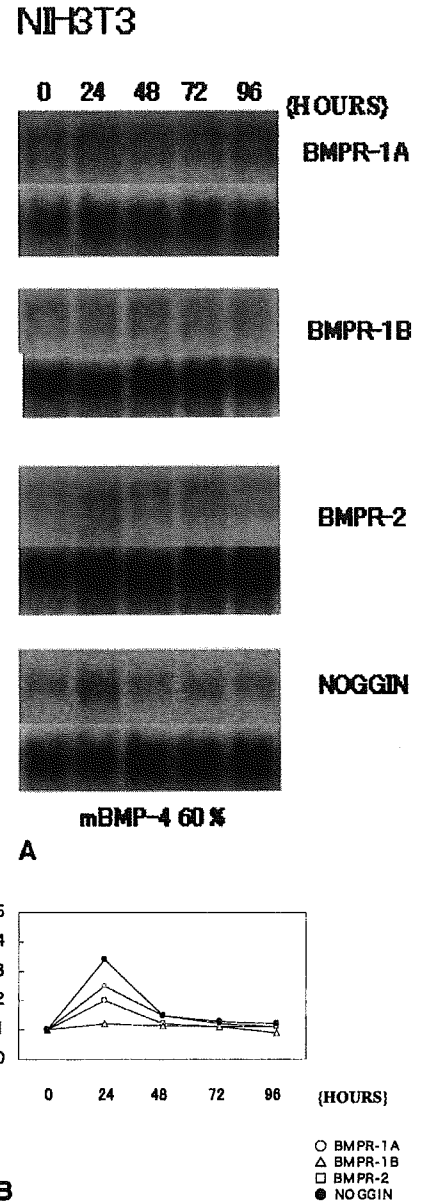


Fig. 8. Gene expression of BMPR-1A, -1B, -2, and Noggin for 0, 24, 48, 72, and 96 h after mBMP-4 (20%) stimulation in NIH3T3 cell line by Northern blot analysis (A) and quantitation of the data of Northern blot analysis by Densitometry (B). G3PDH mRNA levels (the bottoms of all lanes are G3PDH) obtained 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 24 h, then decreased gradually. Noggin was moderately induced after stimulation showed maximal expression at 24 h, 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.

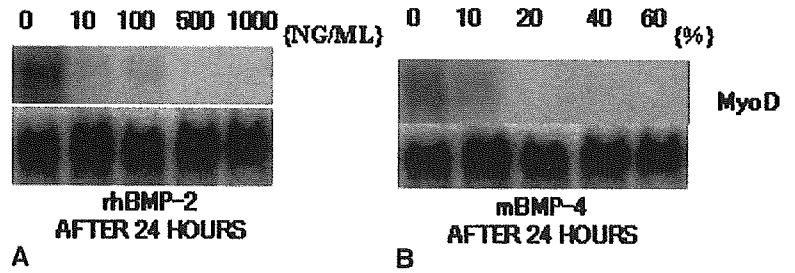


Fig. 9. The expression of MyoD in muscle-derived primary culture cells by Northern blot analyses. G3PDH mRNA levels obtained by Northern blotting were used for normalization. The expression of

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

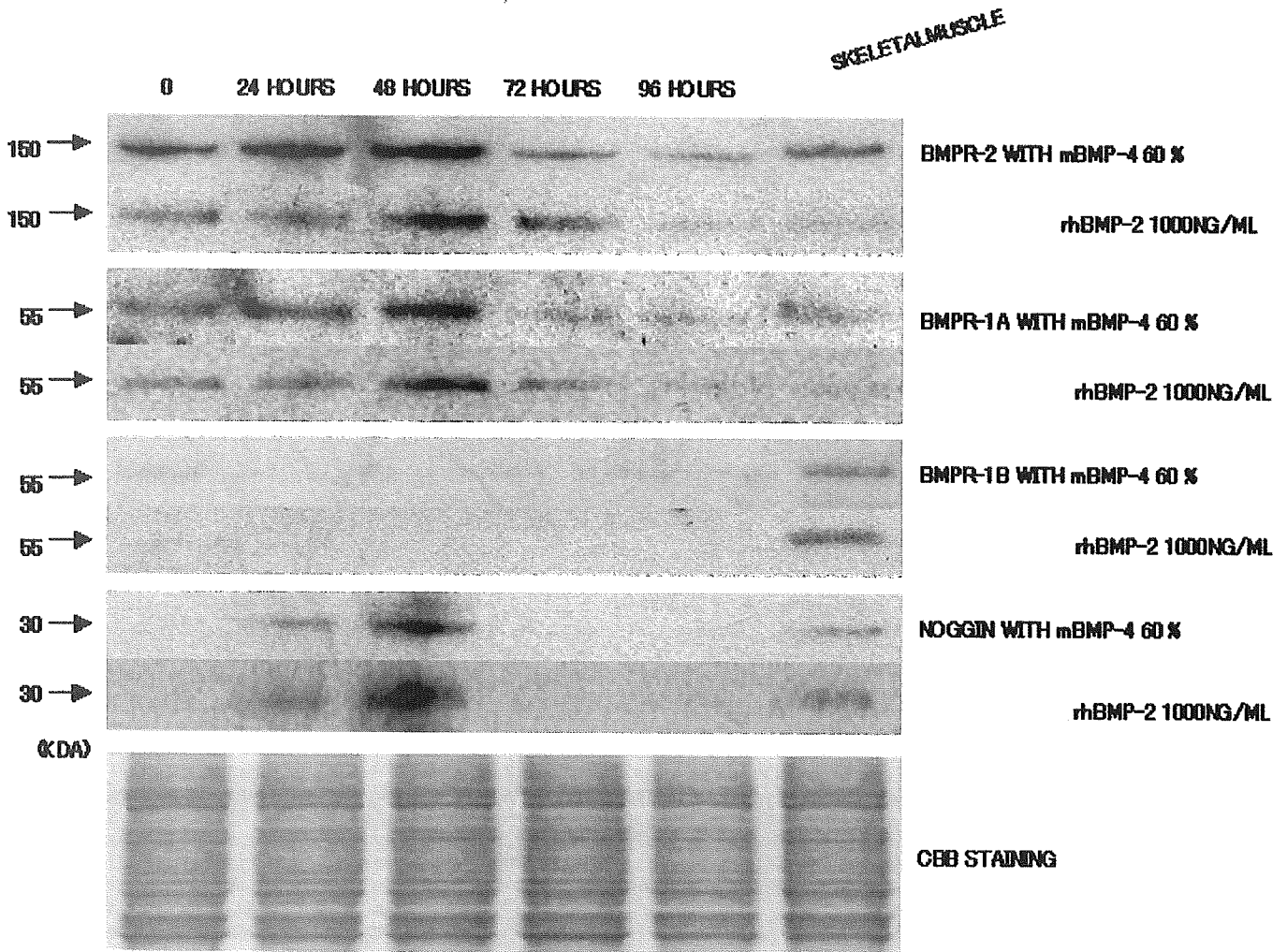


Fig. 10. Western blot analysis of BMPR-1A, -1B, -2, and Noggin after 60% mBMP-4 or 1000ng/ml rhBMP-2 stimulation in muscle-derived primary culture cells. Equivalent loading and integrity of protein were confirmed by Coomassie brilliant blue staining on the gel (*lower panel*). Mouse skeletal muscle proteins were used as positive controls. BMPR-1A and -2 were detected at 0h, induced at 24 h, peaked at 48 h, 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 24 h, 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 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 postnatal rat embryos. Cells were propagated in vitro in monolayer culture for 10 days and packed within diffusion chambers (10^6 /chamber) together with type I collagen (CI) and 0, 1, or 10 μg 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 μg 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 μg rHuBMP-2 or in the absence of rHuBMP-2. Defects receiving cartilage engineered with 10 μg 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-Na₄ (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-Na₄ and packed within diffusion chambers (10⁶ 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, 10⁶ 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'-TCTGCCCCAAGGGTTCTG-3' (reverse); for Col2A1, 5'-GCTCGAGGAGACTGGTG-3' (forward)

and 5'-ACCTGGGGGACCATCAGA-3' (reverse); for Col9A1, 5'-GGTCTCCGGGGAAGCCT-3' (forward) and 5'-CCAACCTCTCCCGCGGT-3' (reverse); for Col10A1, 5'-CGAGGTCTTGTGGCCCTAC-3' (forward) and 5'-CCTGGGTCTGTCCGCT-3' (reverse); for Col11A1, 5'-ATTGCCACCAGTCAACTGCT-3' (forward) and 5'-TTGACTGTGCCTCCGTC-3' (reverse); for MyoD1, 5'-ACTACAGCGGCGACTCAGAC-3' (forward) and 5'-GTG-GAGATGCGCTCCACTAT-3' (reverse); and for Cbfa1/Runx2, 5'-TGCTTCATTCGCCTCACAAAC-3' (forward) and 5'-TAGAACTTGTGCCCTCTGTTG-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'-AGGCGCTCTGTGTAACCCA-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

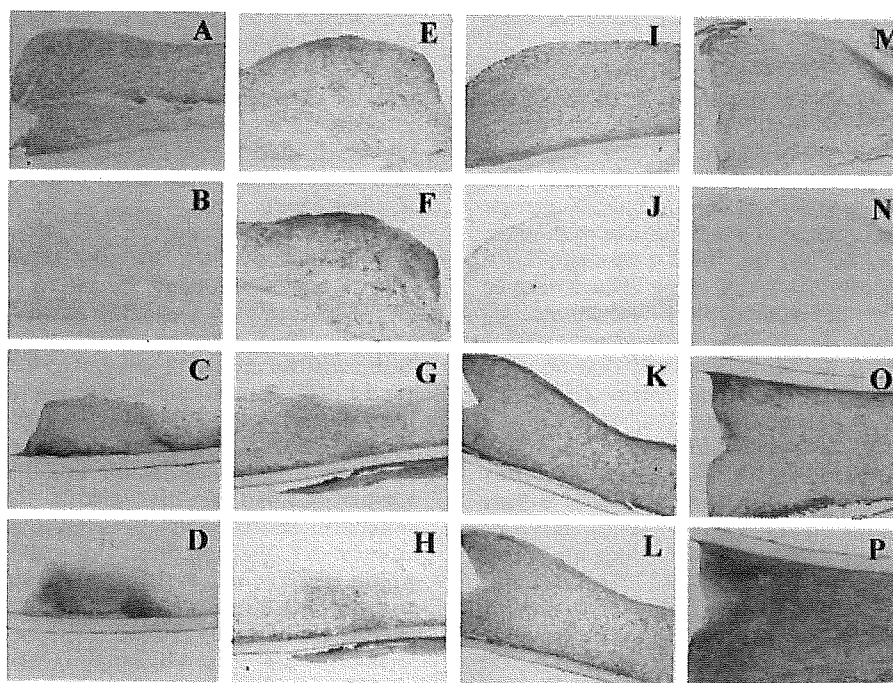


Figure 1. Cartilage formation in the diffusion chamber. Tissue pellets in diffusion chambers were examined at 3 weeks (A–D), 4 weeks (E–H), 5 weeks (I–L), and 6 weeks (M–P) postimplantation, in group B0 (without recombinant human bone morphogenetic protein 2 [rHuBMP-2]) (A, B, E, F, I, J, M, and N) compared 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 $\times 40$.)

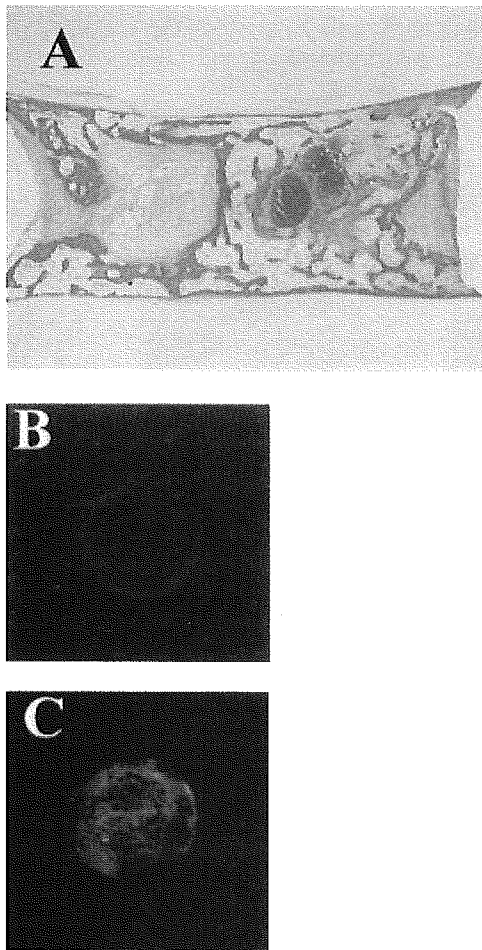


Figure 2. Histologic and radiologic evaluations of engineered cartilage tissue. For the tissue pellet in the diffusion chamber with an accidental hole on the filter (at 5 weeks posttransplantation; obtained from group B10), the normal histologic appearance of bone is clearly visible (stained with hematoxylin 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.

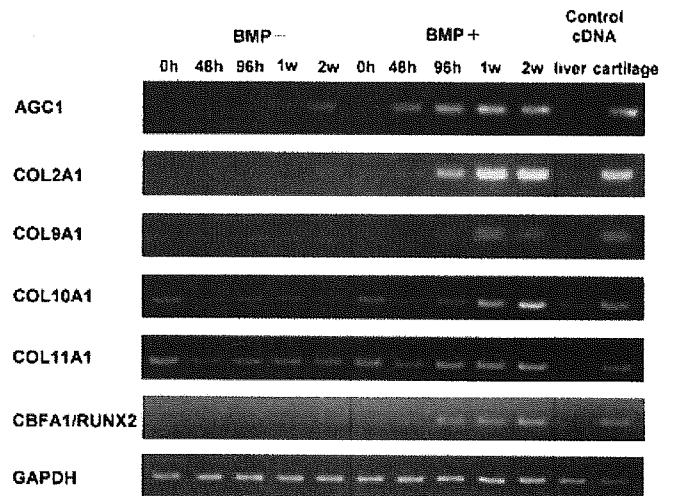


Figure 3. Reverse transcription-polymerase chain reaction analysis. Expression of types X and XI collagen (COL10A1 and COL11A1, respectively) was detected consistently in both groups (with $10 \mu\text{g}$ recombinant human bone morphogenetic protein 2 [BMP+; group B10] and without [BMP-]) throughout 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

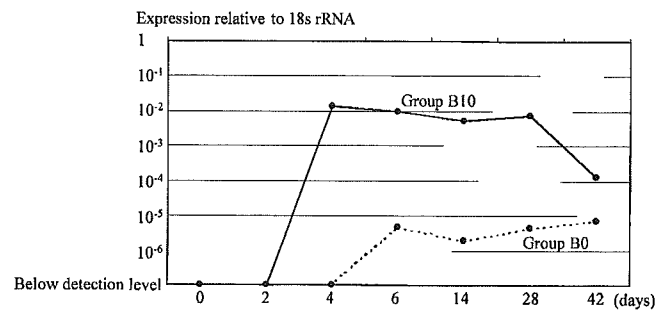


Figure 4. Real-time polymerase chain reaction analysis for type II collagen mRNA. After 4 days, expression of type II collagen mRNA was markedly increased in group B10 (with $10 \mu\text{g}$ recombinant human bone morphogenetic protein 2 [rHuBMP-2]). Group B0 = without rHuBMP-2; rRNA = ribosomal RNA.