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Bone morphogenetic protein activities are enhanced by 3′,5′-cyclic adenosine monophosphate through suppression of Smad6 expression in osteoprogenitor cells

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Abstract

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor (TGF)-β superfamily, and some display potent osteogenic activity both in vivo and in vitro. The BMP signaling cascade involving BMP receptors at the cell membrane and intracellular messengers (Smads) has been elucidated, but the regulatory mechanisms of BMP signaling have not been clarified. We previously found that pentoxifyline (PeTx), a nonspecific inhibitor of phosphodiesterase (PDE), and rolipram, a PDE-4-specific inhibitor, enhance BMP-4-induced osteogenic differentiation of mesenchymal cells, probably through the elevation of intracellular cyclic adenosine monophosphate (cAMP) accumulation and modulation of BMP signaling pathways as enhanced BMP-4 action was reproduced by addition of dibutylyl-cAMP (dbcAMP). However, the precise mechanisms underlying the enhancing effects of those agents on BMP signaling were not completely revealed. As already reported, BMPs utilize a specific intracellular signaling cascade to target genes via R-Smads (Smad1,5,8), Co-Smad (Smad4) and I-Smads (Smad6,7). One possibility for cAMP-mediated effects on BMP signaling might be suppression of I-Smads expression since these proteins form a negative feedback loop in BMP signaling. To examine this possibility, changes in I-Smad (Smad6) expression on addition of dbcAMP or PeTx were examined in a bone-marrow-derived osteogenic cell line (ST2). Alkaline phosphatase activity in ST2 cells was consistently induced by BMP-4 treatment (300 ng/ml), and Smad6 mRNA expression was also induced by BMP-4 treatment. Although concurrent treatment of ST2 cells with BMP-4 and dbcAMP elicited further activation of alkaline phosphatase, addition of dbcAMP reduced BMP-4-induced Smad6 expression in a dose-dependent manner. Furthermore, detection of phosphorylated Smad1/5/8 on Western blotting analysis was prolonged, suggesting prolonged kinase activity of BMP receptors through suppressed expression of Smad6. Elevated intracellular cAMP might thus enhance BMP signaling by suppressing Smad6 induction and prolonging intracellular BMP signaling.

Keywords: Cyclic adenosine monophosphate; Osteoprogenitor cells; Bone morphogenetic protein; Smad6; Pentoxifyline

Introduction

The bone-inducing activity of bone morphogenetic proteins (BMPs) was originally identified in bone matrix through ectopic bone formation after implanting decalcified bone matrix into rodent muscle. A group of BMP molecules belonging to the transforming growth factor (TGF)-β superfamily has since been identified and recognized as multi-functional growth factors.

In bone biology, BMPs are considered crucial in bone and cartilage formation for embryonic development, postnatal bone metabolism and repair of damaged bone. From a clinical perspective, BMPs and related molecules regulating BMP activity are expected to offer powerful tools for the treatment of systemic or local skeletal disorders like osteoporosis and repair of fracture or bone defects associated with bone tumor excision or trauma. For clinical purposes, BMP-2 and BMP-7/osteogenic protein

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(OP)-1 have been synthesized using DNA recombination and utilized in a limited number of human cases in combination with local delivery systems [1]. Gene therapy using BMP genes has also been attempted in experimental animals [2], but efficacious and safe vectors for delivering BMP genes in gene therapy have remained problematic for gene therapy.

When considering the efficient use of BMPs either systemically or locally, one basic and important problem is the low responsiveness of human mesenchymal cells to BMPs [3]. Large doses (in the order of milligrams) of costly BMP are thus required for local lesions in each patient. To overcome such problems and enable more widespread and effective use of BMP molecules, additional agents or methods that intensify BMP activity are desirable. In this context, we screened the phosphodiesterase (PDE) inhibitors pentoxifyline (PeTx, a non-specific inhibitor of PDE) and rolipram (a PDE-4-specific inhibitor), revealing increases in BMP-2-induced bone mass following systemic daily injection of these agents [4,5]. However, the precise pharmacological basis of these effects was uncertain. Elevation of intracellular levels of cyclic adenosine monophosphate (cAMP) may have been involved and might intensify the intracellular BMP signaling cascade. One possible mechanism for cAMP to intensify BMP signaling would be interference with the negative feedback mechanism in BMP signaling formed by inhibitory Smads (I-Smads). The present study investigated changes in mRNA expression of major I-Smads, Smad6 and phosphorylated receptor-regulated Smads (R-Smads) levels after treatment with BMP-4 and dibutylyl-cAMP (dbcAMP), a cell-membrane permeable analog of cAMP, in lined murine osteogenic ST2 cells.

Materials and methods

Reagents

PeTx (1-(5-oxohexyl)-3,7-dimethylxanthine) and dbcAMP were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PeTx and dbcAMP were prepared at 90 mM and 100 mM, respectively, as stock solutions in culture medium.

As a source of mouse BMP-4, conditioned medium from Chinese hamster ovary (CHO) cells transfected with murine BMP-4 (mBMP-4) cDNA was used. Details of the CHO cell have been described in our previous publication [6]. Briefly, cells transfected with mBMP-4 cDNA or mock vector (for controls) were inoculated at a density of 1.0×10^6 cells/100 mm plastic dish (Falcon #3003; Becton Dickinson Labware, Tokyo, Japan) in 10 ml of α-minimal essential medium (α-MEM; Sigma Chemical Co.) with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and antibiotics/antimycotics (100 U/ml penicillin; 100 μg/ml streptomycin; and 0.25 μg/ml amphotericin B, Sigma Chemical Co.) at 37°C in 5% CO_2 atmosphere. Conditioned medium was collected after 5 days incubation, filtered through a membrane filter (Corning, NY, USA; pore size 0.22 μm) and stored at 4°C. Under these conditions, judging from the induction of alkaline phosphatase (ALP) activity in osteoprogenitor cells, a 5% mixture of conditioned medium corresponded to approximately 100 ng/ml of recombinant human BMP-2 (generously provided by Yamanouchi Pharmaceutical, Tokyo, Japan) (data not shown). For experiments, conditioned medium from cells transfected with mock vector was used for negative controls.

Cell culture

Mouse mesenchymal cell line C3H10T1/2 [7], mouse bone marrow stromal cell line ST2 [8] and mouse osteoblastic cell line MC3T3-E1 [9] were obtained from the Riken Cell Bank (Ibaraki, Japan). The C3H10T1/2 and ST2 cell lines are widely regarded as osteogenic precursors as cells go on to exhibit osteoblastic phenotypes under the control of BMPs [10,11]. Cells were seeded at a density of 3×10^5 cells/100-mm plastic dish and cultured in α-MEM containing 10% FBS and antibiotics/antimycotics at 37°C in 5% CO_2 humidified air. Upon reaching confluence, cells were used in the following experiments.

Determination of alkaline phosphatase activity

Alkaline phosphatase activity was measured as a marker of osteoblastic differentiation in response to BMP-4 or other agents. Cells were seeded at a density of 1×10^5 cells/well in 48-well plates ($n = 4$ per group). Upon achieving confluence, medium was replaced by fresh medium containing 5% FBS and pre-incubated for 1 h. BMP-4 and other chemicals were then added to the cultures, and incubation was continued for 3 more days. Cells were pre-incubated with or without PeTx/dbcAMP for 15 min before treatment with BMP-4. After 3 days of incubation, medium was removed, and cells were washed 3 times with saline then lysed in 250 μl of 0.2% Triton-X (Sigma Chemical Co.) with saline. Plates were frozen at −80°C and dissolved again then sonicated for 40 s. After centrifugation, supernatant was collected and used for ALP assay and protein assay. For ALP assay, samples were incubated with 0.5 mM *para*-nitrophenol phosphate, 0.5 mM $MgCl_2$ and 0.5 M Tris–HCl for 30 min at 37°C. To stop the reaction, a quarter volume of 1 N NaOH was added, and then absorbance was read at 405 nm. As a standard, 5 mM *para*-nitrophenol was used. Protein concentration was measured using a DC protein assay kit (Bio-Rad Laboratories, CA, USA). Experiments were performed independently in triplicate.

Determination of intracellular cAMP

When cultures maintained in 24-well plates ($n = 4$ per group) reached confluence, medium was replaced using fresh medium of the same composition. After 1 h of pre-incubation, BMP-4 and other chemicals were added to the cultures, and incubation was continued for 5 or 15 min. The medium was then removed, and cells were overlaid with 250 μl of 0.1 N HCl for 10 min at room temperature, collected by scraping and then centrifuged. Supernatant was then collected and used for cAMP assay, which was performed using a cAMP Kit (R&D Systems, Minneapolis, USA) according to the instructions of the manufacturer. The cAMP assay is based on the competitive binding technique, in which cAMP present in a sample competes with a fixed amount of ALP-labeled anti-cAMP rabbit polyclonal antibody.

Northern blot analysis

Cells were seeded in 100-mm diameter dishes and cultured until confluence. Upon achieving confluence, medium was replaced by fresh medium containing 5% FBS and cultured for 1 h. Cells were then treated with BMP-4 (300 ng/ml) or dbcAMP (2 mM) for 24 h. Cells were pre-incubated with or without dbcAMP for 15 min before treatment with BMP-4.

Total RNA was isolated from cells using Isogen (Wako Pure Chemical Industries, Osaka, Japan), and poly(A)$^+$ RNA was obtained using Poly(A) Quik mRNA Isolation Kit (Stratagene, CA, USA) according to the instructions of the manufacturer. Poly(A)$^+$ RNA (2 μg) was electrophoresed in 1.0% agarose gels in the presence of 1.1 M formaldehyde and blotted to Hybond N membranes (Amersham Bioscience, NJ, USA). The complete coding regions of mouse Smad6 cDNA were labeled by [α-^{32}P]-dCTP using a Random Primer Labeling Kit (Takara Biochemicals, Otsu, Japan). Hybridization was performed in a solution containing 0.5 M phosphate buffer, 1 mM ethylene diamine tetra-acetic acid (EDTA) and 7% sodium dodecyl sulfate (SDS) at 65°C overnight, and the filter was washed at 65°C with 6× standard saline citrate (SSC), 0.1% SDS for 15 s, 2× SSC and 0.1% SDS for 10 min twice. Filters were stripped using boiled distilled water containing 0.1% SDS and rehybridized. Amounts of mRNA were verified by hybridizing filters with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe. Signals were detected using a BAS-2500 image analysis system (Fuji Photo Film Co., Tokyo, Japan).

Smad6 cDNA was generously provided by T. Imamura (Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan), and GAPDH cDNA was generated by reverse transcription polymerase chain reaction (RT-PCR) using specific primer sets as follows: for GAPDH, sense primer 5′-ATGGTGAAGGTCGGTGTGAA-3′ and antisense primer 5′-CTCTTGCTCAGTGTCCTTGCT-3′.

Quantitative real-time reverse transcription polymerase chain reaction

When cultures maintained in 6-well plates ($n = 3$ per group) became confluent, medium was replaced by fresh medium containing 5% FBS and cultured for 1 h, then BMP-4 and other chemicals were added to cultures. In the case of combined treatment, BMP-4 was added 15 min later than other chemicals. Total RNA was isolated from cells treated with each chemical for each time period using NucleoSpin RNA II (Macherey-Nagel, Duren, Germany) according to the instructions of the manufacturer. A total of 1 μg of total RNA was reverse-transcribed into first-strand cDNA with an oligo-dT primer using Superscript II reverse transcriptase (Invitrogen, CA, USA). Real-time RT-PCR was performed according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA, USA). TaqMan fluorogenic probes for Smad6 were purchased from Applied Biosystems. Real time RT-PCR for GAPDH was performed using SYBR Green Supermix (Bio-Rad Laboratories). Experimental samples were matched to a standard curve generated by amplifying serially diluted products using the same PCR protocol. Amplified GAPDH cDNA was used for internal control. To correct for variability in RNA recovery and efficiency of reverse transcription, GAPDH cDNA was amplified and quantitated in each cDNA preparation. Normalization and calculation steps were performed as described by Pfaffl [12]. Experiments were performed on 3 independent occasions, and each experiment comprised of 3 samples.

Western blot analysis

Cells were plated at a density of 2 × 10^4 cells/well on 6-well plates and cultured until confluence. At 1 h after replacing with fresh medium containing 5% FBS, BMP-4 (300 ng/ml) and dbcAMP (2 mM) were added to the media, and cells were cultured for designated periods. Dibutylyl cyclic AMP (dbcAMP) was added 15 min prior to BMP-4. The resultant sample was boiled for 5 min in 20 μl of sample buffer for SDS polyacrylamide electrophoresis (SDS-PAGE) as described previously with minor modifications [13]. Equal amounts of protein samples were applied and run on each lane of SDS 5–20% acrylamide gels (40 mA, low voltage, 90 min) and ultimately blotted to ECL™ nitrocellulose membrane (Amersham Bioscience). Membranes were developed using ECL™ plus reagent (Amersham Bioscience). Signal intensities were measured according to staining of each band using a Chemi Doc XRS-J digital densitometer (Bio-Rad Laboratories).

Statistical analysis

Data are expressed as mean ± SD for each group. Differences between treatment groups were analyzed using

Fisher's PLSD test. Values of $P < 0.05$ were considered statistically significant.

Results

ALP induction by BMP-4, PeTx and dbcAMP

PeTx, a methylxanthine-derived nonspecific inhibitor of PDEs, had no effect on ALP activity in ST2 cells at PeTx concentrations of 0.225–0.9 mM. In contrast, PeTx enhanced BMP-induced ALP activity in a dose-dependent manner (Fig. 1A), indicating a specific enhancing effect of PeTx on BMP-2 activity. Intracellular levels of cAMP increased about 7-fold in 15 min after the addition of PeTx (0.9 mM). Levels of cAMP were unaffected by addition of BMP-4 (50 ng/ml) with PeTx (Fig. 1B).

The potentiating effect of PeTx on BMP action in ST2 cells was mimicked by the addition of dbcAMP (Fig. 1C). Similar results were obtained using C3H10T1/2 and MC3T3-E1 cells (data not shown).

Smad6 mRNA induced by BMP-4 stimulation

Fig. 2 shows dose- and time-dependent changes in Smad6 mRNA expression following BMP-4 treatment, as determined using quantitative real-time RT-PCR methods. In ST2 cells, Smad6 mRNA expression was significantly up-regulated by mBMP-4 treatment in a dose-dependent manner over the BMP-4 dose range from 50 ng/ml to 300 ng/ml in 6 h (Fig. 2A). Up-regulated expression of Smad6 mRNA peaked in 3 h and lasted up to 24 h after BMP treatment (300 ng/ml) then gradually reduced to the baseline level within 72 h (Fig. 2B).

Suppression of Smad6 expression by cAMP

In order to elucidate how cAMP accelerates osteoblastic differentiation in ST2 cells, the expression of Smad6 mRNA after treatment with dbcAMP and BMP-4 was analyzed. Smad6 mRNA expression was induced by BMP-4 stimulation, but addition of dbcAMP significantly ameliorated BMP-4-induced Smad6 expression (Fig. 3).

Quantitative real-time RT-PCR of Smad6 mRNA expression treated with or without BMP-4 (300 ng/ml) and various doses of dbcAMP (100–2000 μM) at 12 h revealed that dbcAMP reduced BMP-4-induced Smad6 expression in a dose-dependent manner (Fig. 4).

Results of quantitative real-time RT-PCR for Smad6 mRNA expression at 6, 12, 24 and 72 h are shown in Fig. 5. At 6, 12 and 24 h, dbcAMP reduced the expression of Smad6 mRNA that was induced by BMP-4 (Figs. 5A–C). Although level of Smad6 mRNA induced by BMP-4 returned to

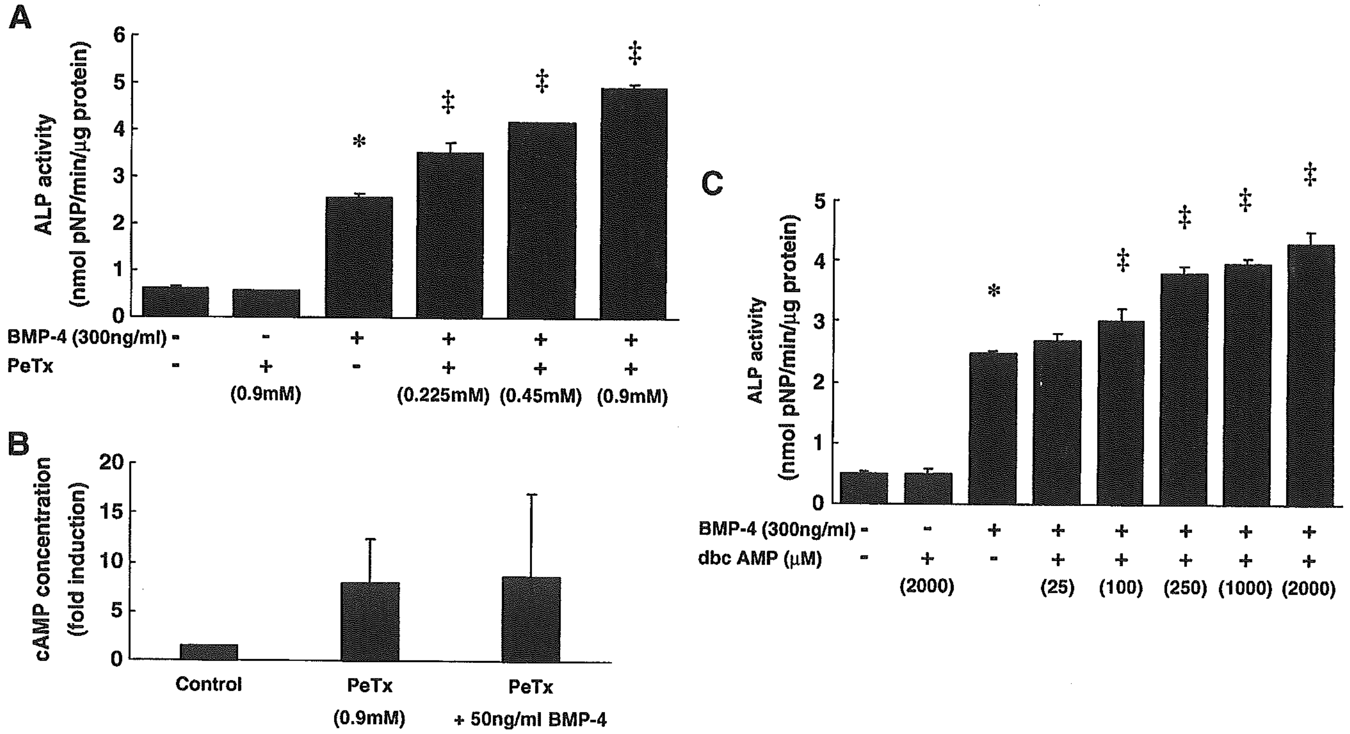

Fig. 1. (A, C) PeTx and dbcAMP intensified BMP-4-induced ALP activation in a dose-dependent manner. ST2 cells were cultured in 24-well plates until confluence then treated with BMP-4, PeTx (A) and dbcAMP (B). After 3 days, ALP activity was measured. (B) Concentration of cAMP in ST2 cells treated with BMP-4 and PeTx for 15 min. Bars and lines represent mean ± SD for 4 wells. $^{*}P < 0.05$, cells treated with BMP-4 compared with untreated cells. $^{‡}P < 0.05$, cells treated with BMP-4/PeTx or dbcAMP compared with BMP-4-treated cells.

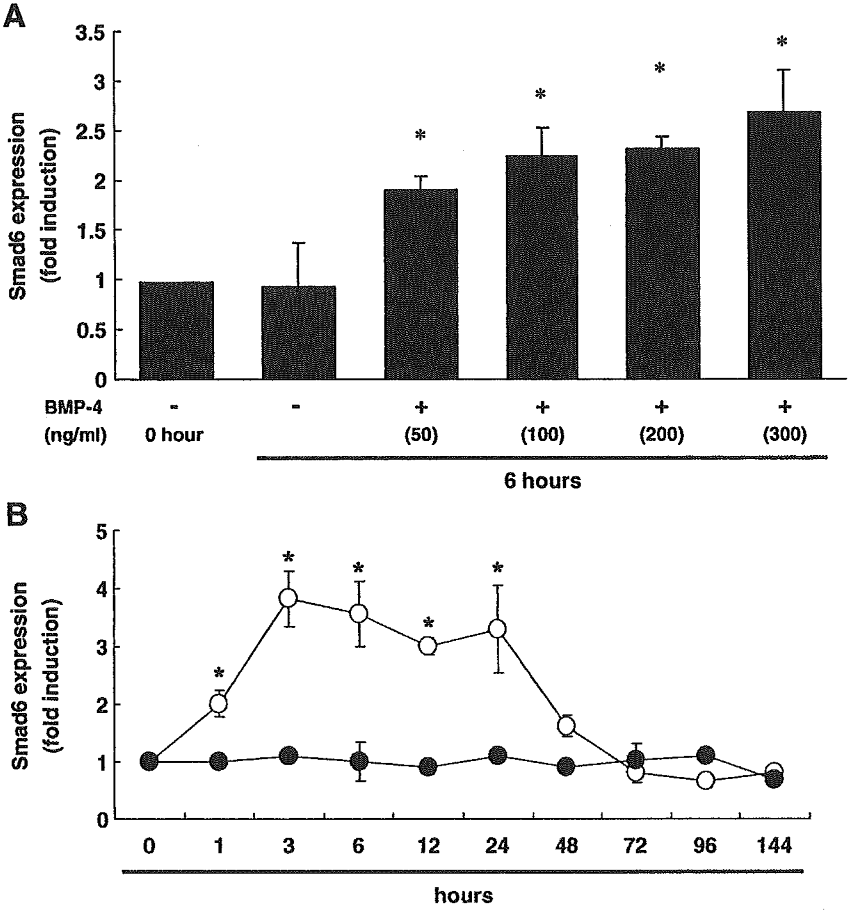

Fig. 2. Effects of BMP-4 on expression of Smad6 in ST2 cells. ST2 cells were cultured in 6-well plates to confluence then treated with BMP-4. After 6 h (A) or other designated times (B), total RNA was extracted, and levels of Smad6 expression were analyzed using real-time RT-PCR, normalized to GAPDH expression and presented as the expression level relative to that in control untreated cells. BMP-4 induced Smad6 expression in a dose-dependent manner at 6 h after stimulation (A). In panel B, closed circles represent Smad6 expression at each time point in untreated cells, and open circles represent expression in cells treated with BMP-4 (300 ng/ml). Bars and lines represent mean ± SD for 3 wells. Similar results were obtained in 3 independent series of studies. *$P < 0.05$, cells treated with BMP-4 compared with untreated cells.

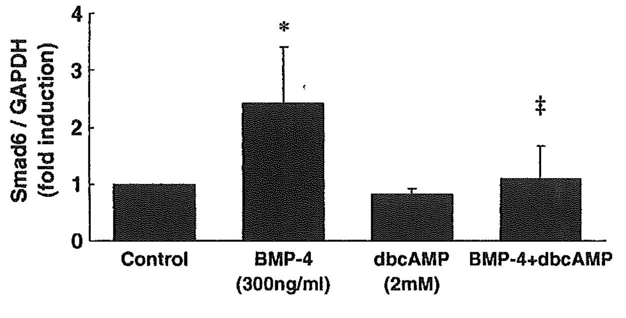

Fig. 3. Cyclic AMP reduced BMP-4-induced Smad6 expression in ST2 cells. Results of Northern blotting. After 24 h of treatment using BMP-4 (300 ng/ml), dbcAMP (2000 μM) or both, mRNA was extracted and analyzed. Messenger RNA obtained using a Poly(A) Quik mRNA Isolation Kit was electrophoresed in agarose-formaldehyde gels, blotted to Hybond N membranes and hybridized with Smad6 and GAPDH probes. Showing the summary of 3 independent experiments. Bars and lines represent mean ± SD for 3 experiments. *$P < 0.05$, cells treated with BMP-4 compared with untreated cells. $^{\ddagger}P < 0.05$, cells treated with BMP-4/dbcAMP compared with BMP-4 treated cells.

baseline by 72 h, dbcAMP still repressed Smad6 mRNA levels (Fig. 5D).

Prolonged phosphorylation of BMP-specific R-Smads by dbcAMP

Phosphorylation of BMP-specific R-Smads by BMP-4 was analyzed by immunoblotting using phospho-Smad1/5/8-specific antibody with or without dbcAMP treatment (Fig. 6). Phosphorylation of Smad1/5/8 after BMP-4 stimulation started from 1 h after BMP-4 stimulation and became undetectable after day 3. Addition of dbcAMP did not exhibit significant effects at 1 or 24 h after treatment, but phosphorylated Smad1/5/8 were detected until day 5.

Discussion

The cyclic nucleotide PDE family includes a large numbers of PDE isomers, which are encoded in at least 13

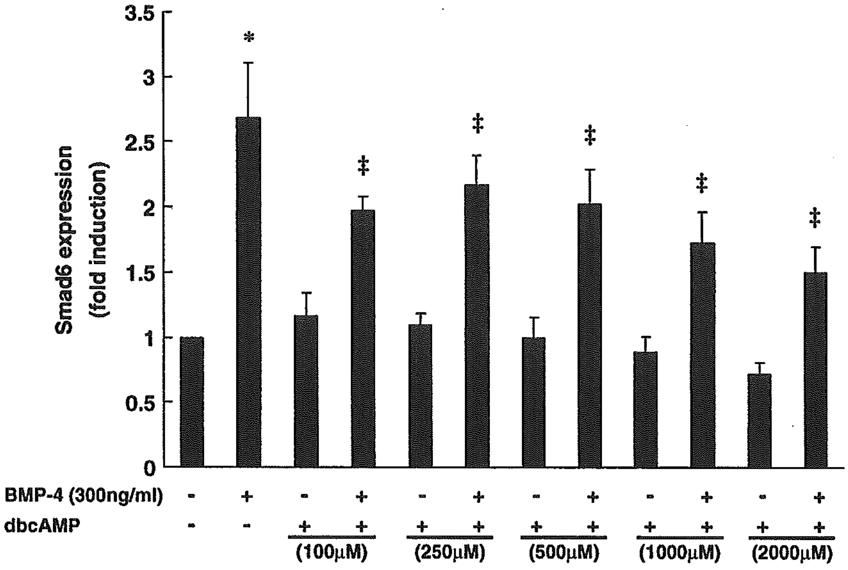

Fig. 4. Cyclic AMP reduced BMP-4-induced Smad6 expression in ST2 cells in a dose-dependent manner. After 12 h of treatment with BMP-4 (300 ng/ml), dbcAMP or both, total RNA was extracted, and expression of Smad6 was analyzed using real-time RT-PCR, normalized to GAPDH expression and presented as the expression level relative to that in control untreated cells. Bars and lines represent mean ± SD for 3 wells. $^*P < 0.05$, cells treated with BMP-4 compared with untreated cells. $^‡P < 0.05$, cells treated with BMP-4/dbcAMP compared with BMP-4 treated cells.

distinct genes and hydrolyze cAMP and/or cyclic guanosine monophosphate (cGMP), thereby contributing to the regulation of intracellular cAMP levels [14]. A number of compounds inhibiting respective PDE subfamily enzymes in a selective or non-selective manner have already been developed [15]. Interestingly, some PDE inhibitors have been reported to stimulate osteoblastic differentiation and inhibit osteoclastic differentiation in vitro [16,17]. We have

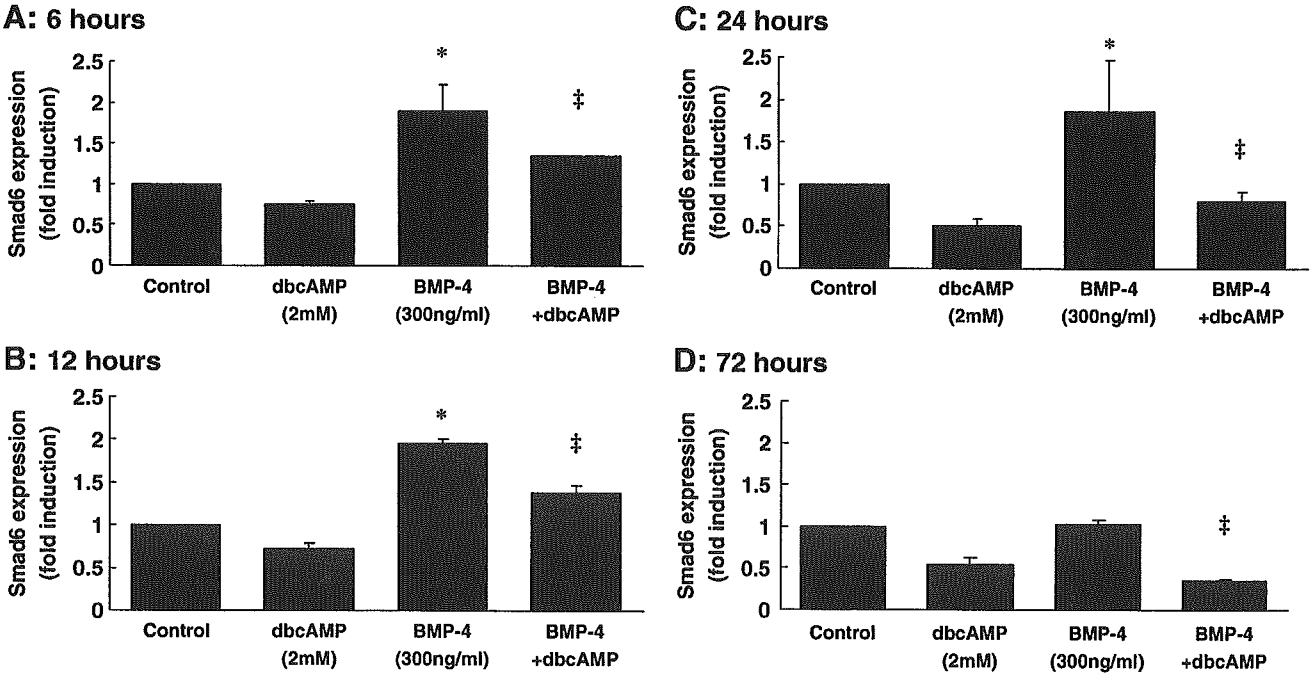

Fig. 5. Cyclic AMP reduced BMP-4-induced Smad6 expression in ST2 cells at 6, 12 and 24 h. ST2 cells were treated with BMP-4 (300 ng/ml), dbcAMP (2 mM) or both for 6 h (A), 12 h (B), 24 h (C) or 72 h (D). Total RNA was then extracted, and expression of Smad6 was analyzed using real-time RT-PCR, normalized to GAPDH expression and presented as expression level relative to that in control untreated cells. Bars and lines represent mean ± SD for 3 wells. $^*P < 0.05$, cells treated with BMP-4 compared with untreated cells. $^‡P < 0.05$, cells treated with BMP-4/dbcAMP compared with BMP-4 treated cells.

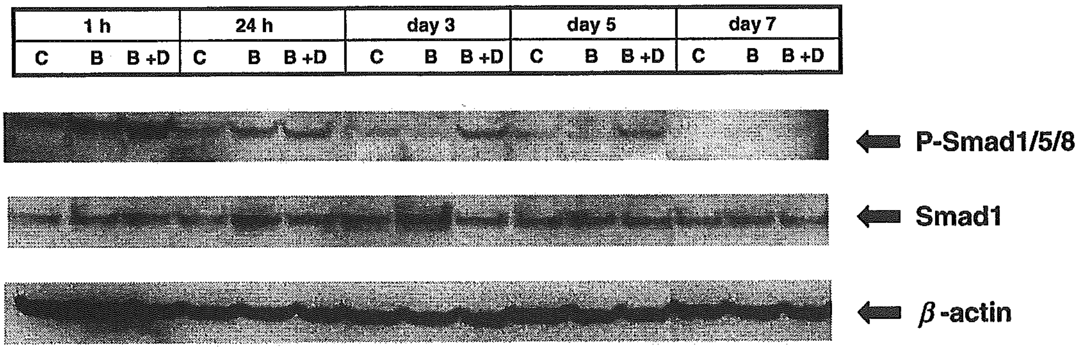

Fig. 6. Effects of dbcAMP on BMP-4-induced Smads signaling in ST2 cells. ST2 cells were pretreated for 1 h in 5% FBS before stimulation. Cells were treated with BMP-4 (300 ng/ml) or BMP-4 and dbcAMP (2 mM). Whole-cell extracts were prepared at indicated time points followed by immunoblotting. Identical amounts of protein samples were applied and run on each lane of a 10% acrylamide gels, blotted to an ECL nitrocellulose membrane, and membranes were developed using ECL plus reagent. Activated BMP-specific R-Smads were detected by anti-phospho-Smad1/5/8 antibody. Bands for Smad1 and β-actin are shown as a loading control. C, control; B, BMP-4; B + D, BMP-4 and dbcAMP.

reported that the PDE inhibitors, PeTx and Rolipram, increase bone mass mainly by promoting bone formation in normal mice [4,18]. Furthermore, PDE inhibitors have been shown to exert therapeutic effects in different experimental osteopenia models [17,19,20]. Although some PDE inhibitors reportedly promote bone formation under both in vivo and in vitro conditions, the precise mechanisms leading to the osteogenic effects of those PDE inhibitors are currently unknown. The present study was performed to gain clues regarding the anabolic effect of the PDE inhibitor PeTx on osteoblastic differentiation of bone marrow stroma cell-derived osteoprogenitor cells, ST2 cells.

Our previous report showed that daily injection of PeTx increased systemic bone mass by enhancing bone formation [18], and it also stimulated BMP-2 induced ectopic bone formation [4]. However, the mechanisms underlying the effects of PDE inhibitors on bone metabolism have remained obscure. Regarding mechanisms of action, one of the basic but major questions has been whether the anabolic effect of PDE inhibitors on bone metabolism is linked specifically to BMP signaling system to induce osteoblastic differentiation or results from functional activation of fully differentiated osteoblasts independent of BMP. Based on the results in our present and previous studies, the effect of PDE inhibitors might be brought about in association with BMP since the effects of PDE inhibitors in enhancing osteoblastic differentiation were not seen in the absence of BMP but were consistently noted in the presence of BMP, indicating a potential function of the PDE inhibitor in enhancing BMP signaling through elevation of intracellular cAMP levels [21]. In addition, intracellular cAMP-elevating agents such as dbcAMP and forskolin also increase BMP-4-stimulated osteoblastic differentiation when estimated by elevating ALP activity [21]. These results indicate that cAMP-elevating agents might enhance BMP signaling pathway to enhance bone formation. Regarding the effects of PDE inhibitors on BMP signaling, the results of a recent study suggest that the anabolic actions of PeTx might be attributable to cross-talk between BMP signaling and protein kinase C (PKC) signaling cascades [22]. Rawadi et al. noted that PeTx could promote osteoblast differentiation not by protein kinase A (PKA) activation, since inhibition of PKA by H-89 (a protein kinase inhibitor) did not alter the PDE-induced osteogenic reaction, but through activation of extracellular signal-regulated kinase (ERK) 1/2 and p38 kinase pathways [22]. However, we could not exclude the possible contribution of cAMP-activated PKA to the acceleration of osteoblastic differentiation by PeTx since inhibition of PKA by H-89 partially abolishes PDE inhibitor-mediated increases in the induction of ALP by BMP-4 (data not shown). Cross-talk between the cAMP/PKA cascade and the Smads-mediated BMP signaling pathway awaits elucidation in future studies.

Among the 3 groups of Smad proteins involved in the BMP signaling pathway, Smad6 and Smad7 are classified as I-Smads. I-Smads interact with activated serine/threonine kinase BMP or TGF-β receptors and compete with R-Smads (Smad1/5/8) to activate the receptors [23]. Smad6 has also been reported to compete with common-partner Smad (Co-Smad, Smad4) in forming complexes with R-Smads. Smad6 might be more crucial in the negative feedback loop as Takase et al. [24] reported that Smad6 mRNA was markedly induced by BMP-2 or BMP-7/OP-1 in various osteoprogenitor cell lines, whereas Smad7 expression remained unchanged in most cells. In this context, Smad6 was targeted to examine involvement of the protein in the mechanism enhancing BMP signaling by cAMP. We confirmed up-regulation of Smad6 expression in response to BMP-4 stimulation in ST2 cells. Interestingly, up-regulated expression of Smad6 by BMP-4 stimulation appears to be partially suppressed by elevated intracellular cAMP levels on addition of cell-membrane-permeable dbcAMP in a dose-dependent manner in ST2 cells. Elevated levels of phosphorylated R-Smads following BMP-4 treatment and dbcAMP lasted longer than that with BMP-4 treatment alone on Western blot analyses. Although dbcAMP significantly suppressed the expression of Smad6 mRNA induced by BMP-4 within 6 h (Fig. 5A), levels of phosphorylated R-Smads induced by BMP-4 at 24 h were not altered (Fig. 6). This discrepancy might be explained by following two points. First, Smad6 was evaluated by mRNA level, whereas phosphorylated R-Smads were determined by protein level. Second, Smad6 binds in a stable manner to serine/threonine kinase receptors and then interferes with phosphorylation of R-Smads by receptors. This negative feedback loop might require the appropriate time lag. These results suggest that the anabolic effects of cAMP on BMP-4-induced osteoblastic differentiation might be partially attributable to suppressed expression of Smad6 in the negative feedback loop and the facilitation of positive BMP-4 signaling in cells. In addition, dbcAMP enhanced the expression of Id-1 mRNA, an inhibitor of myogenesis, induced by BMP-4 (data not shown). These findings taken together suggest that cAMP might be a modulator of BMP signaling. Ishida et al. identified the 28-base pair regions responsible for transcriptional activation by BMPs in the mouse Smad6 promoter [25]. Ionescu et al. characterized a putative cAMP response element (CRE) site in the adjacent 17-base pair [26]. In that report, dominant negative cAMP response element binding protein reduced BMP-2-stimulated Smad6 gene transcription [26], but these results were not consistent with our current results. Potential causes for these inconsistencies include interactions of other transcriptional factors and differences in cells and BMPs. Similar mechanisms were reported to explain signal cross-talk between BMP and TGF-β [27]. Further studies are needed to elucidate relationships between cAMP and BMPs.

Smad proteins play important roles in BMP signaling and display characteristic pathways. Only a limited number of previous reports have examined relationships between the Smad pathway and cAMP. On addition of dbcAMP, BMP-4-induced Smad6 expression was significantly suppressed (Fig. 4). These results indicate that dbcAMP enhances BMP-4 osteogenic activity through the suppression of a self-regulated negative feedback loop. Parathyroid hormone (PTH) or its amino-terminal fragment is known to enhance systemic bone formation following daily subcutaneous injection and is currently utilized to recover bone mass in osteoporotic patients without the precise mechanisms of action being understood. As PTH also elevates intracellular levels of cAMP in cells with PTH-specific receptors, cAMP interference in the BMP negative feedback might be involved in PTH-enhanced systemic bone formation. Additional studies confirming suppression of I-Smads by PTH treatment are necessary to elucidate the mechanisms of action underlying the anabolic effects of PTH.

In conclusion, suppression of BMP-4-induced Smad6 expression appears to represent one of the mechanisms by which BMP action is enhanced by PeTx and dbcAMP treatments. Manipulation of the BMP signaling loop may also provide new insights into enhancing the efficacy of BMP-mediated local new bone formation for the treatment of damaged bone.

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ORIGINAL ARTICLE

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

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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 15min, or 1, 2, 4, or 8h, or autoclaved at 120°C for 15min. 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, 5μg rhBMP-2 samples were impregnated into porous collagen disks (6mm in diameter, 1mm 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 100ng/ml to cultures of MC3T3-E1 cells. After 48h, 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 8h, 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

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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 (1mg rhBMP-2/ml). This was diluted 3-fold (15μl saline containing 5μg rhBMP-2) and heated at 50, 70, 90, or 100°C for 15min, or 1, 2, 4, or 8h using a heat block (Dry Thermo Unit, Taitec, Saitama, Japan), or autoclaved at 120°C for 15min. 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% CO_2.

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 100ng/ml. After 48h, the osteoblastic cells were washed twice with phosphate-buffered saline, scraped from each well into 0.3ml 0.5% NP-40 containing 1mM $MgCl_2$ and 10mM Tris (pH 7.5), and sonicated twice for 20s with a sonicator (model W-220; Wakenyaku, Kyoto, Japan). The cell lysates were then centrifuged for 10min 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 Phospha 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 ± 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 (6mm in diameter, 1mm thick), soaked in the sample solutions containing 5µg 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 5µg rhBMP-2 that had never been heated (control), or heated at 50, 70, 90, or 100°C for 15min, or for 1, 2, 4, or 8h, or autoclaved at 120°C for 15min. 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 5µm 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 2h were dissolved into 20µl sample buffer solution (0.5µM, pH6.8 Tris-HCl buffer solution/0.2% SDS/10% glycerol/0.01% bromophenol blue) with or without 100mM dithiothreitol (DTT, a disulfide-bond reducing agent) and boiled for 5min. Each sample was run on a SDS gel at 40mA for 60min. The gel was then stained with Comassie brilliant blue and destained in 10% acetic acid solution.

Statistical analysis

Quantitative data were expressed as the mean ± 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.

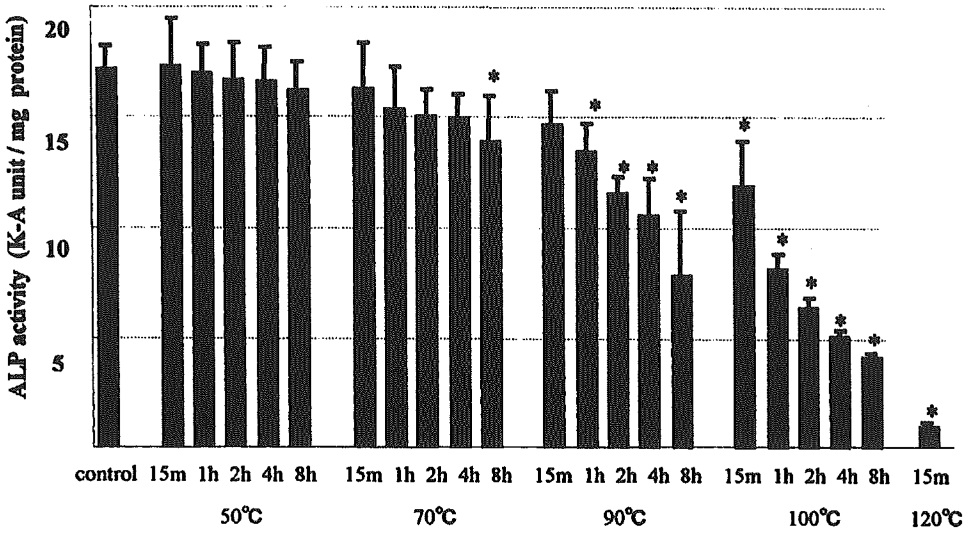

Fig. 1. The influence of heat treatment on rhBMP-2-induced alkaline phosphatase (*ALP*) activity of MC3T3-E1 cells. Data are means ± SD of three culture wells. A significant difference from the control is indicated as * ($P < 0.05$)

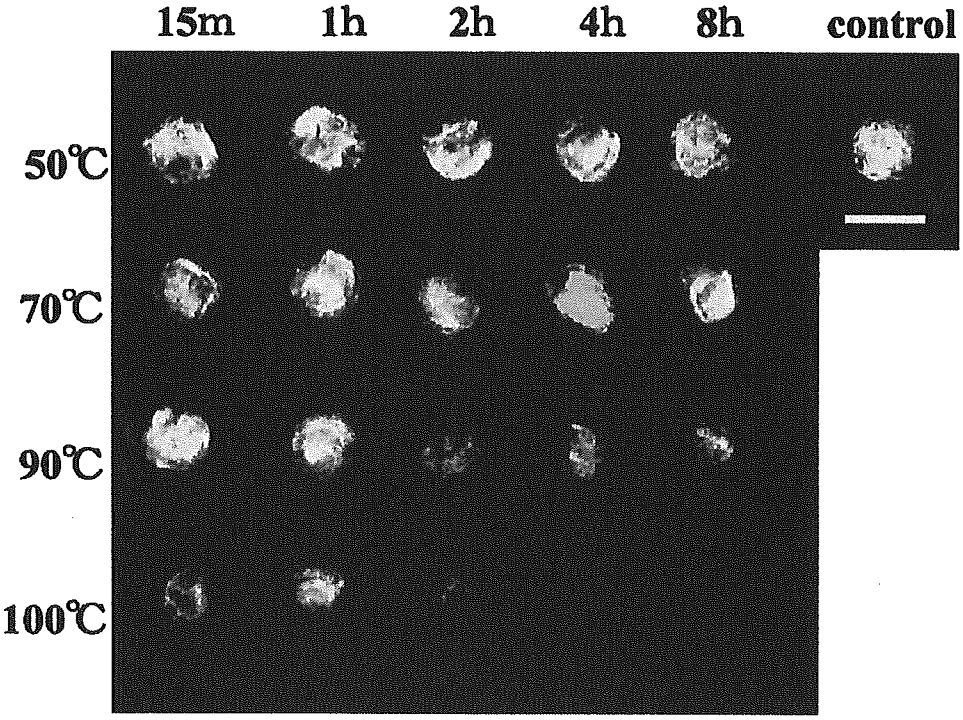

Fig. 2. Soft X-ray photograph of an ossicle formed at 3 weeks after implantation. A typical implant from each group is shown. *Scale bar* = 6 mm. There is no difference in the trabecular pattern within ossicles from each group. However, a reduction in size was observed over 90°C in a time-dependent manner. 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 μg 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

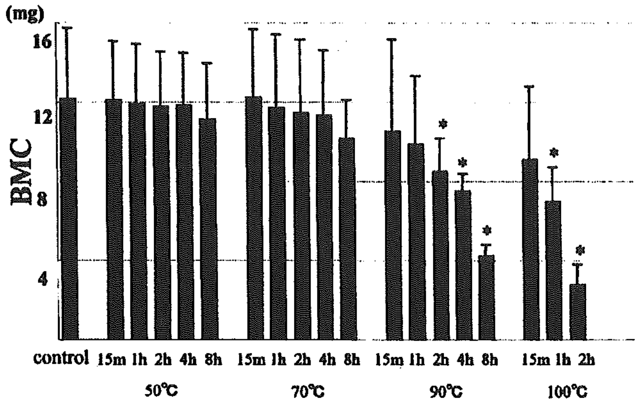

Fig. 3. The bone mineral content (*BMC*) of the tissues harvested at 3 weeks after surgery. Data are means ± SD of 8 samples. A significant difference from the control is indicated as * ($P < 0.05$). There was no significant difference between the BMC of the 50°C and 70°C groups and the control group. However, the mean BMCs of groups heated at 90°C for 2, 4, or 8h and the one heated at 100°C for 1, or 2h ($P < 0.01$) were significantly lower than that found in the control group. In the groups heated at 100°C for 4h, at 100°C for 8h, and at 120°C for 15min, 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 2h or more. After treatment for more than 4h at 100°C or for 15min 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 (±SD) BMC of the control group was 12.2 ± 3.56mg. In the group treated at 50°C for 8h, the mean BMC was 11.2 ± 2.81mg, 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 15min was 12.2 ± 3.45mg. 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 15min or 90°C for 1h were not significantly different from those of the control group. However, the mean BMC values of the groups treated at 90°C for 2h (8.48 ± 1.68mg), at 90°C for 4h (6.98 ± 1.06mg), or at 90°C for 8h (4.26 ± 0.53mg) were significantly reduced when compared with those of the control group ($P < 0.01$). Again, in the group treated at 100°C for 15min, 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 1h (6.96 ± 1.71mg) or at 100°C for 2h (2.80 ± 0.99mg) 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).

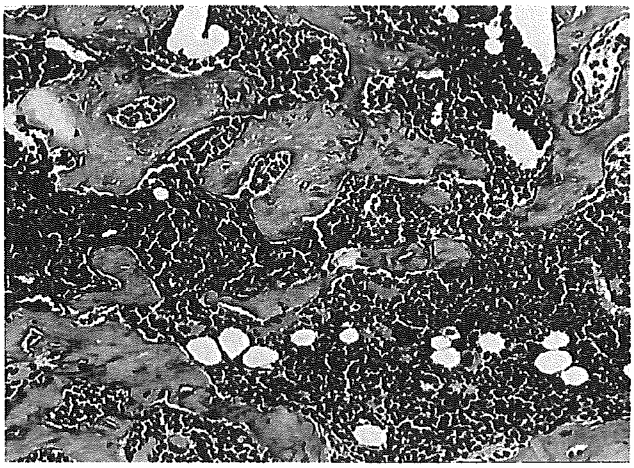

Fig. 4. Photomicrograph of the tissue harvested at 3 weeks after implantation (heated to 50°C for 8h) (H&E, ×200). Normal bone histology with hematopoietic marrow and bony trabeculae was observed

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 2h, 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 2h, 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

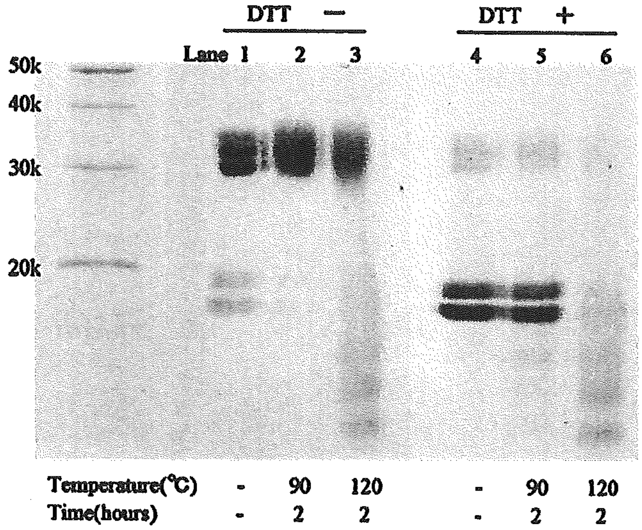

Fig. 5. SDS-PAGE profiles of rhBMP-2 before (*lane 1*) and after (*lane 2*) heating at 90°C and at 120°C (*lane 3*) for 2h. *Lanes 4, 5,* and *6* show the respective samples under reducing conditions in the presence of dithiothreitol (DTT, a disulfide-bond reducing agent). The original rhBMP-2 showed 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 autoclaving of rhBMP-2 causes this protein to lose its biological activity based on the results of the present study.

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ORIGINAL ARTICLE

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

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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].

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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 70ng/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 10ml 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 1500ng/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% CO_2 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 144h with each medium containing rhBMP-2 (0, 10, 100, 500, 1000, or 1500ng/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 96h with each medium containing rhBMP-2 (1000 or 1500ng/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 30min at 42°C. Aliquots of the cDNA pool obtained were subjected to PCR and amplified in a 20μl 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 3min, 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 10min. 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 [^{32}**P**]-labeled mouse BMPRs, Noggin, OC, Smad-4, and MyoD cDNA fragment probes at 65°C for 3h in hybridization buffer (50mM Tris-HCl (pH 7.5), 1mg/ml denatured salmon sperm DNA, 1% SDS, 1M NaCl, 10mM EDTA, 0.2% Ficol 400, 0.2% polyvinylpyrolidone, and 0.2% bovine serum albumin) and washed three times with 0.1 × SSC and $NaDodSO_4$ for 1h 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 positive control) were homogenized and dissolved in 0.5ml sample buffer (0.05M Tris-HCl (pH 6.8), 2% SDS, 6% β-mercaptoethanol, and 10% glycerol) and centrifuged at 12000*g* for 5min 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 280nm. Anti-mouse Noggin antibody (R&D Systems, Minneapolis, MN, USA) was used at 1μg/ml. Polyclonal goat antibodies for BMPRs (Santa Cruz, San Diego, CA, USA) were also used at a dilution of 1μg/ml. Aliquots of protein solution (5μl) were adjusted to 1μg/μl, mixed with 1% BPB (1μl), and then boiled for 2min and loaded onto each lane of SDS (10%–20%) acrylamide gradient gels (35mA, low voltage, 90min). 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 1h at room temperature, the membranes were washed with PBS for 5min, and then incubated for 1h 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 1h. 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 24h 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 1500ng/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 (1000ng/ml), rhBMP-2 (1500ng/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 1000ng/ml dose of rhBMP-2 and 60% mBMP-4. The mRNA expression of these molecules was readily detected at the 10ng/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 (100ng/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 (1000ng/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 24h is shown in Fig. 9A or 9B.

Protein expression levels

Western blotting on the muscle-derived primary culture cells using either 1000 or 1500ng/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 1000ng/ml and 1500ng/ml doses of rhBMP-2, and at 60% or 80% concentrations of mBMP-4. Therefore, rhBMP-2 (1000ng/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

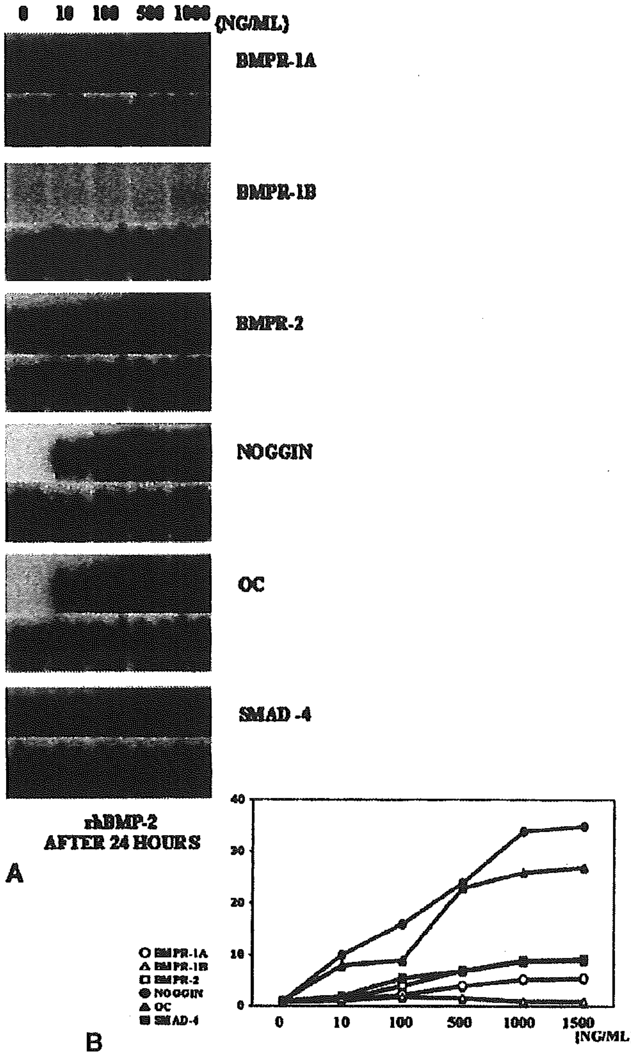

Fig. 1. Gene expression level of BMPR-1A, -1B, -2, Noggin, OC, and Smad-4 after 24-h stimulation of rhBMP-2 (0, 10, 100, 500, 1000 ng/ml) in muscle-derived primary culture cells by Northern blot analysis (**A**) and quantitation of the data of Northern blot analysis by Densitometry (**B**). G3PDH mRNA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

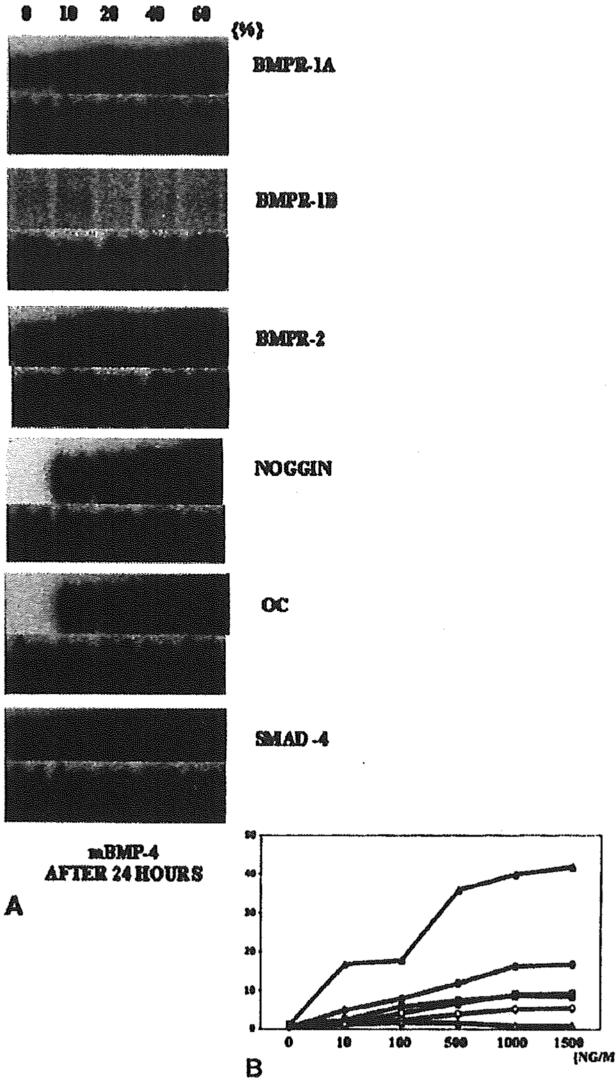

Fig. 2. Gene expression level of BMPR-1A, -1B, -2, Noggin, OC, and Smad-4 after 24-h stimulation of mBMP-4 [0%, 10%, 20%, 40%, and 60% (v/v) conditioned media] in muscle-derived primary culture cells by Northern blot analysis (**A**) and quantitation of the data of Northern blot analysis by Densitometry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

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 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