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Hepatocyte Growth Factor Contributes to Fracture Repair by Upregulating the Expression of BMP Receptors

Yuuki Imai,[1] Hidetomi Terai,[1] Chizumi Nomura-Furuwatari,[1] Shinya Mizuno,[2] Kunio Matsumoto,[2] Toshikazu Nakamura,[2] and Kunio Takaoka[1]

ABSTRACT: Hepatocyte growth factor (HGF) is activated and the expression of BMP receptors (BMPRs) is induced around the fracture site during the early phase of fracture repair. HGF facilitates the expression of BMPRs in mesenchymal cells. This study suggests that HGF contributes to fracture repair by inducing the expression of BMPRs.

Introduction: The precise mechanisms that control the upregulation of BMP, BMPRs, and other molecules involved in bone repair are not completely understood. In this study, we hypothesized that HGF, activated through the action of thrombin on the HGF activator, may enhance BMP action through the local induction of BMP or BMPRs.

Materials and Methods: Callus samples from tibial fractures in mice were harvested for immunohistochemical analysis of HGF and phosphorylated c-Met, for in situ hybridization of BMPRs, and for real-time RT-PCR analysis for the expression of HGF, c-Met, and BMPRs. To study the changes in gene expression of BMPRs in response to HGF, C3H10T1/2 cells were cultured with or without HGF and harvested for real-time RT-PCR and for Western blot analysis. To evaluate the contribution of HGF to the biological action of BMP2, C3H10T1/2 cells and primary muscle-derived mesenchymal cells were precultured with HGF and cultured with BMP2. In addition, the expression of the *luciferase* gene linked to the *Id1* promoter containing the BMP responsive element and alkaline phosphatase (ALP) activity were assayed.

Results: Positive immunostaining of HGF and phosphorylated c-Met was detected around the fracture site at 1 day after the fracture was made. mRNA expression of BMPRs was increased 1 day after fracture and localized in mesenchymal cells at the fracture site. From an in vitro study, the expression of mRNA for BMPRs was elevated by treatment with HGF, but the expression of BMP4 did not change. Western blot analysis also showed the upregulation of BMPR2 by HGF treatment. The results from the luciferase and ALP assays indicated increased responsiveness to BMPs by treating with HGF.

Conclusions: This study indicates that HGF is activated and expressed at the fracture site and that HGF induces the upregulation of BMPRs in mesenchymal cells. Furthermore, HGF may facilitate BMP signaling without altering the expression of BMP molecules.
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Key words: fracture, BMP receptors, hepatocyte growth factor, injury, upregulation

INTRODUCTION

T HE REGULATION OR promotion of repair in fractures or damaged bone is one of the most important subjects in the basic research and clinical practice areas of orthopedic surgery.[1] Classically, it has been recognized that fracture repair is achieved by local new bone or callus formation, which is attributed to the regenerating potential inherent to skeletal tissues. Attempts have been made to enhance the regeneration potential of bone to promote bone repair. To devise a more effective method to achieve this goal, it is

important to gain a more precise understanding of the biological mechanisms underlying the repair reaction in skeletal tissues. For many years, the precise molecular and cellular events involved in bone repair have remained a mystery. Fortunately, recent advances in molecular biology and related technologies have provided new approaches and insights into our understanding of how bone is repaired. Previous studies had linked fracture repair with the molecule(s) responsible for the regenerating potential of bone.[1,2] Because the discovery of bone-inducing activity in organic bone matrix, the sources have been identified (BMP-2, -4, and -7) and are now currently produced by the use of recombinant DNA technology.[3] Thereafter, recep-

tors for the BMPs (type I and type II serine/threonine kinase receptors) and Smad-dependent pathways involved in the cascade of BMP-related intracellular signaling have also been identified.[4] In terms of fracture repair, it has been shown that the expression of BMPs and BMP receptors (BMPR1 and 2) is upregulated in cells surrounding the fracture site during the initial phase of the repair process.[5] However, the trigger for the regulation of BMP expression elicited by the onset of fracture remains unknown.

Hepatocyte growth factor (HGF) was originally cloned as a potent growth factor for hepatocytes in the regenerating liver.[6] Subsequent extensive studies of HGF revealed a variety of biological activities (motogenic, mitogenic, or morphogenetic potential) that could contribute to the regenerating reaction in a broad range of damaged organs such as liver,[7] heart,[8] lung,[9] kidney,[10] and blood vessels[11] or other organs[12,13] by binding with its receptor, c-Met, and by phosphorylation of a tyrosine residue in its intracellular domain with kinase activity. However, the involvement of HGF in the bone repair reaction has, to date, not been explored. This report describes the results of a study into the role of HGF in the fracture healing process and, specifically, the effects of this growth factor on the biological activities and signaling mechanism of BMPs in in vivo and in vitro experimental systems.

MATERIALS AND METHODS

Experimental models

The right tibias of 65 ICR male mice were fractured by manual bending under anesthesia by methyl ether inhalation. The mice were maintained and monitored in cages with free access to water and food. Thirteen mice were killed by anesthesia at scheduled intervals (0, 1, 3, 7, and 14 days after the onset of the fracture), and nine of the fractured tibias with soft tissue around the fracture site were harvested: six were fixed in 10% neutral buffered formalin for in situ hybridization and immunohistochemical analysis of phosphorylated c-Met, and three were fixed in 70% ethanol for immunohistochemical analysis of HGF. The four remaining tibias were harvested for RNA extraction. This experimental protocol was approved by the Institutional Committee of Animal Care and Experiments of Osaka City University.

Histological sections

The specimens were decalcified in 0.5 M EDTA, dehydrated through a graded ethanol series, and embedded in paraffin. Sections of 5 μm thickness were prepared using a microtome and processed for routine hematoxylin/eosin staining, immunohistochemistry, and in situ hybridization.

Immunohistochemistry

Polyclonal antibody against rat HGF (cross-reacts with murine HGF) and anti-c-Met (pYpYpY1230/1234/1235) phospho-specific antibody were obtained from the Institute of Immunology (Tokyo, Japan) and BioSource International (Camarillo, CA, USA), respectively, and were diluted to 10 μg/ml and 1:50, respectively, as described pre-

viously.[14] Sections were deparaffinized and treated with 0.3% hydrogen peroxidase for 30 minutes at room temperature to block endogenous peroxidase. The sections were treated with 3% defatted dried milk in PBS for 30 minutes. After blocking with the dried milk solution, the sections were incubated with or without the primary antibody overnight at 4°C, washed three times with PBS for 5 minutes, and incubated with biotinylated horseradish peroxidase–conjugated anti-rabbit second antibody (DakoCytomation, Glostrup, Denmark) for 30 minutes at room temperature. After washing three times with PBS for 5 minutes, the sections were incubated with the Vectastain Elite ABC visualization system (Vector Laboratories, Burlingame, CA, USA), and the color reaction was developed by diaminobenzidine (DAB) followed by washing with distilled water. Finally, sections were counterstained with methyl green for 5 minutes at room temperature.

In situ hybridization

A 0.50-kb fragment of mouse BMPR1A cDNA and a 0.55-kb fragment of mouse BMPR2 cDNA were used as templates to synthesize RNA probes. They were subcloned into pBluescript SK(−) plasmid (Stratagene, La Jolla, CA, USA). The cDNA encoding the mouse BMPRs were obtained by RT-PCR, and the primers of BMPRs for PCR were selected as described previously.[15] In situ hybridization was carried out as described previously.[16]

Cell cultures

The mouse fibroblastic cell line C3H10T1/2 was obtained from the RIKEN Cell Bank (Tsukuba, Japan). Primary muscle-derived mesenchymal cells were prepared from the hindlimb of mice embryo (E15.5) as described previously without using collagenase treatment.[17] Cells were seeded at a cell density of 3×10^5 cells per 100-mm plastic dish and cultured in α-MEM (Sigma, St Louis, MO, USA) containing 10% (vol/vol) heat-inactivated FBS (Gibco, Grand Island, NY, USA) for growth or 2.5% FBS for examination at 37°C in 5% CO_2 humidified air. On reaching confluency, the cells were used in the subsequent experiments. C3H10T1/2 cells maintained between passages 7 and 12 were used for the in vitro experiments.

3-[4,5-Dimethylthyazol-2-yl]-2,5-diphenyl tetrazolium bromide assay

Cell proliferation was evaluated with an assay kit as described previously[18] (Promega). Fifteen microliters of dye solution was added to the cells on each well of a 96-well tissue culture plate and incubated at 37°C for 4 h. One hundred microliters of Solubilization/Stop solution was added to each well, and the plate was incubated at 37°C for 1 h and mixed thoroughly. The plates were read on a microplate reader at a wavelength of 595 nm.

Real-time RT-PCR

Total RNA was prepared from cells treated with or without 5 ng/ml of recombinant human HGF (rhHGF) for 0, 1, 3, 6, and 12 h on a 10-cm dish or from homogenized fractured tibias using ISOGEN (Wako, Osaka, Japan). One microgram of total RNA was reverse-transcribed into first-

strand cDNA with oligo dT primer using Superscript II reverse transcriptase (Invitrogen). Real-time RT-PCR was performed according to the manufacturer's instructions. Sequences for primers and TaqMan fluorogenic probes (Applied Biosystems, Foster City, CA, USA) were as follows: BMPR1A, forward primer, 5'-GGATCTCTCTATGA-CTTCCTGAAATGT-3', reverse primer, 5'-CAGC-AGAATAAGCTAACTTGAGTAGGG-3', TaqMan probe, 5'(FAM)-CCACACTAGACACCAGAG-(TAMRA)3'; BMPR2, forward primer, 5'-GCCA-AGATGAATACAATCAATGCA-3', reverse primer, 5'-CTTCTACCTGCCACACCATTCATA-3', TaqMan probe, 5'(FAM)-AGAGCCTCATGTGGTGAC-(TAM-RA)3'. TaqMan probes for HGF, BMP-4, and GAPDH were purchased from Applied Biosystems. Real-time RT-PCR for c-Met 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. To correct for variability in RNA recovery and efficiency of reverse transcription, GAPDH cDNA was amplified and quantified in each cDNA preparation. Normalization and calculation steps were performed as described previously.[19] For the in vitro study, experiments were performed on three separate test occasions with an n of 3 for each test occasion.

Immunoprecipitation and Western blot analysis

Cells were plated at a density of 1–2 × 10^4 cells/cm^2 on 100-mm plates and cultured for 2–3 days until a confluence of 80–90% was reached. rhHGF (5 ng/ml) was added to the media, and the cells were cultured for 12, 24, 48, or 72 h to examine the time dependency in response to HGF. To examine the dose-dependent response to HGF, different concentrations of HGF (0, 1, 5, 10, and 20 ng/ml) were added to the plates, and the cells were cultured for 72 h. After HGF treatment, immunoprecipitation was performed using the immunoprecipitation kit, Immunoprecipitation Starter Pack (Amersham Bioscience) according to the manufacturer's instructions. The polyclonal goat antibodies against mouse BMPR2 (1 μg for each sample; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. The resultant sample was boiled for 5 minutes in 20 μl of sample buffer for SDS-PAGE as described previously.[15] Equal amounts of protein samples were applied and run on each lane of an SDS 10% acrylamide gel (40 mA, low voltage, 90 minutes), and ultimately blotted to an enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham). The membranes were developed with ECL plus reagent (Amersham). To examine the possible contribution of platelet growth factors to modulate or upregulate expression of the BMPRs, the heterodimer of platelet-derived growth factor AB (PDGF-AB; R&D Systems) was used at a dose of 5 ng/ml using the same protocol described for rhHGF. We selected PDGF-AB instead of PDGF-BB based on a previous report indicating the negative effect of PDGF-BB on osteogenic differentiation.[20] The intensity of staining of each band was calculated using a digital densitometer (Bio-Rad).

Relative luciferase activity assay

To detect the changes in intensity of BMP signaling after treatment with HGF, the C3H10T1/2 cells and primary muscle-derived mesenchymal cells were transfected with 1 μg of the luciferase gene linked to the Id1 promoter containing the BMP responsive element or a mutated form without the promoter function (kindly provided by Dr T Katagiri, Saitama Medical School Research Center for Genomic Medicine, Saitama, Japan). Each construct was used together with 250 ng of the control luciferase vector (pRL) that was used as an internal control to calculate transfection efficiency by the calcium phosphate/DNA precipitation method. For the transfection, 5 × 10^3 cells/well were plated on 48-well plates for 1 day, and medium was changed 2 h before transfection. Twelve hours later, cells were washed with Hanks' balanced salt solution (Sigma) twice, and cells were treated with or without 5 ng/ml of rhHGF for 0, 12, 24, 48, or 72 h. The other group was treated with medium containing 1 μg/ml of the anti-c-Met antibody (R&D Systems) before pretreatment with HGF for 72 h. The culturing of the cells was continued with or without the addition of 100 ng/ml of rhBMP-2 (Yamanouchi Pharmaceutical Co., Tokyo, Japan) for an additional 24 h. Luciferase activity was determined by a Dual-Glo Luciferase Reporter Assay System (Promega, Madison, WI, USA) as previously described.[21]

Protocols for assay for alkaline phosphatase activity

Alkaline phosphatase (ALP) levels in C3H10T1/2cells and primary muscle-derived mesenchymal cells were assayed to check for the effects of HGF on ALP induction by BMP signaling at the translational level. Both types of cells were seeded at a density of 1 × 10^4 cells/well in 48-well plates ($n = 8$ per group). On achieving confluency, the cells were pretreated with 5 ng/ml of rhHGF in culture medium for 0, 12, 24, 48, and 72 h, washed twice with PBS, and treated with medium plus 100 ng/ml of rhBMP-2 for 2 days. The other group was treated with medium containing 1 μg/ml of the anti-c-Met antibody as described above. The cells were washed twice with normal saline, and ALP activity was assayed as described previously using p-nitrophenylphosphate as the substrate.[22] The effects of HGF on BMP-induced ALP activity were normalized by protein. Experiments were performed in triplicate independently.

Statistical analysis

Data are expressed as the mean ± SD for each group. Statistical differences among treatment groups were analyzed using Fisher's protected least significant difference (PLSD) test. Values of $p < 0.05$ were considered significant.

RESULTS

Immunohistochemical detection of endogenous HGF and phosphorylated c-Met at the early phase of fracture repair

Immunohistochemical analyses using anti-rodent HGF and phosphorylated c-Met revealed positive signals especially around the fracture sites 24 h after the onset of the fracture. The HGF$^+$ cells were predominantly localized in
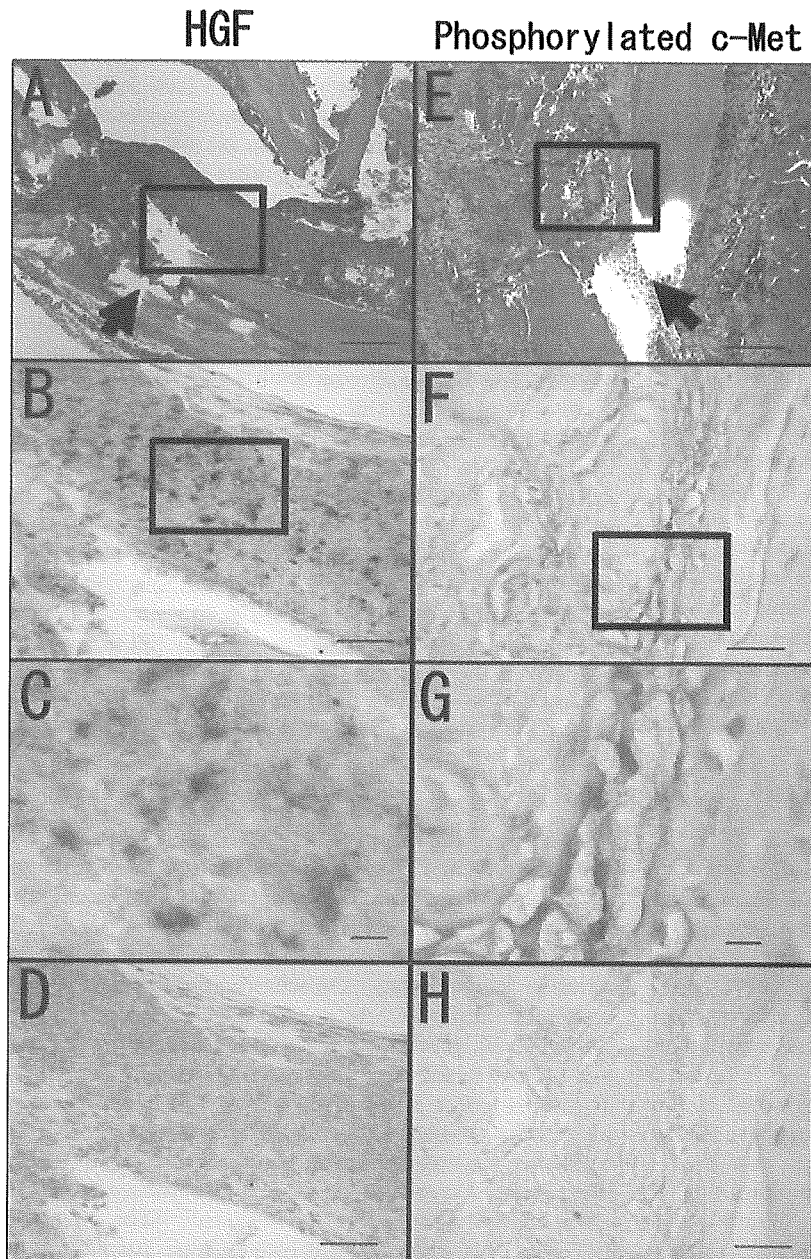
HGF

Phosphorylated c-Met

FIG. 1. Localization of HGF and phosphorylated c-Met at 24 h after the onset of fracture. Immunohistochemical localizations of HGF and phosphorylated c-Met were detected with (B and C) anti-rodent HGF antibody and with (F and G) anti-tyrosine (pYpYpY1230/1234/1235)-phosphorylated c-Met antibody, respectively. (B and F) High magnification of the boxed areas of A and E. (C and G) High magnification of the boxed areas of B and F. (D and H) Negative controls stained without primary antibodies at the same magnification of B and F. Arrows indicate the fracture sites. Bars = 200 (top panels), 50 (second and bottom panels), and 10 μm (third panels).

stromal cells around the fracture site (Figs. 1B and 1C), whereas c-Met phosphorylation was noted mainly in parenchymal areas nearby the fracture site (Figs. 1F and 1G). In contrast, there was no apparent signal in the negative controls stained without primary antibodies (Figs. 1D and 1H). Of note, immunopositive signals for HGF and phosphorylated c-Met were not detected around the fracture site immediately after the onset of the fracture and were only weakly visible 3 days after fracture (data not shown). This result indicates a specific activation of HGF/c-Met signals that is caused by the injury of the bone. Based on the histological data, we hypothesized that the paracrine delivery system of HGF toward mesenchymal cells may be critically involved in the initial phase of fracture repair. Furthermore, the upregulation of HGF and c-Met mRNA expression was

detected in the early phase of fracture repair by real-time RT-PCR (Fig. 2). Based on previous results indicating upregulated HGF and c-Met expression by activated HGF,[23] these results also provide evidence of immediate HGF activation and activity around the fracture site.

Upregulation of BMPR mRNA expression at the fracture site

In situ hybridization at the fracture site showed mRNA expression of BMPRs in the mesenchymal cells around the fracture site in the early phase of fracture repair, chondrocyte-like cells in the callus at day 7, and osteoblastic cells in newly formed bone at day 14 (Fig. 3). We could not detect the positive staining cells in the specimens using sense
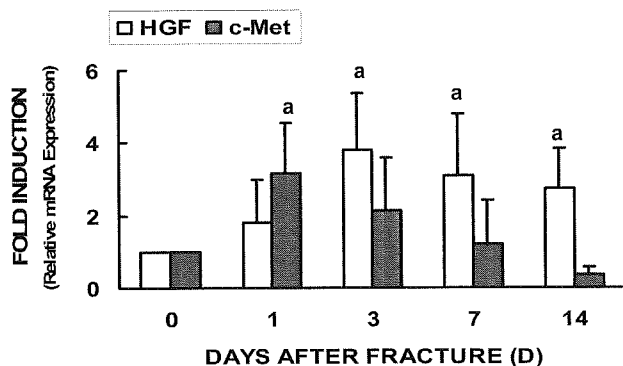
FIG. 2. Relative expression levels of HGF (open bars) and c-Met (patterned bars) mRNA analyzed by real-time RT-PCR. One microgram of total RNA extracted from the fracture site at days 0, 1, 3, 7, and 14 was used for examination. Relative mRNA expression of HGF and c-Met normalized with those of amplified GAPDH signals are indicated. Value of day 0 after fracture is indicated as 1. Significant increase of HGF and c-Met mRNA expression is shown ([a]$p < 0.05$ compared with day 0). Error bars indicate SD values from experiments carried out in triplicate.

probes at any time-point (data not shown). Real-time RT-PCR for BMPRs using extracted total RNA from the fracture site showed a statistically significant 4-fold increase in BMPR1A mRNA expression at day 1 that was maintained up to day 14. The significantly increased BMPR2 mRNA expression was also detected at day 1, and it increased a further 6-fold by day 14 (Fig. 4).

Upregulated expression of c-Met, BMPR1A, and BMPR2 by HGF in an in vitro system

The effects of HGF on cell proliferation under in vitro conditions were examined by MTT assay. There was no significant proliferation effect of HGF on the cells at the concentrations used in this experiment (data not shown). All in vitro experimental results were normalized by values calculated from the MTT assay.

Based on the above results observed in an in vivo system, the effects of HGF in relation to BMP expression and the BMP signaling system were analyzed in an in vitro system using a cell line of mesenchymal origin (C3H10T1/2 cells) and various molecular biological methods.

Real-time RT-PCR analysis showed expression of c-Met in the C3H10T1/2 cells stimulated by exposure to exogenous rhHGF for >1 h (Fig. 5A), thereby mimicking the in vivo result described above.

Although the qualitative RT-PCR analysis indicated the constitutive expression of BMPR1A and BMPR2 in the cells (data not shown), the real-time RT-PCR analysis showed a significant elevation in the expression of the BMP-receptors at 6 h. Thereafter, a decrease in BMPR1A and BMPR2 expression was noted after the addition of rhHGF relative to the control where there was no HGF treatment (Fig. 5B). However, no change in the level of BMP4 expression was noted after treatment with HGF (Fig. 5B).

To verify that the upregulated translation resulted from upregulation of transcription of the BMP receptors, we ran

a Western blot analysis for BMPR2. The results confirmed the upregulated translational expression of BMPR2 after addition of rhHGF in a time- (Fig. 5C) and dose- (Fig. 5D) dependent manner. The BMPR2 synthesis was not affected by PDGF-AB at the concentration of 5 ng/ml that we used in this study (Fig. 5C).

Effects of HGF on signaling of BMP

The relative luciferase expression assay in C3H10T1/2 cells (data not shown) and primary muscle-derived mesenchymal cells using the *Id1* promoter containing the BMP responsive element showed elevation of transcriptional activity of luciferase by pretreatment with HGF for >48 h followed by treatment with BMP compared with the group that did not receive either HGF or BMP (Fig. 6A). Furthermore, increased transcriptional activity obtained by HGF pretreatment was abolished by addition of the anti-c-Met neutralization antibody. ALP activities in primary muscle-derived mesenchymal cells also were significantly elevated in the group treated with rhHGF for 24 h or more in comparison with the group treated with BMP only. The enhanced BMP-2–induced ALP activity by HGF was also blocked by addition of the anti-c-Met antibody (Fig. 6B). In C3H10T1/2 cells, almost the same results were obtained (data not shown). These results indicate that HGF and its receptor are involved in a mechanism to regulate BMP signaling through the transcriptional regulation of BMPRs.

DISCUSSION

In this experimental study, we investigated the contribution of HGF to the healing reaction in fracture. The results indicate that localization and activation of HGF occur around the fracture site during the early phase of the healing process and that the expression of HGF is upregulated at the fracture site during the same time. This early upregulation of expression of HGF mRNA resembles that of pulmonary ischemia-reperfusion injury.[24] The observations in healing bone are consistent with previous studies that have shown that HGF is linked with the regenerative processes in a wide range of organs and tissues. As already reported, HGF is excreted from cells in a latent form and is converted to the active form by HGF activation factor (HGF-AF), which in turn is activated by thrombin, a blood coagulation factor.[25] In this type of pathological condition, HGF has multiple roles in the repair of injured tissues, for example, by inducing angiogenesis. The active form of HGF was not detected directly at the fracture sites in this study. However, we could confirm the phosphorylation of c-Met (Fig. 1), which is essential for the biological activity of HGF and mRNA expression of HGF itself and of c-Met (Fig. 2), which could be induced by HGF as previously reported,[23] instead of detection of the active form of HGF,[23] although it could not be easily compared at each time-point because of the heterogeneity of cells in the fracture site. In terms of activation of the locally produced or circulating HGF, the presence of thrombin in the hematoma formed at the fracture site just after the onset of the fracture might contribute through the activation of HGF-AF and HGF. Thus, a nonspecific circulatory disruption at
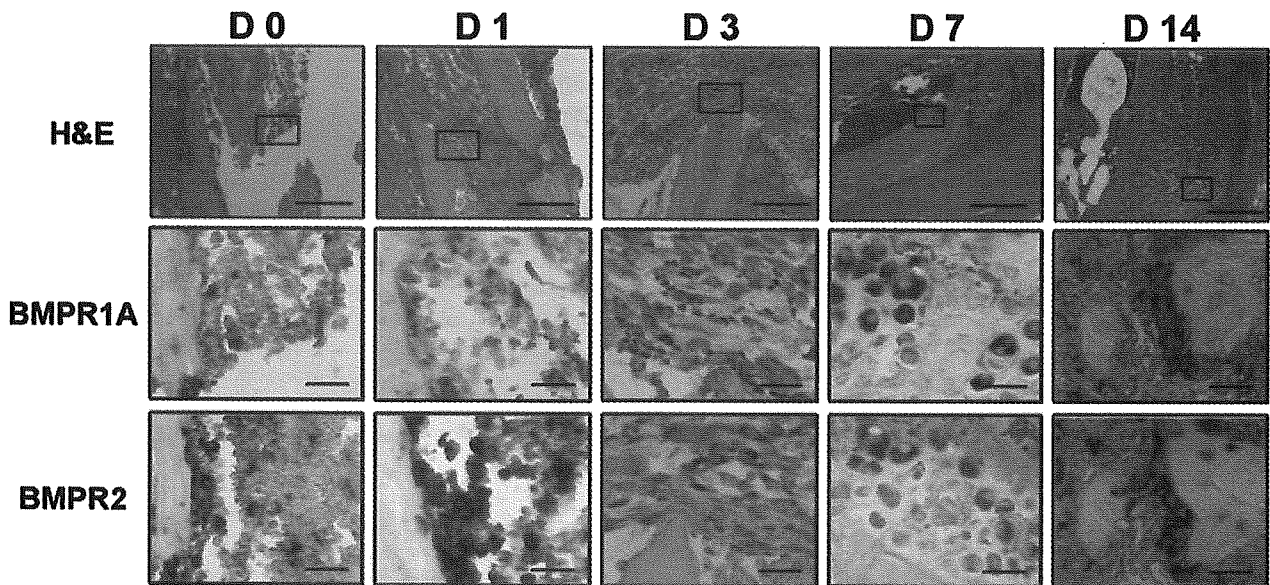
FIG. 3. Localization of BMPR1A and BMPR2 mRNA in the early phase of fracture repair. The top panels of H&E staining indicate the histology of the fracture site. The second and third panels indicate the results of in situ hybridization using antisense probes of BMPR1A and BMPR2, respectively. Bars = 500 (top panels) and 20 μm (other panels).
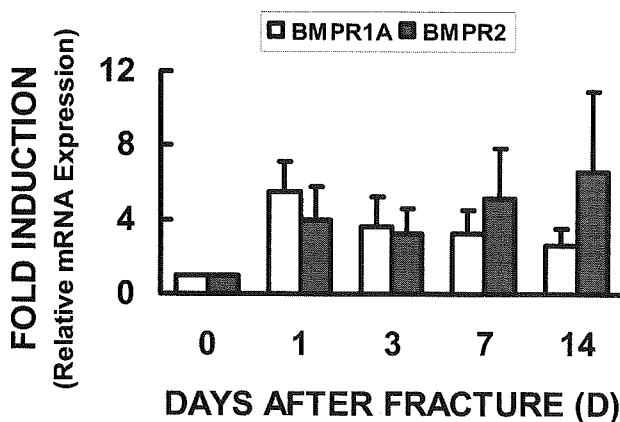

FIG. 4. Relative expression levels of BMPR1A (open bars) and BMPR2 (striped bars) at the fractured site examined by real-time RT-PCR as described in Fig. 2. Significant increase of BMPR1A and BMPR2 mRNA expression is shown in all samples compared with day 0 ($p < 0.05$). Error bars indicate SD from experiments carried out in triplicate.

the onset of injury to any tissue might enable the local activation of HGF and initiate the tissue repair reaction. The results of in situ hybridization of BMPRs (Fig. 3) indicate that the fracture healing process begins as a result of the immediate mRNA expression of BMPRs in the cells around the fracture site. It is possible that the immediate reactions mentioned above do not occur by newly induced molecules, but instead by activated molecules at the fracture site. From this perspective, we tried to study the relationship between HGF, the activation of which is related to the hemorrhage, and the expression of BMPRs.

In this study, the upregulated expression of BMPRs at the transcriptional and translational levels was confirmed by real-time RT-PCR and Western blotting, respectively.

These results have revealed the potential contribution of HGF to the fracture healing process. The HGF receptor, c-Met, has a tyrosine kinase activity at its intracellular domain and acts downstream of the MAPK cascade. Furthermore, the activating protein-1 binding element has been located in the promoter region of both BMPR1A and BMPR2. These facts suggest that mRNA expression of BMPRs is able to be induced by HGF. However, we have not shown that the transcriptional regulation of BMPRs occurs as a consequence of intracellular signaling by c-Met.

Although BMP molecules were reported to be upregulated in adjacent periosteal cells during the early phase of fracture repair at the fracture site,[1] this type of change in BMP expression was not noted after stimulation by HGF in the in vitro system. However, BMP receptors in the mesenchymal cells were upregulated by HGF and could potentially contribute to healing of the fracture by amplifying BMP signal transduction and promoting fracture healing reactions during the initial phase of fracture repair. This response seems to be specific to HGF, because PDGF, the receptors of which also have tyrosine kinase domains, did not significantly induce expression of BMPRs for C3H10T1/2 in our study. However, the in vitro studies are limited by the clonal nature of the cell types involved in these studies and the absence of circulating hormones.

From our in vivo study, the expression levels of BMPRs were elevated for several days during fracture repair (Fig. 4); however, from our in vitro study, HGF transiently induced mRNA expression of BMPRs (Fig. 5B). This discrepancy can be explained by the following two points: (1) the stimulation to the multipotent cells by HGF may continue during the early phase of fracture repair in vivo, although the stimulation was transient in vitro, and (2) because the in vivo studies take place in an environment different from the in vitro studies, there may be molecules,
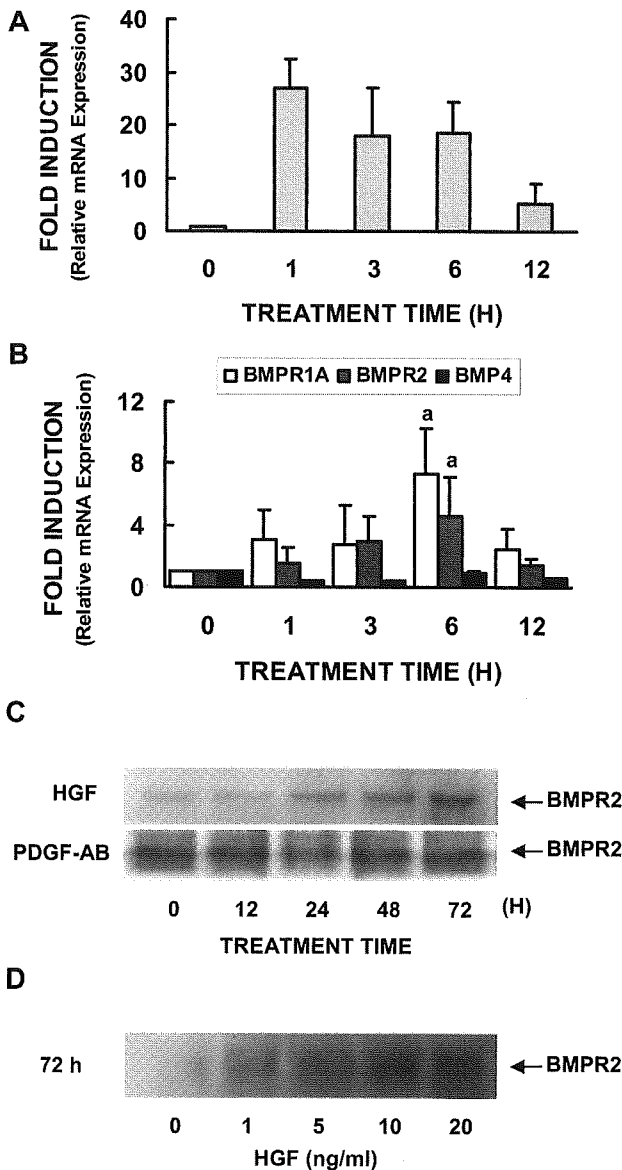
FIG. 5. (A) Changes in the relative expression levels of c-Met mRNA analyzed by real-time RT-PCR. One microgram of total RNA was extracted from C3H10T1/2 cells treated with 5 ng/ml of rhHGF for 0, 1, 3, 6, and 12 h and were used for analysis. Value of day 0 after fracture is indicated as 1. Significant increase of c-Met mRNA expression is shown in all samples compared with hour 0 ($p < 0.05$). (B) Changes in the relative expression levels of BMPR1A (open bars), BMPR2 (striped bars), and BMP4 (closed bars) mRNA analyzed by real-time RT-PCR. The RNA was prepared as described in A. The mRNA expression of BMPR1A and BMPR2 is significantly increased in the group treated with HGF for 6 h compared with hour 0 ([a]$p < 0.05$); however, BMP4 mRNA expression was not affected. Error bars indicate SD from experiments carried out in triplicate. (C) Western blot analysis for BMPR2 using immunoprecipitated products of C3H10T1/2 cells treated with or without 5 ng/ml of HGF (top panel) and with or without 5 ng/ml of PDGF-AB (bottom panel) for several hours. (D) Same as C but treated with HGF for 72 h in a dose-dependent manner.

for example, BMPs,[15] which can induce the expression of BMPRs around the fracture site. Therefore, our results suggest that the effect of HGF in the expression of BMPRs has
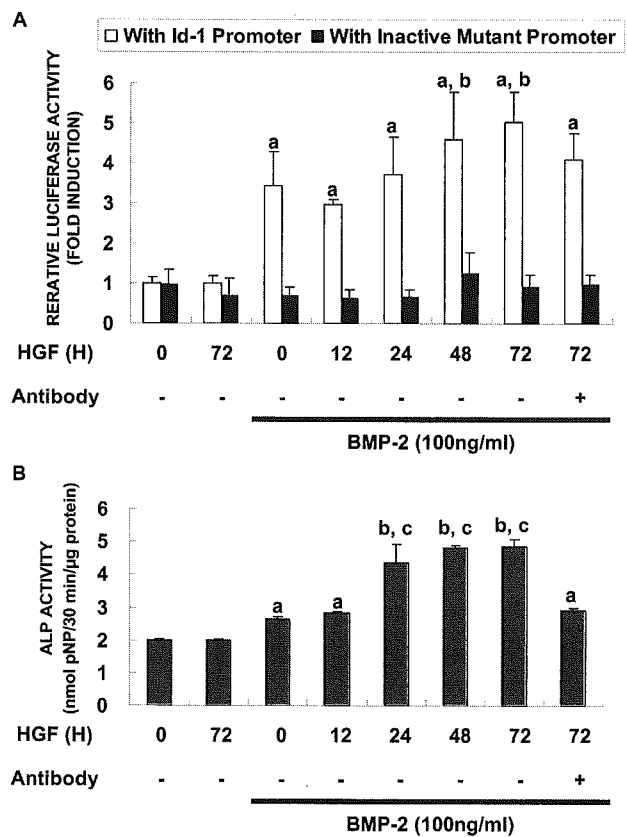
FIG. 6. (A) Relative luciferase activity in the cells transfected with Id1 promoter-containing plasmids. Primary muscle-derived mesenchymal cells were pretreated with or without HGF and after treatment with BMP2. Relative luciferase activity of the groups treated with 100 ng/ml of BMP2 (groups 0, 12, 24, 48, 72) is shown. In the groups with wildtype Id1 promoter-containing plasmids (open bars), the relative luciferase activity of the groups pretreated with 5 ng/ml of HGF for >48 h before treatment with BMP2 are significantly increased compared with the group that was not treated with HGF ([a]$p < 0.05$ to the group treated without HGF or BMP; [b]$p < 0.05$ to the group treated with BMP2 only). The relative luciferase activities of the groups with mutant Id1 promoter-containing plasmids (patterned bars) are not altered in the presence or absence of BMP and/or HGF treatment. The effects of HGF and BMP2 are abolished by addition of anti-c-Met neutralization antibody. Error bars indicate SD from experiments carried out in triplicate. (B) ALP activities of the groups treated with 5 ng/ml of HGF for >24 h followed by treatment with 100 ng/ml of BMP2 are significantly elevated compared with the control group that is treated only with BMP2 ([a]$p < 0.05$ and [b]$p < 0.01$ to the group treated without HGF or BMP, respectively; [c]$p < 0.01$ to the group treated with BMP only). The effect of HGF was abolished by addition of anti-c-Met neutralization antibody.

a significant role as the trigger of fracture repair by BMP signaling despite the effect being transient and mild.

To further understand the interaction between the activation of HGF and the expression of BMPRs, it will be necessary to form a system for the local administration of HGF into the fracture site and use a null animal model. Unfortunately, the null mouse of c-Met[26] and HGF[27,28] are embryonic lethal as previously reported, so siRNA intervention or a conditional knockout animal model will be required. On the other hand, to clarify the precise mecha-

nism of fracture repair, further study will be necessary to determine the identity and molecular mechanisms by which other factors regulate expression of BMP molecules during the early phase of fracture healing.

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Address reprint requests to:
Yuuki Imai, MD
Department of Orthopaedic Surgery
Osaka City University Graduate School of Medicine
Asahimachi 1-4-3, Abenoku
Osaka 545-8585, Japan
E-mail: imai@med.osaka-cu.ac.jp

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

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

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

(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].

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% 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 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 μl reaction mixture using Taq polymerase (Takara Shuzo). Amplifications were performed in a Program Temp Control System (PC800; ASTEC, Fukuoka, Japan) for 30 cycles after an initial denaturation step at 94°C for 3 min, denaturation at 94°C for 30 s, annealing for 30 s at 60°C, and extension at 72°C for 90 s, with a final extension at 72°C for 10 min. Reaction products were electrophoresed in a 1.5% agarose gel, and the amplified DNA fragments were visualized by ethidium bromide staining under UV light. PCR products were subcloned and sequenced using a DNA sequencing kit (Applied Biosystems, Warrington, UK). The primers of Noggin, Smad-4, OC, and MyoD for PCR were set as described previously [10,13,15]. The primers of BMPRs for PCR were set as follows: BMPR-1A, 5′-CTCATGTTCAAGGGCAG-3′ (5′ sense) and 5′-CCCCTGCTTGAGATACTC-3′ (3′ antisense; 346–362 and 850–833, respectively); BMPR-1B, 5′-ATGTGGG CACCAAGAAG-3′ and 5′-CTGCTCCAGCCCAATGC T-3′ (215–231 and 681–664, respectively); BMPR-2, 5′-GTGCCCTGGCTGCTATGG-3′ and 5′-TGCCGCCTC CATCATGTT-3′ (47–64 and 592–575, respectively). Nucleotide sequences of the cDNA fragments were checked and found to be identical to mouse BMPRs (BMPR-1A, NM009758; BMPR-1B, NM007560; BMPR-2, NM007561). The specificity of these cDNAs was confirmed by sequencing using an autosequence analyzer (ABI Prism 310 Genetic Analyzer; Perkin-Elmer Japan, Tokyo, Japan).

Northern blot analysis

Twenty micrograms of total RNA were separated by electrophoresis on a 1.0% agarose–formaldehyde gel and blotted onto Hybond-N^+ membrane (Amersham Intl., Piscataway, NJ, USA) for Northern blotting. Filters were hybridized overnight with random-primed [^{32}P]-labeled mouse BMPRs, Noggin, OC, Smad-4, and MyoD cDNA fragment probes at 65°C for 3 h in hybridization buffer (50 mM Tris-HCl (pH 7.5), 1 mg/ml denatured salmon sperm DNA, 1% SDS, 1 M NaCl, 10 mM EDTA, 0.2% Ficol 400, 0.2% polyvinylpyrolidone, and 0.2% bovine serum albumin) and washed three times with 0.1 × SSC and $NaDodSO_4$ 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 12000 g 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 μg/ml. Polyclonal goat antibodies for BMPRs (Santa Cruz, San Diego, CA, USA) were also used at a dilution of 1 μg/ml. Aliquots of protein solution (5 μl) were adjusted to 1 μg/μl, mixed with 1% BPB (1 μl), and then boiled for 2 min and loaded onto each lane of SDS (10%–20%) acrylamide gradient gels (35 mA, low voltage, 90 min). After running the gels, BMPR-1A, -1B, -2, and Noggin proteins in mouse embryo muscle-derived cells, and MC3T3-E1 and NIH3T3 cells, and mouse skeletal muscle tissue were stained with Coomassie brilliant blue (Sigma Chemical). The protein bands were then transferred to polyvinylidene difluoride membrane (Immunobilon-P Transfermembrane, Millipore, Bedford, MA, USA) according to the manufacturer's instructions. After treatment with Blocking Reagent (Nippon Roche, Tokyo, Japan) for 1 h at room temperature, the membranes were washed with PBS for 5 min, and then incubated for 1 h with primary antibody (BMPRs, 1:200; Noggin, 1:100). After two 5-min washes with PBS, the membranes were incubated with peroxidase-conjugated rabbit anti-goat antibody (1:50; Histofine, Nichirei, Tokyo, Japan) for 1 h. After two further 5-min washes with PBS, the immunoblot was developed using an ImmunoStar Kit for Rabbit (Wako Pure Chemical Industries, Tokyo, Japan) to detect biotin and chemiluminescence.

Results

Expression level of messenger RNA (mRNA)

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

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

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

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

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

Protein expression levels

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

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

Discussion

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

0 10 100 500 1000 {NG/ML}

BMPR-1A

BMPR-1B

BMPR-2

NOGGIN

OC

SMAD -4

rhBMP-2
AFTER 24 HOURS

A

O BMPR-1A
△ BMPR-1B
□ BMPR-2
● NOGGIN
▲ OC
■ SMAD-4

B

0 10 100 500 1000 1500
{NG/ML}

0 10 20 40 60 {%}

BMPR-1A

BMPR-1B

BMPR-2

NOGGIN

OC

SMAD -4

mBMP-4
AFTER 24 HOURS
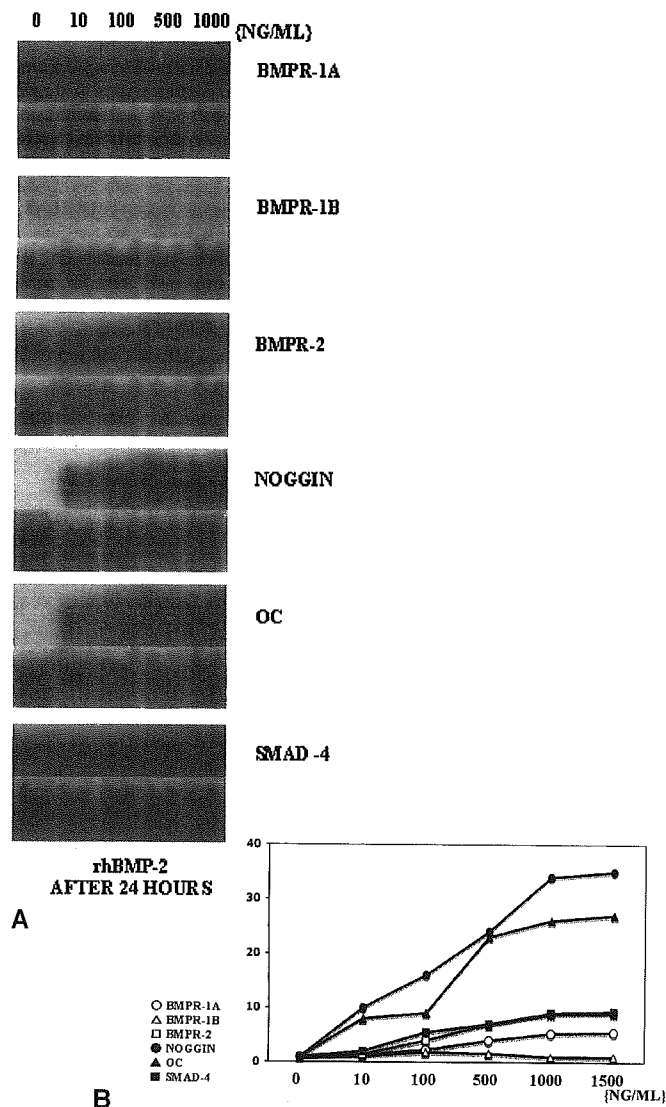
A

B

0 10 100 500 1000 1500
{NG/ML}

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)
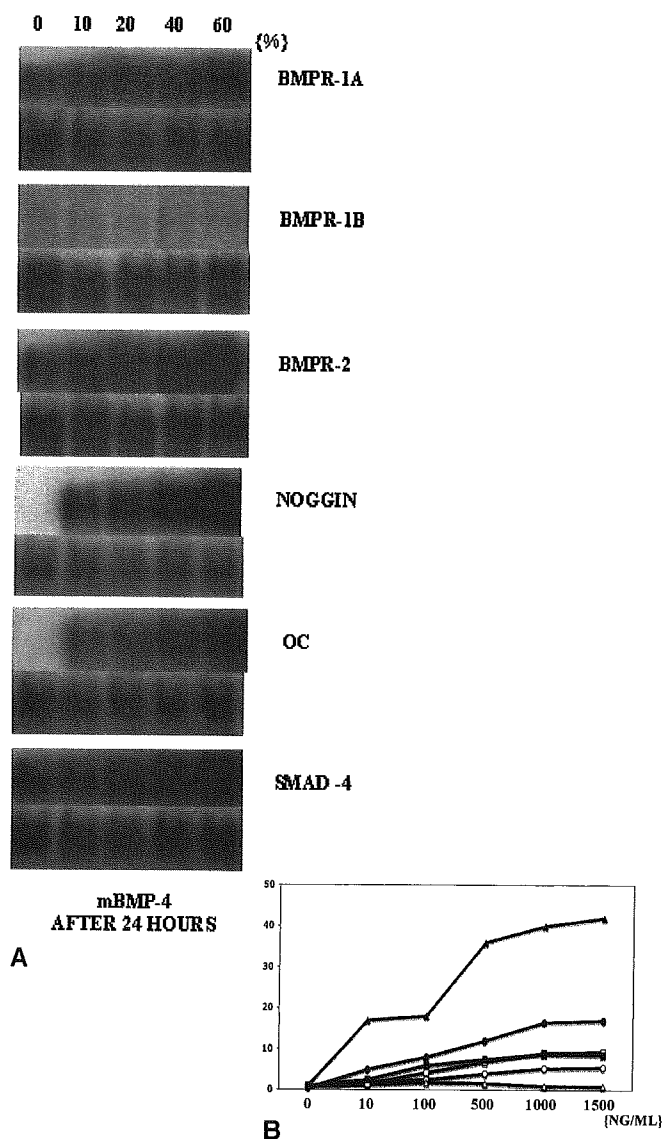
Fig. 2. Gene expression level of BMPR-1A, -1B, -2, Noggin, OC, and Smad-4 after 24-h stimulation of mBMP-4 [0%, 10%, 20%, 40%, and 60% (v/v) conditioned media] in muscle-derived primary culture cells by Northern blot analysis (A) and quantitation of the data of Northern blot analysis by Densitometry (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
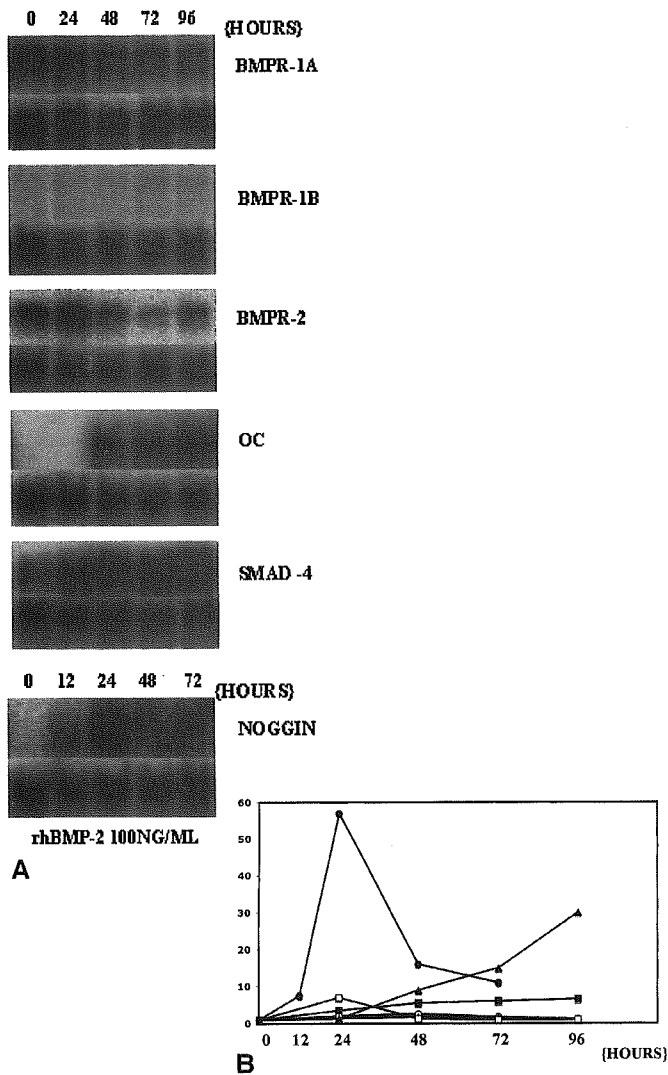
430

0 24 48 72 96 {HOURS}

BMPR-1A

BMPR-1B

BMPR-2

OC

SMAD-4

0 12 24 48 72 {HOURS}

NOGGIN

rhBMP-2 100NG/ML

A

B

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

0 24 48 72 96 {HOURS}

BMPR-1A

BMPR-1B

BMPR-2

OC

SMAD-4

0 12 24 48 72 {HOURS}

NOGGIN

mBMP-4 20 %

A

B

O BMPR-1A
△ BMPR-1B
□ BMPR-2
● NOGGIN
▲ OC
■ SMAD-4

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

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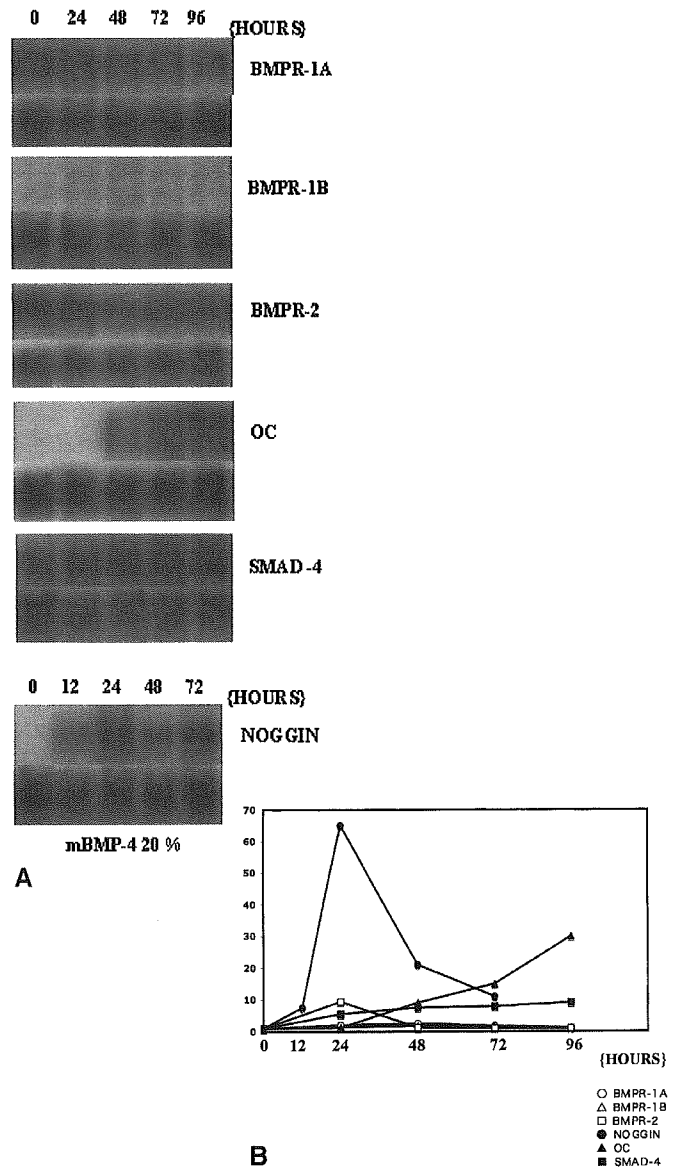
Noggin might act as a negative regulator of the BMP-induced cellular reactions, and consequently reduce the susceptibility of the cells to BMPs.

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-
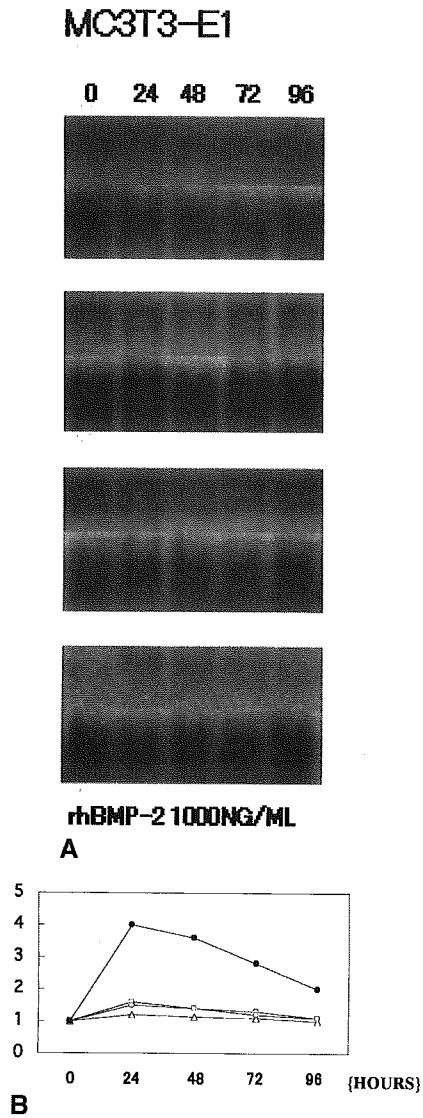
MC3T3-E1

0 24 48 72 96

rhBMP-2 1000NG/ML

A

B

0   24   48   72   96   (HOURS)

MC3T3-E1

0 24 48 72 96 (HOURS)

BMPR-1A

BMPR-1B

BMPR-2

NOGGIN

mBMP-4 60 %

A

B

0   24   48   72   96   (HOURS)

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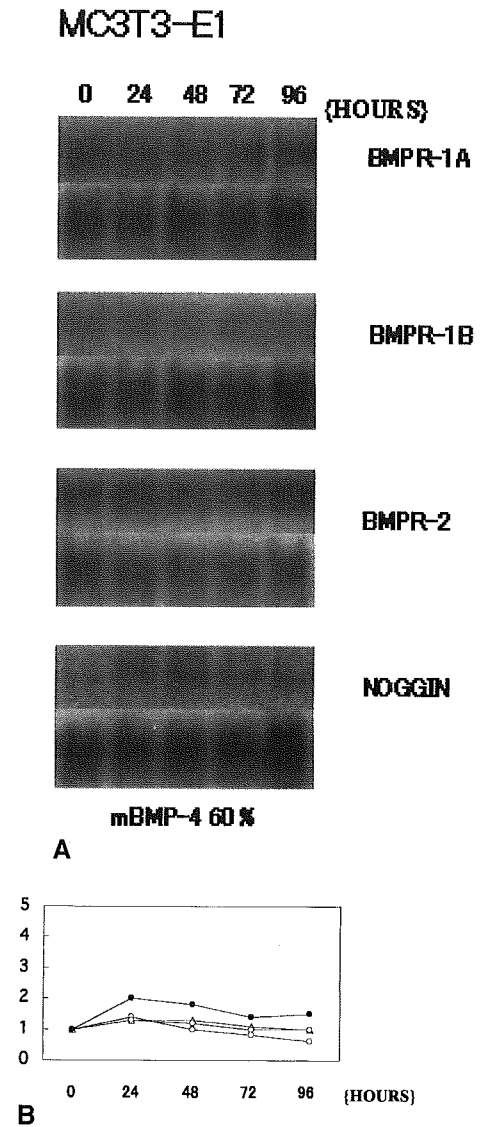
muscle-derived primary culture cells might lose the muscle phenotype after BMP exposure.

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
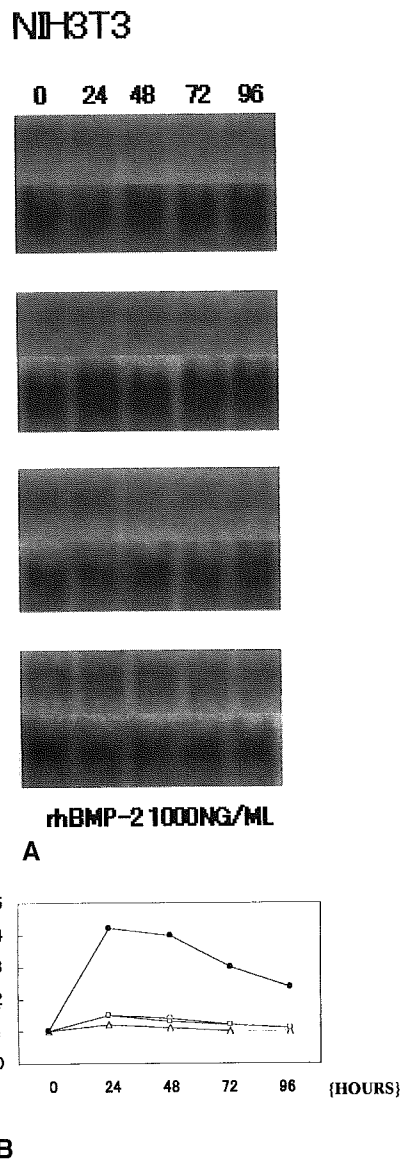
NIH3T3

0 24 48 72 96

rhBMP-2 1000NG/ML

A

5
4
3
2
1
0

0   24   48   72   96   {HOURS}

B

NIH3T3

0 24 48 72 96 {HOURS}

BMPR-1A

BMPR-1B

BMPR-2

NOGGIN

mBMP-4 60 %

A

5
4
3
2
1
0

0   24   48   72   96   {HOURS}

○ BMPR-1A
△ BMPR-1B
□ BMPR-2
● NOGGIN

B

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

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 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.
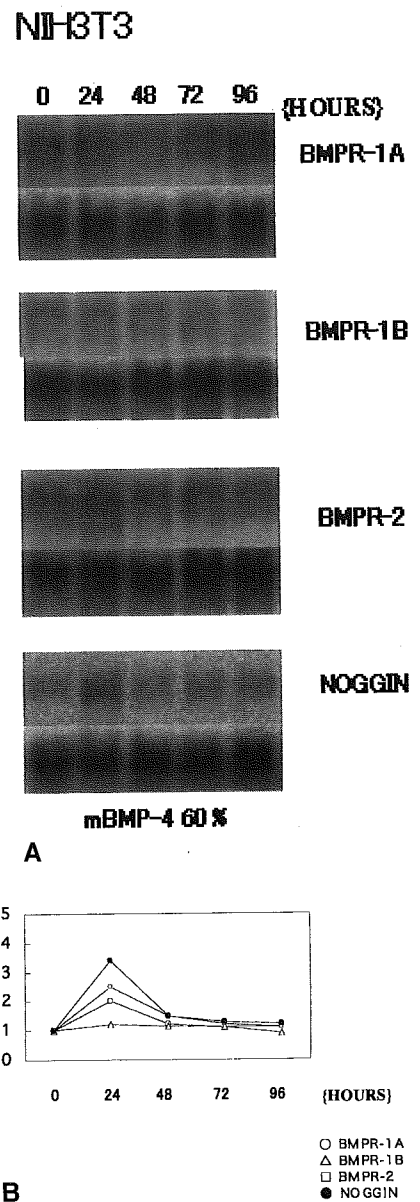
The majority of undifferentiated mesenchymal cells in muscle-derived primary culture cells showed a fibroblastic appearance. These cells are considered to be heterogenous, and contain some kinds of precursor cells such as bone, cartilage, and muscle. They differentiate into each phenotype when they are placed in each differentiation condition.
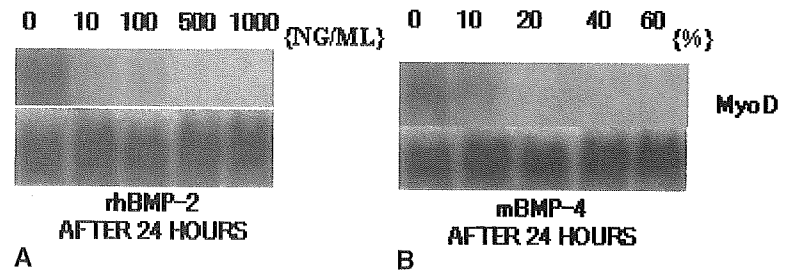
Fig. 9. The expression of MyoD in muscle-derived primary culture cells by Northern blot analyses. G3PDH mRNA levels obtained by Northern blotting were used for normalization. The expression of MyoD mRNA was not detected after BMP-2 or -4 exposure, and the expression was detected only at 0 and 24h, and not after 24h BMP stimulation
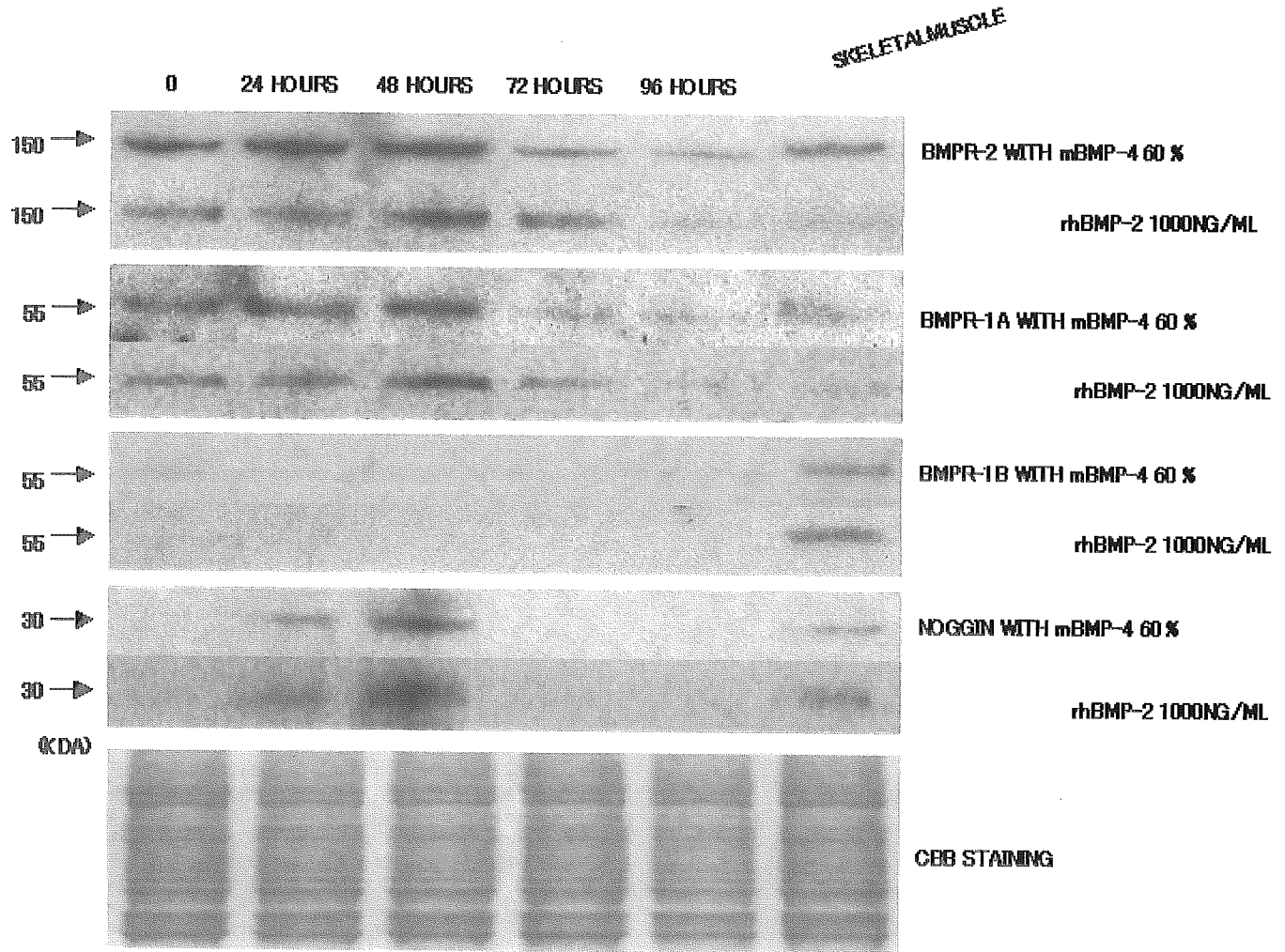

Fig. 10. Western blot analysis of BMPR-1A, -1B, -2, and Noggin after 60% mBMP-4 or 1000ng/ml rhBMP-2 stimulation in muscle-derived primary culture cells. Equivalent loading and integrity of protein were confirmed by Coomassie brilliant blue staining on the gel (*lower panel*). Mouse skeletal muscle proteins were used as positive controls. BMPR-1A and -2 were detected at 0h, induced at 24h, peaked at 48h, and then gradually decreased in both 60% mBMP-4 and 1000ng/ml rhBMP-2 stimulation groups. Expression was greater for BMPR-2 than for BMPR-1A. BMPR-1B was not detectable during any stages in either treatment group. Noggin was not detected at 0h, was up-regulated at 24h, peaked at 48h, and decreased thereafter

In our study, BMPs stimulated them to upregulate the expressions of a bone marker (OC) and cartilage markers (type II collagen and aggrecan, data not shown), but not the muscle marker examined previously. However, it is unclear whether bone and cartilage phenotypes were induced by BMPs in separate cells or in a single cell.

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

434

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

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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

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

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.

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

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% 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.