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Augmentation of bone morphogenetic protein-induced bone mass by local delivery of a prostaglandin E EP4 receptor agonist

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Abstract

Recombinant human bone morphogenetic protein (rhBMP) is viewed as a therapeutic cytokine because of its ability to induce bone. However, the high doses of rhBMP required for bone induction in humans remain a major hurdle for the therapeutic application of this protein. The development of a methodology that would effectively overcome the weak responsiveness to human BMP is highly desired. In the present study, we investigate the ability of a prostaglandin E EP4 receptor selective agonist (EP4A) to augment the bone-inducing ability of BMP in a biodegradable delivery system. A block copolymer composed of poly-D,L-lactic acid with random insertion of *p*-dioxanone and polyethylene glycol (PLA–DX–PEG, polymer) was used as the delivery system. Polymer discs containing rhBMP-2 and EP4A were implanted into the left dorsal muscle pouch of mice to examine the dose-dependent effects of EP4A. Fifty mice were divided into 5 groups based on the contents of rhBMP and EP4 in the polymer (group 1; BMP 5 μg EP4A 0 μg, group 2; BMP 5 μg EP4 3 μg, group 3; BMP 5 μg EP4 30 μg, group 4; BMP 5 μg EP4 300 μg, group 5; BMP 0 μg EP4 30 μg, $n = 10$ each). All implants were harvested, examined radiologically, and processed for histological analysis 3 weeks after surgery. On dual-energy X-ray absorptiometry (DXA) analysis, the bone mineral content (BMC) of the ossicles was 6.52 ± 0.80 (mg), 9.36 ± 1.89, 14.21 ± 1.27, and 18.75 ± 2.31 in groups 1, 2, 3, and 4 respectively. In terms of BMC, the values of groups 3 and 4 were significantly higher than those of group 1. The mean BMC value of group 4 was approximately 3 times higher than that of group 1. No significant difference in body weight was noted among the groups during the experimental period. In summary, the presence of a prostaglandin E EP4 receptor selective agonist in the carrier polymer enhanced the bone-inducing capacity of rhBMP-2 with no apparent systemic adverse effects.

Keywords: Bone morphogenetic proteins; Bone metabolism; Bone volume; Bone mineral density; Biomaterials

Introduction

Bone has an inherent regenerating potential, and damaged bone or fractures are repaired by local new bone (callus) formation in a period of several weeks after an injury. The regenerating potential of bone has been attributed to factors or molecules with the biological capacity to induce mesenchymal cells to differentiate into bone- or cartilage-forming cells (osteoblasts and chondrocytes) and thereby form the callus. Bone morphogenetic proteins (BMPs) were originally isolated on the basis of their ability to induce ectopic cartilage and bone formation via an endochondral cascade when implanted in experimental animals [1]. Because of the specific biological activity of BMPs and the successful generation of synthetic BMPs by DNA recombination, there is tremendous interest in using these proteins for bone repair and reconstructive surgery in a clinical setting [2]. However, 2 problems need to be addressed before we can witness the widespread clinical use of rhBMPs. One issue involves the use of a carrier material that has adequate safety and efficacy for BMP delivery. Currently, bovine collagen is used clinically as a carrier for rhBMPs, but use of this material comes with the risk of contracting bovine spongiform encephalopathy (BSE) or Creutzfeldt–Jacob disease (CJD). These diseases are

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potentially transmitted by prion proteins through cattle-derived foods and implant materials. Another problem is the high dose of rhBMP required for clinical efficacy in human patients. For example, to achieve a single level of spinal fusion, several to 10 mg of rhBMP are required. This results in the high cost and limited use of BMP as a substitute for bone autograft. Large doses of BMP may also increase the risk of potential adverse events in patients [3–6].

To address the issue of finding a suitable carrier, we have developed new biodegradable synthetic polymers that work effectively to deliver rhBMP and elicit new bone formation consistently at the implanted sites. The combination of rhBMP-2 and the polymers has enabled the successful regeneration of critical-size bone defects in experimental animals [7–10].

To improve the performance of rhBMP, we have sought agents to reinforce the bone-inducing activity of the protein and increase the induced bone mass. To this end, we have examined phosphodiesterase (PDE) inhibitors (pentoxifylline, rolipram) and a compound (ONO-4819), which is a prostaglandin (PG) EP4 receptor selective agonist (EP4A) [11–13]. PGE produced by cells of the osteoblastic lineage has been implicated as a regulator of bone metabolism through stimulation of either bone formation or resorption [14–16]. Exogenously applied PGE, either systemically or locally, also has enhanced bone formation in in vivo experimental models [17–19]. These biological effects of PGE are mediated through PGE receptors, which have been classified into 4 sub-types, EP1 through EP4. These EP receptors are encoded by distinct genes and are expressed in a tissue-specific manner [20–25]. In general, PGE mediated via EP1 increases intracellular Ca^{2+} concentration, EP2 and EP4 increase cAMP, and EP3 reduces cAMP and modulates down-stream signaling [25]. Knockout mouse studies have revealed that EP4 is the major receptor that mediates the PGE_2-induced anabolic action in bone [26–30]. Systemic administration of an EP4 agonist (ONO-4819) enhanced new bone formation in mice, and an EP4 antagonist suppressed the increase in trabecular bone volume induced by PGE_2 [13,30–33]. In our previous study, the systemic administration of these drugs by daily injection for 1 week during the initial phase of BMP-induced bone formation led to a significant augmentation of ossicle mass [13]. These results suggest that the efficient local release of these activators for BMPs could induce augmented bone formation without adverse effects due to high dose and long-term administration. Therefore, we examined the effects of adding a low dose of ONO-4819 to the BMP delivery system on new bone formation.

Materials and methods

Drugs/chemicals/materials

The prostanoid receptor EP4-selective agonist (ONO-4819), methyl 7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(*E*)-(3*S*)-3-hydroxy-4-(*m*-methoxymethylphenyl)-1-butenyl]-5-oxocyclopentyl]-5-thiaheptanoate (Patent Cooperation Treaty publish No. WO 00/03980), was obtained from Ono Pharmaceutical (Osaka, Japan) and dissolved in phosphate-buffered saline prior to use.

rhBMP-2 was produced by the Genetics Institute (Cambridge, MA) and donated to us through Yamanouchi Pharmaceutical Co. (Tokyo, Japan). The rhBMP-2 was supplied in a buffer solution (5 mmol/l glutamic acid, 2.5% glycine, 0.5% sucrose, and 0.01% Tween-80) at a concentration of 1 μg/μl after filter sterilization.

Poly-D,L-lactic acid–*p*-dioxanone–polyethylene glycol block copolymer (PLA–DX–PEG) (MW; 9800, PLA/DX/PEG molar ratio; LA/Dx/E0 = 43/14/43) was synthesized and provided to us by Taki Chemicals Co. (Kakogawa, Japan). The structural formula of the polymer is shown in Fig. 1. The polymer has a sticky gel-like character at room temperature and turns into a soft gel at 50°C. The physicochemical characteristics and the efficacy of this polymer as a carrier material for rhBMP-2 have been described by our group in previous reports [9,10]. The minimal optimal content of rhBMP-2 required to induce new bone formation was approximately 1 μg in 20 mg of the polymer (0.005%) in mice, 0.02% in rabbits, and 0.04% in dogs based on our previous experimental data [8,10,34].

Animals

One hundred and ten closed colony male ICR mice (4-weeks old; Nippon SLC, Hamamatsu, Japan) were housed and acclimated in cages with free access to food and water for 1 week. Experiments were carried out in strict accordance with the Institutional *Guidelines for the Care and Use of Laboratory Animals* of Osaka City University.

Preparation of PLA–DX–PEG polymer implants containing rhBMP-2 and ONO-4819

To prepare a single implant, 30 mg of the PLA–DX–PEG polymer was softened by heating to 37°C, mixed with an aliquot of either the rhBMP-2 solution (0.5 μg/5

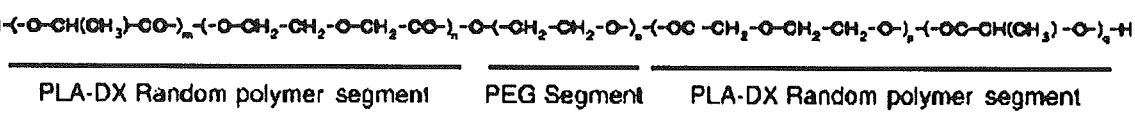

Fig. 1. Structural formula of PLA–DX–PEG polymer. Structural formula of the poly-D, L-lactic acid with random insertion of *p*-dioxanone and polyethylene glycol block copolymer (PLA–DX–PEG). The subscripts m, n, o, p, and q represent variable numbers of these units.

μl or 5 μg/5 μl) or rhBMP-2 and ONO-4819 solution (3 μg/3 μl, 30 μg/3 μl, 300 μg/3 μl) and then fabricated into a disc (6 mm diameter, Fig. 2). In summary, 0, 3, 30, or 300 μg of ONO-4819 was mixed with the polymer plus 5 μg of rhBMP-2 and implanted into mice in each group (5 mice in each group and 1 implant/mouse). To examine the effects of ONO-4819 alone, 30 μg/8 μl was added to the polymer without rhBMP-2. All procedures were carried out under sterile conditions. The implants were stored at −40°C in a freezer until required for implantation.

Experimental design

To examine the dose-dependent effects of the EP4 receptor agonist on ectopically induced bone formation by rhBMP-2, 50 mice were divided into 5 groups (10 mice per group). The mice were anesthetized by diethyl-ether gas inhalation, and the PLA–DX–PEG polymer discs prepared as described above were surgically implanted into the left dorsal muscle pouches (one pellet per animal) of the mice. In group 5, polymer discs containing 30 μg of ONO-4819, but no rhBMP-2, were implanted in the same manner.

1. 5 μg of rhBMP-2 per animal
2. 5 μg of rhBMP-2 and 3 μg ONO-4819 per animal
3. 5 μg of rhBMP-2 and 30 μg of ONO-4819 per animal
4. 5 μg of rhBMP-2 and 300 μg of ONO-4819 per animal
5. 30 μg of ONO-4819 per animal

At 1, 2, and 3 weeks after surgery, the body weight of each mouse was measured and recorded. Three weeks after surgery, the mice were sacrificed, and the implants were harvested and processed for histological analysis following morphological and radiological examination.

Radiological and histological analyses for rhBMP-2 induced ectopic bone

All harvested tissues were radiographed with a soft X-ray apparatus (Sofron Co., Ltd., Tokyo, Japan). The bone mineral content (BMC) (milligrams per ossicle) of each ossicle was measured by dual-energy X-ray absorptiometry (DXA) using a bone mineral analyzer (DCS-600EX, Aloka Co., Tokyo). The ossicles or tissue mass from each group was then fixed in neutralized 10% formalin, decalcified with K-CX (Fujisawa Pharmaceutical Co., Ltd. Japan), dehydrated in gradient ethanol series, and embedded in paraffin wax. Sections of 3 μm thickness were cut, stained with hematoxylin–eosin, and observed under a light microscope.

Bone metabolic markers in mice

To investigate the anabolic effects of ONO-4819 on systemic bone metabolism, an additional 60 mice were divided into 3 groups as follows: sham-operated mice that received sham operation and lacking implants (10 mice per group), group 1: 5 μg of rhBMP-2 per animal (5 mice per group) and group 3; 5 μg of rhBMP-2 and 30 μg of ONO-4819 per animal (5 mice per group). Blood samples were collected from mice of each group at 1, 2, and 3 weeks. The samples were stored at −80°C until biochemical analysis. Serum osteocalcin was measured by immunoradiometric assay (IRMA) using a commercial kit (Immutopics, Inc. San Clemente, CA) according to the manufacturer's instructions. Total alkaline phosphatase (ALP) activity, calcium (Ca), and phosphate (P) in serum were also measured in each group with commercially available kits.

Statistical analysis

Data are presented as mean ± SE. The degree of significance was determined by post hoc testing using the

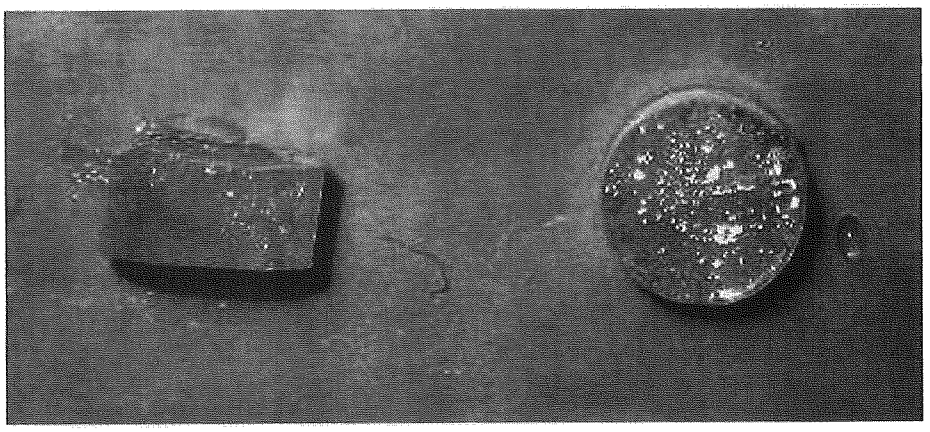

Fig. 2. PLA–DX–PEG polymer disc. Photograph of 6-mm-diameter PLA–DX–PEG polymer disc. The polymer has a hard sticky gel-like property at room temperature and softens when heated to 50°C.

Bonferroni method. An associated probability (P value) of <0.05 was considered significant.

Results

Body weight changes in animals

In our previous experiments, mice that received systemic injection of an excessive dose (100 μg/kg) of ONO-4819 every 8 h for 3 weeks showed a significant decline in body weight gain. In the current experiments, no significant difference in body weight gain was noted among the groups that received implants with or without local release of ONO-4819 (Fig. 3).

Radiological and histological evaluations

Pieces of hard tissue were harvested from the implantation sites of mice from groups 1, 2, 3, and 4 at 3 weeks after implantation. In group 5 (ONO-4819, 30 μg without BMP-2), no evidence of hard tissue formation was found at the implantation sites. On soft X-ray radiograms, the calcified samples retrieved from the mice revealed a trabecular network encased within a shell-shaped bone layer (Fig. 4). Histological sections of these samples showed normal characteristics of bone with trabeculae and hematopoietic marrow in the inter-trabecular space, findings that were also common to ossicles from groups 1, 2, 3, and 4. (Fig. 5) Radiological images indicated that the ossicles from group 3 (rhBMP-2, 5 μg + ONO-4819, 30 μg) and 4 (rhBMP-2, 5 μg + ONO-4819, 300 μg) were larger than those observed from control group 1 (rhBMP-2, 5 μg without ONO-4819).

On DXA analysis, the bone mineral content (BMC) of the ossicles containing ONO-4819 increased in a dose-dependent manner (3, 30, and 300 μg groups were 9.36 ± 1.89 mg, 14.21 ± 1.27 mg, and 18.75 ± 2.31 mg, respectively) Ossicles from group 1 mice (without ONO-4819) had a BMC of 6.52 ± 0.80 mg. In terms of BMC, the values of groups 3 and 4 were significantly higher than those of group 1. The mean BMC value of group 4 (BMP-2, 5 μg + ONO-4819, 300 μg) ossicles was approximately 3 times higher than that of the control group (Fig. 6).

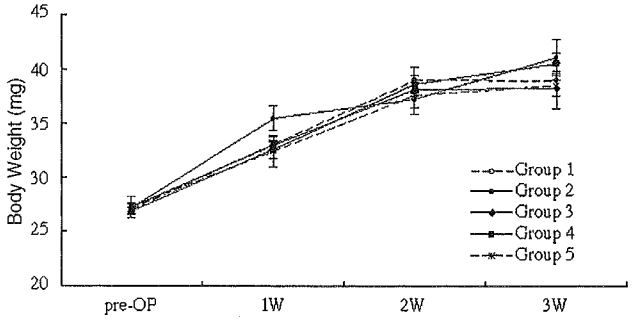

Fig. 3. Body weight. No significant difference in body weight was noted among the groups with implants with or without ONO-4819.

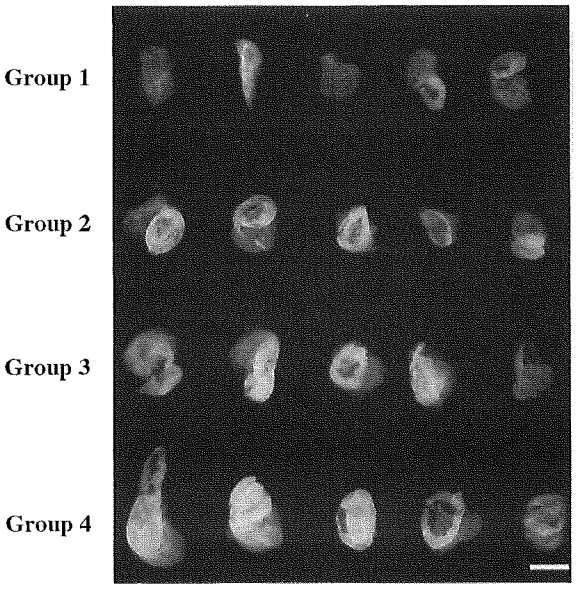

Fig. 4. Radiographic findings. Soft X-ray photograph of the ossicles harvested at 3 weeks after implantation (bar = 5 mm). A typical implant from each group is shown (groups 1, 2, 3, and 4). Both the radio-opaque areas and radiological densities of the ossicles on the radiogram were larger in groups 3 and 4 than in control group 1.

Serum osteocalcin and ALP activity assay

At 1 week, both serum osteocalcin (299.8 ± 24.4 ng/ml) and ALP activity (495.2 ± 32.0 IU/l) levels significantly increased in group 3 compared to the sham-operated animals (osteocalcin 208.6 ± 25.6 ng/ml, ALP activity 356.0 ± 39.8 IU/l). At 2 weeks, serum ALP activity (439.0 ± 76.8 IU/l) levels had increased significantly when compared to the sham-operated animals (ALP activity 313.2 ± 12.1 IU/l) (Fig. 7A). However, there were no significant differences among the groups at 3 weeks after implantation (Fig. 7B). In addition, there was no significant increase in serum calcium and phosphate level among them at any time point (data not shown). No significant changes in serum osteocalcin and ALP levels from the baseline were recorded in the groups that received implants containing ONO-4819.

Discussion

Based on these data, EP4A was examined for its ability to enhance BMP-induced bone formation and improve rhBMP-2 performance. In our previous study, systemic subcutaneous injections of the EP4A (ONO-4819) for 3 weeks increased bone mass induced by rhBMP-2 and caused a decline in body weight gain in the experimental animals [13]. To achieve the anabolic action and avoid the systemic adverse effect, low doses of the drug were added to the degradable polymer carrying the rhBMP-2 and implanted into the host mice. In this study, in a very encouraging response, ONO-4819 significantly increased the BMP-induced bone mass in dose-dependent manner

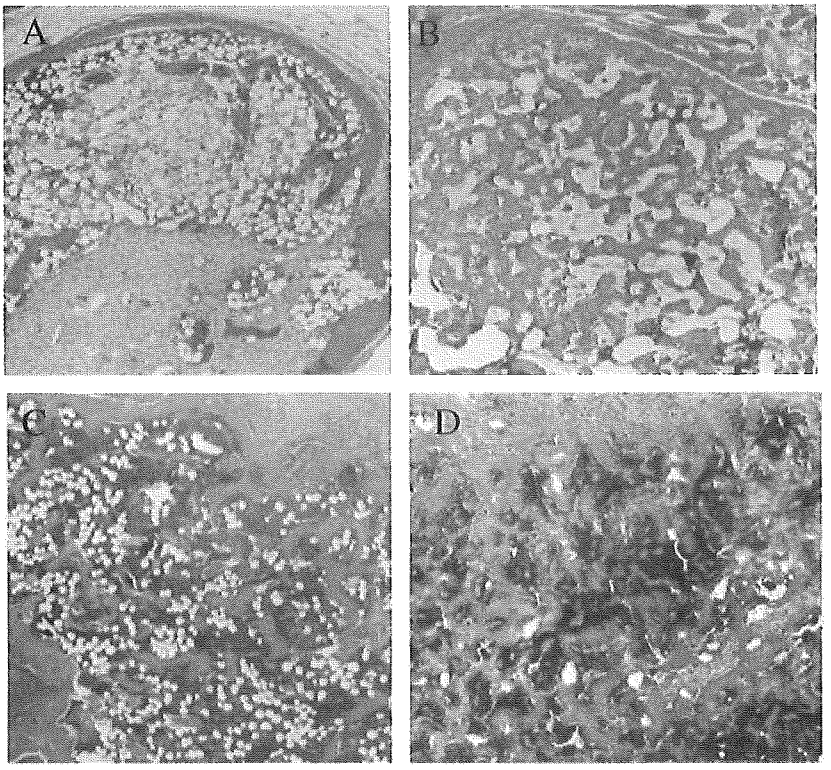

Fig. 5. Histology. Histological sections of the ossicles at 3 weeks after implantation are shown (hematoxylin–eosin stain; original magnification ×40). (A) group 1: 5 μg of rhBMP-2, (B) group 2: 5 μg of rhBMP-2 and 3 μg of ONO-4819, (C) group 3: 5 μg of rhBMP-2 and 30 μg of ONO-4819, (D) group 4: 5 μg of rhBMP-2 and 300 μg of ONO-4819. New bone formation with hematopoietic marrow and bony trabeculae was visible in the rhBMP-2-induced ossicles. In groups 3 and 4, there were visible increases in the number and thickness of bony trabeculae when compared to the ossicles from group 1.

without significant body weight loss. The total dose of ONO-4819 required for a doubling of the BMP-induced bone mass was reduced when compared to the dose required using consecutive systemic administration (3 injections/day for 3 weeks) of the drug.

Enhanced bone formation by systemic administration of the EP4A over an experimental period of 3 weeks was essentially reproduced by the local release of the agent over the first week following implantation. This is the period when young mesenchymal cells most likely migrate, proliferate, and infiltrate the BMP/polymer composite implants before new bone formation gets underway

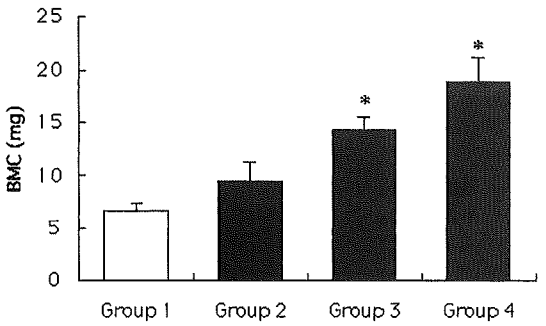

Fig. 6. Bone mineral content. The bone mineral content (BMC) of the ossicles at 3 weeks after implantation. BMC of ossicles was dose-dependently higher in groups 2, 3, and 4 than those in the group 1. Data expressed as mean ± SE. *Significantly different from controls ($P < 0.05$).

[7,9,10]. It is possible that these young mesenchymal cells were responsible for the bone formation enhanced by EP4A. Therefore, a low dose of the EP4A, ONO-4819, delivered locally and concurrently with rhBMP enhanced new bone formation and significantly increased bone mass. The effective period of local release of the EP4A is not greater than 2 weeks based on the degradation rate of the polymer [9,10]. Therefore, one possible explanation for the bone mass increased by EP4A is that EP4A works first in osteoblast precursors with a potential for chondro-osseous differentiation in the early phase of the bone-forming reaction. In the previous study, due to identifying the time phase when ONO-4819 exerts its pharmacological effects, EP4A was systemically administered for 1 week over pre (−1–0 week), initial (0–1 week), middle (1–2 week), or late (2~3 week) phase, respectively. The anabolic effects of EP4A were seen in mice that received EP4A exclusively in the initial phase. This result might also indicate that EP4A and BMP work cooperatively to stimulate osteoblastic differentiation in its early stage at the interface to the BMP-retaining pellets. Previous in vitro studies support our consideration. Suda et al. reported that EP2/EP4 seems to be involved in osteoblastic differentiation, and EP1/EP3 is likely to be associated with their proliferation [35]. Weinreb et al. described that PGE_2 stimulates osteoblastic differentiation through an anabolic effect in rat bone marrow cultures mediated by activation of EP4, probably

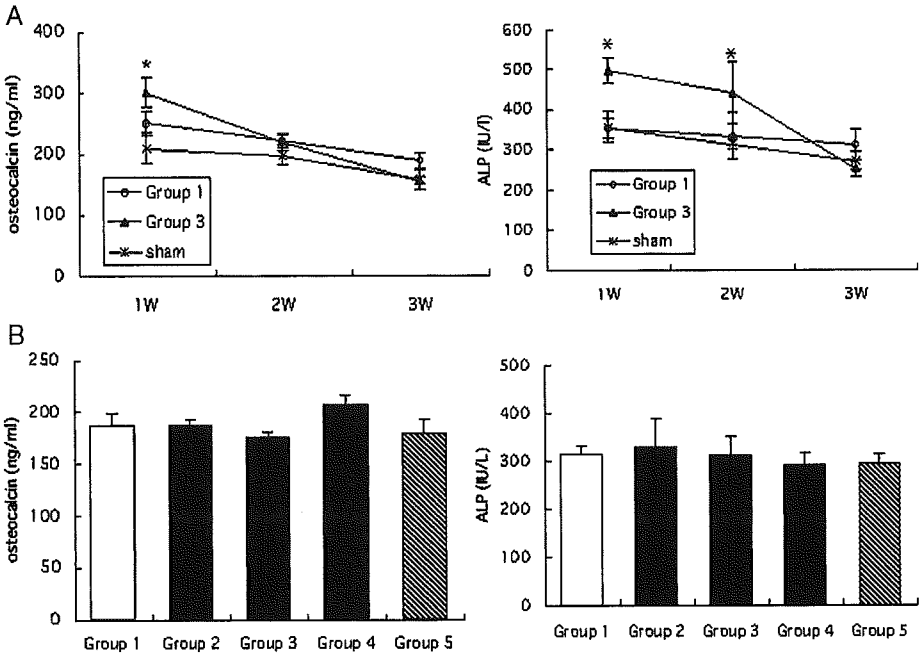

Fig. 7. Serum osteocalcin and ALP. Serum osteocalcin and ALP levels. (A) Serum osteocalcin and total ALP activity from group 3 with rhBMP-2 and ONO-4819 pellets were significantly increased compared to the sham group at 1 week. Total ALP activity from group 3 with rhBMP-2 and ONO-4819 pellets was significantly increased compared to the sham group at 2 weeks. (B) There were no significant differences in serum osteocalcin and ALP levels among the groups at 3 weeks after implantation.

by recruiting noncommitted osteogenic precursors [36,37]. Yoshida et al. described that PGE_2 induced the expression of core-binding factor alpha-1 (Runx2/Cbfa1) and enhanced the formation of mineralized nodules in a culture of bone marrow cells from wild-type mice, both of which were absent in a culture of cells from EP4 knockout mice. EP4 activation increased the number of Runx2 positive cells [30]. EP4 exerts this effect by inducing osteoblast differentiation. On the other hand, several studies indicate that EP4 is essential for PGE_2-induced bone resorption. Suzawa et al. described that, in mouse calvaria cultures, EP4A markedly stimulated bone resorption, and in calvaria culture from EP4 knockout mouse, a marked reduction in bone resorption to PGE_2 was found. EP4A induced cAMP production and the expression of osteoclast differentiation factor mRNA in osteoblastic cells [27]. Stimulation of osteoclastogenesis in cocultures of osteoblasts and spleen cells in response to PGE_2 is markedly decreased when the osteoblasts are derived from cells lacking the EP4 receptor [26–29]. These in vitro studies indicate that PGE_2-EP4 signaling works first in osteoblast precursors to induce osteoblast for bone formation and then works in mature osteoblasts to induce osteoclasts on newly formed bone. Further studies are required to elucidate the detailed mechanism of action of the EP4 receptor agonist in in vitro systems using less differentiated osteogenic cells.

The anabolic effect of PGE_2 on bone was exhibited through the activation of EP2 or EP4 and consequent elevation of intracellular cAMP level [23]. In this respect, the action of an EP4 agonist may be similar to that of PTH, PDE-4, which also promotes bone formation and intercellular cAMP accumulation. Daily subcutaneous injection of parathyroid hormones (PTH) is known to enhance systemic bone formation, and daily systemic injection of phosphodiesterase-4 (PDE-4)-selective inhibitor, rolipram, can enhance BMP-2-dependent ectopic new bone formation in mice [11,38]. Although the detailed mechanisms of cAMP signal on bone formation have been unclear, these results might indicate that cAMP functionally has a key role in the regulation of the BMP action in osteoblast differentiation, and further studies are required.

Another possible mechanism of the anabolic effect of EP4A on the BMP-induced bone formation comes from studies involving cyclooxygenase-2 (COX-2). Zhang et al. showed the complementary effect of BMP-2 in a bone marrow cell culture from COX-2 knockout mice and suggested that BMP-2 is a target gene for PGE_2-induced bone formation [39]. Chikazu et al. reported that BMP-2 transcriptionally induces COX-2 expression, which in turn regulates, via the Runx2 binding site, production of PGE_2 and promotion of osteoblastic differentiation [40]. These results indicate that BMP and PGE_2 might have complementary or cooperative anabolic effects on mesenchymal cells to stimulate the early phase of osteoblastic differentiation.

Potent bone anabolic activity of EP4A is expected from clinical application for fractures and bone defects in patients. Development of a more effective way of exposing responding cells and tissues to EP4A is likely to be needed for cost effectiveness, clinical efficacy, and long-term safety.

In cases with a longer fracture healing time, such as in humans, a carrier might be necessary for the sustained release of EP4A to be effective. The property of this polymer would allow retention of rhBMP-2 for a period that is significant to elicit new bone formation and thereby provide a scaffold for further bone growth. Retention of the proteins at the implantation site for a sufficient period to promote progenitor cell migration and cell proliferation has been shown to enhance osteoinductive activity. Our results show that local administration of ONO-4819 using PLA–DX–PEG polymer can mimic the local bone anabolic effect of PGE_2 without an excessive dose. The ability to deliver a molecule so that it will induce a specific biologic effect is critical to the success of pharmacological agent therapy.

In conclusion, a new EP4 receptor agonist compound (ONO-4819) can enhance the bone-inducing activity of rhBMP-2 when administered using a local polymer-based carrier with no apparent systemic adverse effects. This compound may be a useful tool for enhancing the performance of rhBMP-2. This could have a significant impact on the costs associated with using this therapeutic cytokine for bone regeneration and repair in clinical practice. Further safety checks are required before ONO-4819 can be used for this purpose.

Acknowledgments

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

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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

THE 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-

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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 previously.[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′-GGATCTCTCTATGACTTCCTGAAATGT-3′, reverse primer, 5′-CAGCAGAATAAGCTAACTTGAGTAGGG-3′, TaqMan probe, 5′(FAM)-CCACACTAGACACCAGAG-(TAMRA)3′; BMPR2, forward primer, 5′-GCCAAGATGAATACAATCAATGCA-3′, reverse primer, 5′-CTTCTACCTGCCACACCATTCATA-3′, TaqMan probe, 5′(FAM)-AGAGCCTCATGTGGTGAC-(TAMRA)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

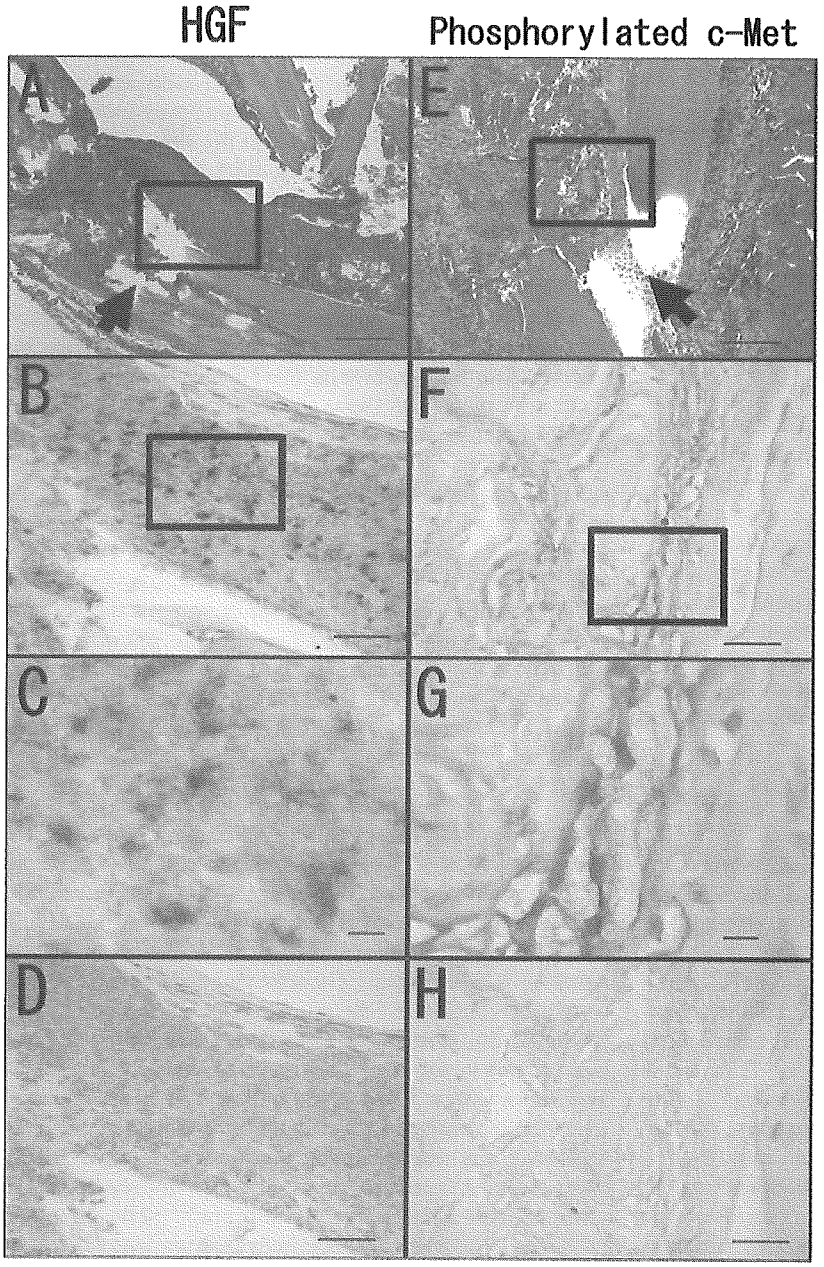

FIG. 1. Localization of HGF and phosphorylated c-Met at 24 h after the onset of fracture. Immunohistochemical localizations of HGF and phosphorylated c-Met were detected with (B and C) anti-rodent HGF antibody and with (F and G) anti-tyrosine (pYpYpY1230/1234/1235)-phosphorylated c-Met antibody, respectively. (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

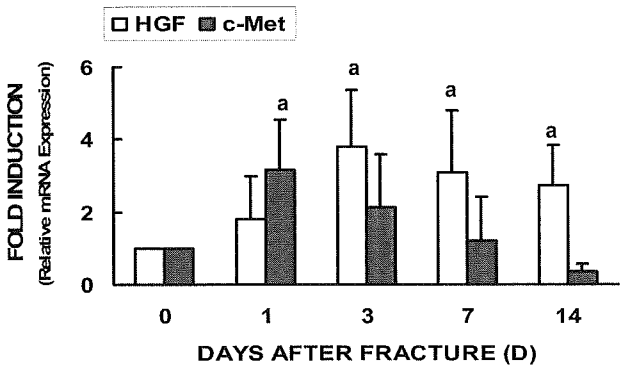

FIG. 2. Relative expression levels of HGF (open bars) and c-Met (patterned bars) mRNA analyzed by real-time RT-PCR. One microgram of total RNA extracted from the fracture site at days 0, 1, 3, 7, and 14 was used for examination. Relative mRNA expression of HGF and c-Met normalized with those of amplified 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

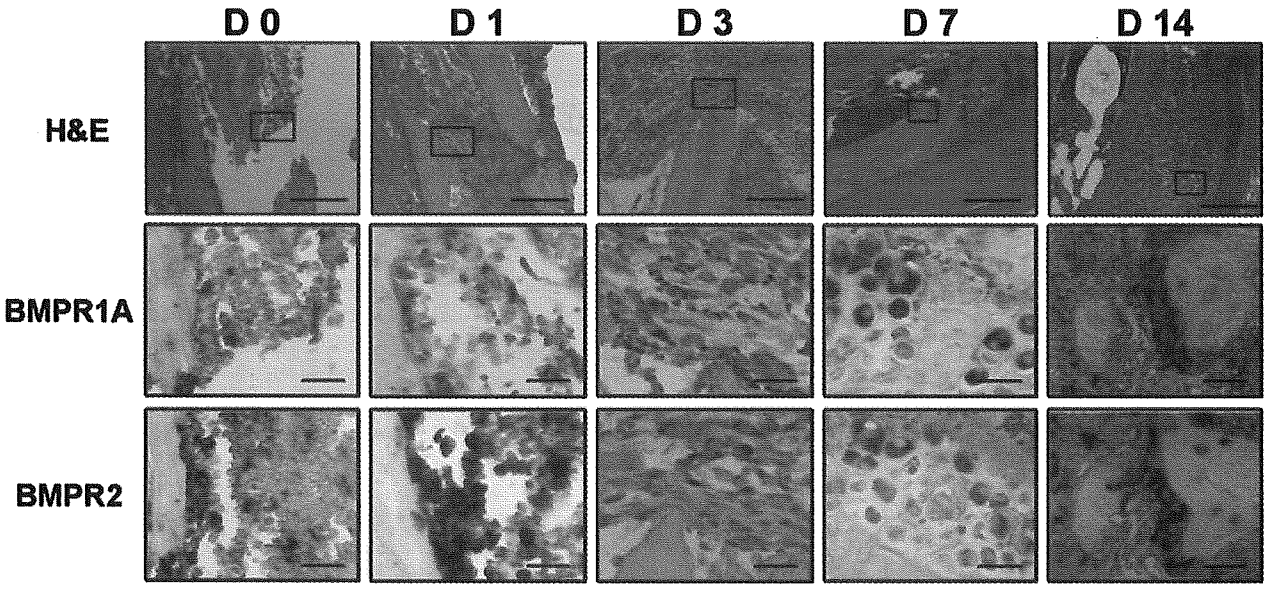

FIG. 3. Localization of BMPR1A and BMPR2 mRNA in the early phase of fracture repair. The top panels of H&E staining indicate the histology of the fracture site. The second and third panels indicate the results of in situ hybridization using antisense probes of BMPR1A and BMPR2, respectively. Bars = 500 (top panels) and 20 μm (other panels).

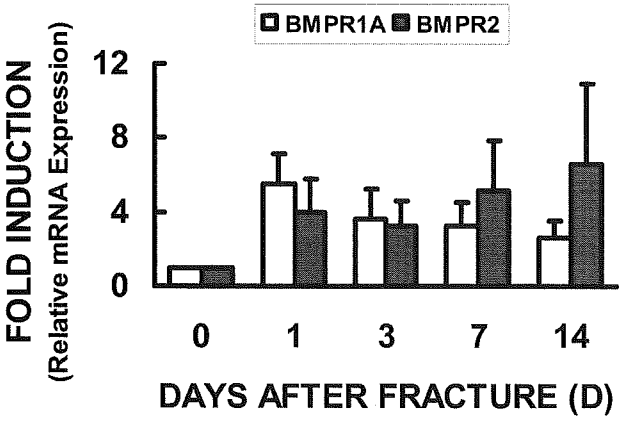

FIG. 4. Relative expression levels of BMPR1A (open bars) and BMPR2 (striped bars) at the fractured site examined by real-time RT-PCR as described in Fig. 2. Significant increase of BMPR1A and BMPR2 mRNA expression is shown in all samples compared with day 0 ($p < 0.05$). Error bars indicate SD from experiments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

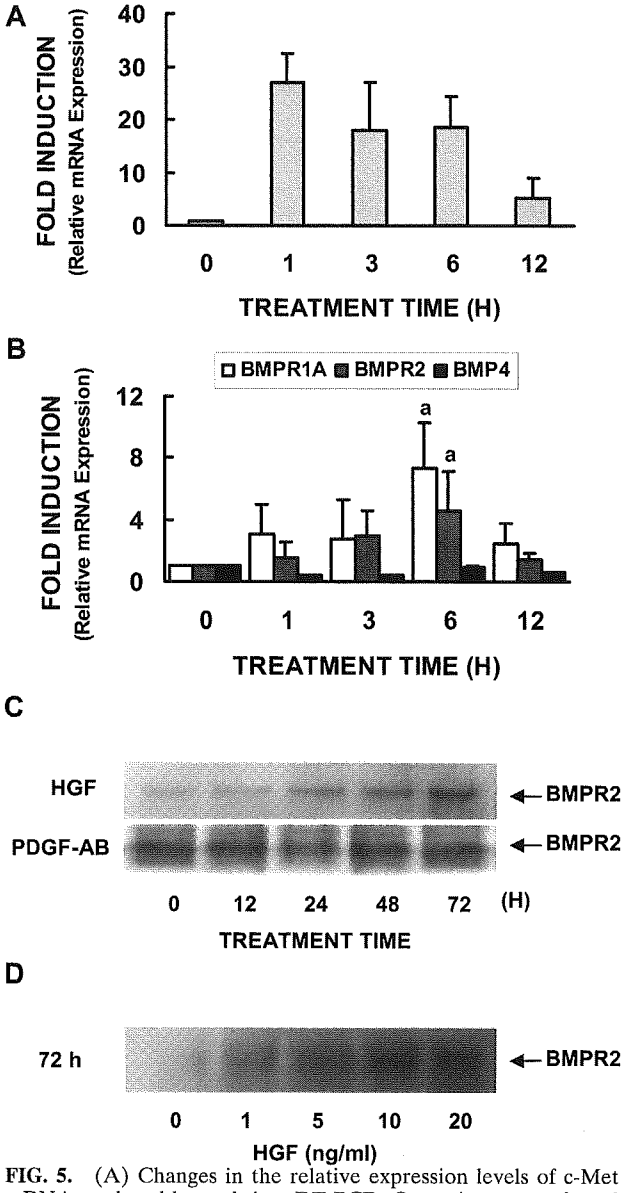

FIG. 5. (A) Changes in the relative expression levels of c-Met mRNA analyzed by real-time RT-PCR. One microgram of total RNA was extracted from C3H10T1/2 cells treated with 5 ng/ml of rhHGF for 0, 1, 3, 6, and 12 h and were used for analysis. Value of day 0 after fracture is indicated as 1. Significant 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.

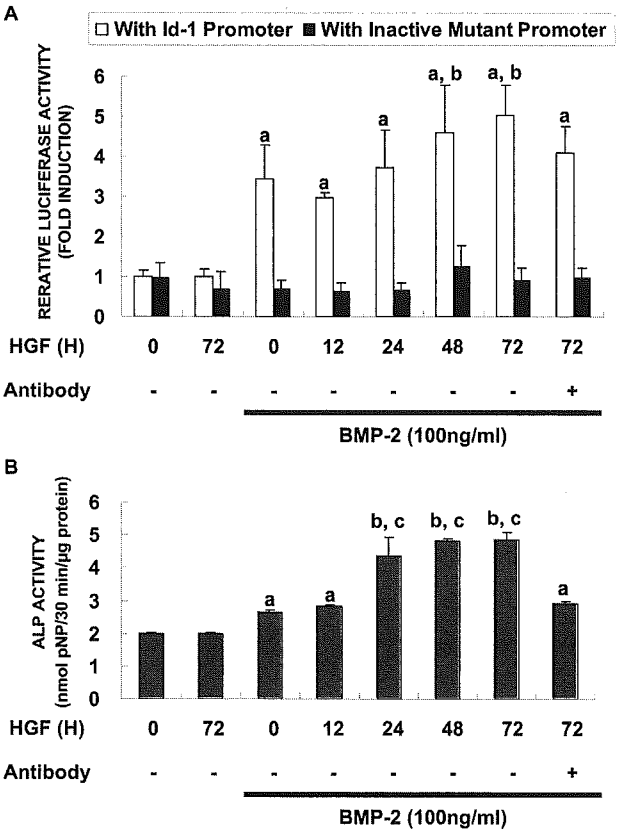

FIG. 6. (A) Relative luciferase activity in the cells transfected with *Id1* promoter-containing plasmids. Primary muscle-derived mesenchymal cells were pretreated with or without HGF and after treatment with BMP2. Relative luciferase activity of the groups treated with 100 ng/ml of BMP2 (groups 0, 12, 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.

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 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.

ACKNOWLEDGMENTS

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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 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

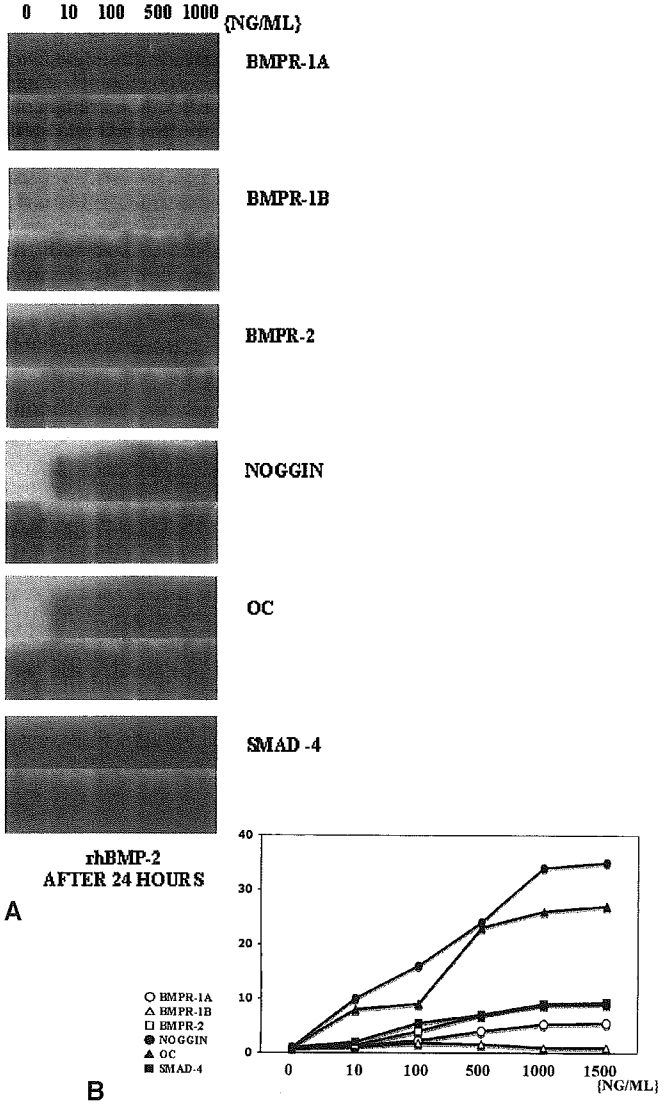

Fig. 1. Gene expression level of BMPR-1A, -1B, -2, Noggin, OC, and Smad-4 after 24-h stimulation of rhBMP-2 (0, 10, 100, 500, 1000 ng/ml) in muscle-derived primary culture cells by Northern blot analysis (**A**) and quantitation of the data of Northern blot analysis by Densitometry (**B**). G3PDH mRNA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

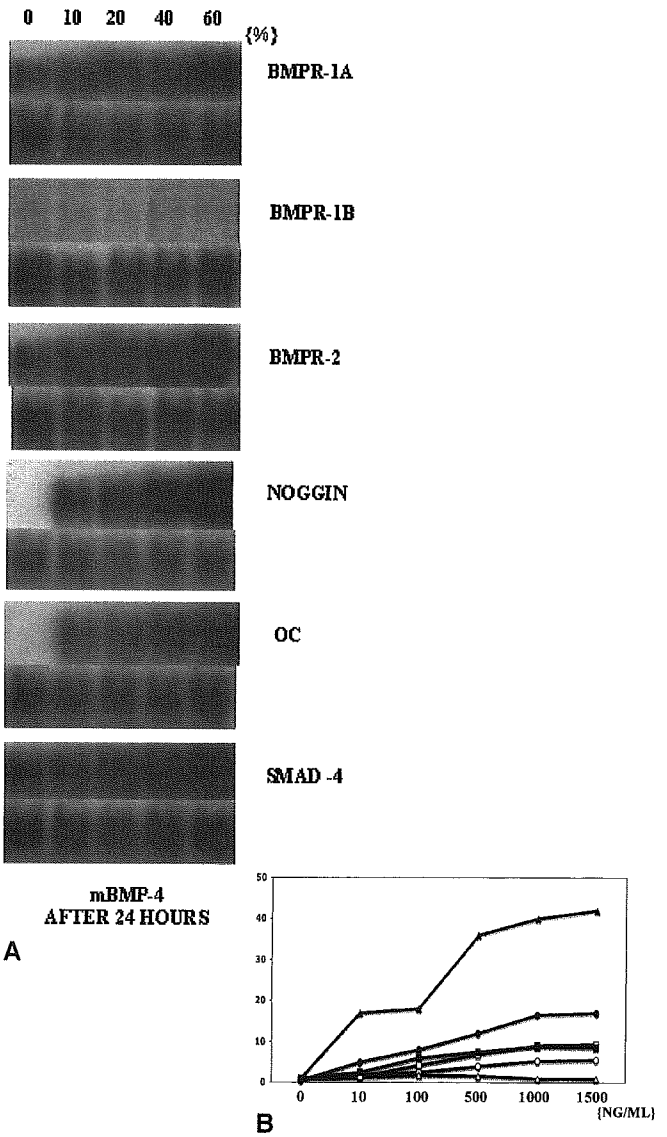

Fig. 2. Gene expression level of BMPR-1A, -1B, -2, Noggin, OC, and Smad-4 after 24-h stimulation of mBMP-4 [0%, 10%, 20%, 40%, and 60% (v/v) conditioned media] in muscle-derived primary culture cells by Northern blot analysis (**A**) and quantitation of the data of Northern blot analysis by Densitometry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