Mechanical Tension-Stress Induces Expression of Bone Morphogenetic Protein (BMP)-2 and BMP-4, but Not BMP-6, BMP-7, and GDF-5 mRNA, During Distraction Osteogenesis

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ABSTRACT

Bone lengthening with osteotomy and gradual distraction was achieved in 57 rats, and the effect of mechanical tension-stress on gene expression of bone morphogenetic proteins (BMPs) was investigated by in situ hybridization and Northern blot analysis using probes of BMP-2, BMP-4, BMP-6, BMP-7, and growth/differentiation factor (GDF)-5. There was a lag phase for 7 days after femoral osteotomy until gradual distraction was carried out for 21 days at a rate of 0.25 mm/12 h using a small external fixator. The signals of the above BMPs mRNA were not detected in the intact rat bone but they were induced after osteotomy except those for BMP-7. By 4 days after osteotomy, BMP-2 and BMP-4 mRNAs were detected in chondrogenic precursor cells in the subperiosteal immature callus. BMP-6 and GDF-5 mRNA were detected in more differentiated cells in chondroid bone. By 7 days after osteotomy, cartilaginous external callus and bony endosteal callus were formed. Meanwhile, the signals of BMP-2 and BMP-4 mRNAs declined to preoperative levels, whereas the signals of BMP-6 and GDF-5 mRNAs were rather elevated. As distraction was started, the callus elongated and eventually separated into proximal and distal segments forming a fibrous interzone in the middle. Expression of BMP-2 and BMP-4 mRNAs was markedly induced at this stage. Their signals were detected widely among chondrogenic and osteogenic cells and their precursor cells sustaining mechanical tension-stress at the fibrous interzone. BMP-6 and GDF-5 mRNAs were detected exclusively in chondrogenic cells at both ends of the fibrous interzone, where endochondral ossification occurred. But neither mRNA was detected in terminally differentiated hypertrophic chondrocytes. As distraction advanced, the cartilage was progressively resorbed from both ends and new bone was formed directly by intramembranous ossification. There was no new cartilage formation in the advanced stage of distraction. The signals of BMP-6 and GDF-5 mRNA declined by this stage, while those of BMP-2 and BMP-4 were maintained at high level for as long as distraction was continued. After completion of distraction, the fibrous interzone fused and the lengthened segment was consolidated. BMP-2, BMP-4, BMP-6, nor GDF-5 was expressed at this stage. The signals of BMP-7 were not detected throughout the experiment. The present results suggest that excellent and uninterrupted bone formation during distraction osteogenesis owes to enhanced expression of BMP-2 and BMP-4 genes by mechanical tension-stress. Abundant gene products of BMP-2 and BMP-4 could induce in situ bone formation by paracrine and autocrine mechanisms. (J Bone Miner Res 1999;14:1084–1095)

INTRODUCTION

Distraction osteogenesis is a recently highlighted method of bone lengthening that is applicable to surgical treatment of congenital and post-traumatic short limbs.[1] A bone is carefully osteotomized, clamped with an external fixation device and subjected to slow progressive distraction at a rate of 0.5–1.0 mm per day. The osteotomy gap is bridged by daily growing bony callus, but it does not fuse until distraction is ceased. After completion of distraction, the lengthened segment is consolidated spontaneously and bone grafting is not necessary with this procedure.

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The process of distraction osteogenesis is clinically divided into three distinct phases; a lag phase for several days after osteotomy, a distraction phase for a number of days depending upon how much lengthening is to be achieved, and a consolidation phase to wait complete bone union. The external fixation device is not removed until bone consolidation is confirmed by radiograph.

Although an increasing number of papers are reporting the clinical results of bone lengthening,[2,3] only limited information is available regarding the cellular and molecular mechanism of distraction osteogenesis.[4–6] It is clear that the initial callus is formed around the site of osteotomy as a process of fracture healing, but the question is why and how the cells maintain osteogenic potential throughout the distraction period? Are there repeated microfractures of the callus, or does mechanical tension-stress stimulate osteogenesis?

We have recently established a rat model of distraction osteogenesis,[5,6] and demonstrated that the mode of ossification changed from endochondral to intramembranous via trans-chondroid bone formation depending on the stage of distraction.[5] It is interesting that young chondrocytes have a capacity to undergo further differentiation into bone cells and switch their collagen phenotypes from cartilage specific-type II to bone-type I under the influence of mechanical tension-stress.[5] Our recent studies also demonstrated that tension-stress affected the cell shape and modulated the phenotypes of chondrocytes to express mRNA for bone matrix proteins, such as osteopontin (OPN) and osteocalcin (OC).[6]

The present study was designed to investigate the effect of mechanical tension-stress on gene expression of bone morphogenetic proteins (BMPs) by the cells involved in distraction osteogenesis. BMPs are the only factors known to be able to induce ectopic bone formation in vivo[7,8] and have important roles during embryogenesis and skeletal development.[9] Recent studies have made clear that BMPs act through a cell surface complex of two types of transmembrane receptors,[10–12] and the signals are transduced by Smads.[13] Since, BMPs play initiative roles of the signaling cascade, we examined the expression of BMPs mRNA in the present study. We demonstrate the temporal and spatial localization of mRNAs of BMP-2, BMP-4, BMP-6, BMP-7, and growth/differentiation factor (GDF)-5 in the rat model of distraction osteogenesis using in situ hybridization and northern blot analysis, and focusing on whether or not mechanical tension-stress influences the endogenous BMPs in the cells participating in bone formation.

MATERIALS AND METHODS

Animal experiments

All experimental procedures were undertaken in compliance with the guidelines for the Care and Use of Animals described in the *American Journal of Physiology*. Male Sprague-Dawley rats, 11 weeks old and weighing about 400 g, were purchased from Charles River Japan (Tokyo, Japan). After 2 weeks of acclimatization, 78 healthy animals were selected for the experiment. The operation was performed under general anesthesia and sterile conditions as previously described.[5,6] Briefly, a monolateral external fixator (Hoffmann Mini Lengthening System 5094–0-202, Howmedica, Jaquet, Geneva, Switzerland) was applied to the lateral aspect of the left femur with four screws. Transverse osteotomy was performed subperiosteally between the second and the third screw using a manual saw. The animals were then divided into two experimental groups; a distracted group and nondistracted control group. For the distracted group, there was a lag phase for 7 days after osteotomy, a distraction phase for 21 days during which distraction was carried out at a rate of 0.25 mm every 12 h (0.5 mm/day), and a consolidation phase during which the external fixator remained in situ to generate bone consolidation. For the nondistracted group, the same operation as in the distracted group was conducted, but distraction was not carried out. There was merely a consolidation phase for 35 days after osteotomy. The process of bone formation was followed by weekly radiographs in both groups. Animals were sacrificed at various postoperative stages for histologic examination and extraction of RNA from osteotomy gap.

Sample preparation

For RNA extraction, the lengthened segment with small pieces of both ends of proximal and distal bone fragments was excised and homogenized. For in situ hybridization, the animals were perfused under general anesthesia and the left femur was excised while the external fixator remained in situ. The bone was fixed and decalcified and histologic sections were prepared as previously described.[14]

Probe preparation

The following complementary DNA (cDNA) clones were used as hybridization probes: mouse BMP-2 containing a 0.6 (827–1409) kb fragment, rat BMP-4 cDNA containing a 0.4 kb (627–1028) fragment, mouse BMP-6 cDNA containing a 0.7 kb (187–893) fragment, rat BMP-7 cDNA containing a 0.63 kb (405–1038) fragment, mouse GDF-5 cDNA containing a 0.27 kb (1837–2103) fragment, rat c-*fos* cDNA containing a 1.25 kb (222–1471) fragment and mouse GAPDH cDNA. These cDNA were obtained by reverse transcription (RT) of mRNA from mouse or rat embryo, followed by a polymerase chain reaction (PCR) and subcloning into *Eco*RV site of pBluescript KS- (Stratagene, La Jolla, CA, U.S.A.). The base sequences were identical to those previously described.[15–22] The specificity of these probes (BMP-2, BMP-4, BMP-6, BMP-7, GDF-5, and c-*fos*) was confirmed by northern blot analysis and the transcripts were identical to those described previously.[15,23–25]

RNA extraction and Northern blot analysis

Total RNA was extracted from normal femur, the distracted and nondistracted femur at various postoperative time points (Table 1). For northern blot analysis, 50 g total RNA was fractionated on a 1% agarose gel and transferred to a Hybond N + nylon membrane (Amersham, Little Chal-

TABLE 1. TIME COURSE AND SEVENTY-EIGHT RATS IN THIS STUDY

	osteotomy		beginning of distraction	early stage	advanced stage	consolidation phase
		lag phase	distraction phase		finish of distraction	
Distracted group						
days after osteotomy	0	4	7	17	28	35
days after distraction			0	10	21	28
elongation (mm)	0	0	0	5	10.5	10.5
number of rats used for histological examination	2	5	5	5	5	0
number of rats used for RNA extraction	2	10	8	6	6	3
Nondistracted group (osteotomy)						
days after osteotomy	0	4	7	17	28	35
number of rats used for histological examination	0	0	0	3	3	0
number of rats used for RNA extraction	0	0	0	5	5	5

font, Buckinghamshire, UK). Membranes were prehybridized and then hybridized with the $[^{32}P]dCTP$-labeled probes, according to the manufacture's instructions. After hybridization, the membranes were washed and the signals were measured by autoradiography. Hybridization signals from autoradiograms were quantitated using a Scanning Densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.) The relative gene expressions were normalized to the GAPDH levels. The basal or normal femur level was then set at 1.0 according to the method described by Harris et al.[23,26]

In situ hybridization

In situ hybridization techniques were carried out as described.[14] Digoxigenin-labeled single-strand RNA probes were prepared for hybridization using a DIG RNA labeling kit (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacture's instructions. Hybridization of BMP-2, BMP-4, BMP-6, BMP-7, and GDF-5 mRNAs was performed at 55°C for 16 h, and the signals were detected using a nucleic acid detection kit (Boehringer Mannheim Biochemica). The controls included hybridization with the sense probes, RNase treatment before hybridization, and use of neither the antisense RNA probe nor antidigoxigenin antibody. All three experiments produced no detectable signal.

RESULTS

Radiological findings

The radiological findings of rat femoral lengthening were described in detail in our previous report.[5] Briefly, imma-

ture callus was formed around the osteotomy site during the lag phase. In the distraction phase, the bony callus separated into proximal and distal segments and elongation occurred at the central radiolucent growth zone that was maintained at a relatively constant thickness. After termination of distraction, the growth zone became calcified, fused and eventually consolidated. In the nondistracted group, the osteotomy site was bridged by bony callus and had united by 28 days after operation.

Histologic findings

Histologic events during the three distinct phases can be summarized as follows. Lag phase: formation of cartilaginous external callus, periosteal and endosteal bony callus; Distraction phase: elongation and separation of external and endosteal callus, formation of fibrous interzone, switch from endochondral ossification to intramembranous ossification; Consolidation phase: formation of bony bridge. These findings have been described in detail.[5,6]

Northern blot analysis

As shown in Fig. 1, no signal of BMP-2, BMP-4, BMP-6, BMP-7, nor GDF-5 mRNA was detected in normal intact bone by northern blot analysis. By 4 days after osteotomy, faint signals of BMP-2, BMP-4, and BMP-6 mRNAs became detectable. The signals of BMP-2 and BMP-4 mRNAs declined by 7 days after osteotomy, while the signal of BMP-6 mRNA rather increased and GDF-5 mRNA became detectable. By 10 days of distraction, the signals of BMP-2 and BMP-4 mRNA were markedly increased, while the signal of BMP-6 and GDF-5 mRNA did not change.
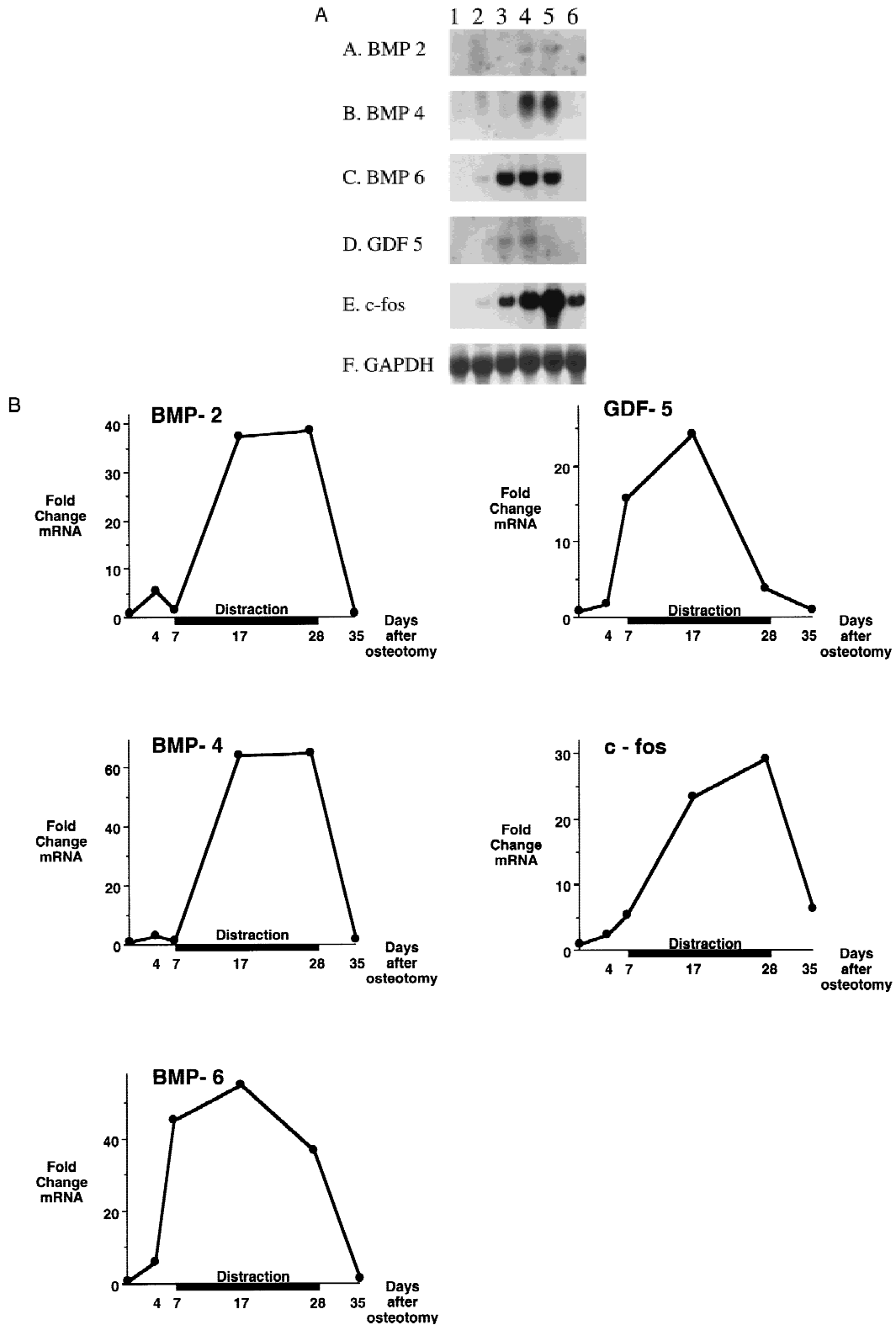
FIG. 1. (A) Northern blot analysis of normal and distracted femur. Lane 1, normal femur; lane 2, four days after osteotomy; lane 3, seven days after osteotomy, just before the beginning of distraction; lane 4, 17 days after osteotomy (10 days of distraction in the distracted group); lane 5, 28 days after osteotomy (21 days of distraction in the distracted group); lane 6, 35 days after osteotomy (7 days after the completion of distraction in the distracted group). (A) Hybridization with the cDNA of BMP-2, (B) hybridization with the cDNA of BMP-4, (C) hybridization with the cDNA of BMP-6, (D) hybridization with the cDNA of GDF-5, (E) hybridization with the cDNA of c-*fos*, (F) hybridization with the cDNA of GAPDH. (B) Quantitation of BMPs mRNA levels. Quantitative GAPDH levels were used to normalize BMPs mRNA levels.
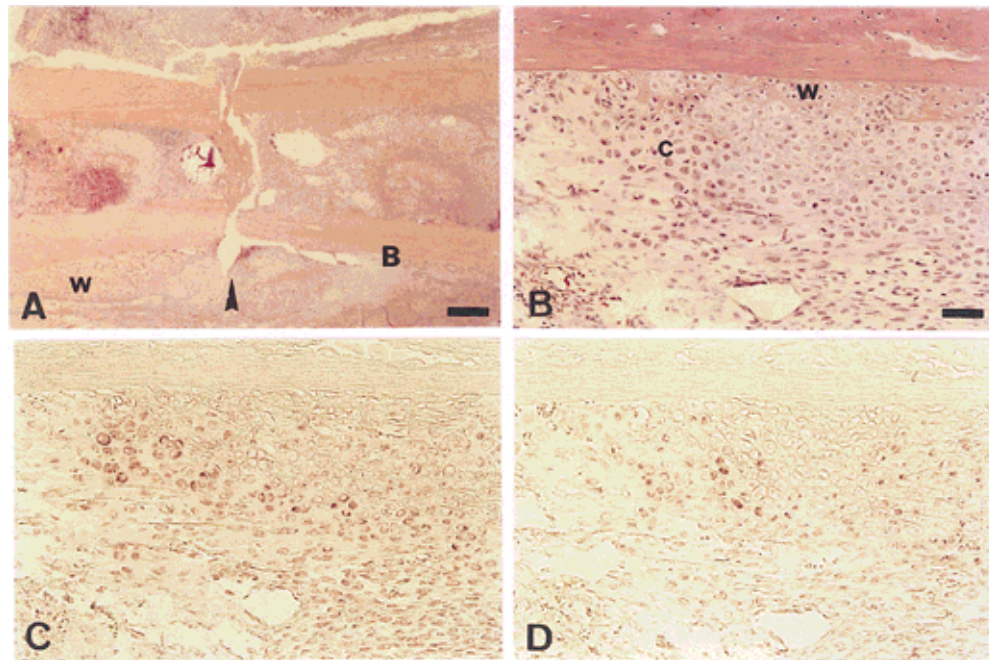
FIG. 2. Histologic appearance and localization of BMP mRNA in a longitudinal section of the femur at four days after osteotomy. (A) Hematoxylin and eosin staining. The osteotomy site (arrow head) is surrounded by cartilaginous external callus, while bone cortex is covered by primitive woven bone. w, woven bone. Bar = 640 μm. (B–D) are sequential sections. (B) Hematoxylin and eosin staining. High power magnification views of the periosteal callus (region "B" in A). c, chondroid bone; w, woven bone. Bar = 60 μm. (C) BMP-4 mRNA signal is present in chondroid bone cells and their precursor cells, but not in woven bone cells. (D) BMP-6 mRNA signal is present in progenies of chondroid bone cells, but not in woven bone cells.

The enhanced expression of BMP-2 and BMP-4 mRNA was maintained until the end of the distraction phase, while the expression of BMP-6 and GDF-5 mRNAs had declined by the end of the distraction phase. None of the BMP mRNAs was detected after completion of distraction. In the nondistracted group, experimental conditions for the initial 7 days after osteotomy were the same as those for lag phase in the distracted group. Signals of BMP-2 and BMP-4 mRNA were not detected at 17, 28 and 35 days after osteotomy without distraction (data not shown). The signals of BMP-6 and GDF-5 mRNAs were detected at 17 days after osteotomy without distraction but had declined in intensity by 28 days after osteotomy (data not shown). Signal of BMP-7 was not detected throughout the experiment in either the distracted or nondistracted group (data not shown). Signal of c-*fos*, which is one of the stress-induced gene,[25] was hardly detected in the normal femora, but the expression was detected at 4 days after the osteotomy. The expression had been strongly induced by the beginning of distraction and peaked during the distraction phase, but decreased after the completion of distraction.

In situ hybridization

During the three distinct phases of distraction osteogenesis, mRNAs of BMP-2 and BMP-4 showed similar patterns of expression, as did mRNAs of BMP-6 and GDF-5. No signal of BMP-7 was detected by in situ hybridization throughout the experiment.

Distracted group

Lag phase: No signal of BMP-2, BMP-4, BMP-6, BMP-7 and GDF-5 mRNA was detected in any type of cell in normal intact bone without osteotomy (data not shown).

Soon after osteotomy, external, periosteal and endosteal callus formed as part of the process of fracture healing (Fig. 2A). By 4 days after osteotomy, external callus contained immature cartilage, while periosteal and endosteal callus consisted of chondroid bone and woven bone (Fig. 2B). Positive signals of BMP-2 and BMP-4 mRNAs were detected in chondroid bone cells and their precursor cells under the periosteum, but not in more differentiated cells in woven bone (Fig. 2C). Faint signals of BMP-6 (Fig. 2D) mRNA were detected in more differentiated cells in chondroid bone, but not in woven bone.

By 7 days after osteotomy, the external callus had obtained its maximum size consisting of metachromatic hyaline cartilage, while the periosteal and the endosteal callus consisted of new bone (Figs. 3A–3D). None of the cells at this stage showed positive signal of BMP-2 and BMP-4 mRNA (data not shown), while the chondrocytes in the external cartilaginous callus expressed BMP-6 and GDF-5 mRNAs (Figs. 3E and 3F).
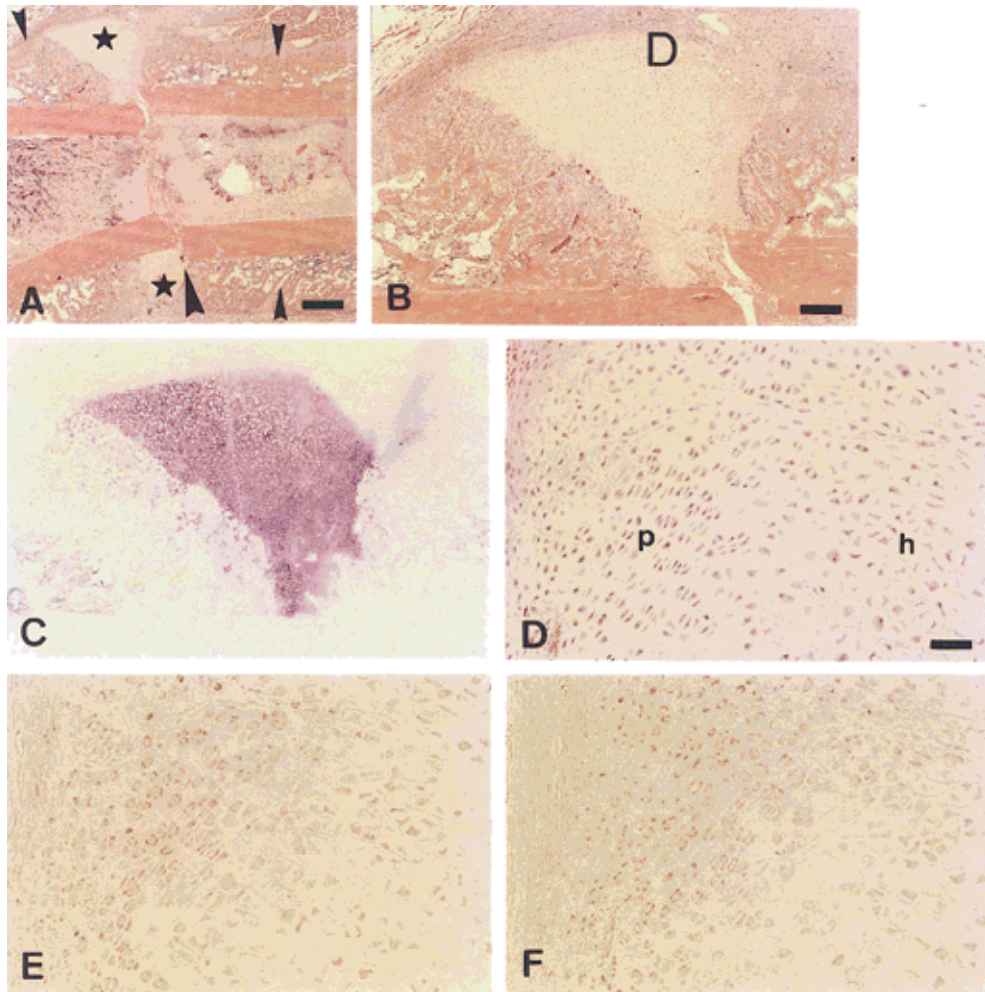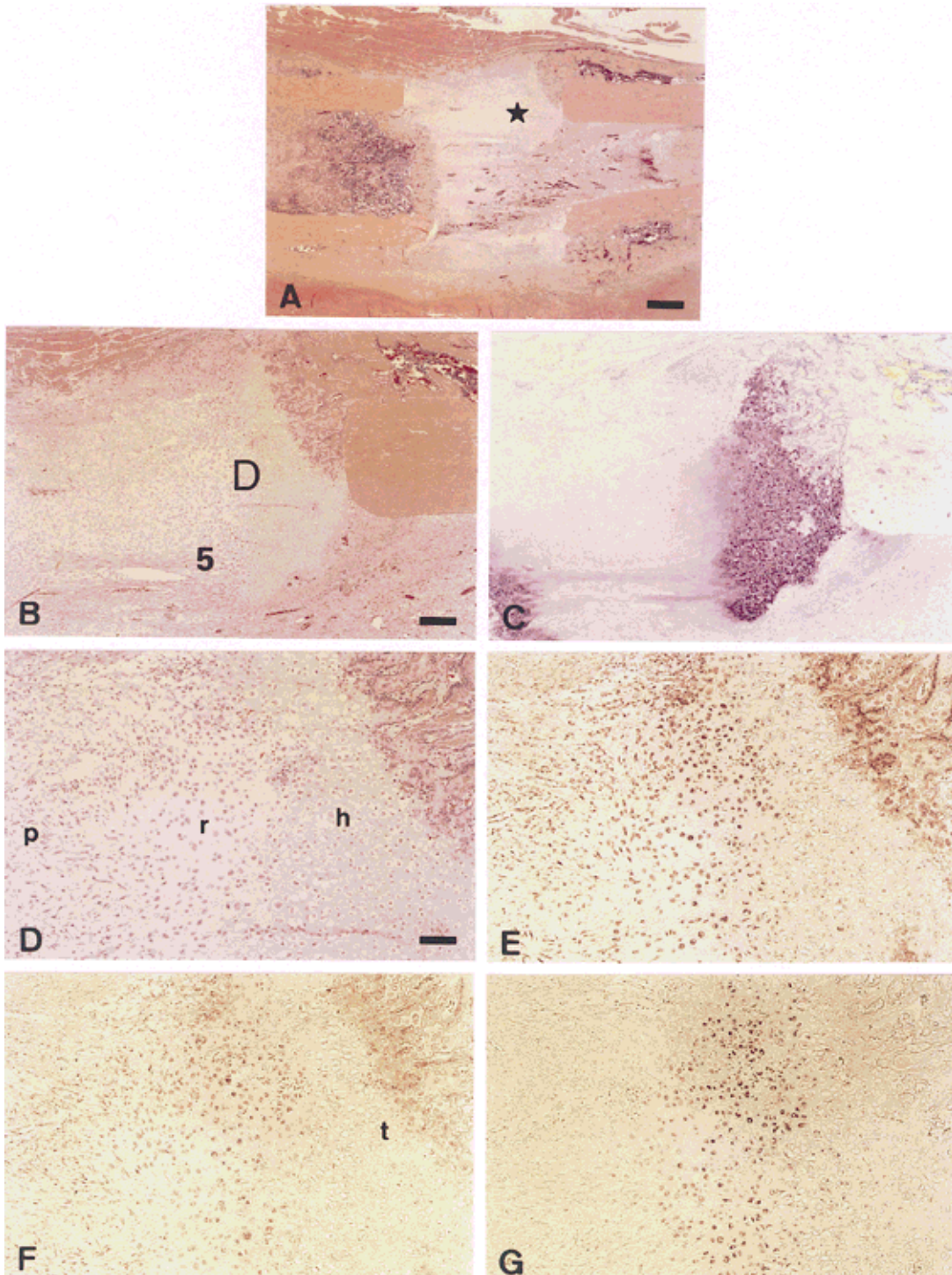
FIG. 3. Histologic appearance and localization of BMP mRNA in a longitudinal section of the femur at seven days after osteotomy, just before the beginning of distraction. (A) Hematoxylin and eosin staining. The osteotomy site (larger arrow head) is surrounded by a cartilaginous external callus (star). Bony hard callus (smaller arrow head) is found between elevated periosteum and bone cortex. Bar = 870 μm. (B, C) are sequential sections. (B) Hematoxylin and eosin staining. High power magnification views of the osteotomy site. (upper star region in A). Bar = 300 μm. (C) Toluidine blue staining. Abundant metachromatic matrix is found. (D–F) are sequential sections. (D) Hematoxylin and eosin staining. High power magnification views of the cartilaginous collar. (region "D" in B). p, proliferative chondrocytes; h, hypertrophic chondrocytes. Bar = 60 μm. (E) BMP-6 mRNA signal in proliferative and hypertrophic chondrocytes. (F) GDF-5 mRNA signal in cell types similar to those expressing BMP-6 mRNA.

Distraction phase: As distraction was started, the external soft callus and the endosteal bony callus were separated into proximal and distal segments forming a fibrous interzone in the middle (Fig. 4A). The cartilage callus at the mouth of the osteotomy site was gradually resorbed and replaced by new bone through endochondral ossification (Figs. 4B–4D). Expression of BMP-2 and BMP-4 mRNAs was markedly enhanced at this stage. The signals were detected widely among the cells participating in chondrogenesis, namely, chondrocytes, prechondrogenic cells and their precursor cells arranging in order of differentiation stage (Fig. 4E). Signals were also found in osteoblasts participating in osteogenesis. The signal of BMP-6 mRNA was detected intensely in round chondrocytes and prehypertrophic chondrocytes, and faintly in prechondrogenic cells and

osteoblasts (Fig. 4F). GDF-5 mRNA, showed more limited localization, being expressed in differentiated chondrocytes and prehypertrophic chondrocytes (Fig. 4G). It was interesting that elongated chondrocytes and spindle-shaped fibroblast-like cells sustaining mechanical tension-stress in the fibrous interzone (Fig. 5A) showed intense signals of BMP-2 and BMP-4 (Fig. 5B) and faint signal of BMP-6 (Fig. 5C). GDF-5 mRNA was detected in chondrocytes but not in fibroblastic spindle cells (Fig. 5D).

As distraction advanced, cartilage callus was completely resorbed and replaced by new bone (Fig. 6A). There was no new cartilage formation, so that new bone was formed directly by intramembranous ossification (Fig. 6B). Strong signals of BMP-2 and BMP-4 mRNAs were detected in osteoblasts at the ossification front, as well as preosteo-

FIG. 4. Histologic appearance and localization of BMP mRNA in a longitudinal section of the femur lengthened 5 mm during 10 days of distraction. (A) Hematoxylin and eosin staining. The cartilaginous callus elongated and separated into proximal and distal segments and a fibrous interzone (star) formed in the middle. Bar = 750 μm. (B, C) are sequential sections. (B) Hematoxylin and eosin staining. High power magnification views of the "star" region in (A). Bar = 270 μm. (C) Toluidine blue staining. Abundant metachromatic matrix is found. (D–G) are sequential sections. (D) Hematoxylin and eosin staining. High power magnification views of region "D" in (B). Endochondral ossification is observed at both ends of the fibrous interzone. p, small polygonal cells; r, round chondrocytes; h, hypertrophic chondrocytes. Bar = 110 μm. (E) BMP-4 mRNA signal in small polygonal cells, round chondrocytes and hypertrophic chondrocytes, and new bone forming osteoblasts. (F) BMP-6 mRNA signal is detectable in round and prehypertrophic chondrocytes, and, to a lesser extent, in small polygonal cells and osteoblasts, but not in terminally differentiated hypertrophic chondrocytes. t, terminally differentiated hypertrophic chondrocytes. (G) GDF-5 mRNA signal is present in round and prehypertrophic chondrocytes, but not in terminally differentiated hypertrophic chondrocytes.
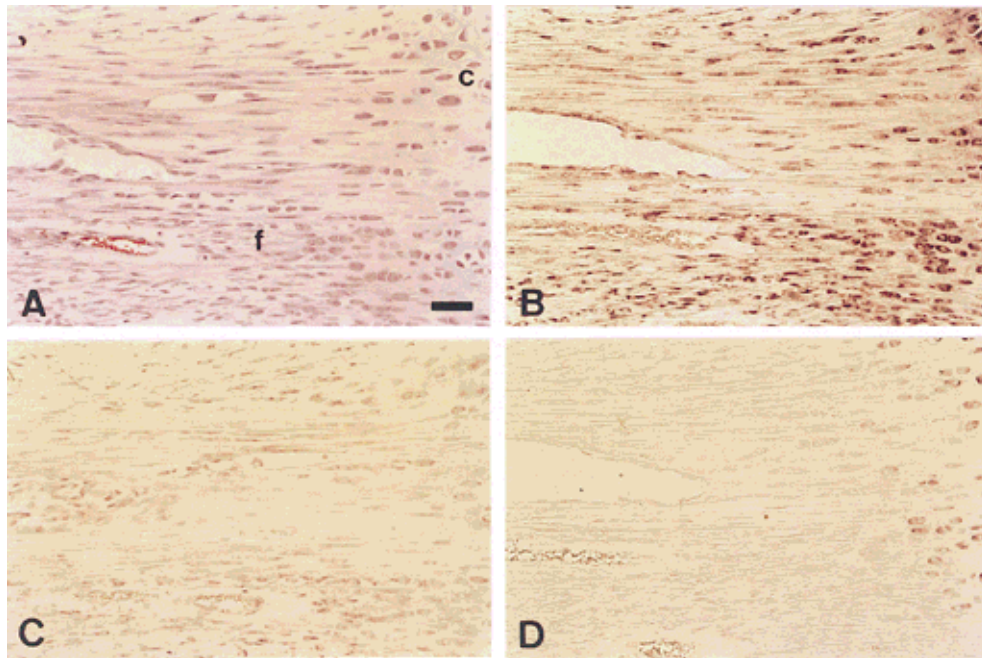
FIG. 5. Region of transition from cartilage to fibrous interzone (region "5" in B). (A–D) are sequential sections. (A) Hematoxylin and eosin. The fibrous interzone contained elongated chondrocytes and fibroblast-like spindle cells. These cells do not resemble osteogenic cells histologically. c, chondrocytes; f, fibroblast-like cells. Bar = 60 μm. (B) Strong signal of BMP-4 mRNA in elongated chondrocytes and fibroblast-like cells. (C) Faint signal of BMP-6 mRNA in the same cells. (D) GDF-5 mRNA signal in chondrocytes.

blasts and their precursor cells arranging along the tension vector in the fibrous interzone (Figs. 6C and 6D). Faint signal of BMP-6 was detected in those osteogenic cells at this stage, but signal of GDF-5 was not detected (data not shown). BMP-7 mRNA was not detected in any specimens.

Consolidation phase

All the signals of BMP-2, BMP-4, BMP-6, and GDF-5 mRNAs declined and became undetectable by 7 days after completion of distraction (data not shown).

Nondistracted group

Histologic events and gene expression taking place in the nondistracted group during the initial seven days were the same as those of lag phase in the distracted group. By 17 days after osteotomy (equivalent in post operative time to 10 days of distraction in the distracted group) the external cartilaginous callus was invaded circumferentially by endochondral ossification and became a small island surrounded by new bone trabeculae (Fig. 7A). Some signals of BMP-6 and GDF-5 mRNA were detected in hypertrophic chondrocytes, but the signals of BMP-2 and BMP-4 mRNA were not detected at this stage (data not shown).

By 28 days after osteotomy (equivalent to 21 days of distraction in the distracted group), the cartilage island had been completely resorbed and the osteotomy gap was

bridged by new bone (Fig. 7B). No signal of BMP mRNA was detected at this stage (data not shown).

DISCUSSION

The results of the present study clearly demonstrated the influence of mechanical tension-stress on BMP gene expression and suggested roles for endogenous BMPs in continuous bone formation during distraction osteogenesis. BMPs are multifunctional growth factors originally identified in bone matrix by their ability to induce ectopic bone formation.[7,8] BMP-induced bone is normal bone, and should contain BMPs as well as other bone matrix proteins. New bone forming cells therefore should express BMP genes at certain stage of differentiation. The present study has demonstrated the expression of endogenous BMPs by the cells participating in bone formation during rat femoral lengthening. Endogenous BMPs were induced both by osteotomy-related stimuli during lag phase, and by mechanical tension-stimulus during distraction phase.

Lag phase

No BMP-2, BMP-4, BMP-6, BMP-7, nor GDF-5 mRNA was detected in normal intact bone without osteotomy in the present study, suggesting that endogenous BMPs do not participate in physiological bone remodeling in young rats.
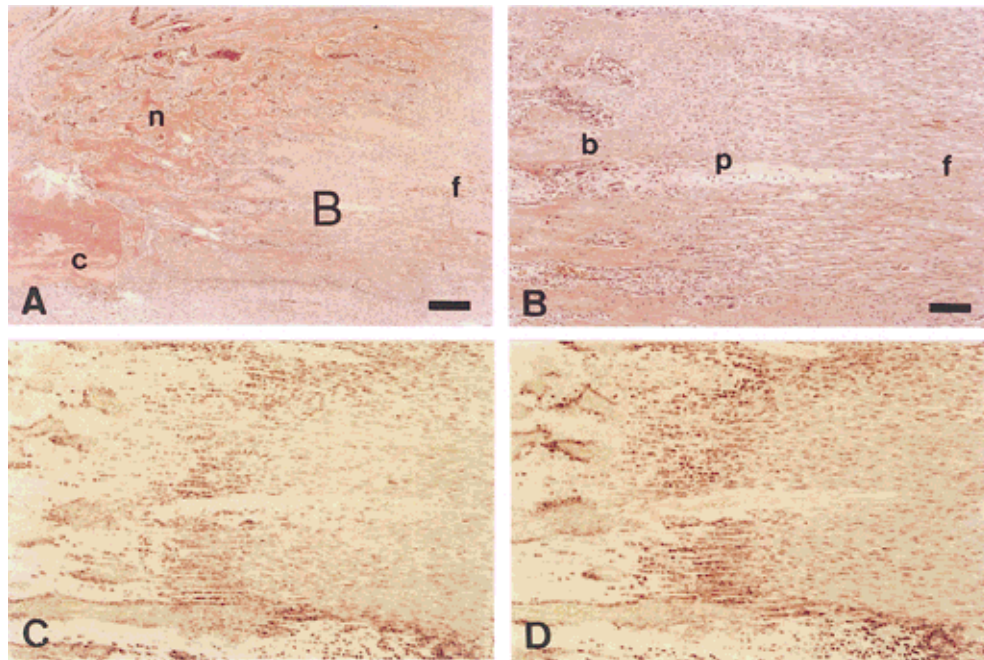
FIG. 6. Histologic appearance and localization of BMP mRNA in a longitudinal section of the femur lengthened 10.5 mm during 21 days of distraction. (A) Hematoxylin and eosin staining. New bone is formed directly from the fibrous tissue. No cartilaginous tissue is found. c, cortex; f, fibrous tissue; n, new bone. Bar $=$ 300 μm. (B–D) are sequential sections. (B) Hematoxylin and eosin staining. High power magnification view of the intramembranous ossification (region "B" in A). Elongated collagen fibers switch into bone spicules without a break. Osteogenic cells arrange longitudinally in order of differentiation stage along the tension vector. b, osteoblasts; f, fibroblast-like cells; p, preosteoblasts. Bar $=$ 120 μm. (C) BMP-2 mRNA signal in the fibroblast-like cells, preosteoblasts and osteoblasts of the new bone columns. (D) BMP-4 mRNA signal in cell types similar to BMP-2 mRNA-expressing cells.
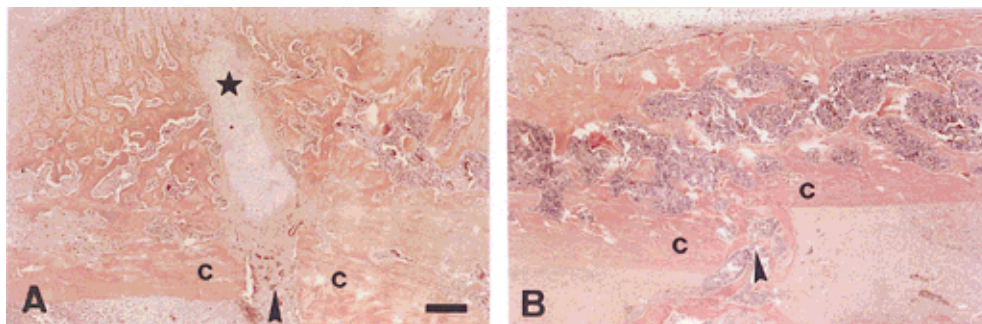
FIG. 7. Hematoxylin and eosin staining of a longitudinal section of the femur in nondistracted rat. (A) The histology at 17 days after osteotomy. The cartilaginous external callus (star) became a small island surrounded by bone trabeculae. The hypertrophic chondrocytes are invaded and replaced by new bone through endochondral ossification. c, cortex; arrow head, the osteotomy site. Bar $=$ 300 μm. (B) The histology at 28 days after osteotomy. The osteotomy site (arrow head) has made bony union. c, cortex.

The molecular events that took place in the lag phase after osteotomy were basically the same as those of the fracture healing process. It is generally accepted that fracture hematoma contains various cytokines and growth factors exudated from broken bone matrix. It is not evident, however, whether bone matrix-derived BMPs are released into hematoma and contribute to induction of the mesenchymal cells into osteoblasts or chondrocytes. In the pres-

ent study, induction of endogenous BMP-2 and BMP-4 was detected by 4 days after osteotomy in immature prechondrogenic cells under the periosteum. It is possible that the gene products of these two BMPs accelerate differentiation of the cells into osteogenic/chondrogenic cells by an autocrine or paracrine mechanism. Transient and localized expression of BMP-4 genes is consistent with previous observations of the fracture healing process.[27] Expression of

BMP-6 and GDF-5 genes was detected after osteotomy in more differentiated chondrocytes and the expression lasted for a few weeks until cartilage callus was resorbed and replaced by bone. The different patterns of expression of BMP-2 and BMP-4 genes and BMP-6 and GDF-5 genes suggested they have independent transactivation pathways. BMP-7 gene was not induced by osteotomy. Probably, endogenous BMP-7 does not participate in the fracture repair process in adult bone.

Distraction phase

More dynamic molecular events characteristic to distraction osteogenesis took place during the distraction phase. Since the expression of c-*fos*, which is generally accepted to be mechanically responsive,[25] was strongly enhanced during the distraction phase, mechanical tension-stress was considered to affect the cells which took part in the distraction osteogenesis. Expressions of BMP-2 and BMP-4 mRNAs, which had declined by the end of the lag phase, were strongly enhanced by 10 days of distraction. Northern blot analysis demonstrated the magnitude of induction of BMP-4 gene by mechanical tension-stimulus was 20-fold higher than that by osteotomy stimuli. (Fig. 1B) The strong signals of BMP-2 and BMP-4 mRNA were detected widely among chondrogenic and osteogenic cells and their precursor cells. There was no histologic evidence of trabecular bone microfracture, and positive signals of BMP-2 and BMP-4 genes were observed not only near the ossification front but also in the fibrous interzone away from bony trabeculae. These results suggest that the continuous process of bone formation during the distraction phase does not depend on repeated microfracture but is mediated by elevation of endogenous BMP-2 and BMP-4. As distraction advanced, cartilaginous callus was progressively resorbed and new bone was formed directly by intramembranous ossification. Meanwhile, expression of BMP-6 and GDF-5 genes gradually declined, whereas expression of BMP-2 and BMP-4 was maintained for as long as distraction continued. These results again suggested the independent modulation of BMP genes. Elevation of BMP-6 and GDF-5 gene expression levels in the early distraction phase were more likely due to osteotomy-related stimuli than tension stress-related stimulus, because the expression patterns of these genes did not change in nondistracted control.

BMPs belong to the TGF superfamily and are currently classified into several subfamilies based on homology of protein structure.[9] BMP-2 and BMP-4 belong to the same subfamily and showed similar expression patterns during the present experiment. BMP-6 and GDF-5 belong to different subfamilies, but showed similar expression patterns in the present study. BMP-7, which belongs to the same subfamily as BMP-6, was not detected in the present experiment. Taking into account previous studies, we next discuss possible functions of each BMP.

BMP-2 and BMP-4

BMP-2 and BMP-4 induce formation of ectopic bone and cartilage when implanted into extraskeletal sites.[8,28–30]

These BMPs are considered to be functionally interchangeable.[31,32] Since mice in which BMP-2 or BMP-4 has been knocked out die between 6.5 and 9.5 days postcoitum before the beginning of chondrogenesis/osteogenesis,[33,34] the physiological functions of these BMPs in adult tissues are not known. The expression of BMP-4 protein and mRNA in fracture healing has been investigated by immunohistochemistry and in situ hybridization.[27,35] BMP-2/-4 immunostaining was most intense in primitive mesenchymal cells, early chondrocytes, and early osteoblasts.[35] BMP-4 mRNA was reported to be expressed in the early phase of fracture repair by osteoprogenitor cells in the cambium layer of periosteum.[27] In in vitro studies, recombinant BMP-2 stimulates osteogenesis,[36–38] inhibits myogenesis,[38] and does not affect chondrogenesis.[36] Moreover, recombinant BMP-2 can accelerate bone formation in vivo in a dose-dependent manner.[30] In the present study, we consider that ample expression of BMP-2 and BMP-4 genes during the distraction phase should enhance BMP-2 and BMP-4 protein synthesis and contribute to uninterrupted bone formation. Since the induction was observed in the distracted group but not in the nondistracted group, BMP-2 and BMP-4 are considered to respond to mechanical tension-stress. Recently, the presence of shear-stress-responsive elements (SSRE) were found in the promoter region of Platelet-derived growth factor B chains (The core binding sequence, GAGACC)[39] and monocyte chemotacting protein-1 (The core binding sequence, TGACTCC; complementary sequence ACTGAGG).[40] It is interesting that SSREs are also located in the promoters of the mouse BMP-2[17] (–240 GAGACC –245) and mouse BMP-4[41] (–1518 GAGACC –1513, –172 ACTGAGG –166). Mechanical tension-stress may act to promote BMP-2 and BMP-4 mRNA expression through SSRE. Further study of the transcriptional regulation of these two factors is required.

BMP-6

CHO cells overexpressing murine BMP-6 gene induce endochondral bone formation when implanted into the subcutaneous tissue of athymic nude mice.[18] BMP-6 mRNA is localized in hypertrophic chondrocytes,[42] and in osteoblastic cells.[43] In the present study, endogenous BMP-6 was induced by osteotomy stimuli in more differentiated chondrocytes. BMP-6 gene did not appear to respond to mechanical tension-stimulus. It is not clear whether BMP-6 has a mechanical stress response element. BMP-6 gene may reflect chondrogenic activity and subsequent endochondral bone formation.

BMP-7

BMP-7 also induces ectopic bone and cartilage formation when implanted into extraskeletal sites.[29] In the present study, endogenous BMP-7 was not detected. It is likely that BMP-7 plays a major role in embryonic development[9,44] but not in adult tissues.

GDF-5

GDF-5 is also known as cartilage-derived morphogenetic protein-1 (CDMP-1). The physiological role of this protein is not well understood, although mutations of GDF-5 gene result in brachypodism, chondrodysplasia Grebe type, Hunter-Thompson chondrodysplasia and brachydactyly type C.[20,45–47] Recently, Erlacher reported CDMP stimulated cartilage matrix synthesis but markedly reduced activity in the promotion of osteogenesis.[48] In contrast to the other members of the BMP family, the expression of GDF-5 mRNA is found predominantly at the stage of precartilaginous mesenchymal condensation in the cartilaginous cores of developing long bones and in the joint interzones.[9,15] In the present study, GDF-5 mRNA expression was enhanced after osteotomy but showed no reaction against mechanical stress, and its pattern was similar to, but more localized than that of BMP-6. Endogenous GDF-5 may reflect chondrogenic activity.

The present study has provided new insights into the molecular mechanism of distraction osteogenesis. It is as yet unclear, however, how the mechanical tension-stress is transmitted to osteogenic/chondrogenic cells that produce BMPs. Transcriptional regulation of BMPs, and the expression of their receptors[49] and signal transduction molecule smads,[13,50] are subjects for further investigation.

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