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Tensile Stress Induces Bone Morphogenetic Protein 4 in Preosteoblastic and Fibroblastic Cells, Which Later Differentiate into Osteoblasts Leading to Osteogenesis in the Mouse Calvariae in Organ Culture

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ABSTRACT

Mechanical stress is an important factor controlling bone remodeling, which maintains proper bone morphology and functions. However, the mechanism by which mechanical stress is transduced into biological stimuli remains unclear. Therefore, the purpose of this study is to examine how gene expression changes with osteoblast differentiation and which cells differentiate into osteoblasts. Tensile stress was applied to the cranial suture of neonatal mouse calvaria in a culture by means of helical springs. The suture was extended gradually, displaying a marked increase in cell number including osteoblasts. A histochemical study showed that this osteoblast differentiation began in the neighborhood of the existing osteoblasts, which can be seen by 3 h. The site of osteoblast differentiation moved with time toward the center of the suture, which resulted in an extension of osteoid. Scattered areas of the extended osteoid were calcified by 48 h. Reverse-transcription polymerase chain reaction (RT-PCR) revealed that tensile stress increased bone morphogenetic protein 4 (BMP-4) gene expression by 6 h and it remained elevated thereafter. This was caused by the induction of the gene in preosteoblastic cells in the neighborhood of osteoblasts and adjacent spindle-shaped fibroblastic cells. These changes were evident as early as 3 h and continued moving toward the center of the suture. The expression of Cbfa1/Osf-2, an osteoblast-specific transcription factor, followed that of BMP-4 and those cells positive with these genes appeared to differentiate into osteoblasts. These results suggest that BMP-4 may play a pivotal role by acting as an autocrine and a paracrine factor for recruiting osteoblasts in tensile stress–induced osteogenesis. (J Bone Miner Res 2001;16:24–32)

Key words: **mechanical stress, osteoblast differentiation, osteogenesis, bone morphogenetic protein 4, osteoblast precursors**

INTRODUCTION

MECHANICAL STRESS is known to influence both bone formation and bone resorption. Tensional stress promotes bone formation, while the lack of physical stress, as in immobilization, results in disuse osteopenia or osteoporosis. Similarly, orthodontic tooth movement necessitates bone resorption at the pressure side with concomitant bone formation at the tension side. Osteoblasts are the bone-forming cells responsible for bone remodeling, a complex

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series of events whereby bone is first resorbed and then new bone is deposited. Because osteoblasts and osteocytes are believed to be cells responsive to mechanical stress, understanding the mechanism(s) by which externally applied forces are transduced to those cells to yield a series of biochemical events such as bone remodeling is, therefore, important.

Recent studies, using in vitro mechanically loaded bone tissues and/or osteoblastic cells, as well as in vivo animal models with repetitive compressive mechanical stress application, have shown responses similar to that observed when cells have been exposed to extracellular ligands such as growth factors, neuropeptides, or hormones, all modulators of cellular phenotype. These changes include production of cyclic adenosine monophosphate (cAMP),[1,2] inositol phosphates,[1,3] 1,2-diacyglycerol,[4] prostaglandins,[5–9] and nitric oxide.[10–12] Activation of protein kinase C[4] and ion channels[9,13,14] and an increase of intracellular calcium[15,16] have been reported as well. It also has been shown that expressions of many genes or gene products are regulated by mechanical stress. These genes include early responsive genes such as c-*fos*[17–19] and *egr-1*,[20,21] genes involved in the eicosanoid metabolism[5] and nitrogen oxide synthesis,[22] and genes encoding growth factors such as insulin-like growth factor I (IGF-I)[17,22,23] and transforming growth factor β (TGF-β).[7,18] However, these responses also have been found in many other tissues and so far no osteoblast-specific events that eventually lead to osteogenesis induced by mechanical stress have been shown. To elucidate the mechanism(s) whereby mechanical stress is transduced into osteogenesis, we selected an organ culture system in which mouse calvarial sutures were incubated with or without tensile stress. This experimental system was reported by Hickory and Nanda,[24] in which tensile stress stimulated bone formation in rat calvarial sutures. We chose this experimental system for the following reasons. First, calvarial sutures contain not only bone tissues but also surrounding soft tissues including fibroblastic cells, which could differentiate into osteoblasts. This system is more complex and a much better model of the in vivo situation compared with the osteoblastic cell culture system. Second, it is less complex and better suited for microscopic observations of cell differentiation compared with in vivo experiments. Third, because osteogenesis of calvariae is devoid of endochondral ossification, the system is simpler than those including other parts of bone such as long bone tissues. Fourth, this organ culture system is free from systemic regulation such as hormonal control.

Using this model, the purpose of the present study was to examine the time course of osteogenesis induced by mechanical stress with particular interest as to which cell or cells can differentiate into osteoblasts, and which genes, which are specific to osteoblast function or osteogenesis, are regulated. Here we present data showing that tensile stress application to mouse calvarial sutures induced differentiation and growth of osteoblasts, which eventually lead to osteogenesis. Before osteoblasts were recruited, bone morphogenetic protein 4 (BMP-4) gene expression was upregulated or induced in the preosteoblast and fibroblastic cells surrounding bone tissues, which was followed by induction of Cbfa1/Osf-2 expression in these cells. Thus, BMP-4 may play a role as an autocrine and paracrine factor in the differentiation of preosteoblasts and fibroblasts into osteoblasts, thereby stimulating osteogenesis induced by tensile stress in the organ culture of the calvarial suture.

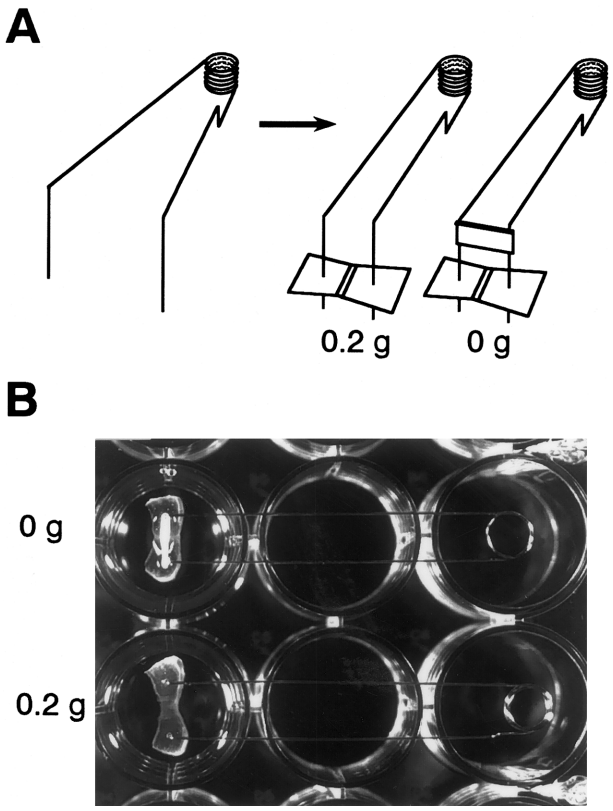


FIG. 1. Application of tensile stress to calvarial sutures in culture. (A) Schematic illustration of the helical springs used. See text for details. (B) Calvarial suture was removed from mice and tensile stress was applied as illustrated. The suture was suspended in the medium of each well.

MATERIALS AND METHODS

Organ culture and mechanical force device

Calvariae were aseptically removed from neonatal ddY mice (3–4 days old) and the cranial suture of each calvaria was incubated in a 24-well plate (Corning Costar, Corning, NY, USA) with or without mechanical stress at 37°C and 5% CO_2 in air in BGJb medium (Gibco BRL, Life Technologies, Inc., Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), penicillin G (100 U/ml; Gibco BRL), and streptomycin (100 μg/ml; Gibco BRL) for the period indicated. Tensile stress was applied to each cranial suture using a helical spring made of Elgiloy orthodontic wire (Elgiloy 0.01 in, semiresilient; Rocky Mountain, Denver, CO, USA) according to the method of Hickory and Nanda[24] with a slight modification (Fig. 1). The magnitude of each helical spring was adjusted to give 0.2 g tensile stress when the distance at the top of each arm (length, 4 cm) was set at 5 mm (Fig. 1). The

magnitude of the spring was decreased at a rate of 0.01 g/mm (data not shown). For the control, springs maintained at 5 mm by means of adhesive tape, thus giving 0 g of tensile strength, were used (Fig. 1). After incubation, cranial sutures were subjected to histochemical analyses, in situ hybridization, or reverse-transcription polymerase chain reaction (RT-PCR).

Histochemical studies

Cranial sutures were fixed for 1 h at 4°C with a fixative containing 4% paraformaldehyde, embedded in optimum cutting temperature (O.C.T.) compound (Tissue-Tek; Miles, Inc., Elkhart, IN, USA) and 8-μm cryosections were cut by Cryotome (Shandon, Elgany, France). The sections were then subjected to histochemical analyses. Alkaline phosphatase activity was detected according to the azo-dye method of Burstone.[25] The same sections were then subjected to von Kossa staining to detect calcification. Methylene blue was used for the counterstaining. To detect cartilage as well as calcified tissues in the samples, some of sections were subjected to alizarin red and Alcian blue staining.

RT-PCR

Cranial sutures were freed from calcified tissues under a dissection microscope and total RNA was extracted using Trizol (Gibco BRL) according to the manufacturer's protocol. Then, first-strand complementary DNA (cDNA) was synthesized using oligo (dT) (Gibco BRL) and Superscript II (Gibco BRL) as a primer and a reverse transcriptase, respectively. For PCR reactions, aliquots of synthesized cDNA were added to a PCR reaction mixture (0.04 μl/μl) containing 3′- and 5′-primer (0.2 μM each), deoxynucleoside triphosphate (dNTP) mixture (0.2 mM each; Gibco BRL), and *Taq* polymerase (0.05 U/μl) in *Taq* polymerase buffer (1×; Gibco BRL). Cycling conditions were 94°C/30 s, 55°C/30 s, and 72°C/60 s for alkaline phosphatase (ALPase), type I collagen, osteocalcin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin for 25 cycles, and 94°C/30 s, 57°C/30 s, and 72°C/60 s for BMP-4 for 32 cycles, respectively (Fig. 2 lower panel). Products were separated on a 1% agarose gel. Primers for PCR are as follows: oligonucleotides 5′-GACTGGTACTCGGATAACGAGATGC-3′ and 5′-TGCGGTTCCAGACATAGTGG-3′ corresponding to the 735–759 nucleotides (nt) and 980–999 nt of mouse ALPase gene, respectively; 5′-ACCATCTGGCATCTCATGGC-3′ and 5′-GCAACACAATTGCACCTGAGG-3′ corresponding to the 666–685 nt and 847–867 nt of mouse type I collagen gene, respectively; 5′-TGCGCTCTGTCTCTCTGACC-3′ and 5′-CTGTGACATCCATACTTGCAGG-3′ corresponding to the 87–167 nt and 422–443 nt of mouse osteocalcin gene, respectively; 5′-CAGCGGTCAGGAAGAAGAATAAG-3′ and 5′-TCTGCACAATGGCATGGTTG-3′ corresponding to the 1615–1638 nt and 1776–1795 nt of mouse BMP-4 gene, respectively; 5′-CAAGAAGGTGGTGAAGCAGG-3′ and 5′-ATTGAGAGCAATGCCAGCC-3′ corresponding to the 814–833 nt and 934–952 nt of mouse GAPDH gene; 5′-CAGGAGATGGCCACTGCCGCA-3′ and 5′-TCCTTCTGCATCCTGTCAGCA-3′ corresponding to the 753–773 nt and 1007–1027 nt of mouse β-actin, respectively (Fig. 2).

PCR primers

specificity	sequence (5′-3′)	product size (bp)
collagen type I	forward: ACCATCTGGCATCTCATGGC reverse: GCAACACAATTGCACCTGAGG	202
ALPase	forward: GACTGGTACTCGGATAACGAGATGC reverse: TGCGGTTCCAGACATAGTGG	265
osteocalcin	forward: TGCGCTCTGTCTCTCTGACC reverse: CTGTGACATCCATACTTGCAGG	357
BMP-4	forward: CAGCGGTCCAGGAAGAAGAATAAG reverse: TCTGCACAATGGCATGGTTG	181
GAPDH	forward: CAAGAAGGTGGTGAAGCAGG reverse: ATTGAGAGCAATGCCAGCC	139
β-actin	forward: CAGGAGATGGCCACTGCCGCA reverse: TCCTTCTGCATCCTGTCAGCA	275

PCR programs

collagen type I ALPase osteocalcin GAPDH β-actin	94 °C 2 min.	94 °C 30 sec.	55 °C 35 sec.	72 °C 1 min.	72 °C 7 min.
		25 cycles			
BMP-4	94 °C 2 min.	94 °C 30 sec.	57 °C 35 sec.	72 °C 1 min.	72 °C 7 min.
		32 cycles			

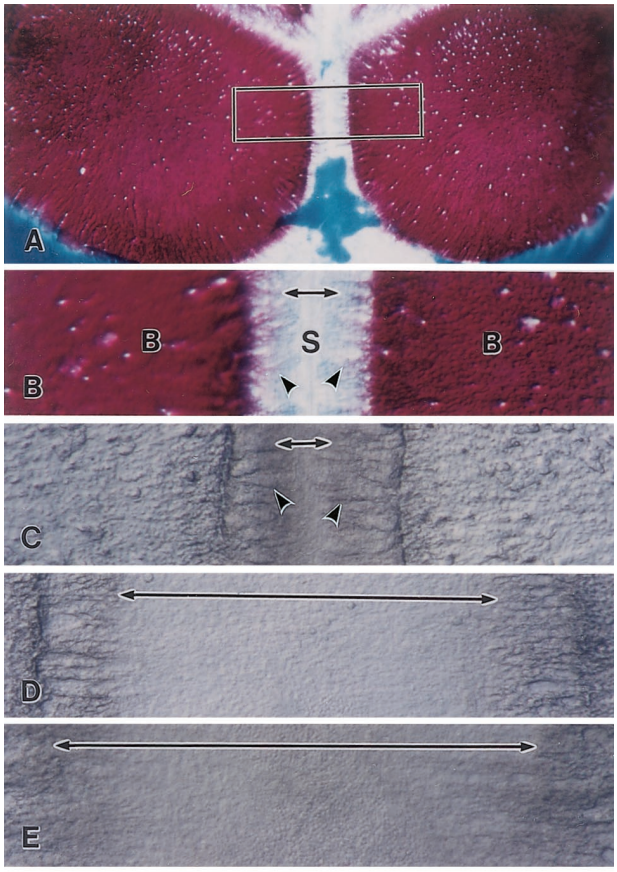
FIG. 2. Primers and conditions used for PCRs. See text for details.

Probe preparation

Digoxigenin (DIG)-labeled sense and antisense single-stranded RNA probes were prepared with a DIG RNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. For BMP-4, a 456-base pair (bp) cDNA fragment (366–821 nt of accession number X56848) was obtained by RT-PCR and subcloned into pGEM-T Vector System (Promega, Madison, WI, USA). After transcription, 40 U of RNAse-free DNAse (Boehringer Mannheim) were added to the reaction and an additional 10-minute incubation was done at 37°C. Transcription products were recovered by ethanol precipitation, and precipitates were washed once in 70% ethanol, air-dried, and resuspended in 100 μl diethylpyrocarbonate (DEPC)-treated water. For Cbfa1/Osf-2 a 0.3-kb fragment of exon I of mouse cDNA[26] was subcloned into pBluescript KS(−) (Stratagene, La Jolla, CA, USA). The subcloned fragments were confirmed to be identical to the respective cDNA sequence by an ABI PRISM 377 DNA Sequencing System (Perkin Elmer Co., Foster City, CA, USA).

In situ hybridization

After incubation, cranial sutures were fixed with 4% paraformaldehyde in DEPC-treated phosphate-buffered sa-



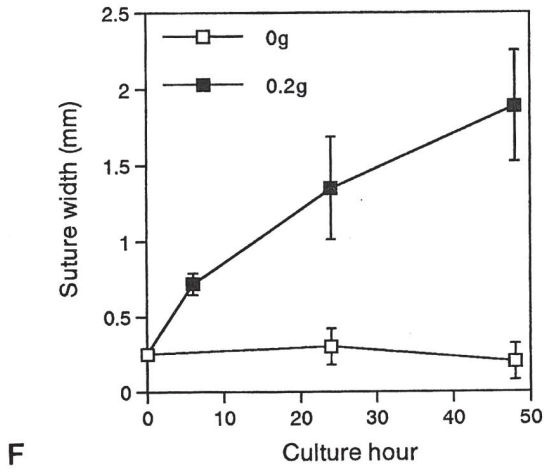


FIG. 3. Tensile stress elongated the calvarial sutures in a time-dependent manner. Calvariae from 3- to 4-day-old mice were cultured as described in the text. (A) Alizarin red and Alcian blue staining of the suture at time 0. Blue staining indicates the presence of cartilaginous tissue (original magnification ×12). (B) Higher magnification of the parietal bone (B) and suture (S) framed in panel A. Note alizarin red–stained short trabeculae extended from the parietal bone (arrowheads; original magnification ×40). (C–E). Calvariae under tensile stress at 0, 24, and 48 h, respectively. The short trabeculae can be seen without staining (arrowheads; original magnification ×40). (F) Suture width was determined as the distance between the two calcified bone edges and plotted against the duration under tensile stress. Each point and bar represents a mean of five experiments ± SD, respectively.

line (PBS) at 4°C overnight, embedded in O.C.T. compound, and 6-μm cryosections were cut by Cryotome. Cryosections were washed in PBS, treated with 0.2N HCl for 10 minutes, and incubated with 0.5 μg/ml proteinase K (Gibco BRL) in 10 mM Tris-HCl (pH 8.0) at 37°C for 30 minutes. Cryosections were then refixed with 4% paraformaldehyde in PBS at room temperature for 20 minutes and further treated with 0.25% acetic anhydride in 0.1 M triethanolamine for acetylation. Acetylated cryosections were hybridized with probes at a final concentration of 1.0 μg/ml in a hybridization mixture containing 50% deionized formamide, 2% blocking reagent (Boehringer Mannheim), 5× SSC (1× SSC = 0.15 M NaCl, pH 7.5, 15 mM sodium citrate), 0.02% sodium dodecyl sulfate (SDS), 0.1% *N*-lauroylsarcosine, 200 μg/ml yeast transfer RNA (tRNA), and 100 μg/ml of salmon sperm DNA at 55°C for 18–20 h, and then washed twice with 0.1× SSC at 60°C for 15 minutes followed by RNAse (Wako Chemicals, Osaka, Japan) treatment at 37°C for 30 minutes. The sections were washed further twice with 2× SSC at 60°C for 15 minutes and then twice with 0.1× SSC at 60°C for 15 minutes. Specific transcripts were detected with anti-DIG–conjugated ALPase antibody by the manufacturer's protocol (DIG Detection Kit; Boehringer Mannheim). The sections were counterstained with nuclear fast red.

RESULTS

Tensile stress induces osteoblast differentiation and osteogenesis in calvarial suture

Figure 3 depicts cranial sutures of calvariae with or without mechanical loading. Short trabeculae[27] stained with alizarin red extend from the edge of the bone (Figs. 3A and 3B, arrows). These trabeculae were visible under a dissecting microscope without staining and they were extended further by mechanical loading (Figs. 3C–3E, arrows; tensile stress was applied outward). Sutures were extended in a time-dependent manner as shown in Fig. 3F.

Figure 4 illustrates cross-sections of calvarial sutures with ALPase activity and von Kossa staining. The calcified bone and partly calcified osteoid were covered with oval-shaped ALPase positive osteoblasts, which were particularly abundant at the growing tip of the osteoid (Fig. 4A). Between the osteoblasts layer and periosteum or dura mater and at the center of the suture, many fibroblastic cells were seen among the fibrous matrices, and none of these cells were ALPase positive (Fig. 4A). Tensile stress increased the width of the suture in a time-dependent manner (Figs. 3 and 4). This increase of suture width was accompanied by cell proliferation evaluated by immunohistochemistry using the proliferating cell nuclear antigen (PCNA) monoclonal antibody (Ab-1; Oncogene Research Products, Cambridge, MA, USA; data not shown). As described previously, the tensile stress application resulted in a continuous time-

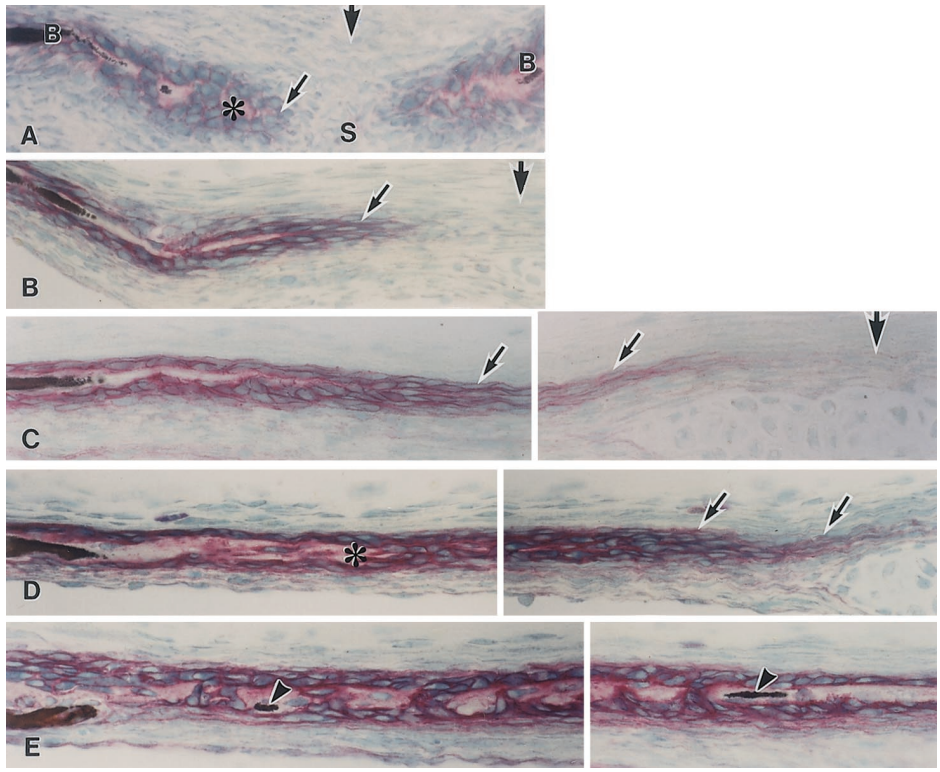


FIG. 4. Tensile stress increased in osteoblast number and tissues as the calvarial sutures increased in size. Cross-sections of calvariae were subjected to ALPase activity and von Kossa staining. (A) Calvaria at time 0. Note the oval-shaped ALPase positive osteoblasts surrounding the bone (B) and osteoid (*) and preosteoblasts (slant arrow; S, suture). (B–C) Calvaria at 1, 6, 24, and 48 h under tensile stress, respectively. (B) At 1 h, osteoblasts, as well as fibroblastic cells, were flattened and elongated. The osteoid and ALPase positive preosteoblast (slant arrow) layer also were extended toward the center of the suture. (C) At 6 h, the flattened ALPase positive cell (slant arrows) layer further extended toward the center and some osteoid is formed. (D) At 24 h, the osteoid (*) and preosteoblast (slant arrows) layer further increased in length. (E) At 48 h, the osteoid thickened, part of which became calcified (arrowheads). The center of the suture is indicated by a perpendicular arrow (original magnification ×280).

dependent elongation of the suture in which most of the cells became flattened and elongated (Figs. 4B–4E). The osteoid covered with ALPase positive cells extended toward the median line of the suture at 1 h after the start of mechanical loading (Fig. 4B). At 6 h, the flattened ALPase positive cell layer extended further toward the center (Fig. 4C), although most parts of the extended cell layer were without osteoid and were thinner compared with the previously existing layer with osteoid (Figs. 4C and 4B), indicating that these ALPase positive cells are preosteoblasts. This also was supported by the fact that they were negative with osteocalcin and osteopontin (data not shown), both of which are typical markers of osteoblasts. By contrast, expression of osteocalcin and osteopontin was detected in the mature osteoblasts on the mineralized bone (M. Ikegame, O. Ishibashi, T. Yoshizawa, H. Ozawa, and H. Kawashima, unpublished data, 1999). At 24 h the extended layer of ALPase positive cells thickened and some new osteoid was added (Fig. 4D). At 48 h the ALPase positive cell layer increased further in length and thickness, which was accompanied by an increase of osteoid (Fig. 4E). Scattered newly calcified areas also were evident in the increased osteoid as evidenced with von Kossa staining (Fig. 4E). Gene expression of osteocalcin and osteopontin was confirmed in these newly recruited osteoblasts (M. Ikegame, O. Ishibashi, T. Yoshizawa, H. Ozawa, and H. Kawashima, unpublished data, 1999). In contrast to these calvarial sutures, those without tensile stress showed practically no significant changes in histological appearance, osteoblast number, and osteoid area from time 0–24 h incubation. However, at 48 h, a minimal increase was observed in the thickness of the bone edge in which part of the osteoid had been calcified without mechanical loading (data not shown). ALPase activity was detected in fibroblastic cells surrounding cartilage and along the dura mater, but these activities did not change with mechanical loading (Figs. 4C and 4D). From these observations, it is possible that a portion of the newly recruited osteoblasts due to tensile stress were differentiated from fibroblastic cells.

Tensile stress up-regulates BMP-4 expression in calvariae

To identify key genes responsible for the differentiation and the growth of osteoblasts induced by tensile stress, calvarial sutures were dissected under a dissecting micro-

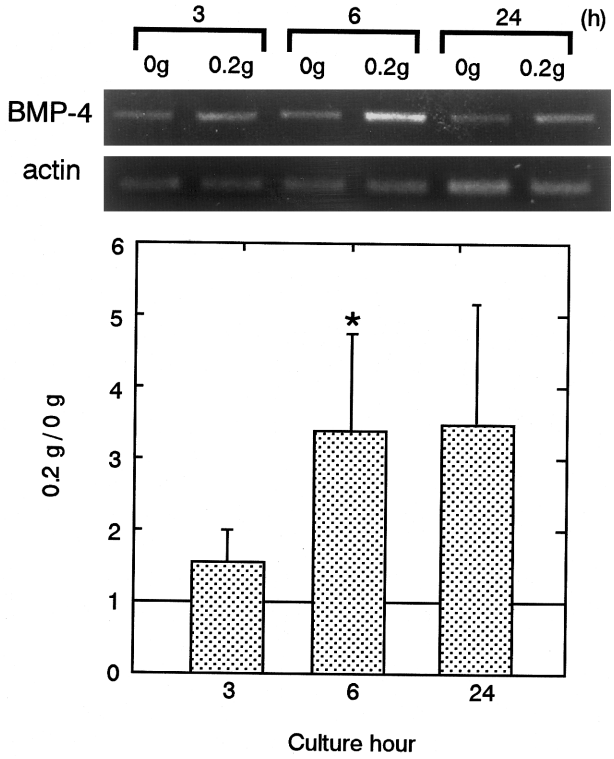


FIG. 5. Tensile stress up-regulated the BMP-4 gene expression in calvariae. Total RNA was extracted from cultured calvariae at each time point indicated and was subjected to RT-PCR. Each column and bar represents a mean of three different experiments and SD. *Significantly different from the control at $p < 0.05$. See text for details.

scope (Fig. 3). Cryosections of the dissected sutures confirmed that the tissues contain only fibrous matrix and osteoid and are devoid of calcified matrix (data not shown). The sutures were then subjected to RNA extraction followed by RT-PCR.

Up to 6 h after the start of tensile stress, no change in the expression of either ALPase, type I collagen, or osteocalcin gene was observed (data not shown). In contrast, PCR products corresponding to BMP-4 slightly increased at 3 h, although this increase was not significant. The expression significantly increased at 6 h reaching approximately 3.5-fold of the control (Fig. 5) and this level of BMP-4 expression was maintained until 24 h. In another set of experiments a similarly elevated expression of BMP-4 gene was observed at 48 h (data not shown). Because the tissue preparation contains heterogenous types of cell (Fig. 4), it is impossible to identify cells in which the BMP-4 expression is up-regulated. To address this question we next examined gene expression in individual cells using an in situ hybridization technique.

Tensile stress sequentially up-regulates BMP-4 and Cbfa1/Osf-2 expression in preosteoblastic and fibroblastic cells in the calvariae

Figure 6 depicts the expression profiles of BMP-4 and Cbfa1/Osf-2 messenger RNAs (mRNAs) in calvariae under tensile stress. Figure 6I illustrates exactly from where Figs. 6A–6F were taken. A schematic illustration of the profiles of Figs. 6A–6F also is given in Fig. 7. BMP-4 expression was detected in osteoblasts surrounding the bone tissues and osteoid, in preosteoblasts and in some of fibroblastic cells near the periosteal surface and the central region of the suture in the control (time 0; Figs. 6B and 7). On the other hand, Cbfa1/Osf-2 expression was localized specifically to osteoblasts and preosteoblasts in the control (Figs. 6A and 7). At 3 h under tensile stress BMP-4 expression was increased in preosteoblastic cells located in front of osteoblasts and was induced in the adjacent spindle-shaped fibroblastic cells near the central region of the suture (Figs. 6D and 7). In contrast, the expression of Cbfa1/Osf-2 did not change significantly (Figs. 6C and 7). At 6 h both preosteoblastic and fibroblastic cells with positive BMP-4 expression further increased in number as the ALPase positive cell layer and osteoid extended (Figs. 4C, 6F, and 7). Cbfa1/Osf-2 expression also was induced in the newly recruited preosteoblasts (Figs. 6E and 7), which were likely to be the fibroblastic cells with positive BMP-4 expression at 3 h (Figs. 6D and 7). To ensure that the expression of BMP-4 precedes the Cbfa1 expression, we counted the number of cells that express BMP-4 or Cbfa1 in four different sets of plates including those shown in Figs. 6A–6F. The actual counting was carried out as follows. The background level was set to the strongest staining on the plate incubated with sense probe, and subtraction was done on a computer screen using the National Institutes of Health (NIH) image 1.60. Then the remaining positive cells were counted. As shown in Fig. 6J, BMP-4 expression slightly increased at 3 h and significantly elevated at 6 h, whereas Cbfa1 expression showed a tendency to increase at 6 h but did not reach a significant level.

DISCUSSION

The present study shows that mechanical stress induces osteoblast differentiation, which then leads to osteogenesis in mouse calvarial sutures in culture. This osteoblast differentiation seemed to be accompanied by an increase in BMP-4 gene expression when evaluated by RT-PCR of the cultured suture. Our study also showed that calvarial sutures express the BMP-4 gene even without mechanical loading. Ever since molecular cloning and characterization of the BMP gene family and its products, one of the major concerns in the field of bone mineral research has been whether or not one or more members of the BMP gene family play a role in modeling or remodeling bone in neonatal and adult life. It is well established that BMP family members play critical roles in patterning and developing of skeletal tissues during embryogenesis.[28,29] Site-specific expression of gene family members also has been shown in the developing bone tissues in the embryo.[30] However, many studies failed to show BMP gene expression in adult bone tissues. The only exception was that the BMP-4 gene was expressed in tissues surrounding healing bone after artificial bone fracture and it disappeared as healing progressed.[31] Our RT-PCR analysis thus provides the first evidence that normal bone tissues from neonatal animals express the BMP-4

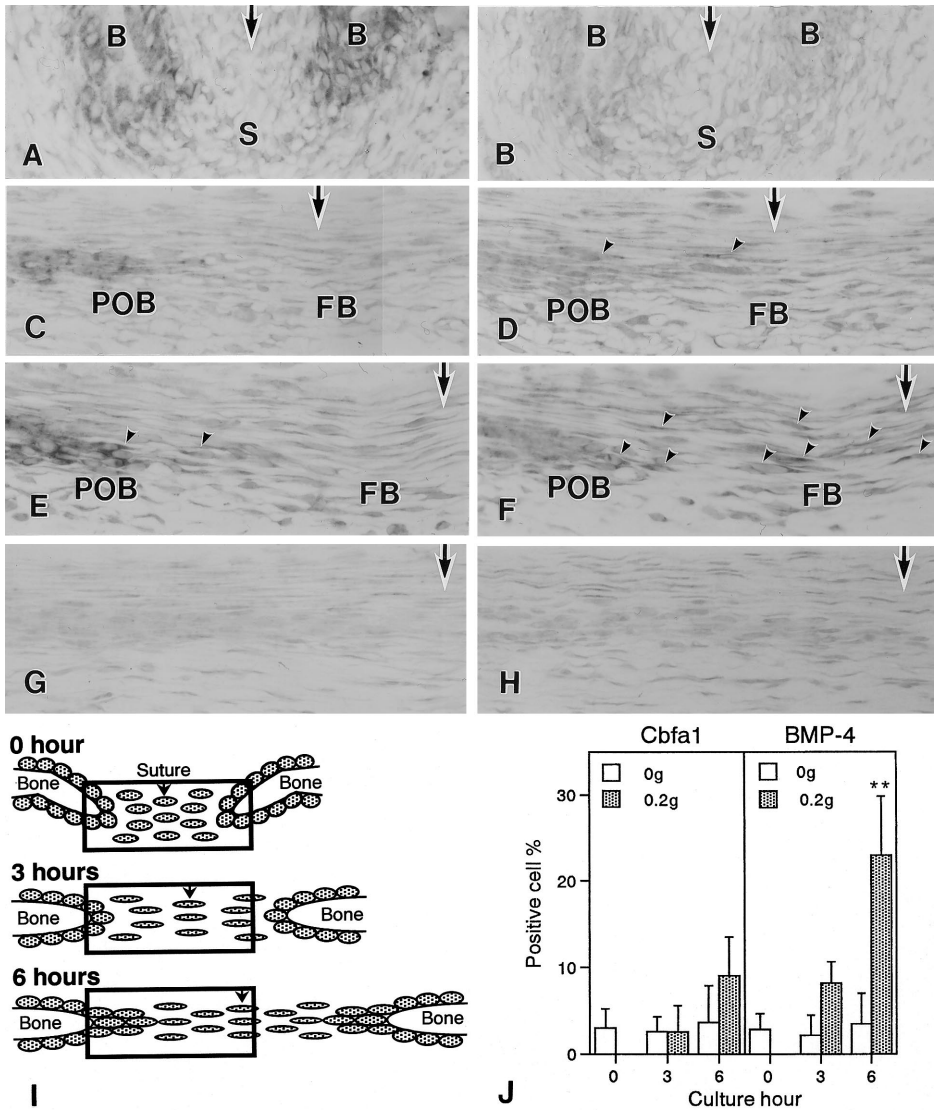


FIG. 6. Sequential up-regulation/induction of BMP-4 and Cbfa1/Osf-2 gene expression preceded osteoblast differentiation in calvariae under tensile stress. (A and B) Time 0; (C and D) 3 h; (E–H) 6 h. (A, C, and E) Antisense Cbfa1/Osf-2 probe; (G) sense Cbfa1/Osf-2 probe; (B, D, and F) antisense BMP-4 probe; (H) sense BMP-4 probe (B, parietal bone; S, suture; POB, preosteoblast; FB, fibroblast; arrows, center of the sutures). (I) Schematic overview showing where panels A–H were taken from. (J) Changes in cell numbers that are positive for BMP-4 or Cbfa1 gene expression. At time 0, BMP-4 expression was detected in osteoblasts surrounding the osteoid and in preosteoblasts and in some of fibroblastic cells (B). On the other hand, Cbfa1/Osf-2 expression was limited to osteoblasts and preosteoblasts (A). At 3 h, BMP-4 expression was induced in preosteoblastic cells and their adjacent spindle-shaped cells (D, arrowheads). Expression of Cbfa1/Osf-2 gene did not significantly change from that at time 0 (C). At 6 h although BMP-4 expression was diminished in some osteoblasts, it was elevated in preosteoblasts and spindle-shaped fibroblastic cells (F, arrowheads). Expression of Cbfa1/Osf-2 also was induced in preosteoblasts and spindle-shaped fibroblastic cells where BMP-4 expression became evident at 3 h (E, arrowheads). The signals of Cbfa1/Osf-2 and BMP-4 disappeared when corresponding sense probes were used (G and H; original magnification $\times 350$). The details as to where pictures of A–H were taken are schematically illustrated by frames in I. Each arrow in the frame indicates the center of the suture. Cell numbers positive for BMP-4 gene expression or for Cbfa1 gene expression were counted in four sets of plates including those shown in A–F and statistically analyzed in J. The counting was carried out for each sample in the area between the right end of the POB layer and the center of the suture. **Significantly different from control (0 g) at $p < 0.01$. POB, preosteoblast.

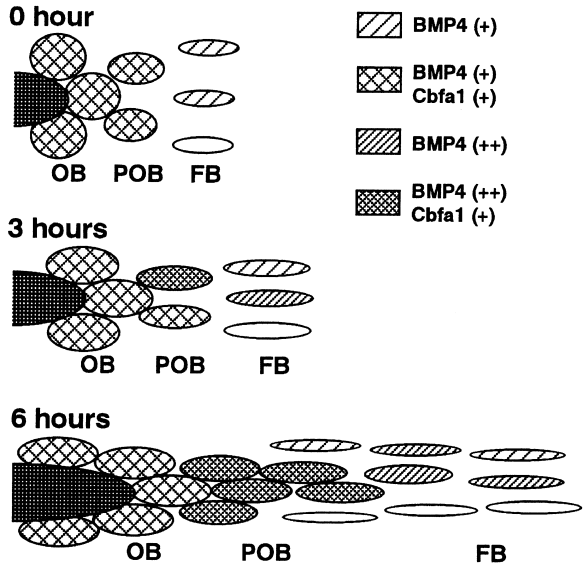


FIG. 7. Schematic illustration of events observed in Fig. 6. OB, osteoblast; POB, preosteoblast; FB, fibroblast.

gene. However, because the suture contained a heterogenous cell population, we next tried to identify cell types that express this gene using an in situ hybridization technique.

As shown in Fig. 6B, BMP-4 gene expression was detected in osteoblasts surrounding osteoid and in their adjacent preosteoblastic and spindle-shaped fibroblastic cells. The expression was increased by tensile stress in preosteoblastic and fibroblastic cells, whereas it remained unchanged or diminished in osteoblasts covering the existing bone tissue. Cell number with elevated BMP-4 expression in both cell types further increased as time of mechanical loading extended. Cbfa1/Osf-2 is an osteoblast-specific transcription factor and under the control of the BMP gene family. The expression of this gene followed the BMP expression under mechanical loading (Figs. 6 and 7). This sequence of events is consistent with the fact that osteoblast differentiation and osteogenesis were observed in Fig. 6.

Based on these observations, we propose that tensile stress increases BMP-4 expression in preosteoblasts and fibroblasts, and BMP-4, in turn, activates Cbfa1/Osf-2 expression, thereby inducing osteoblast differentiation. Thus, BMP-4 may act as an autocrine and paracrine factor in the osteogenesis induced by tensile stress. Fibroblastic cells that became BMP-4 positive do not necessarily differentiate into osteoblasts. Cells that can differentiate into osteoblasts appeared to be limited to the spindle-shaped cells that lined along the extension of existing bone tissue in the direction of the center of the suture, while BMP-4 positive fibroblastic cells at the periosteal surface did not appear to change their fate. Therefore, there must be determinants other than BMP-4 that control the fate of cells to differentiate into osteoblasts. Thus, the exact mechanism by which tensile stress induces BMP-4 expression and how BMP-4 stimulates osteogenesis are subjects of future study.

While we were preparing this article, a paper was published showing that BMP-2 and BMP-4 but not BMP-6, BMP-7, and growth and differentiation factor-5 (GDF-5) expression were induced during distraction osteogenesis in rats.[32] Our data are consistent with this report, although we could not detect up-regulation of BMP-2 expression by RT-PCR. The time frame of the BMP-4 expression in the present study and that in the distraction study cited previously are different; in our study BMP-4 gene expression began to rise at 6 h under tensile stress and remained elevated until 48 h, whereas in the distraction study the gene expression was slightly elevated at 4 days after osteotomy and it declined at 7 days. By this time the soft external callus and the periosteal as well as endosteal bony callus were formed. Distraction caused separation of those calluses, between which a fibrous interzone was formed. The cartilage callus at the mouth of osteotomy site was resorbed gradually and replaced by new bone through endochondral ossification. The BMP-4 expression was elevated at this stage (10 days after distraction started) and the expression remained elevated throughout the distraction phase. In contrast to the osteotomy followed by distraction, application of tensile stress in the present study does not involve any trauma and it does not cause any serious damage to sutures. In addition, osteogenesis in the calvaria is not endochondral but membranous ossification. Therefore, times required for the gene expression and osteogenesis in these two systems may not be the same. Nonetheless, BMP-4 expression appears to play an important role in both membranous and endochondral ossification induced by tensile stress. Further study is needed for a clear understanding of the exact role of BMP-4 in these systems.

In conclusion, we showed that tensile stress induces osteoblast differentiation and osteogenesis in mouse calvarial sutures in culture and also induces BMP-4 expression in preosteoblasts and fibroblasts, which appeared to differentiate eventually into osteoblasts. BMP-4 may act as an autocrine and paracrine factor in tensile stress–induced osteogenesis.

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