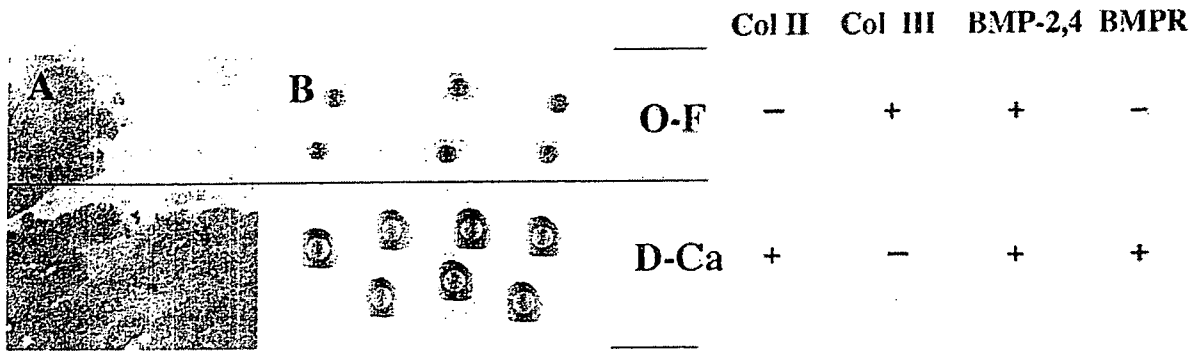

Fig. 12. **A** Histology and **B** illustrations, showing the localization of BMP-2 and -4 in the cartilage cap of osteochondroma. The cartilage cap consists of an outer fibrous (*O-F*) layer and a deep cartilage (D-Ca) layer. The OF layer is composed of fibroblastic cells positive for type III collagen mRNA (*Col III*), and the D-Ca layer is composed of chondrocytes positive for type II collagen mRNA (*Coll II*). BMP-2 and -4 mRNAs and protein are localized in cells in the O-F and D-Ca layers, whereas BMPR (*BMP receptor type IB*) mRNA is localized in cells in the D-Ca layer, but not in the O-F layer. **A** Safranin-O and fast green staining. ×40; *dark area* indicates cartilaginous matrix

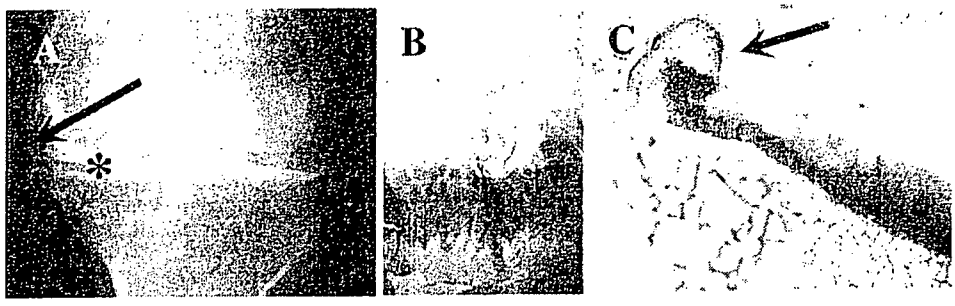

Fig. 13. **A** Radiograph and **B** and **C** histologic characteristics of osteoarthritis (OA). **A** X-ray showing osteophyte formation (*arrow*), as well as narrowing of the width of the articular cartilage *asterisk*. **B** and **C** Histology, showing degenerative changes of OA cartilage (**B**) and newly formed osteo- and chondrogenic tissue, called osteophyte formation (*arrow* in **C**). **B** and **C** Safranin-O and fast green staining (B: ×40, C: macroscopic magnification)

Table 1. Expression and localization of bone morphogenetic protein (BMP)-2 mRNA in osteoarthritis (OA) cartilage cells

Grade of OA cartilage	Zones		
	Upper	Middle	Deep
Moderately damaged	++ to +++	++ to +++	− to +
Severely damaged	No upper zone	++ to +++	++ to +++

cartilage. However, BMP-2 and BMP-4 mRNA and protein were localized in chondrocytes in damaged OA cartilage (Table 1). In moderately damaged OA cartilage, BMP-2 and -4 were localized in OA cells in the upper and middle zones. However, in severely damaged OA cartilage, they were detected in OA cells even in the deep zone. These findings suggest that the zone-specific distribution of BMP-2 and -4 may be dependent on the degree of OA damage, and that BMP-2 and -4 may be induced in response to the damage to articular cartilage (Fig. 14) [49]. Current findings indicate the induction of the *BMP* gene in cells in damaged OA cartilage. Our unpublished observations, by RT-PCR and western blotting using specific probes, have confirmed the expression and the presence of BMP-2 in OA cartilage.

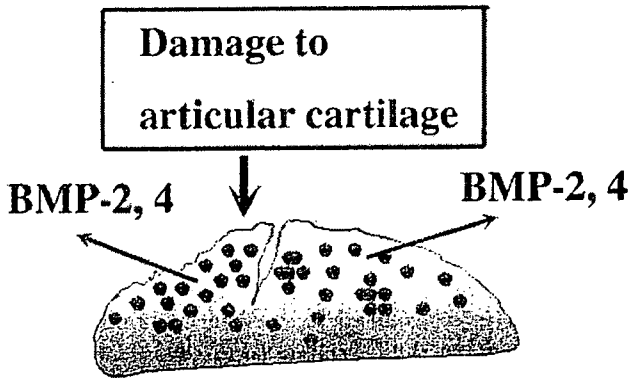

Fig. 14. Proposed mechanisms of BMP induction in damaged OA cartilage. BMP-2 and -4 are induced in OA cells in response to the damage to OA cartilage

Such induction of the *BMP* gene in response to stimuli to cartilage is similar to that observed in fracture healing. Unlike the case in bone repair, spontaneous repair of damaged articular cartilage is rarely observed. The activation of BMP in damaged OA cells does not lead to cartilage repair, despite BMP's chondrogenic activity. Further investigations may aid in our understanding of the downstream mechanisms involved in BMP signaling.

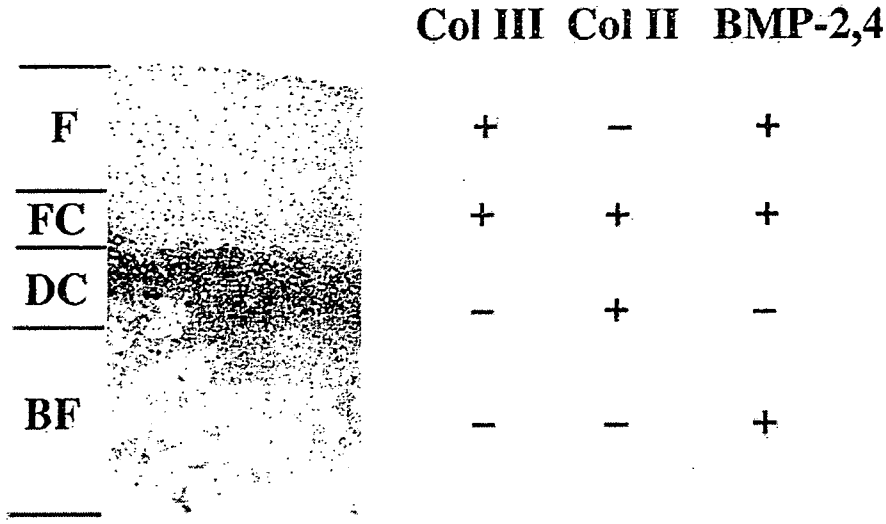

Fig. 15. Localization of BMP-2 and -4 in osteophyte cells. The osteophyte is composed of four distinct layers, i.e., the fibrous (*F*) layer, fibrocartilage (*FC*) layer, deep cartilage (*DC*) layer, and the bone-forming (*BF*) layer. The F layer is composed of fibroblastic cells positive for type III collagen (*Col III*), the FC layer is composed of fibrochondrocytes positive for type II (*Col II*) and type III collagen (*Col III*), the DC layer is composed of chondrocytes positive for type II collagen (*Col II*), and the BF layer is composed of osteoblasts. BMP-2 and -4 are localized in cells in the F, FC, and BF layers, but not in cells in the DC layer

Histologically, findings in the osteophyte are characterized by new bone/cartilage formation resembling endochondral ossification. BMP-2 and -4 mRNAs and proteins are detected in fibroblastic cells, fibrochondrocytes, immature chondrocytes and osteoblasts within the osteophyte tissue in human specimens. BMP-2 and -4 were not detected in mature chondrocytes [49]. In osteophytes, the fibroblasts and fibrochondrocytes were positive for collagen III mRNA; the fibrochondrocytes were also positive for collagen II mRNA, as were immature chondrocytes (Fig. 15). These findings suggest possible roles of BMP-2 and -4 in the early chondrogenic reactions of osteophyte formation.

Conclusion

Several members of the BMP family, such as BMP-2, -4, -7 (OP-1), and GDF-5 (CDMP-1), as well as their signaling molecules, have been identified during the process of skeletal repair and regeneration. These BMPs are induced by stimuli such as the impact of fracture and mechanical stress just at the regions undergoing the formation of skeletal tissues. The BMPs have also been identified in several pathological skeletal disorders showing the formation of bone and cartilage. In particular, BMP-2 and -4 may act as mediators of early skeletal repair and regenerative reactions, and CDMP-1 may play rather distinct roles, such as the promotion of chondrogenesis and tendon repair.

Such information regarding the involvement of BMPs in skeletal repair and regeneration, together with the actions of BMPs shown by in vivo and in vitro studies, holds the key to the formulation of new strategies for the development of BMP-based orthopedic treatment systems. The possible roles of BMPs in both physiological and pathological conditions provide evidence for the possible application of BMP technology in orthopedic medicine, and indicate a pathway for new advanced approaches in the treatment of orthopedic disorders.

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Bone Morphogenetic Proteins in Bone Stimulate Osteoclasts and Osteoblasts During Bone Development

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ABSTRACT: In this study, overexpression of noggin, a BMP antagonist, in developing bone caused significantly decreased osteoclast number as well as bone formation rate, resulting in increased bone mass with immature bone quality. BMP signaling plays important roles in normal bone development and regulation of bone resorption.

Introduction: Bone morphogenetic proteins (BMPs) act on various types of cells. Although involvement of BMP signals in osteoblast differentiation has been studied extensively, the effects of BMPs on osteoclasts have not been widely researched. Consequently, the net effects of BMPs on bone remain unclear. The purpose of this study was to delineate more fully the role of BMPs in skeletal biology.
Materials and Methods: We generated transgenic mice that express BMP4 or noggin in bone under the control of the 2.3-kb α1(I) collagen chain gene (*Col1a1*) promoter, and analyzed their bone phenotype. We also analyzed bone of transgenic mice expressing BMP4 specifically in cartilage.
Results: Mice overexpressing BMP4 in bone developed severe osteopenia with increased osteoclast number. Mice overexpressing noggin, a BMP antagonist, in bone showed increased bone volume associated with decreased bone formation rate and decreased osteoclast number. The noggin-transgenic tibias exhibited reduced periosteal bone formation and reduced resorption of immature bone in marrow spaces, associated with frequent fractures at the diaphysis. Co-culture of primary osteoblasts prepared from noggin-transgenic calvariae and wildtype spleen cells resulted in poor osteoclast formation, which was rescued by addition of recombinant BMP2, suggesting that noggin inhibits osteoclast formation by attenuating BMP activities in noggin-transgenic mice. The expression levels of *Rankl* were not decreased in primary osteoblasts from noggin transgenic mice. Immunoblot analysis showed increased phosphorylation of Smad1/5/8 in osteoclast precursor cells after 20-minute treatment with BMPs, suggesting that these cells are stimulated by BMPs. Mice overexpressing BMP4 in cartilage had enlarged bones containing thick trabeculae, possibly because of expansion of cartilage anlagen.
Conclusions: Overexpression of noggin in bone revealed that BMP signals regulate bone development through stimulation of osteoblasts and osteoclasts.
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Key words: bone morphogenetic proteins, noggin, osteoclasts, endochondral bone formation, transgenic mice

INTRODUCTION

BONE MORPHOGENETIC PROTEINS (BMPs) were originally identified as secreted substances capable of inducing ectopic formation of cartilage and bone when implanted subcutaneously or in muscle pouches.[1] Subsequent molecular cloning studies have revealed that BMPs comprise a large subfamily of the TGF-β superfamily.[2] BMPs bind to BMP receptor types I and II, and their signal is mediated by phosphorylation of receptor-regulated Smads (R-Smads) such as Smads 1, 5, and 8.[3] Phosphorylated R-Smads form heteromers with Smad4, which is a common-partner Smad (Co-Smad), and the heteromers translocate into the nucleus. BMP signaling is delicately regulated at multiple levels: extracellularly, at the membrane site, and intracellularly.[4] In the extracellular space, several molecules antagonize BMPs. One of those antagonists is noggin, which binds to BMPs 2, 4, and 7 and prevents them from interacting with their receptors.[4,5] Recent studies of co-crystal structure clearly show that noggin very specifically inhibits BMPs.[6] Noggin has been used to block BMP action and study its role in certain tissues.

Limb bones are formed through a process called endochondral bone formation. During this process, mesenchymal cells first differentiate into chondrocytes, which form the cartilage anlagen of the bones. Then, the central region of each cartilage anlage is invaded by blood vessels, osteoblasts, osteoclasts, and hematopoietic cells, resulting in for-

The authors state that they have no conflicts of interest.

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mation of primary ossification centers. Bone gradually replaces the cartilage. Osteoblasts form woven bone on the remnant of the calcified cartilage matrix, modeling the trabeculae of the primary spongiosa. Trabecular bone then undergoes remodeling consisting of resorption by osteoclasts and apposition of newly formed bone matrix by osteoblasts, creating secondary spongiosa.[7] Bone remodeling continues in postnatal life to maintain bone mass. Osteoblasts develop from mesenchymal cells, whereas osteoclasts develop from hematopoietic cells of the monocyte/macrophage lineage. Osteoclastic bone resorption and osteoblastic bone formation are coupled on the surface of trabeculae of secondary spongiosa. Osteoblasts and stromal cells regulate osteoclast differentiation by producing RANKL (which supports osteoclast differentiation) and osteoprotegerin (OPG; which inhibits RANKL function by competing with RANK for RANKL).[8]

Conventional gene knockout experiments have shown that BMPs have diverse biological activities during early embryogenesis and various aspects of organogenesis, mediated by their ability to regulate proliferation, differentiation, and apoptosis of various types of cells.[9] Recent osteoblast-specific downregulations of BMP signals in mice have clarified the role of BMPs in osteoblast differentiation. In 4-week-old to 6-month-old mice, osteoblast-specific expression of dominant-negative BMP receptor type IB[10] and osteoblast-specific gene ablation of BMP receptor type IA[11] cause inhibition of osteoblast differentiation and a decrease in bone volume but do not change the osteoclast number, indicating that BMP signals are important for maintenance of bone mass by osteoblasts in postnatal life. In 10-month-old mice, osteoblast-specific gene ablation of BMP receptor type IA causes a decrease in osteoclastic bone resorption activity. Such findings have led to speculation that loss of BMP signaling in osteoblasts leads to impairment of osteoclast-supporting activities, causing downregulation of osteoclast function as the mice age.[11] In mice older than 4 weeks, overexpression of noggin in mature osteoblasts under the control of the osteocalcin promoter sequence causes osteopenia (bone loss) and reduction of the bone formation rate, but does not change the osteoclast number.[12] Thus, BMP signals are important for osteoblast differentiation and function.

On the other hand, several reports indicate that osteoclasts express BMP receptors and that BMPs directly stimulate osteoclast differentiation in vitro.[13–15] Osteoclast differentiation supported by macrophage colony-stimulating factor (M-CSF) and RANKL is enhanced in the presence of BMPs. However, there have been no reported studies of the effects of BMPs on osteoclasts during bone formation in vivo. The process of endochondral bone formation during skeletogenesis is recapitulated in fracture repair.[16,17] BMPs have been clinically used to promote healing of fractures.[18–20] Further clarification of effects of BMPs in bone is needed to improve our understanding of skeletal biology and improve the efficacy of BMPs in bone repair.

The aim of this study was to examine more fully roles of BMPs in skeletal biology. We generated transgenic mice overexpressing BMP4 or noggin under the control of the bone-specific 2.3-kb α1(I) collagen chain gene (*Col1a1*) promoter sequence. Transcriptional activity of the *Col1a1* promoter in osteoblasts is much stronger than that of the osteocalcin promoter.[21] Also, we examined bones of mice with cartilage-specific overexpression of BMP4 to assess the stage-specific effects of BMP4 on endochondral bone formation.

MATERIALS AND METHODS

Generation of transgenic mice

The *Col1a1-LacZ*, *Col1a1-noggin*, and *Col1a1-Bmp4* transgene constructs were created by replacing the *Col11a2* promoter sequence of the *Col11a2-LacZ*, *Col11a2-noggin*, and *Col11a2-Bmp4* transgene constructs,[22] respectively, with the 2.3-kb *Col1a1* promoter sequence.[23] The plasmids were digested to release the inserts from the vector backbone and the *Col11a2* enhancer sequences. The *Col11a2-Bmp4* insert was prepared as described previously.[22] Transgenic mice were produced by microinjecting each of the inserts into the pronuclei of fertilized eggs from F1 hybrid mice (C57BL/6 × DBA) as described previously.[24] Wildtype littermates were used as controls. All present animal experiments were approved by the institutional review board of Osaka University Graduate School of Medicine.

Real-time RT-PCR analysis

Total RNA was extracted from various tissues of 3-week-old mice and primary osteoblasts using Isogen (Wako Pure Chemical Co., Osaka, Japan). The RNA was digested with DNase to eliminate any contaminating genomic DNA before real-time quantitative RT-PCR. RNA samples were further purified using RNeasy Mini Kits (Qiagen, Santa Clarita, CA, USA). Real-time RT-PCR was performed as described previously.[25] The primer pair for noggin was as follows: up, 5′-CGGCCAGCACTATCTACACA-3′; down, 5′-GCGTCTCGTTCAGATCCTTC-3′. The product size for noggin was 116 bp. Primer pairs for *Rankl* and *Opg* were prepared using previously reported methods.[26] The quantified individual RNA expression levels were normalized to the respective tubulin expression levels.

μCT analysis

The tibias were dissected and scanned using a microfocus X-ray CT system (SMX-100CT-SV; Shimadzu, Kyoto, Japan). The proximal metaphyseal region and the diaphyseal region where the fibula attaches to the tibia were scanned at the following resolutions: 2.4 μm for 17.5 days postcoitum (d.p.c.) tibia; 3.3 μm for 2-week-old tibia; 10.7 μm for 3-week-old tibia; 10.6 μm for 8-week-old tibia. The data were reconstructed to produce images of the tibia, using 3D visualization and measurement software (Vay Tek).

Histological analysis and histomorphometry

Mice were dissected using a stereomicroscope, and tissue samples were fixed in 4% paraformaldehyde and dehydrated. Samples from mice older than 0 days were decalcified. Samples were processed, embedded in paraffin, and sectioned. Each serial section was stained using one of the

following procedures: H&E staining; the von Kossa reaction; or TRACP staining, using a TRACP staining kit (Hokudo). Immunohistochemistry and in situ hybridization were performed as previously described.[25] Dynamic histomorphometric indices were determined by double fluorescence labeling in tibias and vertebral bodies. Three-week-old wildtype and transgenic mice were administered tetracycline (20 mg/kg body weight, IP; Sigma-Aldrich), followed 2 days later by administration of a calcein label (10 mg/kg body weight; Wako). At 48 h after calcein administration, the mice were killed. Bones were fixed with ethanol, embedded in methylmethacrylate, and sectioned. Sections were examined using a fluorescence microscope. The histomorphometric analyses were performed by staff at the Niigata Bone Science Institute (Niigata, Japan).

Osteoblast and osteoclast culture

Primary osteoblasts were isolated from calvariae of neonatal mice, using previously described methods.[27] Co-culture experiments were performed using previously described methods.[28] Briefly, primary osteoblasts (1×10^4 cells/cm^2) prepared from wildtype or *Col1a1-noggin* transgenic mice were co-cultured with spleen cells (5×10^5 cells/cm^2) prepared from wildtype mice in α-MEM containing 10% FCS and 10^{-8} M 1α,25$(OH)_2D_3$-dihydroxyvitamin D_3 in 48-well plates. Co-cultures containing transgenic osteoblasts were also performed in the presence of recombinant human BMP2 proteins (rhBMP2; AstellasPharma) at various concentrations. Osteoclast differentiation was evaluated by TRACP staining. Multinucleated TRACP$^+$ cells with more than three nuclei were counted under a microscope. To test resorption activity, co-cultures were performed on 16-well hydroxyapatite-coated slides (Osteologic; Becton Dickinson) for 14 days, and the resorption area was calculated by computer-assisted image analysis. Primary osteoblasts were cultured for 3 days in α-MEM containing 10% FCS and 10^{-8} M 1α,25$(OH)_2D_3$ in the absence or presence of various concentration of rhBMP2, followed by analysis of *Rankl/Opg* expression of the osteoblasts by real-time RT-PCR.

Smad pathways in macrophages

Nearly pure macrophages were prepared from mouse bone marrow cultures treated with M-CSF, using previously described methods.[14] Macrophages were incubated for 20 minutes in the presence or absence of rhBMP2 at various concentrations. The macrophages were lysed and subjected to Western blotting using a rabbit polyclonal antibody against phospho-Smad1/5/8 (Cell Signaling Technology) and an anti-Smad 1 antibody (Calbiochem).

Statistical analysis

Results are expressed as mean ± SD. The unpaired *t*-test was used to compare data between wildtype and transgenic mice. A p value of <0.05 was considered to indicate significance.

RESULTS

Overexpression of BMP4 in bone caused severe bone loss associated with increased osteoclast number during endochondral bone development

When ligated to the *LacZ* reporter gene, the 2.3-kb *Col1a1* promoter sequence directed expression specifically to bone at birth (Figs. 1A and 1B), as previously reported.[23] The pattern of *LacZ* expression indicated by X-gal staining was specific to ossification centers of skeletal components in limbs, rib bones, and calvariae. *LacZ* was not expressed in cartilage or other tissues. We prepared the *Col1a1-Bmp4* transgene by ligating the promoter sequence to the *Bmp4* cDNA (Fig. 1C) and injected the construct to pronuclei of ova. The *Col1a1-Bmp4* transgenic mice died shortly after birth, probably because of impaired locomotion caused by fragility of bones. We obtained five transgenic founder embryos that exhibited similar obvious abnormalities. X-ray photographs taken 18.5 d.p.c. showed irregularly shaped bones in *Col1a1-Bmp4* transgenic embryos (Fig. 1D). Bones, including the humeri and femora, of transgenic mice were more radiolucent than the wildtype. Alcian blue and alizarin red staining showed deformity of bones including humeri (Figs. 1E and 1F). μCT analysis revealed osteopenia (loss of bone) in the ossification center of humeri of *Col1a1-Bmp4* transgenic mice (Fig. 1H) compared with the wildtype (Fig. 1G). Trabecular bone was almost completely absent from the marrow cavities of transgenic mice (Fig. 1H).

Histological analysis (von Kossa staining of semiserial sections of H&E-stained sections [Figs. 2A and 2B]) of proximal humeri at 18.5 d.p.c. showed reduced calcification in ossification centers of *Col1a1-Bmp4* transgenic mice (Fig. 2D) compared with the wildtype (Fig. 2C). Magnified images of bone marrow cavities showed absence of bony matrix in *Col1a1-Bmp4* transgenic mice (Fig. 2F), whereas solid bone matrix was clearly present in the wildtype (Fig. 2E). When the semiserial sections were immunostained using anti-phospho-Smad1/5/8, cells in bone marrow cavities of *Col1a1-Bmp4* transgenic mice (Fig. 2H) exhibited greater immunoreactivity than their wildtype counterparts (Fig. 2G), suggesting that BMP signals were overactivated in bone marrow of transgenic mice. There were many TRACP$^+$ osteoclasts in bone marrow cavities of *Col1a1-Bmp4* transgenic mice (Fig. 2J) compared with the wildtype (Fig. 2I). Because the *Col1a1-Bmp4* transgenic mice had markedly lower bone surface area than the wildtype, the number of TRACP$^+$ cells per bone surface area was markedly greater in the transgenic mice. These results suggest that BMP4 overexpression in bone of *Col1a1-Bmp4* transgenic mice caused osteopenia associated with enhanced Smad phosphorylation in various cells and increased number of TRACP$^+$ cells in bone marrow cavities.

Col1a1-noggin transgenic mice exhibited increased bone volume associated with decreased osteoclast number from embryonic stage

Figure 3A shows the procedure in which the *Col1a1-noggin* transgene was constructed by ligating the 2.3-kb

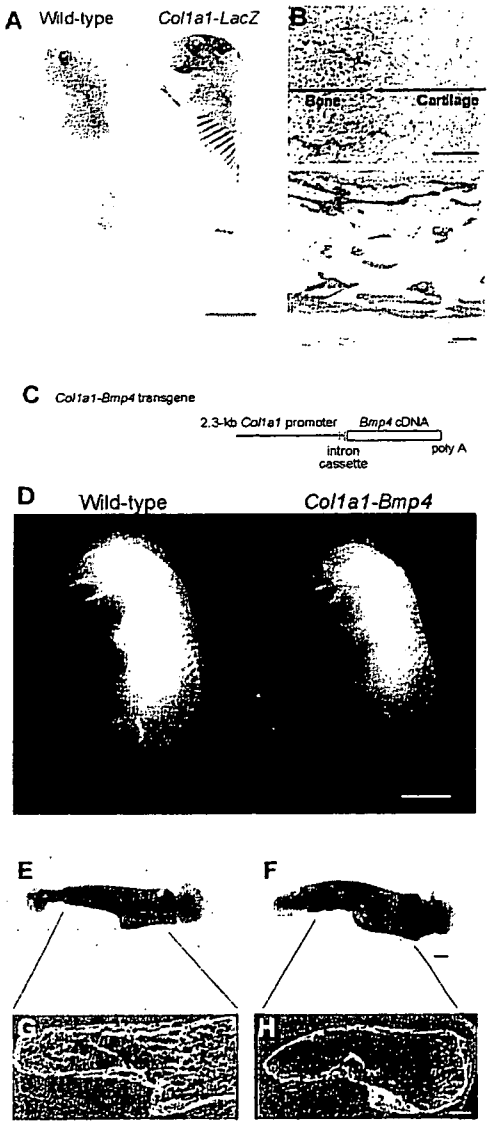

FIG. 1. Osteopenia in *Col1a1-Bmp4* transgenic mice. (A) X-gal staining of wildtype and *Col1a1-LacZ* transgenic mice at birth. The 2.3-kb *Col1a1* promoter sequence directed *LacZ* reporter gene expression specifically in bone. Scale bar, 2 mm. (B) Sagittal sections of *Col1a1-LacZ* transgenic tibias stained with X-gal. Note the X-gal staining in osteoblasts on the trabecular bone in distal metaphysis (top) and osteoblasts around cortical bone in diaphysis (bottom). No X-gal staining in chondrocytes (top). Counterstained with eosin. Scale bars, 100 μm. (C) Schematic representation of *Col1a1-Bmp4* transgene construct. (D) X-ray photograph of wildtype embryo (left) and *Col1a1-Bmp4* transgenic embryo (right) at 18.5 days postcoitum (d.p.c.). Bones of *Col1a1-Bmp4* transgenic embryo were irregular and radiolucent. Scale bars, 2 mm. (E and F) Alcian blue and alizarin red staining of humerus from (E) wildtype embryo and (F) *Col1a1-Bmp4* transgenic embryo at 18.5 d.p.c. Scale bar, 200 μm. (G and H) μCT images of ossification center of humerus of (G) wildtype and (H) *Col1a1-Bmp4* transgenic mice at 18.5 d.p.c. Reconstructed sagittal views. Scale bar, 200 μm.

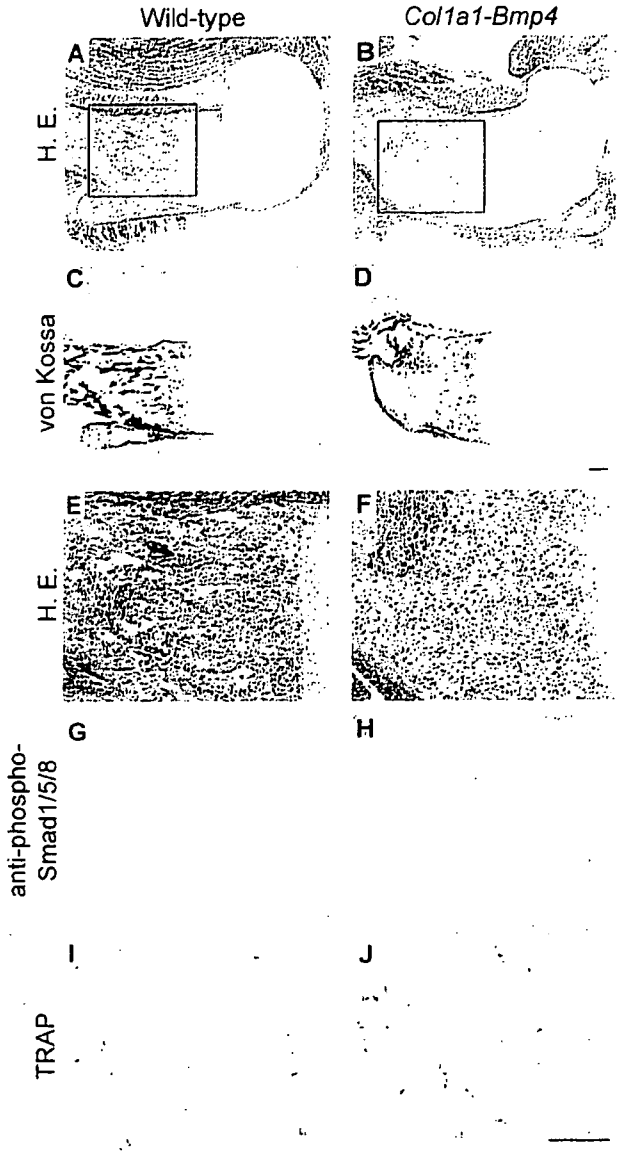

FIG. 2. Histological analysis of proximal humerus of *Col1a1-Bmp4* transgenic mice at 18.5 d.p.c. (A, C, E, G, and I) Wildtype mice and (B, D, F, H, and J) *Col1a1-Bmp4* transgenic mice. (A and B) H&E staining. Semiserial sagittal sections of A and B, stained using von Kossa method, are shown in C and D, respectively. Magnification of boxed regions in A and B are shown in E and F, respectively. Semiserial sagittal sections of E and F, immunostained using anti-phospho-Smad1/5/8 antibody, are shown in G and H, respectively. This antibody recognizes only phosphorylated forms of Smad1/5/8. Semiserial sections of E and F, stained for TRACP, are shown in I and J, respectively. Scale bar, 200 μm.

Col1a1 promoter to noggin cDNA. *Col1a1-noggin* transgenic mice were viable and fertile, and 2 *Col1a1-noggin* transgenic lines were established. The phenotypes of the two transgenic lines were similar, and one line was subjected to close examination. Until 1 week after birth, *Col1a1-noggin* transgenic mice were not visibly distinguishable from the wildtype. Staining of skeletal components of transgenic embryos with Alcian blue at various stages showed that the shapes and sizes of primordial cartilage were normal (data not shown). One week after birth, transgenic mice began to develop dwarfism, which was clearly evident in 3-week-old mice (Fig. 3B). X-ray photographs showed that *Col1a1-noggin* transgenic mice had thicker tra-

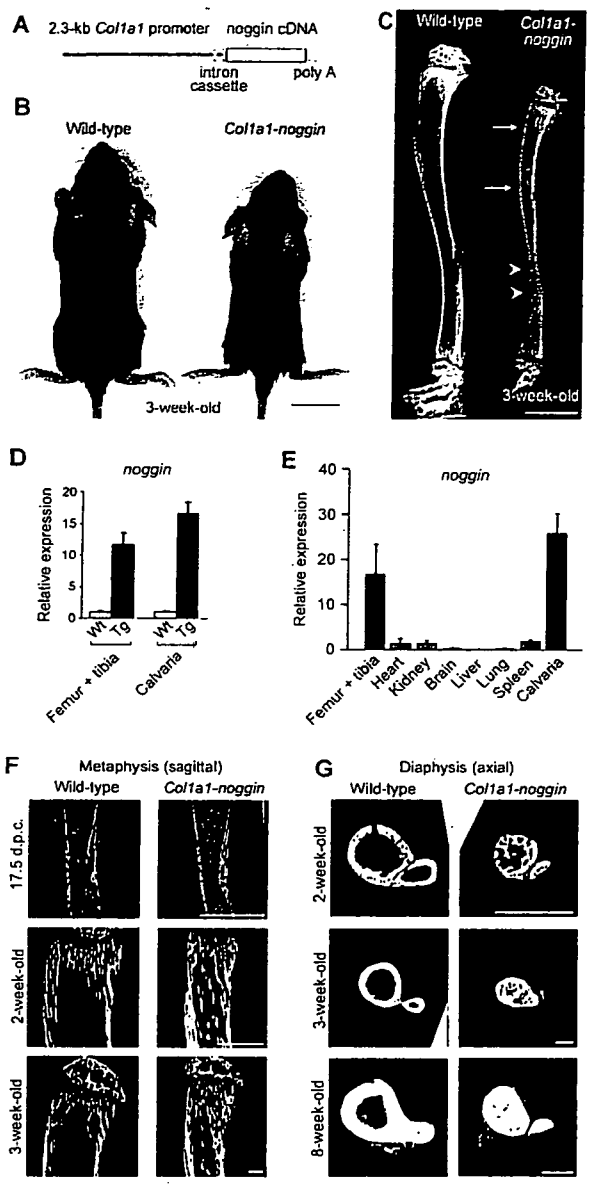

FIG. 3. Phenotype and noggin expression in *Col1a1-noggin* transgenic mice. (A) Schematic representation of the *Col1a1-noggin* transgene construct. (B) Three-week-old wildtype and *Col1a1-noggin* transgenic mice. Transgenic mice developed postnatal dwarfism. Scale bar, 1 cm. (C) X-ray image of the tibia at 3 weeks of age. Note thick trabecular (arrows) and cortical (arrowheads) bone in *Col1a1-noggin* transgenic mice, compared with wildtype mice. Scale bar, 2 mm. (D) Relative expression levels of noggin mRNA in total femur + tibia and in calvaria at 3 weeks of age, measured by real-time RT-PCR. Real-time RT-PCR was performed three times. Data are presented as mean ± SD. Wt, wildtype mice; Tg, *Col1a1-noggin* transgenic mice. The wildtype expression level was designated as 1. (E) Relative expression levels of noggin mRNA in various tissues from 3-week-old *Col1a1-noggin* transgenic mice, measured by real-time RT-PCR. RT-PCR was performed three times. Data are presented as mean ± SD. (F) μCT images of proximal metaphysis of tibia of wildtype and *Col1a1-noggin* transgenic mice at 17.5 d.p.c., 2 weeks of age, and 3 weeks of age. Sagittal views were reconstructed. Scale bar, 500 μm. (G) Axial views of μCT images of distal one third of diaphysis of tibia associated with fibula of wildtype and *Col1a1-noggin* transgenic mice at 2, 3, and 8 weeks of age. Scale bar, 500 μm.

becular bone and cortex than the wildtype, especially in tibias (Fig. 3C). Real-time RT-PCR using total RNA extracted from femur + tibia or calvaria showed that *Col1a1-noggin* transgenic mice had much higher expression levels of noggin mRNA than the wildtype (Fig. 3D). It seems that the *Col1a1-noggin* transgene was expressed at much higher levels in bone (femur + tibia or calvaria) than in other tissues (Fig. 3E). μCT analysis revealed that from the embryonic stage (17.5 d.p.c.) to 3 weeks after birth, the tibias of *Col1a1-noggin* transgenic mice (Fig. 3F) had a greater volume of trabecular bone than the wildtype. In the diaphyseal region of transgenic tibias, marrow cavities progressively filled with cortical bone from 2 to 8 weeks of age (Fig. 3G).

In histological analysis of primary ossification centers of tibias, there was no marked difference in the number of osteoblasts expressing *Col1a1* mRNA (a marker of osteoblasts) between *Col1a1-noggin* transgenic mice and wildtype mice, whereas there were fewer $TRACP^+$ osteoclasts in transgenic mice than in wildtype mice at 17.5 d.p.c. (Fig. 4A). In the primary ossification centers of tibias at birth and the proximal metaphyseal region of tibias at 3 weeks after birth, the number of $TRACP^+$ cells was lower in transgenic mice than in wildtype mice (Figs. 4B and 4C). At the diaphysis of growing long bones, an increase in diameter is the result of deposition of new bone at the outer (periosteal) surface and is accompanied by enlargement of the marrow cavity caused by resorption exceeding formation at the inner (endosteal) surface. In wildtype mice, $TRACP^+$ osteoclasts were located at the inner surface of the cortex, whereas no $TRACP^+$ cells were observed in transgenic mice (Fig. 4D). These results suggest that overexpression of noggin under the control of *Col1a1* promoter in developing bone causes thickening of trabecular bone and elimination of marrow cavities in cortical bone, associated with a reduced number of osteoclasts.

Col1a1-noggin transgenic mice exhibited increased bone volume, reduced bone formation rate, and reduced osteoclastic bone resorption

Figure 5 shows the results of bone histomorphometric assays. At the metaphyseal region of the proximal tibia at 3 weeks of age, trabecular bone volume was significantly greater in *Col1a1-noggin* transgenic mice than in wildtype mice (Fig. 5A). The osteoblast number (osteoblast surface area per bone surface area) was greater in transgenic mice than in wildtype mice (Fig. 5B). Significantly increased trabecular bone volume/tissue volume was also noted in lumbar vertebral bodies of *Col1a1-noggin* transgenic mice at 3 weeks of age (10.2 ± 2.9% in wildtype and 13.6 ± 4.7% in *Col1a1-noggin* transgenic mice, $n = 6$, $p = 0.01$). We analyzed dynamic changes in bone remodeling by injecting tetracycline and calcein at 2-day intervals. Vertebral bodies were subjected to dynamic analysis because of their sufficient areas of spongiosa for analysis at 3 weeks of age. The distance between the two consecutive labels in lumbar vertebral bodies was significantly less in transgenic mice (Fig. 5D), as indicated by a decreased mineral apposition rate (Fig. 5C), compared with wildtype mice. There was no sig-

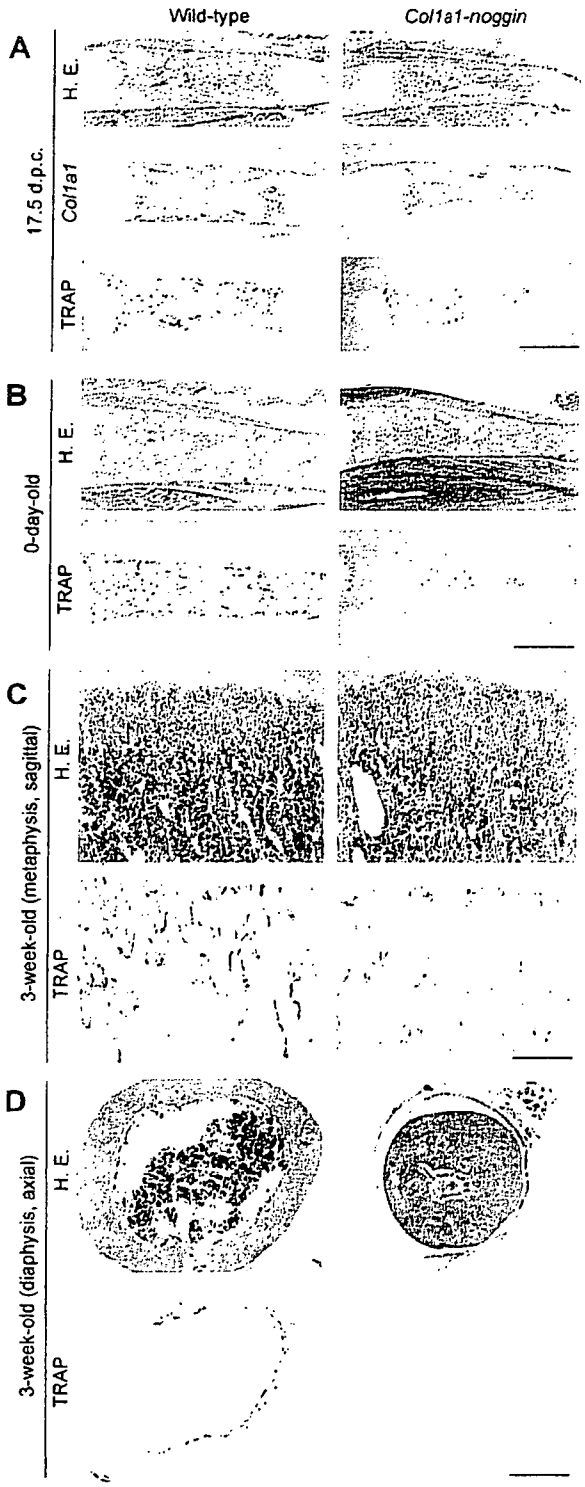

FIG. 4. Histological analysis of tibia of wildtype and *Col1a1-noggin* transgenic mice. (A) Semiserial sagittal sections prepared from 17.5 d.p.c. mice were stained with H&E, hybridized with *Col1a1* antisense cRNA probe (*Col1a1*), and stained for TRACP. Scale bar, 500 μm. (B) Sagittal sections from mice at birth. Semiserial sections were stained with H&E and for TRACP. Scale bar, 500 μm. (C) Sagittal sections of proximal metaphysis of tibias from 3-week-old mice. Semiserial sections were stained with H&E and for TRACP. Scale bar, 200 μm. (D) Axial sections of diaphysis of tibias from 3-week-old mice. Semiserial sections were stained with H&E and for TRACP. Scale bar, 200 μm.

nificant difference in the mineralization surface area per bone surface area (Fig. 5E). The data indicate that the bone formation rate was significantly decreased in the transgenic mice (Fig. 5F). These results suggest that overexpression of noggin in bone disturbs the function of osteoblasts.

The transgenic mice had a significantly lower osteoclast number per bone surface area and a significantly lower erosive surface area per bone surface area (Figs. 5G and 5H), suggesting that overexpression of noggin in bone inhibited osteoclastic bone resorption. The increased bone volume in *Col1a1-noggin* transgenic mice indicates that the decrease in bone resorption was greater than the decrease in bone formation.

Cortex of Col1a1-noggin transgenic mice was thick, but was woven and frequently suffered fractures

In the morphometric assays using the distal one third of the diaphysis of tibias of 3-week-old mice, total tissue volume was lower in *Col1a1-noggin* transgenic mice than in wildtype mice (Fig. 5I), but the cortical area in this region was significantly greater in transgenic mice (Figs. 5J and 5L). The marrow area was markedly lower in transgenic mice (Figs. 5K and 5L). Microscopic examination using polarized light revealed that the cortical bone of transgenic mice consisted mainly of immature bone (also known as woven bone, in which collagen fibers run in all directions), rather than the mature lamellar bone (which contains highly ordered parallel collagen fibers) found in the wildtype mice (Fig. 5L). Dynamic histomorphometric assays using consecutive labeling with tetracycline and calcein showed that bone formation rates were significantly decreased at the periosteal (Fig. 5M) and endosteal (Fig. 5N) surfaces of the diaphysis of tibias in 3-week-old *Col1a1-noggin* transgenic mice compared with wildtype mice. Together with the reduced osteoclast number at the endosteal surface of the noggin-transgenic tibial cortex (Fig. 4D), these results suggest that the presence of woven bone in the diaphyseal marrow spaces of noggin-transgenic mice was caused by failure to resorb initially formed immature bone and that the reduced cortical expansion in noggin-transgenic mice was caused by decreased periosteal modeling. Transgenic mice frequently suffered fractures at this region of the tibial shaft in the later stages of life, suggesting mechanical weakness of the bone (Fig. 5O). X-ray imaging of 8-week-old mice revealed that 7 of the 12 tibias of transgenic mice were broken, whereas none of the 12 tibias of wildtype mice were broken. This bone fragility in noggin-transgenic mice may be caused by a combination of impaired architecture and retention of immature woven bone.

Impaired osteoclast formation caused by noggin overexpression was rescued by BMP2 administration in vitro

To study the effects of noggin expressed by transgenic osteoblasts on osteoclast formation, we performed co-culture experiments using primary osteoblasts prepared from calvariae of wildtype or *Col1a1-noggin* transgenic neonates and spleen cells from wildtype mice. The number of $TRACP^+$ osteoclasts (Figs. 6A and 6B) and the resorp-

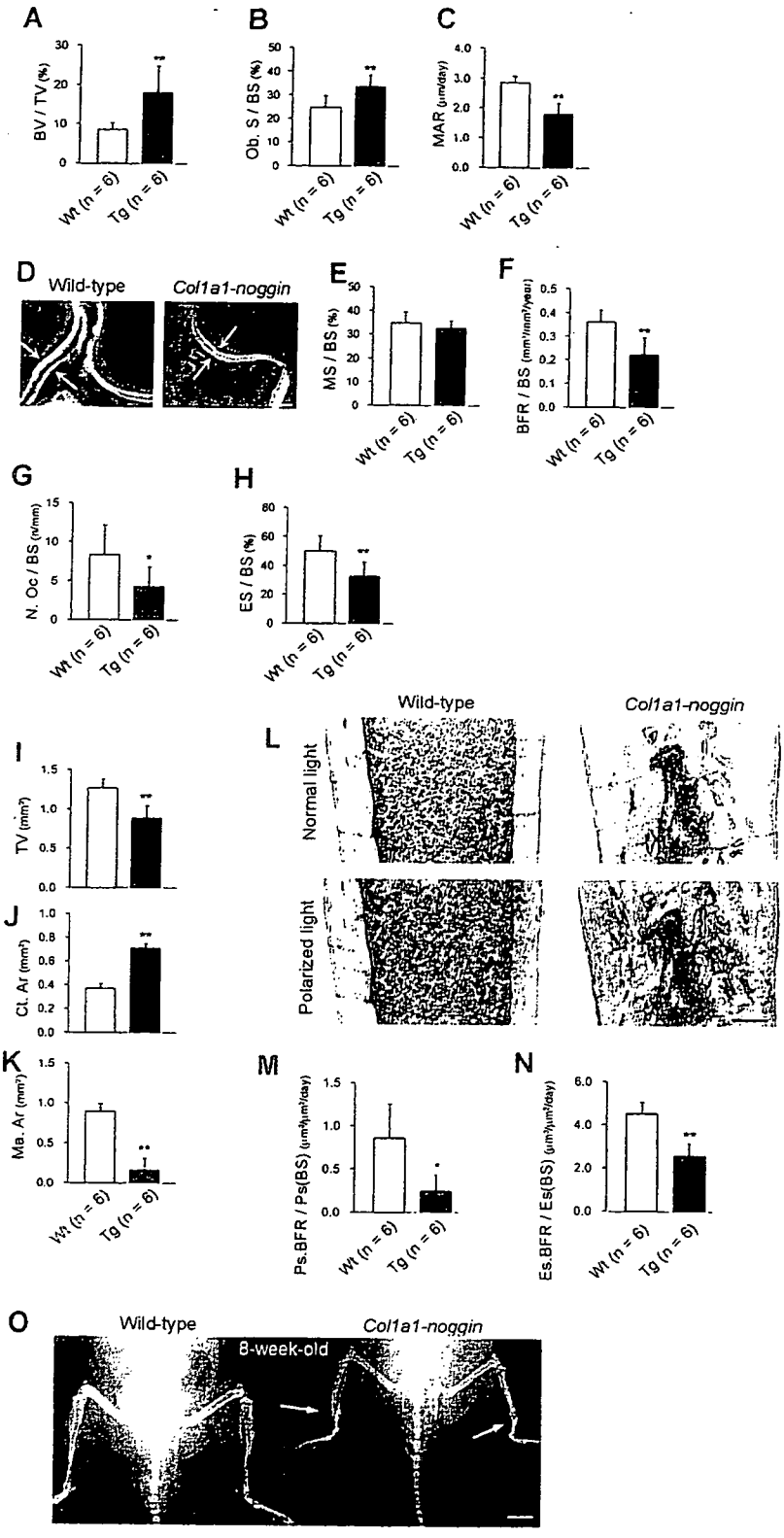

FIG. 5. Bone turnover and remodeling in wildtype and *Col1a1-noggin* transgenic mice. (A and B) Bone histomorphometric analysis of proximal metaphysis of tibia of 3-week-old mice. (A) Trabecular bone volume per tissue volume (BV/TV) and (B) osteoblast surface area per bone surface area (Ob.S/BS). (C–H) Bone histomorphometric analysis of fourth lumbar vertebral bodies of 3-week-old mice. (C) Mineral apposition rate (MAR). (D) Fluorescent micrograph of labeled mineralization fronts in the fourth lumbar vertebral bodies. *Arrows* indicate distance between the two consecutive labels. Scale bar, 20 μm. (E) Mineralizing surface area per bone surface area (MS/BS), and (F) bone formation rate per bone surface area (BFR/BS). (G) Osteoclast number per bone surface area (N.Oc/BS), and (H) eroded surface area per bone surface area (ES/BS). (I–N) Bone histomorphometric analysis of distal one third of diaphysis of tibias of 3-week-old mice. (I) TV, tissue volume; (J) Ct. Ar, cortical area; (K) Ma. Ar, marrow area. Sagittal histological sections of distal one third of diaphysis of tibias of 3-week-old mice were subjected to Villanueva bone staining. (L) Sections were viewed under a microscope using normal light and polarized light. Scale bar, 100 μm. (M) Periosteal bone formation rate per periosteal bone surface area [Ps. BFR/Ps(BS)]. (N) Endosteal bone formation rate per endosteal bone surface area [Es. BFR/Es(BS)]. (O) X-ray images of hind limbs of 8-week-old mice. Note fractures of tibias of *Col1a1-noggin* transgenic mice (white arrows). Scale bar, 5 mm. Error bars indicate means ± SD. $*p < 0.05$ and $**p < 0.01$ between wildtype and transgenic mice, as determined by *t*-test. Wt, wildtype mice; Tg, *Col1a1-noggin* transgenic mice.

tion of hydroxyapatite (Fig. 6C) were significantly lower for transgenic osteoblasts than for wildtype osteoblasts. In co-cultures with transgenic osteoblasts, numbers of TRACP$^+$ cells and osteoclastic resorption of hydroxyapatite were increased by addition of rhBMP2 to the medium in a dose-dependent manner. These results suggest that noggin inhibits its osteoclastogenesis by attenuating BMP activity in *Col1a1-noggin* transgenic mice.

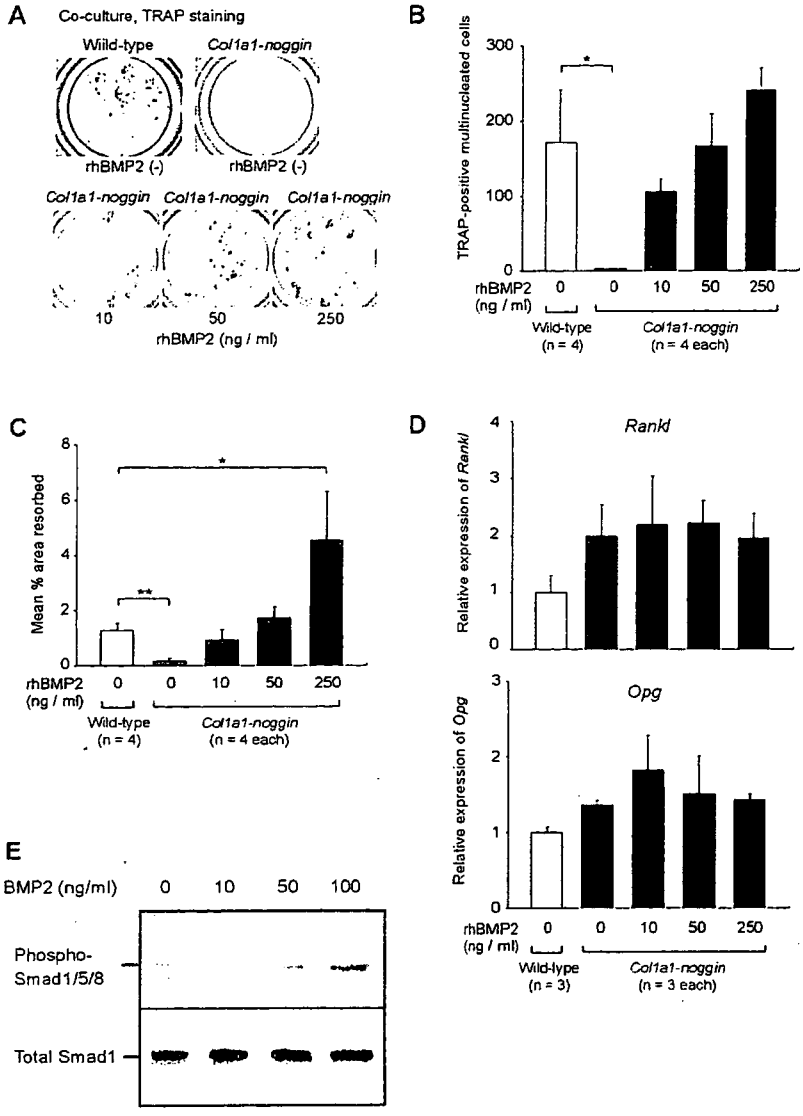

FIG. 6. Analysis of osteoclastogenesis in vitro using samples prepared from wildtype and *Col1a1-noggin* transgenic mice. (A) TRACP staining of osteoclasts in co-cultures of wildtype or *Col1a1-noggin* transgenic primary osteoblasts and wildtype spleen cells. Co-cultures containing transgenic osteoblasts were also performed in the presence of rhBMP2 at various concentrations. (B) Number of multinucleated $TRACP^+$ cells in co-cultures. Error bars indicate means ± SD. $*p < 0.05$, as determined by *t*-test. (C) Resorption of hydroxyapatite in co-cultures. Error bars indicate means ± SD. $*p < 0.05$ and $**p < 0.01$ between wildtype and transgenic mice, as determined by *t*-test. (D) Relative expression levels of *Rankl* and *Opg* mRNA in primary osteoblasts prepared from calvariae of neonate wildtype and *Col1a1-noggin* transgenic mice, measured by real-time RT-PCR. Expression levels of *Rankl* and *Opg* mRNA was also examined in *Col1a1-noggin* transgenic primary osteoblasts cultured in the presence of rhBMP2 at various concentrations for 3 days. The wildtype expression level was designated as 1. RT-PCR was performed three times. Data are presented as mean ± SD. (E) Activation of Smad pathways in bone marrow macrophages by BMP stimulation. Western blots of lysates from bone marrow macrophages incubated with rhBMP2 at various concentrations for 20 minutes. Blots were probed with antibodies against phospho-Smads 1/5/8 and Smad 1.

To examine whether the RANKL/OPG system was involved in changes in osteoclast numbers in *Col1a1-noggin* transgenic mice, we measured expression of *Rankl* and *Opg* mRNA in primary osteoblasts prepared from calvariae by performing real-time RT-PCR three times (Fig. 6D). Primary osteoblasts from *Col1a1-noggin* transgenic calvariae did not exhibit decreased expression of *Rankl* mRNA, and they exhibited only slightly increased expression of *Opg* mRNA. Treatment with rhBMP2 at various concentrations for 3 days did not much affect levels of *Rankl* or *Opg* mRNA in noggin-transgenic primary osteoblasts.

BMPs increased phosphorylation of Smads 1/5/8 in macrophage

We examined whether BMPs stimulate cells in the osteoclastic lineage by analyzing phosphorylation of R-Smads in those cells. Western blot analysis showed that bone marrow macrophages prepared by M-CSF treatment expressed Smad1 proteins. Amounts of phosphorylated Smads 1/5/8 in bone marrow macrophages were increased by 20-minute treatment with rhBMP2 in a dose-dependent manner (Fig. 6E), suggesting that BMPs directly activate Smad pathways in macrophages. In addition, immunohistochemistry using anti-phospho-Smads 1/5/8 (Fig. 2H) showed that in *Col1a1-Bmp4* transgenic mice, Smads 1/5/8 were phosphorylated in various types of cells including osteoclasts.

Cartilage-specific expression of BMP4 causes enlargement of bone and thickening of trabeculae during endochondral bone development

It has generally been believed that BMPs induce cartilage formation.[29] We previously reported that transgenic mice overexpressing growth and differentiation factor 5 (GDF5, also termed CDMP1, a member of BMP family) or BMP4 in chondrocytes exhibited expansion of cartilage.[22,30] To assess the effects of BMPs on bone formation when applied to cartilage during endochondral ossification, we examined bone of transgenic mice overexpressing BMP4 in chondrocytes under the control of the α2(XI) collagen promoter/

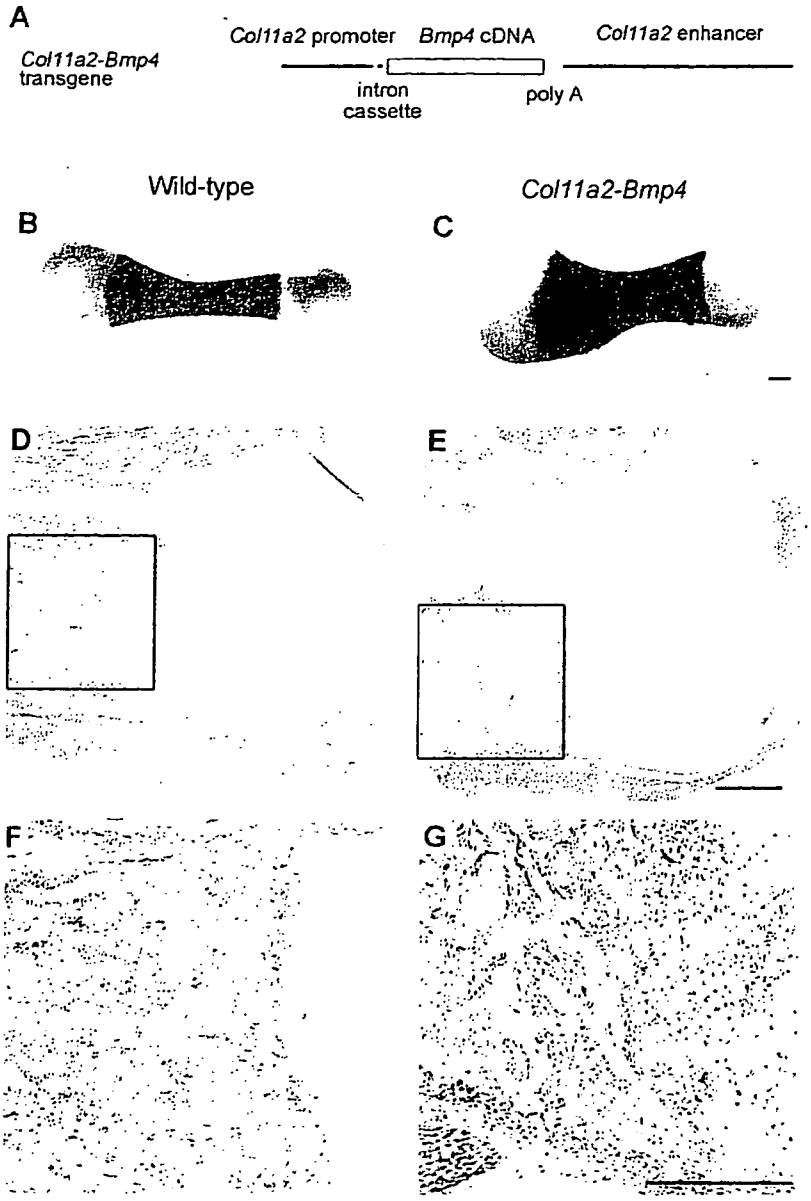

FIG. 7. Bone enlargement with thick trabeculae in *Coll1a2-Bmp4* transgenic mice. (A) Schematic representation of *Coll1a2-Bmp4* transgene construct. Humeri of (B) wildtype and (C) *Coll1a2-Bmp4* transgenic mice at 18.5 d.p.c. were stained with Alizarin red and Alcian blue. Scale bar, 200 μm. Histological analysis of proximal humerus of (D and F) wildtype and (E and G) *Coll1a2-Bmp4* transgenic mice at 18.5 d.p.c. H&E staining. Magnification of boxed regions in D and E are shown in F and G, respectively. Scale bar, 200 μm.

enhancer sequences (*Coll1a2-Bmp4* transgenic mice)[22] (Fig. 7A). At 18.5 d.p.c., epiphyseal cartilage of humeri in *Coll1a2-Bmp4* transgenic mice (Fig. 7C) had expanded compared with the wildtype (Fig. 7B). Ossification centers in the transgenic mice also expanded (Figs. 7B and 7C). Histological analysis further revealed that transgenic mice had thicker trabecular bone in marrow cavities than wildtype mice (Figs. 7D–7G). It seems that the large ossification centers with thick trabeculae in *Coll1a2-Bmp4* transgenic mice might be the result of an expanded cartilage template. These findings suggest that BMPs expressed in cartilage induce expansion of cartilage anlagen, resulting in expansion of bone.

DISCUSSION

In this study, we generated transgenic mice expressing BMP4 or noggin in osteoblasts under the control of the *Col1a1* promoter sequence. BMP overexpression in bone caused severe osteopenia, whereas noggin overexpression in bone resulted in thickening of trabecular and cortical bone. BMPs and noggin are secreted proteins and they diffuse in extracellular spaces. Thus, in *Col1a1-Bmp4* and *Col1a1-noggin* transgenic mice, in addition to autocrine action, BMP4 and noggin, respectively, produced by osteoblasts may also directly act on various cells in bone marrow cavities such as osteoclasts, stromal cells, and hematopoietic cells. In *Col1a1-noggin* transgenic mice, the bone formation rate was decreased, but the number of osteoblasts did not decrease, suggesting impairment of osteoblast function. Impairment of osteoblast function has previously been observed in mice in which BMP signaling in osteoblasts is blocked (e.g., mice with targeted disruption of BMP receptor type IA in osteoblasts)[11] and mice expressing dominant-negative BMP receptor type IB in osteoblasts.[10]

Those findings and these results suggest that, in *Col1a1-noggin* transgenic mice, noggin acts through an autocrine mechanism by preventing BMPs from interacting with BMP receptors on osteoblasts that overexpress noggin.

In addition to impairment of osteoblast function, *Col1a1-noggin* transgenic mice exhibited a significant decrease in osteoclast number. In a previous study, exogenous recombinant noggin attenuated osteoclast formation in stromal cell/hematopoietic cell co-cultures, and this effect is mediated by osteoblasts/stromal cells; this suggests that BMPs act on osteoclasts indirectly through osteoblasts or stromal cells.[31] In mice older than 10 months, conditional disruption of BMP receptor type IA in osteoblasts causes a decrease in the osteoclast number.[11] On the other hand, the osteoclast number was not decreased by expression of dominant-negative BMP receptor type IB in osteoblasts under the control of the 2.3-kb *Col1a1* promoter sequence that we used to direct noggin expression in this study.[10] In those mice, BMP signals may not be blocked in cells other than osteoblasts. Several in vitro studies suggest that BMPs also act on osteoclasts directly and that osteoclasts express BMP receptors.[13–15] Such findings suggest that, in *Col1a1-noggin* transgenic mice, noggin overexpressed by osteoblasts also acts on osteoclasts through paracrine action by preventing BMPs from interacting with BMP receptors on osteoclasts. This hypothesis is consistent with the present finding that *Rankl* expression in *Col1a1-noggin* transgenic primary osteoblasts was not decreased compared with wild-type and was not increased by incubation with rhBMP2 for 3 days or 6 h (data not shown), although it is possible that the *Rankl* expression level changed at other time-points during incubation with rhBMP2. This hypothesis is supported by the present finding that exogenous BMP2 increased phosphorylation of Smad1/5/8 in cultured bone marrow monocytes/macrophages. We speculate that BMPs also stimulate osteoclasts directly in vivo.

Impaired osteoclast formation in co-culture with *Col1a1-noggin* transgenic osteoblasts/spleen cells was rescued by adding rhBMP2, suggesting that noggin exerted their effects by attenuating BMP activity. Noggin binds with various degrees of affinity to BMPs 2, 4, 5, 6, and 7, growth differentiation factor 5 (GDF5), GDF6, and Vg1, but not to other members of the TGF-β family.[5,32,33] Noggin binds to BMP2 and BMP4 effectively and to BMP7 less tightly. Bone phenotype of *Col1a1-noggin* transgenic mice might be mainly caused by blocking activities of BMP2 and BMP4, although it is possible that blockage of activity of other BMPs contributed to abnormalities in *Col1a1-noggin* transgenic mice. These results do not exclude the possibility that some of the effects of noggin are independent of BMPs.

It has been reported that 4-week-old transgenic mice overexpressing noggin under the control of the 1.7-kb rat osteocalcin promoter exhibit decreased bone mass.[12] Transgenic mice overexpressing noggin under the control of the 1.3-kb murine osteocalcin promoter develop normally until they are 4 months old and exhibit decreased bone mass at 8 months of age.[34] The phenotypic difference between those mice and the present *Col1a1-noggin* transgenic mice may be caused by differences in transcriptional activity between osteocalcin and *Col1a1* promoters. The osteocalcin promoter directs noggin expression only in mineralizing osteoblasts, which represent a minor and localized fraction of all osteoblastic cells in situ.[34] The osteocalcin promoter directs strong expression at 4 and 8 weeks of age.[12] In contrast, the 2.3-kb *Col1a1* promoter sequence directs expression in most osteoblasts[23] beginning in the embryonic stage (Figs. 1A and 1B). This helps explain why bone abnormalities in the present *Col1a1-noggin* transgenic mice were detectable beginning in the embryonic stage at 17.5 d.p.c. It has been reported that, in osteoblasts, transcriptional activity of the *Col1a1* promoter is much stronger than that of the osteocalcin promoter.[21] From these lines of observation, we speculate that strong activities of the *Col1a1* promoter sequence might be important for disclosure of the effect of BMPs on osteoclasts, especially for direct effects through paracrine mechanism.

The reduced bone formation and resorption associated with frequent fractures in *Col1a1-noggin* transgenic mice suggests important functions of BMP signals in bone. Because noggin overexpression affected both osteoblast function and osteoclast number in this study, we speculate that a physiological function of BMPs in bone is acceleration of bone turnover, which improves the quality and mechanical strength of bone. Strict control of BMP activity may be necessary for formation of high-quality bone, as suggested by the present finding that both *Col1a1-noggin* and *Col1a1-Bmp4* transgenic mice exhibited fragile bone.

In this study, *Col11a2-Bmp4* transgenic mice exhibited expanded cartilage as well as expanded bone containing thick trabeculae. *Col11a2* promoter/enhancer sequences direct expression in mesenchymal condensation and cartilage, but not in bone.[24,25] These results are consistent with our previous report that noggin overexpression in cartilage under the control of the *Col11a2* promoter/enhancer sequences caused cartilage and bone to become very hypoplastic.[22] We have not analyzed the mechanism by which expansion of cartilage led to bone enlargement and thickening. In addition to its effects as anlagen, expanded cartilage may produce signaling molecules that promote enlargement of bone. The events observed in the skeleton of the present *Col11a2-Bmp4* transgenic mice may resemble the processes that occur during healing of fracture treated with BMPs. BMPs have been used to promote fracture healing.[18–20] BMPs applied to fracture sites may act on mesenchymal cells and chondrocytes, causing formation of a large cartilaginous callus that promotes solid bone formation. Exogenous BMP also contribute to bone formation by stimulating osteoblast function and remodeling. The bone phenotype of *Col1a1-Bmp4* transgenic mice suggest that persistent application of large amount of BMP4 to bone stimulate osteoclastic bone resorption continuously and cause bone loss. These finding may be helpful in planning schedule of BMP application to further improve clinical results of fracture treatment.

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Paraparesis due to exacerbation of preexisting spinal pseudoarthrosis following infliximab therapy for advanced ankylosing spondylitis

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Abstract

BACKGROUND CONTEXT: Recent reports have described the long-term efficacy and safety of infliximab as a treatment for ankylosing spondylitis (AS). The most important adverse effects of infliximab are infections, malignancies, autoimmunities, and hypersensitivity reactions. There has never been a reported case of paraparesis after infliximab therapy for AS.

PURPOSE: To describe a case with paraparesis caused by rapid exacerbation of preexisting spinal pseudoarthrosis after infliximab therapy for advanced AS.

STUDY DESIGN/SETTING: Case report/Osaka University Graduate School of Medicine, Suita, Japan.

PATIENT SAMPLE: A 55-year-old man with a 27-year history of AS.

OUTCOME MEASURES: Case report.

METHODS: A 55-year-old man with a 27-year history of AS was treated with infliximab, which provided considerable pain relief and improvement of activities of daily living. However, as the patient resumed vigorous daily activity, he felt back pain and subsequently developed paraparesis. Radiographs showed rapid exacerbation of preexisting spinal pseudoarthrosis at the T11–T12 level after infliximab therapy.

RESULTS: After laminectomy and posterolateral fusion, the back pain and paraparesis improved sufficiently to allow independent walking, but moderate bladder dysfunction persisted.

CONCLUSIONS: Although this patient could have certainly become myelopathic over time without undergoing infliximab therapy, the patient's history and radiographic course suggest that suppression of inflammation by infliximab improved his activities of daily living, which paradoxically exacerbated preexisting spinal pseudoarthrosis and quickened the onset of subsequent myelopathy.

Keywords: Ankylosing spondylitis/complications; Monoclonal antibodies/therapeutic use; Antirheumatic agents/therapeutic use; Paraparesis/etiology

Introduction

Infliximab (Remicade; Centocor, Inc., Malvern, PA) is a chimeric IgG_1 monoclonal antibody that binds to tumor necrosis factor alpha (TNFα) and inhibits its biological effect. The therapeutic effect of infliximab has been established for rheumatoid arthritis, psoriasis, and Crohn's disease [1]. Recently, infliximab has also been approved for treatment of ankylosing spondylitis (AS) in Europe, after confirmation of the long-term efficacy and safety of infliximab for treatment of AS [1–3]. Infections, autoimmunities, and hypersensitivity reactions are well-known major adverse effects of infliximab [1–3]. However, paraparesis has never been observed as an adverse effect of infliximab therapy for AS. This report describes a rare case of paraparesis following treatment with infliximab for advanced AS.

Case report

A 55-year-old man with a 27-year history of AS was referred to our orthopedic clinic with progressive gait

FDA device/drug status: not applicable.

Nothing of value received from a commercial entity related to this manuscript.

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disturbance and urinary retention. Three months before referral, he began receiving intravenous infliximab at a dose of 5 mg/kg every 2 weeks (induction phase) to treat refractory AS. Infliximab provided considerable pain relief and suppression of disease activity. However, as the patient resumed vigorous daily activities, he began to feel backache radiating to his left flank and crepitation in his back at movement without any preceding trauma. Over a 3-week period, his gait gradually deteriorated, and he developed urinary retention and paresthesia in the sacral area (Fig. 1).

At clinical examination, the patient reported percussion tenderness in the thoracolumbar transitional area. He could hardly walk without the support of handrails or a cane. Neurological examination revealed hyperreflexia and moderate motor weakness in his lower extremities. The patient had hypesthesia in the lower extremities and anesthesia in the sacral area. Anteroposterior and lateral radiographs showed severe spinal pseudoarthrosis at the T11–T12 level, with vacuum phenomenon in the intervertebral disc space and sclerotic change of the vertebral bodies adjacent to the diseased disc, and all other segments were ankylosed (Fig. 2a, b). Hypermobility between T11 and T12 was observed on the lateral radiographs in flexion and extension position (Fig. 2c, d). Retrospective examination of radiographs revealed that initial signs of spinal pseudoarthrosis were present 2 months before the beginning of infliximab therapy (Fig. 2e, f). Magnetic resonance imaging showed decreased signal intensity in the disc space and the adjacent margins of T11 and T12 on both T1- and T2-weighted images. The spinal cord sustained compression by protrusion of the disc anteriorly and by hypertrophy of yellow ligaments posteriorly (Fig. 3a, b). Computed tomography showed a fracture of the left pars interarticularis at T12 and calcification of the hypertrophied yellow ligaments (Fig. 3c).

At surgery, the nonankylosed right facet joint at T11–T12 and pseudoarthrosis at the left pars interarticularis of the T12 lamina were confirmed; these conditions resulted in considerable instability at the T11–T12 level. Laminectomy of T11 and T12, and posterolateral fusion from T10 to L1 were performed using a pedicle screw system (Fig. 4a, b).

After surgery, the patient reported great pain relief in his back, and exhibited full recovery of motor strength in the lower extremities. At the latest follow-up 4 months after surgery, he could walk independently without pain, although moderate bladder dysfunction persisted.

Discussion

Since the first description of a destructive discovertebral lesion complicating AS, by Andersson in 1937 [4], such destructive lesions, known as spinal pseudoarthrosis, have come to be recognized as one of the most important complications of advanced AS [4–22]. The exact pathogenesis of these lesions remains controversial. There is debate as to whether inflammatory changes or mechanical stresses play the key role in the development of these lesions [5–9,15,17,19,21,22].

In the present case, inflammatory activity of AS was apparently controlled by administration of infliximab, which provided dramatic pain relief and improvement of activities of daily living. Unfortunately, the patient's increased activity most likely led to spinal instability at the remaining

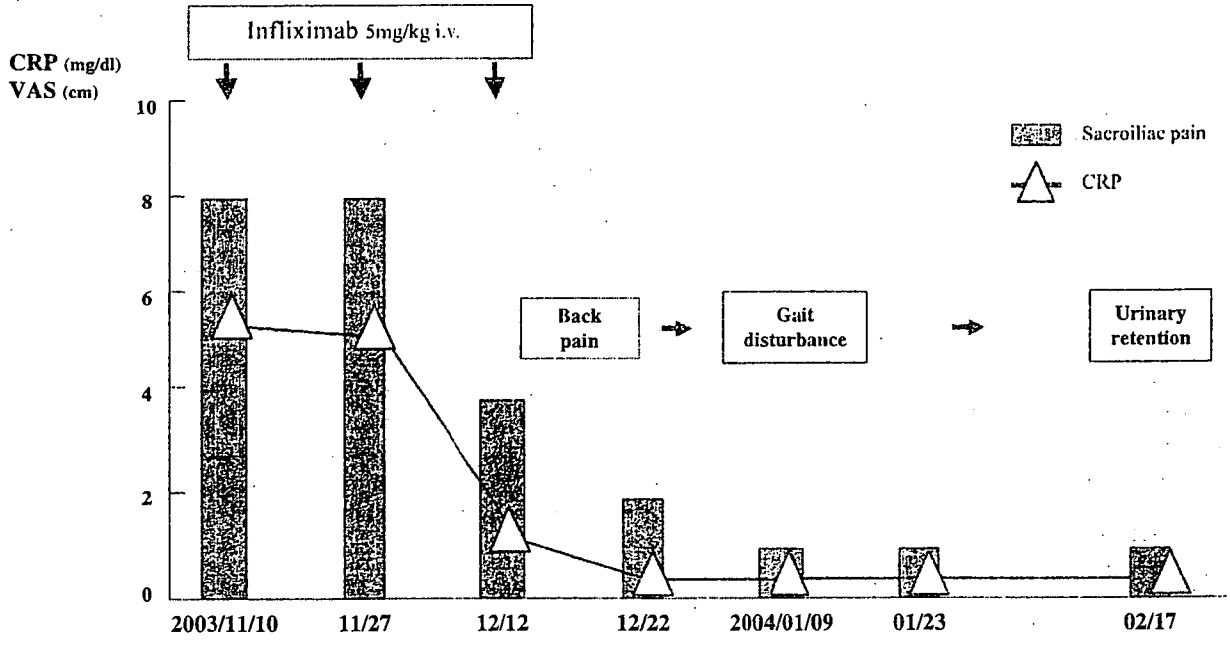

Fig. 1. Clinical course following infliximab therapy is summarized. After the decrease in sacroiliac pain and serum C-reactive protein (CRP), the patient developed back pain and subsequently paraparesis. VAS=visual analogue scale.

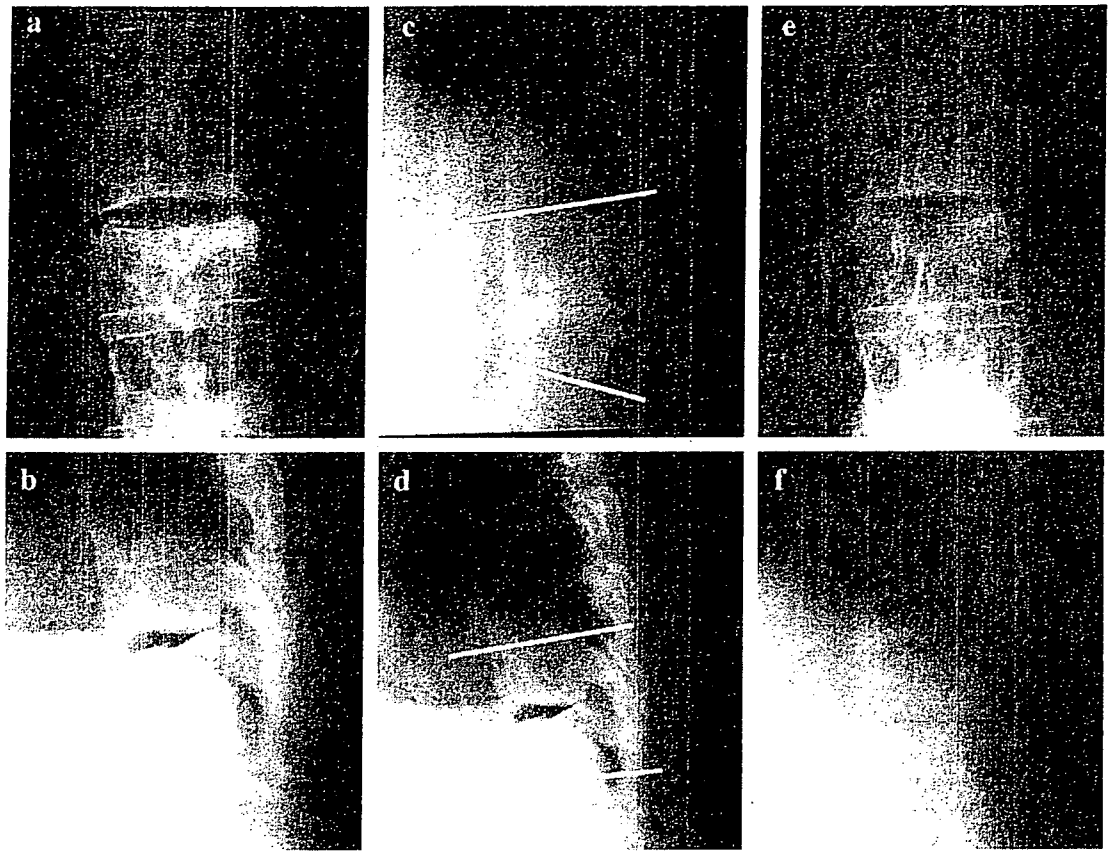

Fig. 2. Radiographs of the thoracolumbar junction. (a, b) At referral (2 months after the third infusion of infliximab). Vacuum phenomenon in the intervertebral space and sclerosis of the adjacent margins of T11 and T12 are visible. (c, d) Lateral radiographs in flexion and extension positions at referral. Hypermobility between T11 and T12 is indicated (range of motion, 12.2°). (e, f) Two months before infliximab therapy. The initial changes of pseudoarthrosis are visible.

mobile segment of the spine, resulting in serious paraparesis. Retrospective examination of radiographs confirmed significant exacerbation of preexisting spinal pseudoarthrosis after the beginning of infliximab therapy. Although this patient could have certainly become myelopathic over time without undergoing infliximab therapy, the patient's history and radiographic course suggest that suppression of inflammation by infliximab improved his activities of daily living, which paradoxically exacerbated the preexisting spinal pseudoarthrosis and contributed to the development of subsequent paraparesis.

Anti-TNFα agents such as infliximab have provided a new approach to the treatment of AS; no disease-modifying antirheumatic drugs are effective against AS [1–3]. Recent reports indicate that infliximab provides long-term efficacy and safety in the treatment of AS [2,3]. Although severe adverse effects of infliximab are rare, some physicians are concerned about the possibility of the following types of adverse events: infections including sepsis and tuberculosis; malignancies such as lymphoma; hematological disorders such as pancytopenia; demyelinating neuropathy; congestive heart failure; elevation of liver enzymes; lupus and vasculitis; autoimmunities and hypersensitivity reactions [1–3]. Braun et al. reported that continuous infliximab treatment for refractory AS for 54 weeks resulted in significant and durable pain relief, improvement in physical function, and decrease in disease activity. Six of their 69 patients discontinued infliximab therapy because of the following adverse events: systemic tuberculosis, leukopenia, possibly antinuclear antibody–associated peripheral arthritis, lupus-like rash, and liver function abnormalities [2]. However, there have been no reported cases of paraparesis due to rapid exacerbation of spinal pseudoarthrosis after infliximab therapy for AS.

Spinal pseudoarthrosis is not an uncommon complication of AS [4–22]; the estimated prevalence of this complication ranges from 1% to 16% [17,22]. Severe neurological deficits are rare clinical manifestations of spinal pseudoarthrosis. Most patients with pseudoarthrosis only exhibit localized backache, which is often misinterpreted as a common symptom of AS. Fang et al. reported that only 4 of their 35 patients with spinal pseudoarthrosis had neurological symptoms, and the remaining 31 patients complained only of back pain [17]. For these reasons, this complication can be easily overlooked by physicians, possibly resulting in more serious consequences. Now that infliximab has

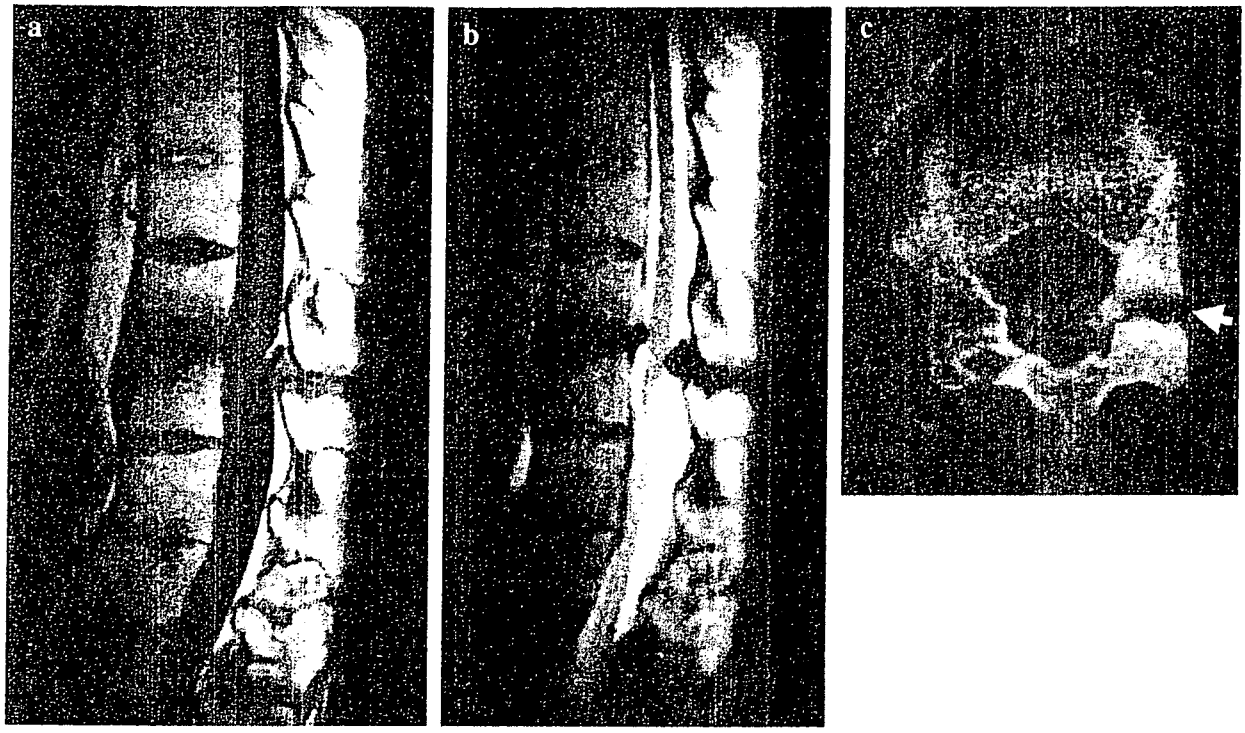

Fig. 3. Before surgery, magnetic resonance imaging showed decreased signal intensity at the disc space and the adjacent margins between T11 and T12 on both the (a) T1- and (b) T2-weighted images. The dural sac was compressed by the protruding disc and the hypertrophied yellow ligaments. (c) Computed tomogram showed a fracture of the left pars interarticularis at T12 (arrow) and calcification of the hypertrophied yellow ligaments.

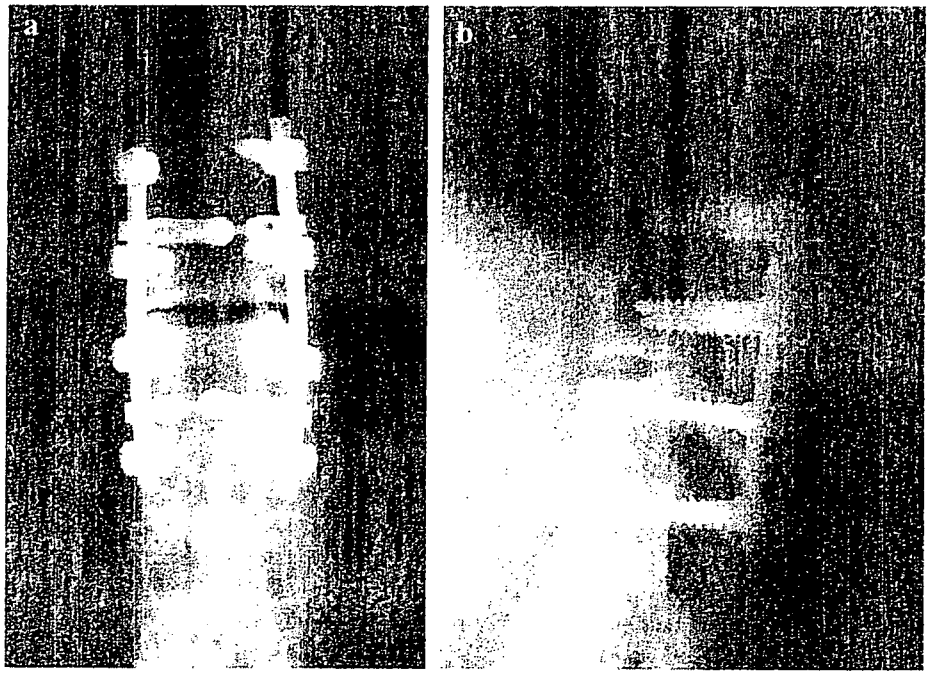

Fig. 4. Postoperative radiographs of the thoracolumbar spine. After laminectomy of T11 and T12, posterolateral fusion from T10 to L1 was performed using a pedicle screw system. (a) Anteroposterior view, (b) Lateral view.

been approved for treatment of AS in Europe, the number of AS patients undergoing infliximab therapy will presumably increase. Therefore, in addition to the known biological adverse events of infliximab, physicians should evaluate all AS patients with severe symptoms for an underlying pseudoarthrosis.

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