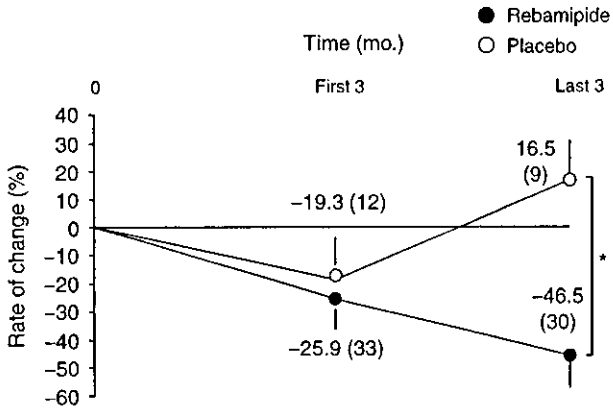

Fig. 6. Rate of mean change in monthly aphthae count score in patients with an aphthae pain score >28 at 1 month before treatment. Data are means ± standard error (SE) [total monthly counts]. * $p < 0.01$ rebamipide vs placebo. Figures in brackets are the numbers of data, and numbers indicate the rates of change.

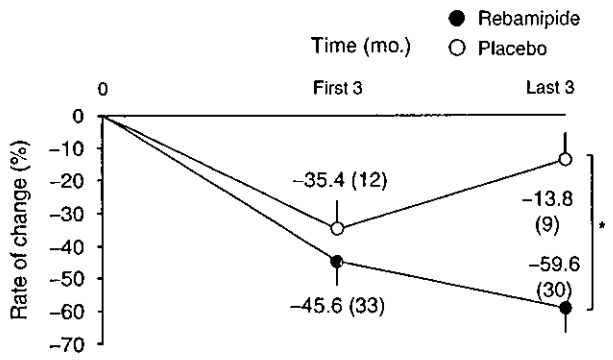

Fig. 7. Rate of mean change in monthly aphthae pain score in patients with an aphthae pain score >28 at 1 month before treatment. Data are means ± standard error (SE) [total monthly counts]. * $p < 0.01$ rebamipide vs placebo. Figures in brackets are the numbers of data, and numbers indicate the rates of change.

tus. There were no serious adverse events that caused concern with long-term use of rebamipide.

Discussion

The results of this randomised, double-blind, placebo-controlled study demonstrate that rebamipide improves aphthae count and pain score in BD patients. It should be pointed out that the placebo group in this study was not strictly a placebo group, in that subjects were allowed to continue their usual treatment. It would thus be more accurate to say that 'usual therapy + placebo' was compared with 'usual therapy + rebamipide'. However, the results were not affected by concomitant use of colchicine, the basic treatment drug for BD, or by corticosteroid-containing ointments and patches, the treatment drugs for aphthae. Of the 11 patients in whom rebamipide was effective, 10 had had aphthae for 10 years or more. The study population was an appropriate sample size for a pilot study. Although the pretreatment observation period was comparatively short at only 4 weeks, comparison of the onset of recurrent oral aphthous ulceration with skin, genital and ocular lesions in these patients indicated that the incidence of aphthae was stable. Thus, the short observation period chosen for this study was not considered to be a problem.

Monthly aphthae count is defined as the total number of aphthae detected daily over 1 month. This count takes into account the duration of individual aphthae. For example, if one aphthae is detected continuously for 5 days, the monthly aphthae count score is 5. The duration of onset of individual aphthae was not observed in this study. However, taking patients' impressions into consideration, a shortened duration between the onset and disappearance of individual aphthae may have contributed to the reduction in monthly aphthae count seen in rebamipide-treated patients. In addition, the improvement in monthly pain score may have resulted from a reduction in the number of aphthae onsets and shortening of the duration of individual aphthae. We suggest that tissue repair mechanisms in rebamipide-treated patients occurred before lesions reached deep tissue areas, which, together with the shortening of the duration of aphthae, prevented the occurrence of severe pain.

Although localised treatments such as corticosteroid-containing ointments and patches and local anaesthetic-containing jellies are currently being used in BD patients, difficulty in applying these treatments in the oral environment is often experienced, thus making their long-term use difficult. No significant adverse events were noted during 6

months of rebamipide treatment. Rebamipide is administered orally, thus the application complications that are encountered with topical treatments could be avoided.

Other BD symptoms, such as uveitis, skin lesions and genital ulcers were not the main evaluation parameters in this study. The three remaining major symptoms were secondary evaluation parameters that were monitored along with the aphthae. The stable incidence that could be seen with aphthae was not seen with the other three main symptoms; therefore, a longer period of administration of 12 months or more would be necessary to assess the efficacy against these symptoms. There was no restriction in drug therapy for the other three main symptoms. There was almost no frequent occurrence of any of these symptoms (0–2 days during 28 days of the pretreatment observation period) . However, if oral administration was found to be effective against aphthae, the drug may be effective against other symptoms too. The difference in the degree of inflammation of each symptom was suggested as the reason for the differences in efficacy. Future studies should address the effects of rebamipide on these symptoms. Furthermore, dose titration and clarification of the mechanism of action of rebamipide on aphthae of BD should be investigated.

Rebamipide showed a mucoprotective action and acceleration of gastric ulcer healing, resulting from an increase in prostaglandins in gastric mucosa via upregulation of cyclo-oxygenase-2 (COX-2) protein[15,16] and endothelial growth factor (EGF) and EGF receptors.[17] EGF and prostaglandin E_2 are present in biological fluids, including saliva, and play a role in maintenance of the epithelial barrier and in healing of damaged mucosa. Wu-Wang et al.[18] suggested that a decrease in EGF and prostaglandin E_2 in the saliva might be associated with ulcer formation in patients with recurrent aphthous stomatitis. In addition to its effect on COX-2 and EGF, rebamipide has a variety of other effects on the gastric mucosa such as the stimulation of mucus secretion,[19,20] inactivation of neutrophils,[21] and scavenging of free radicals.[12,13,22] Accordingly, these factors and/or other unknown targets cannot be ruled out as potential mechanisms for the healing effect of rebamipide.

The effective concentration of rebamipide was shown to be in the range of 1–1000μM from the results of several *in vitro* studies,[23] and differs according to cell and mechanism. The effective concentration in gastric mucosa for the treatment of *in vivo* gastric ulcer and gastritis was shown to be within this concentration range for both animals and humans.[24,25] On the other hand, the effective concentration in the blood was decreased approximately one-tenth or more compared with the concentration in the gastric mucosa.[24,25] However, for example, the oral dose of rebamipide that shows antiulcer action in the rat has been reported to be effective in the rat model of acetic acid-induced buccal mucosal ulcers,[26] and hepatic injury,[27] and the same dose was effective even in human pharyngeal granuloma.[28] From these observations, it was assumed that the action of rebamipide depends on the concentration in the blood and target organs. Further studies are necessary to confirm the mechanism of action of rebamipide.

The treatment of oral aphthae in BD is frustrated by a lack of effective drugs because local treatment only is not satisfactory. Although this was a small pilot study, it provides information on another treatment strategy for mucosal ulceration in BD.

Conclusion

Oral administration of rebamipide 300 mg/day for 12–24 weeks was expected to have an anti-inflammatory effect. Thus the efficacy of the drug in the treatment of recurrent aphthae, which is a major symptom of BD disease, was investigated in a placebo-controlled, double-blind comparative study. When drug efficacy against aphthae count and pain score were evaluated, the rate for moderate or greater improvement was 36% (5/14 subjects) and 65% (11/17 subjects) for the placebo and rebamipide groups, respectively .

Rebamipide reduced oral aphthosis pain and the number of aphthae in BD disease. No serious adverse events that would be problematic during long-term administration were observed. Further studies of long-term administration in a higher number of subjects are needed to follow up on this preliminary study.

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Bone Morphogenetic Protein Signals Are Required for Cartilage Formation and Differently Regulate Joint Development During Skeletogenesis

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ABSTRACT

The bone morphogenetic protein (BMP) family consists of a large number of members and has diverse biological activities during development. Various tissues express pleural BMP family members, which seem to cooperatively regulate developmental events. Here, multiple BMP signals were inactivated in chondrocytes to clarify the function of BMPs during skeletogenesis. To obtain tissue-specific inactivation, Noggin gene (*Nog*) was overexpressed in cartilage under the control of α2(XI) collagen gene (*Col11a2*) promoter/enhancer sequences. The resultant transgenic mice lacked most of their cartilaginous components, suggesting that cartilage does not develop without BMP signals. These effects seem to be mediated through down-regulation of *Sox9* expression. Conversely, specific BMP signals were activated in the skeleton by targeted expression of *Bmp4* in cartilage and the resultant phenotype was compared with that of transgenic mice expressing growth and differentiation factor-5 (GDF-5), another BMP family member. Overactivity of *Bmp4* in the skeleton caused an increase of cartilage production and enhanced chondrocyte differentiation, as *GDF5* expression did, but it did not disturb joint formation as *GDF5* did. During skeletogenesis, unique roles of each BMP may reside in the regulation of joint development. Together with the common effect on the cartilage overproduction by *Bmp4* and *GDF5* overactivation, loss of cartilage by inactivation of multiple BMPs in Noggin transgenic mice indicates that signals for cartilage production are reinforced by multiple BMPs exclusively. These conclusions may account for the reason why multiple BMPs are coexpressed in cartilage. (J Bone Miner Res 2002;17:898–906)

Key words: bone morphogenetic protein, Noggin, cartilage, joint formation, transgenic mice

INTRODUCTION

CARTILAGE SERVES as the template for the development of skeletal components. Formation of the skeleton is initiated by mesenchymal cell condensation, forming primordial cartilage followed by endochondral ossification. This process includes proliferative and hypertrophic chondrocytes. As a final step in endochondral bone formation, the hypertrophic cartilage is invaded by blood vessels and osteoprogenitor cells, and the calcified cartilage is subsequently replaced by bone.

Bone morphogenetic proteins (BMPs) were originally identified as secreted signaling molecules that could induce endochondral bone formation.[1] Subsequent molecular cloning studies[2] have revealed that the BMP family consists of various molecules, including members of the growth

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and differentiation factor (GDF) subfamily. BMP family members have diverse biological activities during the development of various organs and tissues, as well as during embryonic axis determination.[3] Expression analysis of BMP family members has revealed that each protein has a unique tissue distribution, with several BMPs being coexpressed in the same tissues. *Bmp2, Bmp4, Bmp7,* and *Gdf5* are expressed in perichondrium and are believed to regulate cartilage formation and development.[4-6] It has been reported that mice lacking Noggin, an antagonist for BMPs, showed oversized cartilage and impairment of joint development.[7] In Noggin-deficient mice, multiple BMPs, including BMP-2, BMP-4, BMP-7, and GDF-5, seem to be overactivated in cartilage, indicating that these BMPs as a group expand cartilage. However, the unique role of each BMP during skeletogenesis still remains obscure.

To unravel the in vivo function of each BMP, knockout mice have been created and analyzed. *Bmp2* and *Bmp4* show early expression in postimplantation embryos, and inactivation of these genes results in death at an early stage of gestation, before the onset of chondrogenesis.[8,9] Embryonic mice lacking BMP receptor type IA (BMPRIA)[10] or type II (BMPRII)[11] also fail to form the mesoderm and die by 9.5 days postcoitus (d.p.c.), before the onset of skeletogenesis, probably caused by impairment of the BMP signal transduction. In contrast, homozygous loss-of-function mutants for *Bmp5*,[12] *Bmp6*,[13] *Bmp7*,[14,15] and *Gdf5* (also known as *Cdmp1* and *Bmp14*)[16] are viable and exhibit limited malformation of skeletal components. Mice with inactivated BMP receptor type IB gene (*BmprIB*) are viable and exhibit defects that are largely restricted to the appendicular skeleton.[17] The relatively minor nature of these developmental defects suggests that other coexpressed BMP family members or their receptors may functionally compensate for the absence of a protein normally expressed in the same tissue. This concept is supported by the early embryonic death of *Bmp5; Bmp7* double-knockout mice.[18] Therefore, function of BMPs during mammalian skeletogenesis is not well understood.

To examine the physiological role of BMPs during skeletogenesis in vivo, we inactivated multiple BMP signals in the skeleton by targeted expression of Noggin, a BMP antagonist, in cartilage. The resultant transgenic mice lacked most of their skeletal components, suggesting that BMP signaling is required for cartilage formation. Conversely, we activated specific BMP signals in skeleton by targeted expression of BMP-4 in mouse cartilage and compared the phenotype with that of transgenic mice expressing GDF-5,[19] another BMP family member. Excess BMP-4 activity in the skeleton caused an increase of cartilage and enhanced chondrocyte differentiation as GDF-5 overactivation did, but did not disturb joint formation as GDF-5 did. This difference indicates that each BMP has a unique role in joint formation during skeletogenesis. We conclude that formation of cartilage is mainly dependent on BMPs and that each BMP has a unique role, possibly explaining the known existence of multiple BMP expression in cartilage.

MATERIALS AND METHODS

Construction of transgenes

The *Col11a2*-based expression vector, *Col11a2-LacZ*, is identical with the 742*lac*ZInt that have been described previously.[20] *Col11a2-LacZ* contains the *Col11a2* promoter (−742 bp to +380 bp), an SV40 RNA splice site, the β-galactosidase reporter gene, and the SV40 polyadenylation signal, as well as 2.3 kilobases (kb) of the first intron sequence of *Col11a2* as an enhancer (Fig. 1A).

A 0.7-kb DNA fragment covering the entire coding region of the mouse *Nog* complementary (c)DNA[21] was generated by polymerase chain reaction (PCR) using a forward primer tagged with *Not*I site (ATAAGAAGCGGCCGCTAGAGTCATTCAGCGGCTGGTCAGAGGATGGAGCGCTGCCCCAGCCTG) and a reverse primer with *Not*I site (ATAGTTTGCGGCCGCGAGTTCTAGCAGGAACACTTACACTC). A 1.2-kb DNA fragment covering the entire coding region of the mouse *Bmp4* cDNA[22] was also generated by PCR using a forward primer tagged with *Not*I site (ATAAGAAGCGGCCGCTAGAGTCATTCAGCGGCTGGTCAGAGGATGATTCCTGGTAACCGAATGCTG) and a reverse primer with *Not*I site (ATAGTTTGCGGCCGCTCAGCGGCATCCACACCCCTCTAC). After digestion with *Not*I, these PCR fragments were cloned into the *Not*I site of *Col11a2-LacZ* by replacing the β-galactosidase gene to create *Noggin*-expression vector, *Col11a2-Nog*, and *Bmp4*-expression vector, *Col11a2-Bmp4*, respectively (Fig. 1A).

The previously reported *GDF5* (*CDMP1*) expression vector, 742-*CDMP1*-Int,[19] was designated as *Col11a2-GDF5*, containing complete human *GDF5* cDNA coding sequences ligated to the promoter and enhancer sequences of the *Col11a2* and SV40 RNA splice site, which are identical with those of *Col11a2-LacZ*, *Col11a2-Nog*, and *Col11a2-Bmp4* (Fig. 1A).

Generation of transgenic mice

The plasmids containing transgene constructs were digested with restriction emzymes to release the inserts from their vector sequences. Transgenic mice were produced by microinjecting each of the inserts into the pronuclei of fertilized eggs from F1 hybrid mice (C57BL/6 × C3H) as described previously.[20] Transgenic embryos were identified by PCR or Southern assays of genomic DNA extracted from the placenta or skin. Previously reported transgenic embryos bearing *Col11a2-GDF5* (742-*CDMP1*-Int)[19] were reproduced for comparison of phenotype.

Staining of skeleton and histology

Cartilage and bones of embryos and newborn mice were stained as previously described.[23] After skin and internal organs were removed, samples were fixed in 96% ethanol for 2 days followed by staining with alcian blue (Sigma-Aldrich, St. Louis, MO, USA) solution (80 ml 96% ethanol, 20 ml acetic acid, and 15 mg alcian blue) for 2 days. The samples were dehydrated in 100% ethanol for 5 days and immersed in 1% KOH for 2 days. The samples were stained with 0.001% alizarin red S (Sigma-Aldrich) solution in 1% KOH for 2 days, dehydrated in graded solutions of glycerin,

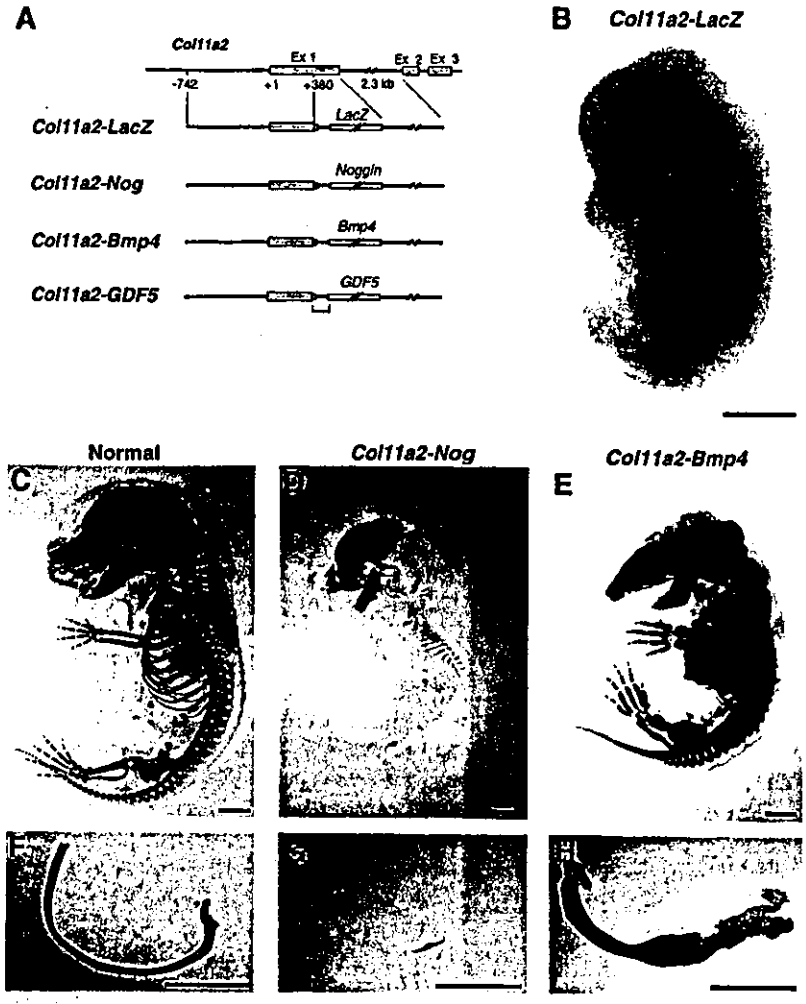

FIG. 1. The structure of each transgene, cartilage-specific expression of the transgenes and the skeletal abnormalities in *Col11a2-Nog* and *Col11a2-Bmp4* transgenic mice. (A) For preparation of the transgenes, a 742-bp promoter sequence and 2.3-kb intron enhancer sequence from the type XI collagen α2 chain gene (*Col11a2*) were ligated to the *LacZ* gene, *Nog* cDNA, *Bmp4* cDNA, and *GDF5* cDNA respectively. Brackets show the SV40 intron cassette. (B) Transgenic mice bearing *Col11a2-LacZ* were stained with X-gal to detect *LacZ* activity at 13.5 d.p.c. Transgene expression directed by the *Col11a2* promoter/enhancer sequences was restricted to the primordial cartilage of the long bones of the limbs and to that of the ribs. Whole skeletons from (C) normal, (D) *Col11a2-Nog*, and (E) *Col11a2-Bmp4* transgenic mice at 16.5 d.p.c. were stained with alcian blue (for cartilage) and alizarin red (for bone). Only traces of cartilage were recognizable in *Col11a2-Nog* transgenic mice, whereas cartilage was expanded in *Col11a2-Bmp4* transgenic mice. The 7th ribs shown are from (F) normal, (G) *Col11a2-Nog*, and (H) *Col11a2-Bmp4* transgenic mice. Scale bars, 2 mm.

and stored in 100% glycerin. For histological analysis, embryos were dissected with a stereomicroscope, fixed in 4% paraformaldehyde, processed, and embedded in paraffin. Serial sections were prepared and stained with hematoxylin and eosin, safranin O-fast green-iron hematoxylin (Sigma-Aldrich).

In situ hybridization and probes

Digoxigenin-11-UTP-labeled single-strand RNA probes were prepared using a DIG RNA labeling kit (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's instructions. cDNAs described below were used to generate antisense and sense probes. Hybridization was performed as described previously.[24] Briefly, after deparaffinization, the sections were treated with 10 μg/ml of proteinase K for 15 minutes at room temperature and subjected to 0.2N HCl to inactivate endogenous alkaline phosphatase. Hybridization was performed at 50°C in 50% formamide, and washes were carried out at a stringency of 2× SSC containing 50% formamide at 55°C. The slides were subjected to 10 μg/ml of RNAse A in TNE (10 mM Tris-HCl [pH 8.0], 500 mM NaCl, and 1 mM EDTA) at 37°C for 30 minutes for digestion of nonhybridized transcripts and washed. A Genius Detection System (Boehringer Manheim) was used to detect signals according to the manufacturer's instructions.

Col2a1 and rat *Sox9* cDNAs were obtained from Y. Yamada (National Institute of Health, Bethesda, MD, USA).[25] Mouse type IIA procollagen cDNA (exon 2) was from L.J. Sandell (Washington University, St. Louis, MO, USA).[26] Mouse *Col10a1* cDNA (pRK26) was provided by K.S.E. Cheah (University of Hong Kong, Hong Kong).[27]

RESULTS

Targeted expression of transgene and transgenic mice expressing Noggin in cartilage

Four DNA constructs were prepared to generate transgenic mice (Fig. 1A). We first expressed β-galactosidase reporter gene (*LacZ*) under the control of the promoter and first intron enhancer sequences derived from α2(XI) collagen gene (*Col11a2*). As reported,[20] the *Col11a2* promoter/enhancer sequences started to direct expression to mesenchymal condensation in limbs at 12.5 d.p.c. At 13.5 d.p.c., the transgenic mice showed clear X-gal staining specifically in the primordial cartilage of the long bones of the limbs and

TABLE 1. PRODUCTION FREQUENCY OF TRANSGENIC MICE

	Number of pups obtained	Number of transgenic mice*	Mice with hypoplastic cartilage†	Mice with expanded cartilage†	Joint fusion‡		
					Complete	Partial	None
Col11a2-Nog	62	11	7	0	0	0	7
Col11a2-Bmp4	92	15	0	12	0	2	10
Col11a2-GDF5	63	12	0	9	7	2	0

* Genotype was analyzed by PCR and/or Southern assays of genomic DNA extracted from the placenta or skin.
† Number of mice with phenotypes are shown.
‡ Number of mice with completely fused and partially fused joints and number of mice with well-formed joints are shown.

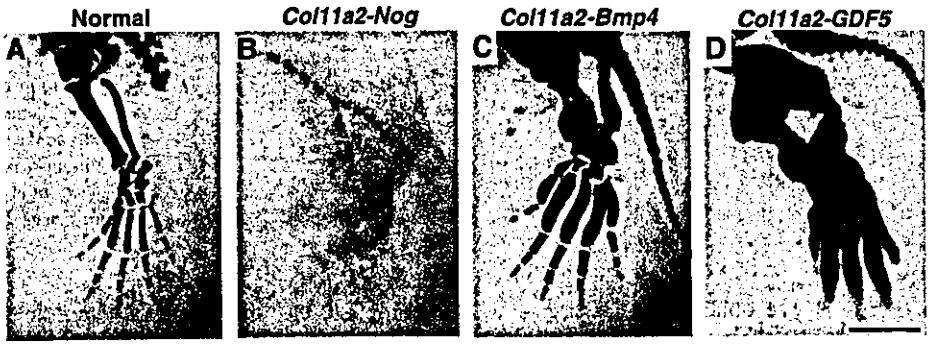

FIG. 2. Hindlimb skeletons of *Col11a2-Nog*, *Col11a2-Bmp4*, and *Col11a2-GDF5* transgenic mice. Hindlimb skeletons from (A) normal, (B) *Col11a2-Nog*, (C) *Col11a2-Bmp4*, and (D) *Col11a2-GDF5* transgenic mice at 16.5 d.p.c. were stained with alcian blue (for cartilage) and alizarin red (for bone). (B) Only traces of cartilage were recognizable in *Col11a2-Nog* transgenic mice. (C) *Col11a2-Bmp4* transgenic mice showed well-formed joints, whereas (D) *Col11a2-GDF5* transgenic mice exhibited skeleton with completely fused joints. Scale bar, 1 mm.

ribs (Fig. 1B), when cells in mesenchymal condensation differentiated into chondrocytes. Cranial components undergoing membranous ossification did not show X-gal staining.

We next tried to inactivate BMP signals in a tissue-specific manner to study the role of BMPs in skeletal development. For this purpose, we expressed *Nog* in cartilage to effectively block multiple BMP signals under the control of the promoter and first intron enhancer sequences derived from *Col11a2*. Noggin has been reported to antagonize the activities of BMP-2, BMP-4, BMP-7, and GDF-5 by binding to these proteins and preventing interaction with their receptors.[21,28] The transgene construct, *Col11a2-Nog*, was introduced into the pronuclei of fertilized eggs to generate transgenic mice. The *Col11a2-Nog* transgenic mice were stillborn due to respiratory failure; therefore, we analyzed pleural generation zero (G_0) embryos (Table 1). We obtained seven mice with abnormal appearances. Average size of crown-rump length of the mice was 10% smaller than that of normal littermates. These mice showed striking skeletal defects, whereas other tissues (including the viscera, muscle, and skin) were histologically normal because transgene expression was restricted to the skeletal tissues (not shown). Alcian blue staining of cartilage showed that all the cartilage components of *Col11a2-Nog* transgenic mice were severely hypoplastic compared with those of normal mice (Figs. 1C and 1D). *Col11a2-Nog* transgenic mice only showed traces of rib cartilage, unlike the rodlike normal rib cartilage (Figs. 1F and 1G). Primordial cartilage of long bones of limbs was very hypoplastic in *Col11a2-Nog* transgenic mice compared with that of normal mice (Figs. 2A and 2B). Alizarin red staining of skeleton showed that the rib bones and the long bones of the limbs were severely hypoplastic in *Col11a2-Nog* transgenic mice compared with those of normal mice. To our analysis, every skeletal components expressing transgene was affected and minimally formed. Alizarin red staining of bone showed that skull was nearly normal, probably because the transgene was not expressed in bone undergoing membranous ossification.

Transgenic mice expressing Bmp4 *and* GDF5 *in cartilage*

We next attempted to activate single BMP signals in cartilage to characterize the action of each BMP ligand during skeletal development. We generated transgenic mice expressing *Bmp4* in cartilage under the control of the *Col11a2* promoter/enhancer sequences. The *Col11a2-Bmp4* transgenic mice were dead at birth due to respiratory failure; therefore, pleural G_0 embryos were analyzed. Activation of BMP-4 signals in cartilage led to an increase of cartilage. Alcian blue staining showed that the entire cartilaginous skeleton was enlarged and thickened when compared with normal mice (Figs. 1C and 1E). The rib cartilage of these *Col11a2-Bmp4* transgenic embryos was three times thicker in diameter on average than normal rib cartilage (Figs. 1F and 1H), resulting in loss of the intercostal spaces in the

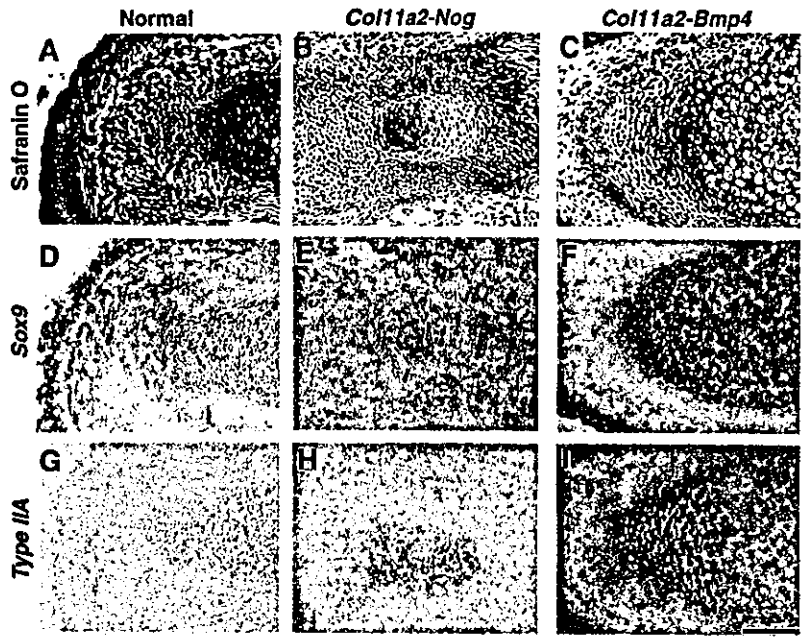

FIG. 3. Expression of *Sox9* and type IIA collagen mRNA in developing cartilage. Sections of the distal phalanges from (A) normal, (B) *Col11a2-Nog*, and (C) *Col11a2-Bmp4* transgenic mice at 16.5 d.p.c. were stained with safranin O to detect the extracellular matrix of cartilage (red). Semiserial sections were used to assess (D–F) *Sox9* expression and (G–I) type IIA collagen mRNA expression by in situ hybridization. *Sox9* is believed to regulate the transcription of genes encoding various extracellular matrix components of cartilage, including types II and XI collagen. The number of cells expressing *Sox9* was decreased in (E) *Col11a2-Nog* transgenic mice and was increased in (F) *Col11a2-Bmp4* transgenic mice when compared with (D) normal mice. (I) Expression of type IIA mRNA (*TypeIIA*), the longer form of type II collagen mRNA generated by alternative splicing, was increased in cells around the primordial cartilage of *Col11a2-Bmp4* transgenic mice, indicating the enhanced commitment of mesenchymal cells to the chondrocytic lineage. Scale bar, 100 μm.

transgenic mice (Fig. 1E). We obtained 12 G_0 transgenic embryos with such changes in their cartilage. Ten of the 12 embryos showed well-formed joints (Fig. 2C), while 2 embryos exhibited partially fused joints (Table 1). To elucidate functional difference between each BMPs, we generated mice expressing *GDF5* under the control of the identical *Col11a2* promoter/enhancer sequences as reported[19] and compared their phenotype with *Col11a2-Bmp4* transgenic mice. The skeletal phenotype of the *Col11a2-Bmp4* transgenic mice was very similar to *Col11a2-GDF5* transgenic mice. Both transgenic mice had a very similar chodrodysplasia-like skeletal phenotype with kyphosis and expansion of cartilage (Fig. 1E).[19] However, joint formation was different between these two types of transgenic mice. For *Col11a2-GDF5* transgenic mice, we obtained nine G_0 embryos with expanded skeleton, and seven of them showed completely fused joints (Table 1, Fig. 2D). These results suggest that BMP-4 and GDF-5 have similar roles in cartilage formation and different functions in joint formation during development.

Cartilage formation and BMP signals

Considering that the final target of BMP signaling in cartilage should be the genes encoding cartilage matrix components, we examined gene expression in the limb cartilage of transgenic mice using in situ hybridization. Transcriptional factor Sox9 binds to the regulatory sequences of the type II collagen gene (*Col2a1*) and *Col11a2* gene to activate their expression.[25,29,30] Therefore, we analyzed expression of *Sox9* in cartilage. The number of *Sox9*-positive cells in *Col11a2-Nog* transgenic mice was dramatically decreased compared with normal mice (Figs. 3D and 3E). In accordance with depletion of *Sox9*-positive cells, expression of *Col2a1* was also decreased in *Col11a2-Nog* transgenic mice compared with normal mice (Figs. 4D and 4E). In addition, histological analysis showed that the staining intensity of cartilage with safranin O was dramatically reduced in *Col11a2-Nog* transgenic mice (Figs. 3A, 3B, 4A, and 4B), suggesting low content of glycosaminoglycan, a component of proteoglycan in cartilage.

Conversely, there was a marked increase of cells expressing *Sox9* in *Col11a2-Bmp4* transgenic mice compared with normal mice (Fig. 3, D and F). Primordial cartilage of *Col11a2-Bmp4* transgenic mice was wider than that of normal mice (Figs. 3A, 3C, 4A, and 4C) and filled with chondrocytes expressing *Col2a1* intensely at epiphyseal regions (Fig. 4F). Widening of primordial cartilage in *Col11a2-Bmp4* transgenic mice may be attributed to increased number of chondroprogenitor cells around cartilage. This idea was tested by expression analysis of type II collagen mRNA. In the early stage of chondrocyte development, two forms of type II procollagen are generated by alternative splicing of the exon 2 sequence.[31] The longer form (type IIA) containing the exon 2 sequence is predominantly expressed by immature chondroprogenitor cells.[26] Expression of type IIA mRNA was greater in the cells around *Col11a2-Bmp4* transgenic cartilage than in cells in wild-type cartilage (Figs. 3G and 3I), suggesting the increased number of chondroprogenitor cells in BMP-4 transgenic cartilage.

Chondrocyte differentiation in transgenic mice

After commitment of mesenchymal cell to chondrocytic lineage, proliferating chondrocytes produce the short form of type II collagen[26] (Fig. 4D) and build an extracellular matrix for cartilage that contains abundant glycosaminoglycans, which can be stained with safranin O (Fig. 4A). Along with differentiation to mature hypertrophic chondrocytes, the expression of *Col2a1* ceases, and the cells begin to express type X collagen gene (*Col10a1*; Fig. 4G), so that the matrix architecture becomes suitable for subsequent bone formation. Compared with normal mice, *Col11a2-Nog*

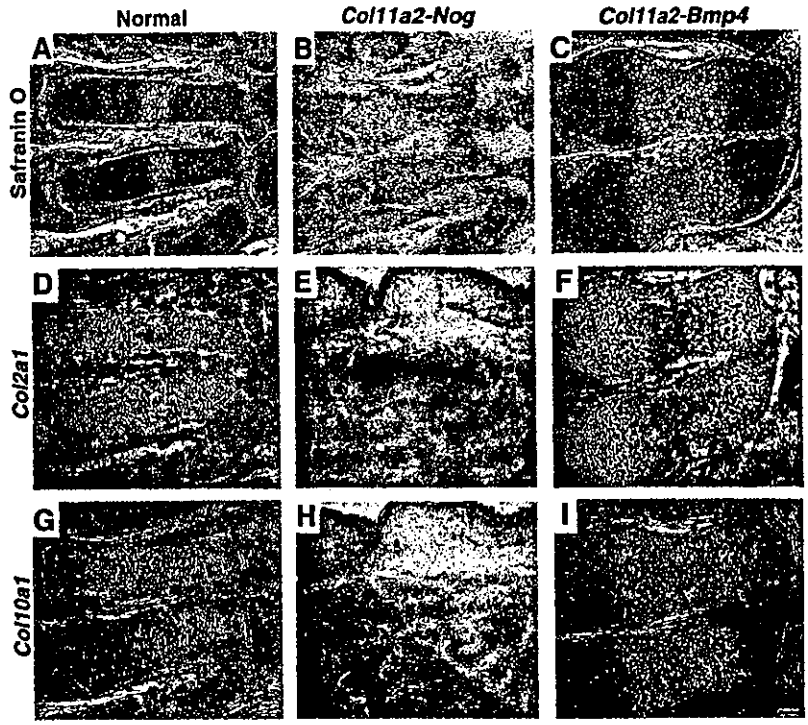

FIG. 4. Influence of the modification of BMP signaling on chondrocyte differentiation. Sections of metacarpals from (A) normal, (B) *Col11a2-Nog*, and (C) *Col11a2-Bmp4* transgenic mice at 16.5 d.p.c. were stained with safranin O to detect the extracellular matrix of cartilage (p, proliferating chondrocytes; h, hypertrophic chondrocytes). Semiserial sections were used to assess (D–F) type II collagen gene (*Col2a1*) and (G–I) type X collagen gene (*Col10a1*) expression by in situ hybridization. In wild-type metacarpals, both ends of each skeletal component consisted of proliferating chondrocytes marked by (D) *Col2a1* expression and the cartilage had (A) abundant extracellular matrix. Differentiation proceeds from the central part of each skeletal component, with chondrocytes becoming (A) hypertrophic and changing from production of (D) type II collagen to (G) type X collagen. In *Col11a2-Nog* transgenic mice, (B) the primordial cartilage was hypoplastic and contained little extracellular matrix with (E) weak *Col2a1* expression. The cartilage only contained *Col2a1*-positive cells and expression of (H) the *Col10a1* was not detected, suggesting impairment of the differentiation process. On the other hand, the primordial cartilage was expanded in *Col11a2-Bmp4* transgenic mice and contained (C) abundant matrix with (F) *Col2a1*-positive cells at both ends of each skeletal component. At the central part of each component, (C) the zone of hypertrophic chondrocytes was markedly wider than normal and (I) expression of the *Col10a1* was prominent indicating the acceleration of chondrocyte differentiation. Scale bar, 100 μm.

transgenic mice had hypoplastic cartilage with weak safranin O staining (Figs. 4A and 4B). In situ hybridization showed that the cartilage of these mice contained cells expressing the *Col2a1* gene (Fig. 4E) but lacked *Col10a1*-positive cells (Fig. 4H). In addition, type IIA mRNA, a marker of immature chondroprogenitor cells, was not only expressed by peripheral cells but also by cells at the center of the primordial cartilage (Fig. 3H). These findings suggest that *Col11a2-Nog* transgenic cartilage was composed of relatively immature chondrocytes.

On the other hand, *Col11a2-Bmp4* transgenic mice showed expansion of cartilage, with an increase in the thickness of the zone of hypertrophic chondrocytes (Fig. 4C). The number of *Col10a1*-positive cells was also increased in *Col11a2-Bmp4* transgenic mice compared with normal mice (Figs. 4G and 4I).

DISCUSSION

To examine the physiological function of BMPs during skeletogenesis in vivo, we generated transgenic mice expressing Noggin or BMPs under the control of the same cartilage-specific promoter/enhancer sequences derived from *Col11a2*. The resultant transgenic mice showed distinct skeletal abnormalities, although their other tissues developed normally because of the high tissue specificity of the promoter/enhancer sequences. These results provide convincing evidence that BMPs play an important role in mammalian skeletogenesis.

BMPs are required for cartilage formation

A striking feature of the *Col11a2-Nog* transgenic mice was the absence of nearly all cartilage. In these mice, Noggin was overexpressed in the mesenchymal condensation at 12.5 d.p.c. and subsequently was overexpressed in the proliferating chondrocytes of all primordial cartilage. Noggin has been reported to antagonize the activities of BMP-2, BMP-4, BMP-7, and GDF-5 by binding to these proteins and preventing interaction with their receptors.[21,28] Therefore, an excess amount of Noggin might have dramatically depressed the activities of these BMPs in the cartilage of *Col11a2-Nog* transgenic mice.

It has been shown that overactivity of BMPs causes expansion of the cartilage in Noggin knockout mice[7] and GDF-5 transgenic mice.[19] In addition to these findings, a definitive assessment of the importance of BMP in normal skeletogenesis might be achieved by loss-of-function studies. Inactivation of *Bmp7* causes fused ribs and polydactyly of the hindlimbs,[14,15] whereas the loss of GDF-5 activity results in shortening of the appendicular skeleton in *brachypod* (bp) mice.[16] In these mice, cartilage is generally formed despite morphological changes to limited parts of the skeleton. Such relatively minor changes of the cartilage raise the possibility that other coexpressed BMP family members can compensate functionally for the absence of a protein in these mice. Our results also support this possibility. Expansion of cartilage in *Col11a2-Bmp4* transgenic mice indicates that BMP-4 has a potent cartilage-forming effect. A study in *Col11a2-GDF5* transgenic mice has shown that GDF-5 also has a potent cartilage-forming activity.[19] Therefore, the signals for formation of cartilage seem to be reinforced by multiple BMPs. Conversely, the absence of most cartilage in *Col11a2-Nog* transgenic mice clearly indicates the importance of BMPs for cartilage formation, because very little cartilage developed without BMPs. From these results, we speculate that multiple BMPs may be expressed in cartilage to ensure its formation during development, because of a highly important role of BMPs in cartilage formation.

Regarding bone formation in *Col11a2-Nog* transgenic mice, the calvarium appeared to be normal because the transgene was not expressed throughout the process of membranous ossification. On the other hand, the ribs and the long bones of the limbs were severely hypoplastic in these mice, with the limb bones being more severely affected (Fig. 1D). During endochondral ossification, *Col11a2* promoter/enhancer sequences are reported to direct expression in proliferating chondrocytes and weakly in hypertrophic chondrocytes, but not in bone.[20] Therefore, hypoplasia of the limb bones and ribs in the transgenic mice was a consequence of hypoplastic primordial cartilage.

BMP-4 and GDF-5 have distinct roles in joint formation

The general skeletal phenotype of *Col11a2-Bmp4* transgenic mice was very similar to that of transgenic mice expressing *GDF5* under the control of the same *Col11a2* promoter/enhancer sequences. *Col11a2-Bmp4* and *Col11a2-GDF5* transgenic mice both had a very similar chodrohysplasia-like skeletal phenotype with kyphosis and expansion of the cartilage (Fig. 1E).[19] These similar gross skeletal abnormalities confirmed that the pattern and level of transgene expression did not differ between *Col11a2-Bmp4* and *Col11a2-GDF5* transgenic mice. However, joint formation showed differences between these two types of mice. *Col11a2-Bmp4* transgenic mice usually had well-formed joints, whereas *Col11a2-GDF5* transgenic mice usually showed fusion of the joints. This difference may suggest the existence of unique signaling pathways in cartilage for each of these BMP family members. Various receptors for BMPs (BMPRs) have been identified so far, and it is known that the affinity for these receptors differs between BMPs.[32] Experiments using retroviral vectors to deliver activated BMPRs in chicks have demonstrated that BMPRIA and BMPRIB regulate distinct processes in the formation and differentiation of cartilage.[5] In addition, various Smads and other molecules may transduce intracellular signals from BMPs. It remains to be determined how each BMP activates specific signals and exerts its unique effect during skeletogenesis.

It has been reported that Noggin-deficient mice showed excess cartilage formation and impaired joint development due to BMP overactivity.[7] As shown in this study, activation of BMP-4 or GDF-5 also caused the expansion of cartilage, but joint formation was much less disturbed in *Col11a2-Bmp4* transgenic mice (Fig. 2C) than in *Col11a2-GDF5* transgenic mice (Fig. 2D). Therefore, the cartilage changes in *Noggin* knockout mice seem to arise from a combination of abnormalities caused by the enhanced signaling of several BMPs, including GDF-5 and BMP-4. Comparison of the phenotype of *Noggin* knockout mice with transgenic mice expressing each *Bmp* in cartilage may help to elucidate the mechanism by which multiple BMPs cooperate in the regulation of skeletal development. We speculate that the joint fusion seen in mice with inactivation of the *Noggin* gene may be caused by GDF-5 among the various BMPs expressed in cartilage.

Recently, it was proposed that Wnt-14 plays a critical role in the initiation of joint development and in the spacing of the joints.[33] Wnt-14 expressed in the developing joint interzone may induce expression of *Gdf5*, which could act on neighboring cartilage elements to prevent the induction of a new interzone. This action seems to be modulated by BMPs that are produced by cells surrounding cartilage elements.[34] BMP-2 and BMP-4 are expressed in perichondrium.[35] Our results support this mechanism for the spacing of the joints. Fused joints in *Col11a2-GDF5* transgenic mice indicated that GDF-5 may inhibit joint formation, whereas well-formed joints in *Col11a2-Bmp4* transgenic mice suggest that BMP-4 may promote joint formation. BMP-4 and GDF-5 may play opposing roles during the process of joint formation in the downstream of Wnt-14 signals.

Mechanism for regulation of cartilage formation by BMPs

Cartilage is composed of chondrocytes embedded in an abundant extracellular matrix. Histological examination of transgenic mice showed that cartilage formation occurred through production of matrix and an increase in the number of chondrocytes. First, we analyzed the expression of the *Col2a1* and *Sox9* genes to examine how BMPs controlled the production of cartilage matrix components. *Sox9* encodes a transcriptional factor that regulates the expression of cartilage-specific collagen genes, including *Col2a1* and *Col11a2*.[25,29,30] In *Col11a2-Nog* transgenic mice (in which BMP activity might be depressed), expression of *Sox9* was decreased, probably resulting in a decrease of *Col2a1* expression. This observation is consistent with the effects of Noggin on the limbs of chicks when delivered with a retroviral vector.[36] On the other hand, overactivation of BMP-4 in *Col11a2-Bmp4* transgenic mice might cause an increase of *Sox9* expression, leading to high levels of

Col2a1 expression. It has been reported that inactivation of *Sox9* results in the abolition of cartilage formation, because there is no cartilage in teratomas derived from $Sox9^{-/-}$ embryonic stem cells.[37] The severe cartilage hypoplasia in *Col11a2-Nog* transgenic mice seems to be a similar result to the effect of inactivating *Sox9* in embryonic stem cells. These observations suggest that BMPs may control cartilage formation by regulating the expression of the *Sox9* gene. In this context, it has also been reported that application of BMP-2 to chick limbs[38] and mesenchymal cells[39] results in the upregulation of *Sox9*.

Next, we examined the mechanism of chondrocyte proliferation. In the early stage of chondrocyte development, two forms of type II procollagen are generated by alternative splicing of exon 2.[31] The longer form (type IIA) containing the exon 2 sequence is predominantly expressed by immature chondroprogenitor cells.[26] Expression of type IIA collagen mRNA was greater in the cells around *Col11a2-Bmp4* transgenic cartilage than in cells in wild-type cartilage (Figs. 3G and 3I). An increase of type IIA mRNA expression is also observed in the cartilage of transgenic mice with overexpression of *GDF5*.[19] In these mice, the activation of BMP signaling in cartilage might enhance the commitment of mesenchymal cells to the chondrocytic lineage, contributing to expansion of the primordial cartilage.

BMP signaling and chondrocyte differentiation

A striking feature in the cartilage of *Col11a2-Bmp4* transgenic mice was the increased thickness of the hypertrophic zone accompanied by a reduced thickness of the proliferating chondrocyte zones. Similar findings were also observed in the cartilage of *Col11a2-Gdf5* transgenic mice.[19] Endochondral bone formation is initiated when chondrocytes in the center of the primordial cartilage proliferate and differentiate into hypertrophic chondrocytes. Hypertrophic chondrocytes are eventually replaced by osteoblasts in the process of bone formation. This change radiates outward with formation of the growth plates at both ends of the primordial cartilage and these events are represented histologically by zones of proliferative and hypertrophic chondrocytes.[40] The reduction in the height of the proliferating zone in *Col11a2-Bmp4* transgenic mice seems to be caused by accelerated differentiation into hypertrophic chondrocytes. It is conceivable that enhanced differentiation of these cells into hypertrophic chondrocytes caused the increased height of the zone of hypertrophy in the transgenic mice. In situ hybridization with *Col10a1*, a marker for hypertrophic chondrocytes, showed enlargement of the hypertrophic zone. Conversely, *Col11a2-Nog* transgenic mice had hypoplastic cartilage that lacked signals for *Col10a1* (Fig. 4H). In addition, type IIA collagen mRNA, a marker of immature chondroprogenitor cells, was expressed by most of the chondrocytes in primordial cartilage (Fig. 3H), suggesting that Noggin may inhibit differentiation and maintain chondrocytes in an immature state. Taken together with the absence of mature hypertrophic chondrocytes in *Col11a2-Nog* transgenic mice, the increase of hypertrophic cells in *Col11a2-Bmp4* (Fig. 4C) and *Col11a2-Gdf5* transgenic mice[19] indicates that activation of BMP-4 or GDF-5 results in the acceleration of chondrocyte differentiation.

Role of BMPs in cartilage development

By overexpressing *Nog* in chondrocytes, we created mice that lacked cartilage, thus showing that BMP signaling is essential for cartilage development. In addition, we characterized the influence of BMP-4 on cartilage by assessing the response to its overactivation in mouse chondrocytes. Activation of BMP-4 resulted in cartilage expansion and promoted chondrocyte differentiation, as did activation of GDF-5. These observations indicate that the signals for cartilage production and chondrocyte differentiation are reinforced by multiple BMPs. In addition, the lack of cartilage in *Col11a2-Nog* transgenic mice suggests that the loss of BMP signals could not be compensated by other growth factors such as fibroblast growth factors (FGFs) or hepatocyte growth factor (HGF), despite possible interactions of the intracellular signaling pathways for these factors.[41] Comparison of skeletal differences between *Col11a2-Bmp4* and *Col11a2-GDF5* transgenic mice suggested the possible existence of unique signaling pathways in the cartilage for each BMP family member. These unique intracellular signaling pathways are subjects for further investigation.

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Distribution of genes for parathyroid hormone (PTH)–related peptide, Indian hedgehog, PTH receptor and patched in the process of experimental spondylosis in mice

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Object. Little is known about the molecular mechanisms underlying the process of spondylosis. The authors determined the extent of genetic localization of major regulators of chondrogenesis such as Indian hedgehog (Ihh) and parathyroid hormone (PTH)–related peptide (PTHrP) and their receptors during the development of spondylosis in their previously established experimental mouse model.

Methods. Experimental spondylosis was induced in 5-week-old ICR mice. The cervical spines were chronologically harvested, and histological sections were prepared. Messenger (m) RNA for PTHrP, Ihh, PTH receptor (PTHR; a receptor for PTHrP), patched (Ptc; a receptor for Ihh), bone morphogenetic protein (BMP)–6, and collagen type X (COL10; a marker for mature chondrocyte) was localized in the tissue sections by performing in situ hybridization.

In the early stage, mRNA for COL10, Ihh, and BMP-6 was absent; however, mRNA for PTHrP, PTHR, and Ptc was detected in the anterior margin of the cervical discs. In the late stage, evidence of COL10 mRNA began to be detected, and transcripts for Ihh, PTHrP, and BMP-6 were localized in hypertrophic chondrocytes adjacent to the bone-forming area in osteophyte. Messenger RNA for Ptc and PTHR continued to localize at this stage. In control mice, expression of these genes was absent.

Conclusions. The localization of PTHrP, Ihh, BMP-6, and the receptors PTHR and Ptc demonstrated in the present experimental model indicates the possible involvement of molecular signaling by PTHrP (through the PTHR), Ihh (through the Ptc), and BMP-6 in the regulation of chondrocyte maturation leading to endochondral ossification in spondylosis.

KEY WORDS • **spondylosis** • **parathyroid hormone–related peptide** • **Indian hedgehog** • **in situ hybridization** • **gene expression** • **experimental model**

MOLECULAR signaling regulating the process of osteophyte formation in spondylosis remains incompletely elucidated. Standard histological examination has shown that the formation of an osteophyte is a novel form of osteochondrogenesis, a process resembling that of endochondral ossification,[14,15] which is observed in bone and cartilage formation during fracture repair[22] and fetal development.[2]

We previously reported the involvement of the molecular signaling of BMP families, one of the major osteoinductive agents, in a mouse spondylosis model.[19] We clarified the involvement of molecular signaling of several BMP members and receptors in the chondrogenesis observed in spondylosis.[19] In our previous publication, however, we were unable to address the molecules regulating endochondral ossification in spondylosis.

Recently, two molecules, PTHrP and Ihh, have been identified as major regulators of endochondral ossification.[4,11,25] The transition of chondrocytes from a state of proliferation to maturation is a critical step in endochondral ossification.[21] Reportedly PTHrP promotes proliferation and inhibits maturation of chondrocytes.[1,4,13,16] It has also been reported that Ihh upregulates PTHrP expression,[25] and in turn, PTHrP downregulates Ihh expression to form a negative-feedback loop.[25] In such a manner, PTHrP and Ihh reportedly control endochondral ossification.[4,11,25] Furthermore, BMP-6 has been recently implicated in the regulation endochondral ossification in a coordinated manner (Fig. 1).[6]

These PTHrP, Ihh, and BMP-6 molecules are reportedly localized in chondrocytes in fetal growth cartilage and at sites of fracture healing,[8,17,23,26] and we recently reported on *BMP-6* gene localization in an experimental spondylosis model in mice.[19] Our observations led us to hypothesize

Abbreviations used in this paper: ALC = anulus-ligament complex; BMP = bone morphogenetic protein; COL10A1 = alpha 1 chain of collagen type X; Ihh = Indian hedgehog; mRNA = messenger RNA; Ptc = patched; PTHrP = parathyroid hormone-related peptide; PTHR = parathyroid hormone receptor.

that PTHrP, BMP-6, and Ihh signaling is involved in spondylosis, possibly occurring specifically at the area of osteophyte formation believed to mimic endochondral ossification. To the best of our knowledge, no authors have published work on the involvement of PTHrP/BMP-6/Ihh signaling in spondylotic processes or osteophyte formation.

The purpose of this study was to extend the findings of our previous study by further analyzing the molecular mechanisms of spondylosis that mimic endochondral ossification. We investigated the signaling-related localization patterns of PTHrP, BMP6, and Ihh in spondylosis by investigating expression of these molecules. Distributions of genes for PTHrP and Ihh together with PTH receptors (PTHR, a receptor for PTHrP,[9] and Ptc, a receptor for Ihh)[24] were examined using in situ hybridization. Messenger RNA for the COL10A1, a marker for maturated chondrocyte,[10] was also examined in this study.

Materials and Methods

Murine Spondylosis Model

We used 27 5-week-old male ICR mice weighing 22 ± 2 g (± standard deviation) as previously described.[19] In 12 mice the spinous processes and related ligaments were resected as previously described.[18,19] Anesthesia was induced, and the cervical spinal sections were removed at 1, 2, 4, and 6 months postoperatively (E1, E2, E4, and E6 [three mice each at each time point]). Age-matched controls underwent the same procedure except that no surgery (no skin incision was performed (C1, C2, C4, and C6 [three mice each at each time point]), as did three 5-week-old mice (E0). All procedures were conducted in accordance with the guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Preparation of Tissue Sections

Tissue samples were prepared as previously described.[20] Briefly, they were fixed in 4% paraformaldehyde (pH 7.4), decalcified in 20% ethylenediaminetetraacetic acid, dehydrated in graded ethanol series, and embedded in paraffin. Sections 4 μm thick were prepared using a microtome for in situ hybridization. Sections were also prepared for safranin-O–fast green staining to distinguish the cartilaginous matrix.

Preparation of Probes

Digoxigenin-labeled single-strand RNA probes were prepared using a specific kit (DIG RNA Labeling Kit; Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) as described previously.[20] Complementary RNA probes used in this study are as follows: murine COL10A1[10] (a gift from Dr. K. S. E. Cheah, University of Hong Kong, China), murine Ihh,[8] murine BMP-6[5] (a gift from Dr. S. E. Gitelman, University of California, San Francisco, CA), murine Ptc[24] (a gift from Dr. M. P. Scott, Stanford University School of Medicine, CA), rat PTHR (PTH1 receptor),[12] and rat PTHrP[12] (rat PTHR and rat PTHrP were gifts from Dr. G. N. Segre, Massachusetts General Hospital, Boston, MA, and Dr. T. Koike, Osaka City University School of Medicine, Osaka, Japan).

In Situ Hybridization

Hybridization was performed as previously described.[20] Briefly, paraffin-embedded sections were dewaxed, fixed with 4% paraformaldehyde, and treated with 0.2 N HCl for 10 minutes to inactivate endogenous alkaline phosphatase; hybridization was performed at 50°C for 16 hours. After hybridization, the slides were washed as previously described.[19,20] Hybridized probes were detected using a nucleic acid detection kit (Boehringer Mannheim GmbH Biochemica) according to the manufacturer's instructions. Controls included 1) hybridization with the sense (mRNA) probes, 2) ribonuclease A treatment (20 μg/ml) before hybridization, and 3) use of neither antisense nor antidigoxigenin antibody. No positive signals were demonstrated in the control specimens. The number of positive cells in each in situ hybridization analysis was based on the cell counts. The total number of positive cells in the anterior one-third portion from cranial to caudal discovertebral junction was counted at × 200 magnification by using a microscope.[7,19]

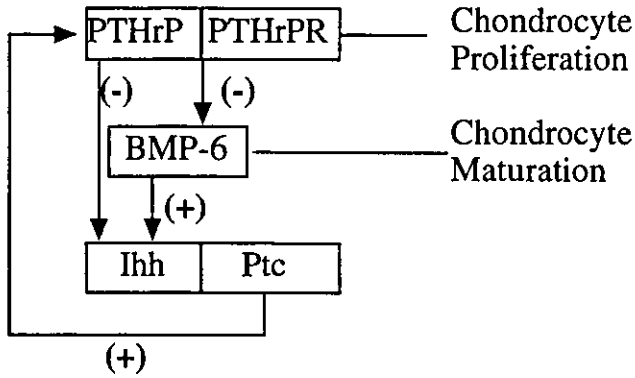

FIG. 1. Diagram showing the negative-feedback loop produced by PTHrP, BMP-6, and Ihh based on previous findings. The PTHrP is a potent promoter of chondrocyte proliferation and inhibits chondrocyte maturation. The BMP-6 is an autocrine stimulator for chondrocyte maturation. Both Ihh and BMP-6 are positive stimulators of PTHrP expression. The PTHrP suppresses BMP-6 and subsequently suppresses Ihh expression. Thus, PTHrP downregulates its own positive stimulators to create a negative-feedback loop.

Results

Histological Findings

Details of the histological findings in this model have been described in our recent report.[19]

Preoperative Stage. Histological examination of the spines in the three mice that did not undergo surgery (E0) showed normal morphological features; the nucleus pulposus was surrounded by many layers of annulus fibrosus, and cells in the annulus fibrosus were spindle shaped (Fig. 2A).

Postoperative Stage. Histological events occurring in the postoperative stage were most clearly observed in the anterior portion of the spines. In the early phase (E1 and E2 mice were categorized into this group; representative, photomicrographs of spinal specimens obtained in E2 mice are shown in Fig. 2B), some of the cells in the outer layer of the annulus fibrosus showed round chondrocyte-like cell morphological features. No prominent osteophyte formation was observed. In the age-matched control murine groups, such metaplasia of the cells in the outer layer of the annulus was not observed.[19] In the late phase (E4 and E6 mice were categorized into this group; representative, photomicrographs of spinal specimens obtained in E6 mice are shown in Fig. 2C), the lamellar structure of the annulus was severely disorganized. An osteophyte containing new bone and cartilage formation was observed in the anterior margin of intervertebral space. Spindle-shaped cells in the annulus were lost and replaced by round chondrocyte-like cells. In the age-matched control murine groups, spindle-shaped cells remained, and there was no evidence of cartilage and osteophyte formation.[19]

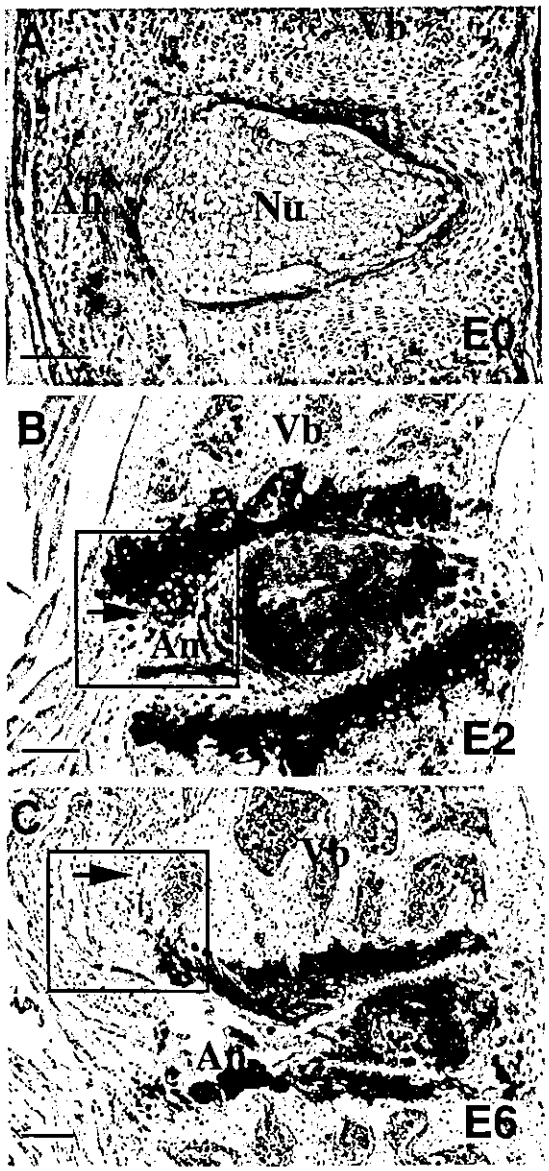

FIG. 2. Photomicrographs showing a sagittal C4–5 section obtained in E0 (A), E2 (B), and E6 (C) mice. The left side indicates anterior; right side, the posterior; upper, the cranial; and lower indicates caudal directions. A: Lamellar structure of annulus fibrosus (An) surrounding nucleus pulposus (Nu) is apparent. B: Part of the annulus shows disorganization, and cells in ALC show round chondrocyte-like morphological features *(arrow)*. The osteophyte is not apparent. C: The lamellar structure of the annulus is severely disorganized. Prominent osteophyte formation is observed in the anterior margin of discovertebral junction *(arrow)*. Safranin-O–fast green staining; cartilaginous matrix is indicated as dark area; bar = 50 μm.

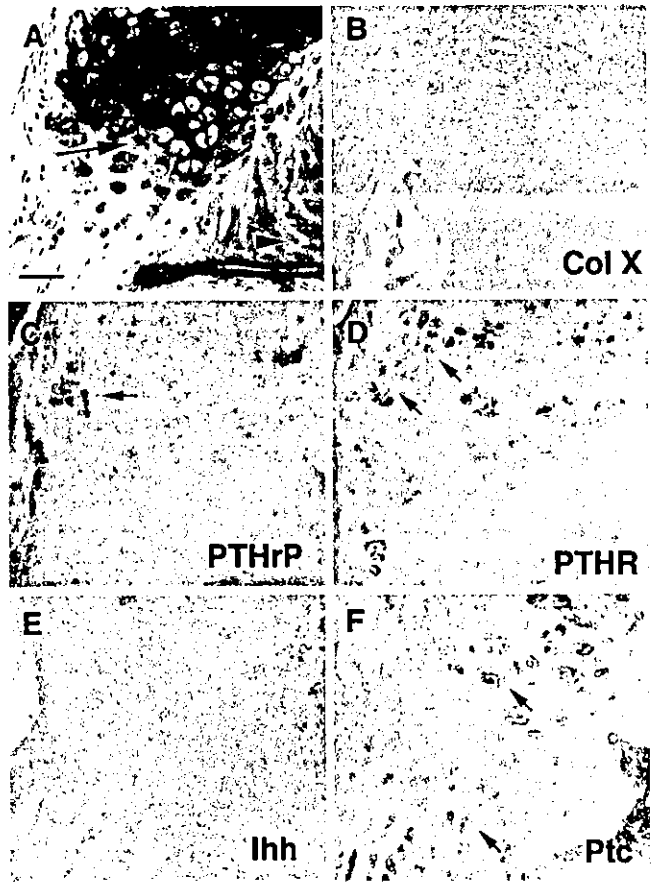

FIG. 3. Photomicrographs showing the histological (A) and distribution patterns of mRNA for COL10A1 (B), PTHrP (C), PTHR (D), Ihh (E), and Ptc (F) in the anterior margin of cervical spine in early-stage experimental spondylosis. All photomicrographs are representative of sagittal C4–5 sections obtained in an E2 mouse. A: Higher magnification of square area shown in Fig. 2B. Metaplasia of spindle-shaped cells to chondrocyte-like cells in the anterior ALC *(arrows)* is apparent. The cell in the inner layer shows spindle-shaped morphology *(arrowheads)*. B–F: Transcripts for COL10A1 (B) and Ihh (E) were not detected, whereas those for PTHrP (C) and PTHR (D) were detected in chondrocyte-like cells in the anterior ALC *(arrows)*. Messenger RNA for Ptc was detected in chondrocyte-like cells in the ALC and the outer layer of annulus *(arrows)*. Safranin-O–fast green staining; bar = 20 μm.

In Situ Hybridization

The photomicrograph in Fig. 3A shows representative histological features of the anterior margin of cervical spine in early-phase spondylosis. In the early phase, COL10A1 mRNA was absent (Fig. 3B). We found PTHrP mRNA in chondrocyte-like cells in the ALC in the anterior margin of the disc (Fig. 3C) together with PTHR mRNA (Fig. 3D), whereas neither Ihh (Fig. 3E) nor BMP-6 mRNA (data not shown) was detected in this early stage. Transcripts for Ptc (Fig. 3F) were localized in chondrocyte-like cells in the ALC and the outer layer of annulus fibrosus in the anterior margin of the disc.

The photomicrograph in Fig. 4A shows representative histological features of the anterior margin of cervical spine in late-phase spondylosis. In the late phase, COL10A1 mRNA began to be detected in round chondrocytes in the area of newly formed osteophyte in the anterior margin (Fig. 4B), and PTHrP mRNA was localized (Fig. 4C) in hypertrophic chondrocytes in the same area. The PTHR mRNA continued to be expressed in chondrocytes (Fig. 4D). The Ihh mRNA began to be detected (Fig. 4E) in hypertrophic chondrocytes, and Ptc mRNA continued to be

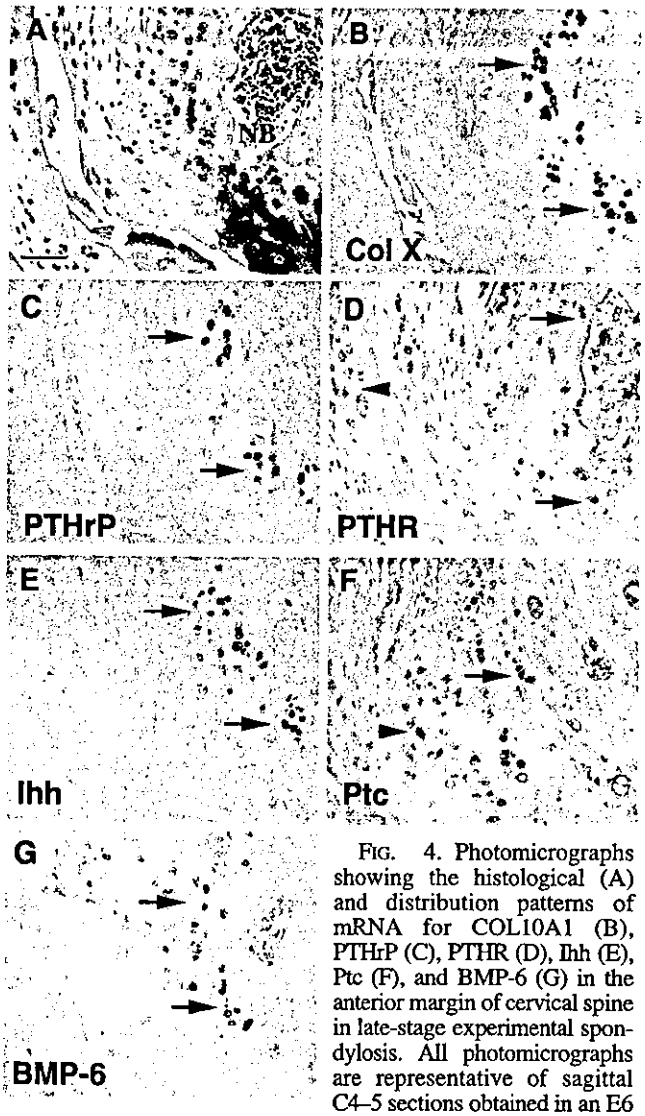

FIG. 4. Photomicrographs showing the histological (A) and distribution patterns of mRNA for COL10A1 (B), PTHrP (C), PTHR (D), Ihh (E), Ptc (F), and BMP-6 (G) in the anterior margin of cervical spine in late-stage experimental spondylosis. All photomicrographs are representative of sagittal C4–5 sections obtained in an E6 mouse. A: Higher magnification of square area in Fig. 2C. Lamellar structure of the anulus is lost and no spindle-shaped cells are observed. Numerous chondrocyte-like cells are apparent in the outer layer of the anulus. Prominent osteophyte formation containing newly formed bone is visible. NB = newly formed bone. B–G: COL10A1 (B), PTHrP (C), Ihh (E), and BMP-6 (G) mRNAs are detected in round, hypertrophic chondrocytes *(arrows)* adjacent to the newly formed bone osteophyte. Both PTHR (D) and Ptc (F) mRNA was detected not only in hypertrophic chondrocytes *(arrows)* but also in other proliferating chondrocytes *(arrowheads)*. Safranin-O–fast green; bar = 20 μm.

expressed in chondrocytes (Fig. 4F). We found evidence of BMP-6 mRNA expression (Fig. 4G). Localization of COL10A1, Ihh, PTHrP, and BMP-6 mRNAs was mainly observed in hypertrophic round chondrocyte-like cells adjacent to newly formed bone. In contrast, mRNA for Ptc and PTHR showed wider distribution.

Messenger RNAs for COL10A1, Ihh, Ptc, PTHrP, PTHR, and BMP-6 were not detected in cells in the intervertebral region of the young control specimens (E0) or of the age-matched control specimens (C1, C2, C4, and C6) (data not shown). The cell count assessment (Table 1) demonstrated that the number of cells positive for Ihh and BMP-6 mRNA was significantly higher in the late stage than in the early stage. Although PTHrP mRNA was expressed both in the early and late stage, its expression was higher in the late stage. Messenger RNAs for Ptc and PTHR were expressed throughout the spondylosis process. These patterns of gene distribution were most clear in the C4–5 and C5–6 regions. Similar trends were also observed in the other sites (C2–4 and C6–7).

Discussion

To the best of our knowledge, this is the first description implicating the involvement of the molecular signaling by PTHrP and Ihh and their receptors in the process of spondylosis. Both PTHrP and Ihh showed specific localization in osteophytic tissue, particularly in the late stage of spondylosis. The present findings of the cellular localization of PTHrP, Ihh, and their receptors do not conflict with those previously published in which endochondral ossification in fracture callus was studied.[8,26] In our most recent study, we reported the pattern of BMP-6 transcript localization in the process of experimental spondylosis.[19] In the age-matched (nonsurgery) groups, genetic expression of *COL10*, *PTHrP*, *Ihh*, *PTHR*, and *Ptc* was absent, indicating that resection of posterior spinal elements in this model may enhance the maturation of chondrocytes and the molecular activation of PTHrP, Ihh, and their receptors.

Early-Stage Spondylosis

Because COL10 is believed to be a marker for chondrocyte hypertrophy leading to endochondral ossification,[10] the absence of COL10A1 mRNA in early-stage spondylosis indicates that chondrocytes were still immature and endochondral ossification had not yet begun. Messenger RNAs for PTHrP and its receptor (PTHR) were detected in chondrocyte-like cells in the ALC. In light of the reported potent effect of PTHrP on chondrocytes,[1,4,16] these findings suggest that PTHrP may promote the proliferation of chondrocytes during early-phase spondylosis. Also at this stage, mRNA expression for the Ihh receptor (Ptc) was localized in the anterior margin of the disc, whereas mRNA for Ihh was not detected in any region. This finding raises the possibility that other ligands for Ptc, such as sonic hedgehog,[3] may be upregulated at this stage, a notion that could not be addressed in the present study.

Late-Stage Spondylosis

In the late stage, the COL10A1 mRNA began to be localized in the area adjacent to newly formed bone in the osteophyte. This observation indicates that round chondrocyte-like cells appearing in late-stage spondylosis have a mature chondrocyte phenotype (hypertrophic chondrocyte). Interestingly, we detected focal localization of mRNAs for PTHrP, Ihh, and BMP-6 in hypertrophic chondrocytes in proximity to the bone-forming area at this stage, suggesting that they play a contributory role in the process of osteophyte formation. Messenger RNA for PTHrP and Ptc continued to be localized even in this late stage. These findings suggest that PTHrP and Ihh act

TABLE 1

*Distribution of mRNA for PTHrP, Ihh, BMP-6, PTHR, and Ptc in the anterior margin of C4–5 throughout experimental spondylosis**

Murine Group	Ligands (%)			Receptors (%)	
	PTHrP	Ihh	BMP-6	PTHR	Ptc
preop (E0)	0	0	0	0	0
early stage (E1 & E2)	7.7 ± 1.6	0	0	29.3 ± 4.2	27.1 ± 2.5
late stage (E4 & E6)	22.0 ± 6.4	25.7 ± 2.2	32.0 ± 3.6	40.4 ± 9.8	35.8 ± 3.8

* All values are presented as the means ± standard deviations.

through PTHR and Ptc, respectively. The colocalization of PTHrP and Ihh ligands and their corresponding receptors (PTHR and Ptc) suggests the possible involvement of local paracrine signalings of PTHrP–PTHR and Ihh–Ptc in late-stage spondylosis.

Contribution of Ihh, BMP-6 and PTHrP Signaling to the Process of Late Spondylosis

Other investigators have recently reported that PTHrP, Ihh, and BMP-6 regulate the pace of endochondral ossification by controlling the rate of chondrocyte maturation[1,4,6,11,13,16,25] (Fig. 1). We recently reported upregulation of the *BMP-6* gene in late-phase spondylosis,[19] and findings in the present study have expanded this knowledge by implicating the colocalization of PTHrP, BMP-6, and Ihh in late-phase spondylosis. Analysis of our findings suggests the existence of a molecular loop involving these three molecules in spondylosis-related osteophyte formation. Whether such a loop is active or how it works, however, remains to be elucidated. Furthermore, whether the same mechanisms are involved in the human is an important issue for future research.

Finally, we have proposed a molecular mechanism of endochondral ossification in spondylosis (Fig. 5). In the early phase, PTHrP may promote early chondrocyte proliferation through PTHR in the ALC in the anterior margin of the disc. In the late phase, at the very least, PTHrP, BMP-6, and Ihh, together with their receptors PTHR and Ptc, are involved in the process of osteophyte forma-

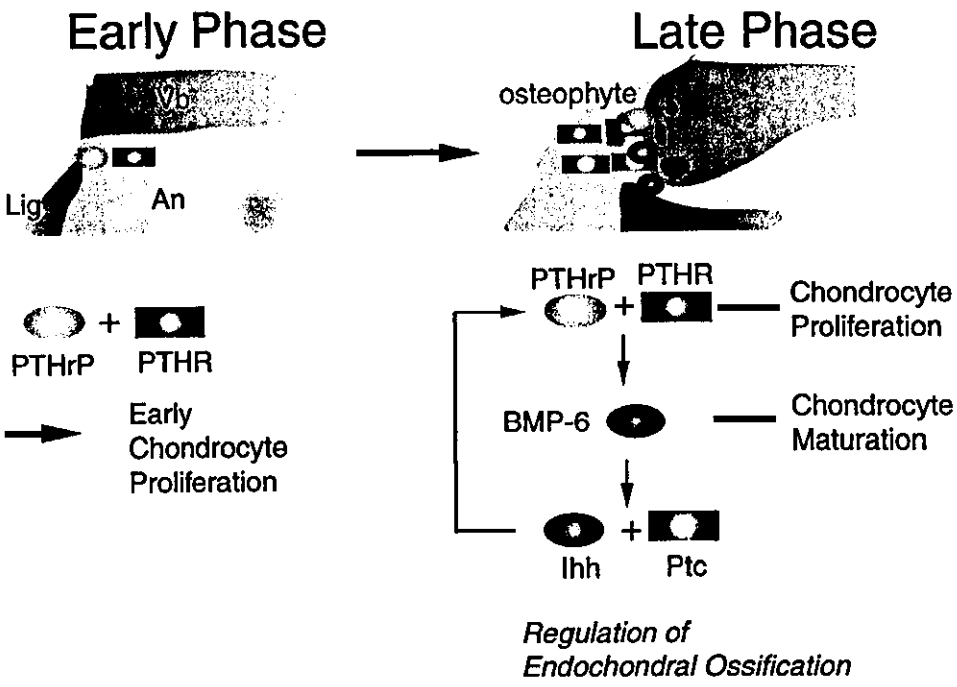

FIG. 5. Proposed model for molecular progress of chondrocyte maturation leading to endochondral ossification in experimental spondylosis. In the early phase, PTHrP may stimulate the early chondrocyte proliferation through the PTHR. In the late phase, PTHrP mRNA was synthesized in the mature chondrocytes adjacent to the osteophyte in the anterior portion of disc and vertebral junction. The PTHrP promotes proliferation of chondrocytes located in proximity to mature chondrocytes through the PTHR. The BMP-6 promotes chondrocyte maturation. Thus, PTHrP is a negative and BMP-6 a positive regulator of chondrocyte maturation. Through its receptor PTHR, PTHrP regulates BMP-6 expression; BMP-6 regulates Ihh expression, and Ihh (through its receptor Ptc) regulates PTHrP expression. In this manner, PTHrP, BMP-6, and Ihh regulate endochondral ossification in late-stage spondylosis. An = anulus fibrosus; Vb = vertebral body; Lig = ligament. *Arrows* indicate the regulation of gene expression.

tion. Chondrocyte maturation may be regulated by PTHrP, BMP-6, and Ihh, leading to endochondral ossification through PTHR and Ptc.

Conclusions

The present data indicate that, postoperatively, transcripts for PTHrP, Ihh, BMP-6, PTHR, and Ptc are focally colocalized in chondrocytes adjacent to newly formed bone in the osteophyte in the murine spondylosis model. These findings suggest the involvement of PTHrP/PTHR signaling in early-stage spondylosis, as well as the activation of signalings by PTHrP (through PTHR), Ihh (through Ptc), and BMP-6 in late-stage experimental spondylosis. We believe the present findings will provide further insight into the molecular mechanisms of spinal disease, such as regulation of the pace and synchrony of chondrocyte maturation leading to osteophyte formation.

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Original Article

Hylonome

Early closure of growth plate causes poor growth of long bones in collagen-induced arthritis rats

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Abstract

Abnormalities of the epiphyseal growth plate that occur in collagen-induced arthritis (CIA) were studied. CIA was induced in 6-week-old Lewis rats by immunization with type II collagen. Radiographic examination revealed the early closure of the epiphyseal growth plate with growth retardation of the femur and tibia. Histological evaluation confirmed the early closure of the epiphyseal growth plate accompanied by decreased intensity of safranin-O staining indicating decreased amounts of proteoglycans in the extracellular matrix (ECM) of the cartilage. Immunohistochemical methods showed that the number of chondrocytes expressing matrix metalloproteinase (MMP)-3 and/or vascular endothelial growth factor (VEGF) increased in the growth plates of CIA rats. This study confirmed that disturbances of long bone growth with early closure of the epiphyseal growth plates occur in CIA. There appeared to be overexpression of MMP-3, which may be involved with proteoglycan degradation. Additionally, VEGF, which is associated with cartilage ossification and angiogenesis, might also play a role in this event. Further clarification of the mechanism of the growth disturbance in CIA may yield clinical benefits, especially in prevention of the premature closure of growth plate that is seen in juvenile rheumatoid arthritis and other diseases.

Keywords: Epiphyseal Growth Plate, Collagen-Induced Arthritis, Rat, Matrix Metalloproteinase, Vascular Endothelial Growth Factor

Introduction

Experimental animal models, such as collagen-induced arthritis (CIA) and adjuvant arthritis, are widely used to examine pathologies of arthritis with immunological abnormalities and evaluate efficacy of therapeutic agents[1-3]. However, few studies have focused on pathological changes in the epiphyseal growth plate in animal models of inflammatory chronic arthritis. Jee et al.[4] reported a decreased thickness and erosion of the epiphyseal growth plate following adjuvant-induced polyarthritis in rats. This was accompanied by an infiltration by inflammatory cells and marked proliferation of osteoclasts. Bunger et al.[5] reported that in carrageenan-induced monoarthritis there were growth disturbances in the bones adjacent to the involved joints. Although these reports indicate that growth abnormality is one of the changes that may develop in rat models of arthritis, details of pathological changes that occur in the growth plates and associated pathogenesis remain unclear.

Juvenile rheumatoid arthritis, juvenile chronic arthritis, and hemophilic arthropathy are sometimes associated with premature closure of juxta-articular epiphyses, resulting in joint malalignment, extremity length discrepancy or short stature[6]. Although inflammatory changes may contribute to the pathogenesis of early epiphyseal closure in such clinical cases, the causality of this relationship has not been proven.

The purpose of this study was to determine if growth retardation occurs at the epiphyseal plates in CIA rats, and to describe the histopathology of the growth plate in this model.

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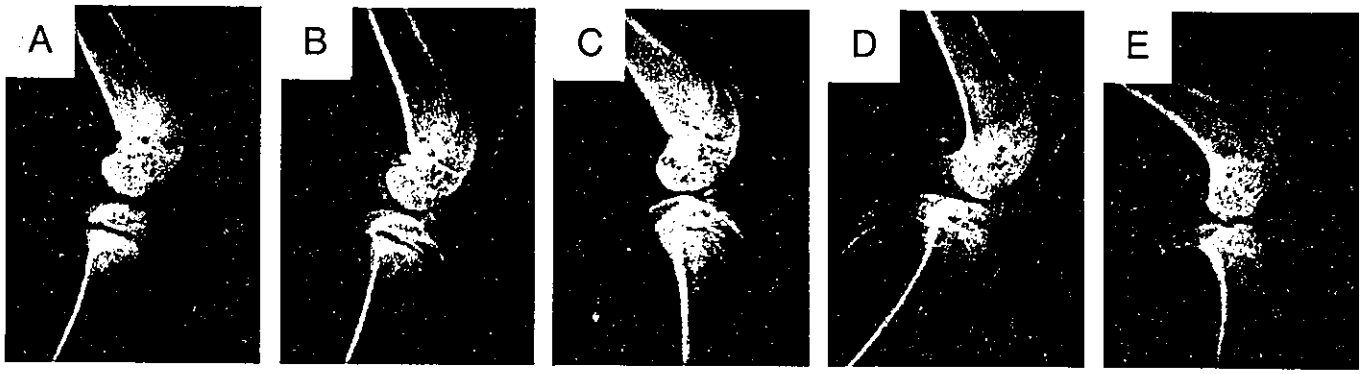

6-week-old normal rat | 12-week-old normal rat | 18-week-old normal rat | 12-week-old CIA rat 6 weeks after immunization | 18-week-old CIA rat 12 weeks after immunization

Figure 1. Radiologic appearance of the knees. Growth plates gradually grew narrower with age, but were still visible in 18-week-old normal rats (A-C). In CIA rats, growth plates rapidly narrowed, and had closed (radiographically observed) 12 weeks after immunization (D, E).

Materials and methods

Induction of CIA

CIA was induced using the modified method described previously[7]. Six-week-old female Lewis rats (Clea Japan, Tokyo, Japan) were immunized intradermally with 0.5 mg of bovine type II collagen (Cosmo Bio, Tokyo, Japan) emulsified in 0.5 ml of Freund's incomplete adjuvant (Difco, Detroit, MI, USA) at 4°C. Seven days after the first immunization, the rats received an intradermal booster injection of half the initial volume of emulsified type II collagen. CIA was detected in all of the animals by day 12-16 after the first immunization. Twenty CIA and 20 normal (control) rats were used in this study.

Radiographic evaluation of the knee and measurement of femoral and tibial length

Twelve CIA and 12 normal rats were used for radiographic and histological evaluations. Two rats from each group were killed by an overdose of anesthesia sequentially at 0, 2, 3, 4, 6 and 12 weeks. The legs were excised, and bones were separated from soft tissues and placed on a radiographic box. Radiography was performed with μFX1000 (Fuji Photo Film, Tokyo, Japan), with 35kV exposure for 12 seconds (Figure 1A-E).

Femoral and tibial lengths of the remaining 8 CIA and 8 normal rats were measured, as previously described[8], under anesthesia every week until 12 weeks after immunization using a FUJIX BIO-IMAGING ANALYSER system (Fuji Photo Film, Tokyo, Japan). The X-ray image was acquired using an Image Reader and displayed on a computer, and lengths of each femur and tibia were measured with an Image Gauge (Figure 2A).

Tissue preparation

Preparation of tissue samples was performed as follows. After radiographic evaluations, excised legs were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). The femurs and tibias were decalcified in 20% EDTA with microwave irradiation for 5 hours, dehydrated in ethanol and embedded in paraffin. Sagittal sections of knee were cut with the plane of section oriented parallel to the longitudinal axis of the bone. Sections 5μm thick were cut with a microtome and stained with hematoxylin and eosin (H & E), (Figure 3). Serial sections were prepared for safranin-O staining (Figure 4).

Immunohistochemistry for MMP-3 and VEGF

Immunohistochemistry was performed using the streptavidin-peroxidase method with Histofine SAB-PO kits (Nichirei, Tokyo, Japan) according to the manufacturer's protocol. Tissue sections were deparaffinized and hydrated in PBS (pH 7.4), then incubated in 0.3% H_2O_2 in methanol for 30 minutes at room temperature to block endogenous peroxidase activity. After a PBS wash, the sections were incubated for 30 minutes at room temperature with 10% normal serum from the same species as the secondary antibody to minimize background staining, then with primary monoclonal antibodies (anti-MMP-3; Fuji Chemical, Toyama, Japan and anti-VEGF; Upstate Biotechnology, Lake Placid, NY, USA) overnight at 4°C. After washing in PBS, the sections were incubated with secondary antibody for 30 minutes at room temperature, then washed in PBS. The color reaction was performed using the substrate reagent 3,3'-diaminobenzidine tetrahydrochloride (Dojindo, Tokyo, Japan). Finally, the slides were counterstained with hematoxylin, dehydrated in graded ethanol series and mounted.

Morphometric analysis of the growth plate

Morphometric analysis was performed as described previously[9-11]. The H & E-stained sections of 4 legs from each group were examined by light microscopy (Nikon ECLIPSE