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Messenger RNA Expression of the Genes Encoding Receptors for Bone Morphogenetic Protein (BMP) and Transforming Growth Factor-β (TGF-β) in the Cells from the Posterior Longitudinal Ligament in Cervical Spine

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Posterior longitudinal ligament (PLL) in cervical spine is one of the sites of ossification in idiopathic hyperostotic diseases. Although the mechanism of the pathological triggering of the disease has not yet been clarified, the cells in PLL have been reported to express osteotropic cytokines such as BMP-2 and TGF-β. However, it has not been known whether the cells in PLL express receptors for these cytokines. We examined the expression of the messenger RNAs of the genes encoding receptors for BMP-2/4 and TGF-β in the PLL cells. Tissues from three OPLL (ossification of the posterior longitudinal ligament) patients who underwent anterior decompression surgery with removal of the ossified PLL were dissected microscopically and were subjected to explant cultures; the cells outgrown from the explants were examined. Type I BMP receptor (BMPR) mRNA was expressed at moderate levels in the cells derived from both ossifying PLL tissues as well as nonossifying adjacent fibrous tissues. Type II TGF-β receptor (TβR) mRNA and α1(I) collagen mRNA were also constitutively expressed in these PLL cells from either regions. Treatment with BMP-2 enhanced the expression of BMPR mRNA in five out of ten of the cell cultures, suggesting that functional BMP receptors were expressed in at least a part of the PLL cells. The BMP-2 effect on BMPR was specific since no such enhancement was observed with regard to the levels of TβR mRNA in all of the ten cultures. These results indicated for the first time that mRNAs of the genes encoding receptors for BMP-2/4 and TGF-β were expressed in the cells derived from human PLL cells.

Key Words: Ossification of the osterior longitudinal ligament (OPLL); Type IA BMP receptor; Type II TGF-β receptor.

Introduction

Posterior longitudinal ligament (PLL) in cervical spine is one of the frequent sites of ectopic bone formation in hyperostotic diseases (Resnick et al., 1978). Ossification of the posterior longitudinal ligament (OPLL) appears as an abnormal radiopacity along the posterior margins of the vertebral bodies on the lateral views of the roentogenogram (Tsuyama, 1984). Many cases of OPLL in cervical spine are associated with ossification of other ligaments and tendons throughout the body, including anterior longitudinal ligaments (Forestier disease) of the cervical spines (Forestier and Lagier, 1971), ligamenta flava of thoracic spines, ligaments in the pelves and hips, and calcaneal enthesis of Achilles tendons (Hukuda et al., 1983). Epidemiological findings show that most patients are over 40 yr old, males are twice more frequently affected than females, and OPLL is much more common among Japanese and other Asian populations than Caucasians (Tsuyama, 1984). In the cases of advanced OPLL (which occupies most of the spinal canal), spinal cords and/or the spinal roots are compressed, resulting in severe myelopathy and/or radiculopathy. Patients with such severe OPLLs are mostly to be subjected to surgical treatments (Yamaura, 1989, 1990).

Several systemic factors have been reported to be related to this abnormal ossification. High incidence of elevated insulin levels in serum was observed in patients with

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paravertebral ligamentous ossification (Takeuchi, 1989). Hypoparathyroidism has been reported to be associated with paravertebral ligamentous ossification (Matsumoto, 1984). Anomalous calcium metabolism also appears to affect the development of OPLL (Seichi et al., 1992). Serum estrogen (Motegi, 1993), growth hormone binding protein (Ikegawa et al., 1993), or fibronectin (Miyamoto et al., 1993) levels were reported to be relatively high in OPLL patients. However, the pathogenesis of OPLL has not yet been elucidated.

Bone formation has been known to be under the control of both systemic and local regulators. Among the local factors, TGF-β and BMP have been thought to play crucial roles in osteogenesis. Transforming growth factor-βs (TGF-βs) constitute a family of multifunctional proteins with a wide range of biological activities (Massague, 1990). In bone, TGF-βs are potent growth regulators for osteoblasts, are produced by these cells, and modulate osteoblastic activities. In vitro studies have revealed a variety of TGF-β actions in osteoblasts (Noda and Rodan, 1986; Centrella et al., 1987; Pfeilschifter et al., 1987; Rosen et al., 1988; Bonewald and Mundy, 1990). Furthermore, TGF-β has been shown to stimulate bone formation in vivo (Noda and Camilliere, 1989). It is well known that bone is the major storage site for TGF-β in the body (Carrington et al., 1990). The observation that osteoblasts produce TGF-β in significant amounts (Robey et al., 1987) suggests that TGF-β would function as an auto/paracrine factors in osteoblasts. TGF-βs exert their effects through their binding to specific cell surface receptors. The type I and II receptors are transmembrane proteins that possess serine/threonine kinase domains (Massague, 1992). Autophosphorylation of the serine and threonine residues in the type II TGF-β receptor kinase has been demonstrated (Lin et al., 1992). Type I receptor requires type II receptor for ligand binding and interaction of both receptor components, including phosphorylation of GS domain of type I receptor by type II receptor, is required for signaling (Wrana et al., 1992).

Bone morphogenetic proteins (BMPs) were originally isolated from bone using an in vivo ectopic cartilage and bone formation assay (Wang et al., 1988). Molecular cloning demonstrated the existence of at least twelve distinct but related genes except BMP-1. These BMPs are the members of TGF-β super family (Wozney et al., 1988; Wozney, 1993). BMP-2 has been shown to induce cartilage and bone formation in both ectopic and orthotopic sites in vivo (Wang et al., 1990), to have many different effects on multiple cell types in vitro (Takua et al., 1991; Thies et al., 1992; Wozney, 1993) and to regulate expression of helix-loop-helix type transcription factors (Ogata et al., 1993; Tamura and Noda, 1994), which are expressed in osteoblasts (Kawaguchi et al., 1992). Genes for several BMP receptors were recently cloned by RT-PCR, where oligonucleotide primers designed based on the conserved regions of serine/threonine kinase domains were used. One of the BMP receptor cDNAs, TFR11 (BMPRIA), was cloned by Suzuki et al. from the MC3T3-E1 cDNA library (Suzuki et al., 1994).

Recently, the presence of BMP-2 and TGF-β ligands was suggested in the ossification area of PLL (Kawaguchi et al., 1992). If PLL cells are the source of osteoblasts, which cause ectopic bone formation in this ligament, the PLL cells should express the receptors for cytokines, which induce differentiation of the ligament cells into osteogenic cells. However, it has been unknown whether the PLL cells do express receptors for these cytokines. The aim of this study was to examine the messenger RNA expression of the genes encoding BMP 2/4 and TGF-β receptors in PLL cells, and to examine the effects of BMP-2 on their expression. We found that PLL cells derived from both ossified and nonossified regions expressed messages for these receptor genes.

Results

Morphological Profile of the Ossification of the Posterior Longitudinal Ligament (OPLL)

Representative morphology of the OPLL in one of the patients (case 3) is presented in Fig. 1. Lateral view of the roentogenogram of the cervical spine showed continuous mass of the OPLL (arrow heads) extending from the third to the fifth cervical spine level (Fig. 1, left panel). X-ray tomography (Fig. 1, middle panel) and computed tomographical myelography (CTM) (Fig. 1; right top, C3/C4 level; bottom, C4 level) of the same patient indicated the centrally located OPLL. The histology of OPLL shown in Fig. 2 indicates continuous ossified mass (asterisks) in the PLL tissue.

Morphology of the PLL Cells

The PLL cells (outgrown from the dissected OPLL tissues, including both ossified and adjacent fibrous tissues) showed elongated shape during the culture either in the early phase or in the later confluent phase (Fig. 3). The morphology of the PLL cells taken from the diverse parts of the tissues was similar. Treatment with 200 ng/mL human recombinant BMP-2 for 24 h did not cause any major morphological change in the cells from any parts of the PLL tissues of the three patients.

Northern Blot Analysis

BMP receptor (BMPR) messenger RNAs were observed as two major bands of approx 6 and 4 kb in size (Fig. 4A). The abundance of the two species was similar in the cells obtained from the three patients (Fig. 4A,B; left, middle, and right sets of panels represent the cells from each of the three patients). The levels of these mRNAs in the cells from the ossified PLL (OPLL) were basically similar when compared to those of nonossifying regions of PLL (PLL) and the adjacent fibrous tissue (FIB). Only in case 3 did we obtain cells from hypertrophied PLL (HPLL).

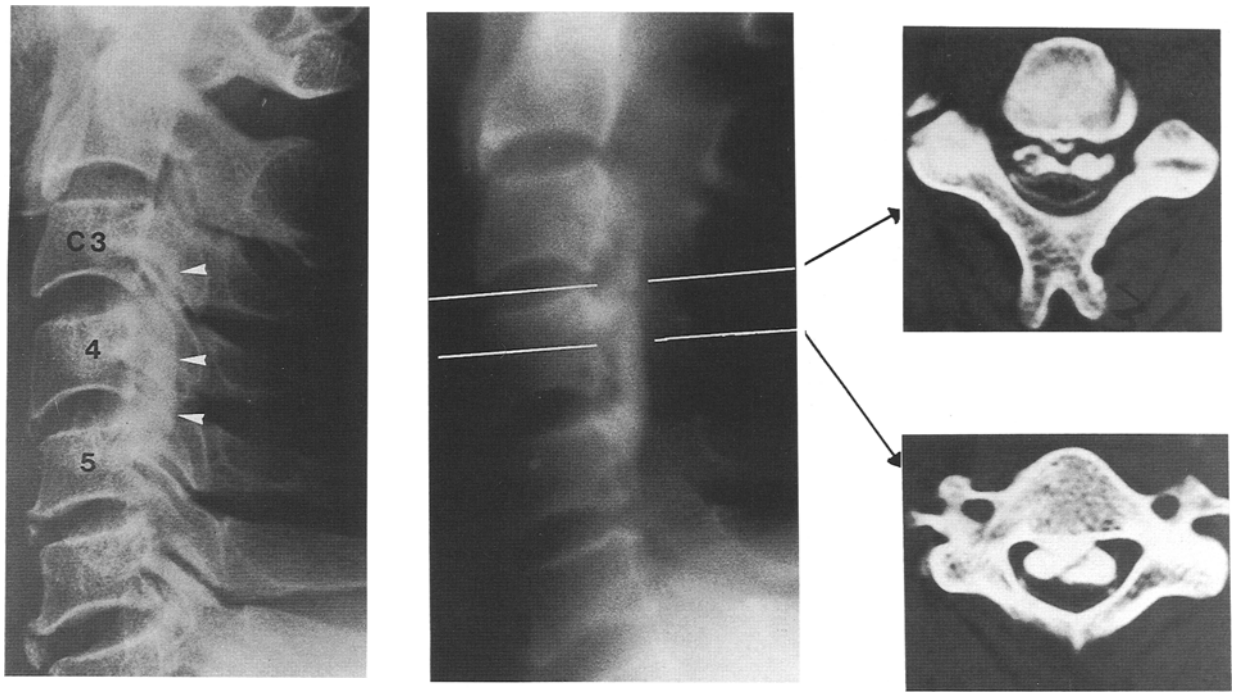

Fig. 1. Morphology of OPLL. (Left column) Lateral view of the roentgenogram of the patient (case 3) showing noticeable abnormal radiopacity of OPLL along the posterior margins of vertebral bodies C3 to C5. (Middle column) Lateral tomogram of the same patient showing segmental OPLL starting at C3 and continuous to C4 and C5. (Right column) Preoperative CT-myelography showing that ossified PLL widely occupied the spinal canal and that the spinal cord was compressed by the ossified PLL. Top, C3/4 level; bottom, C4 level.

BMP-2 treatment enhanced BMPR mRNA levels in the cultures of the PLL and OPLL cells by 43% (PLL) and 38% (OPLL) in case 1, and by 33% (PLL) and 58% (OPLL) in case 3, respectively (Fig. 4A,B; compare – vs + for PLL and OPLL in the left panel for case 1; and – vs + for PLL and OPLL in the right panel for case 3). The OPLL cells from case 2 did not show response to the treatment with BMP-2 (Fig. 4A,B; middle panel PLL – vs +).

The BMP-2 effect on the levels of BMPR mRNA was specific since the abundance of TβR mRNA was similar, regardless of the treatment with BMP-2 or not in the same Northern blot filters (Fig. 5A,B; compare – vs + for PLL and OPLL in the left, middle, and right panels corresponding to cases 1, 2, and 3, respectively). TβR mRNA was observed as an approx 5 kb molecular-weight species. The abundance of the TβR mRNA was moderate and similar, regardless of the regions of the dissected tissues from which the cells were derived (Fig. 5A,B).

α1(I) collagen mRNA was expressed as two major 5.2- and 4.8-kb bands (Fig. 6A,B). The relative abundance of the expression of these two RNA species was similar. The cells from the three patients exhibited varying levels of α1(I) collagen mRNA expression in each region of the tissue. With regard to the response to the treatment with BMP-2, moderate enhancement of α1(I) collagen mRNA abundance was observed in the cells outgrown from the OPLL in all of the three patients (13, 42, and 29% for cases 1, 2, and 3, respectively, Fig. 6A,B; OPLL), whereas consistent effect was not observed in the other cultures (Fig. 6A,B; PLL and FIB).

Discussion

In this report we have observed for the first time the expression of mRNA species, which hybridizes to type IA BMP receptor (BMPR) cDNA (TFR11) in the cells derived from the human posterior longitudinal ligaments (PLLs). Although OPLL tissues were reported to express bone-inducing cytokines, such as BMP (Kawaguchi et al., 1992), it has not been known whether the cells from the posterior longitudinal ligament could express the genes for the cognate receptors for these cytokines. Although preliminary, our results regarding the effects of BMP-2 on BMPR mRNA levels also suggested the presence of functional BMP receptors in the cells derived from PLL tissues. However, whether BMP-based autocrine mechanisms could be involved in the pathogenesis of this disease through the anomaly in the quality or the quantity of the receptors is still to be elucidated by comparing tissues from patients with and without OPLL.

In contrast to the previous reports that the cells derived from OPLL expressed higher alkaline phosphatase activity than those from nonossifying area (Ishida and Kawai, 1993), our preliminary Northern blot analysis did not detect mRNAs for alkaline phosphatase and osteocalcin (data not shown) in the cells. This may be owing to the dominance of more precursor-like cell populations in the cultures of out-

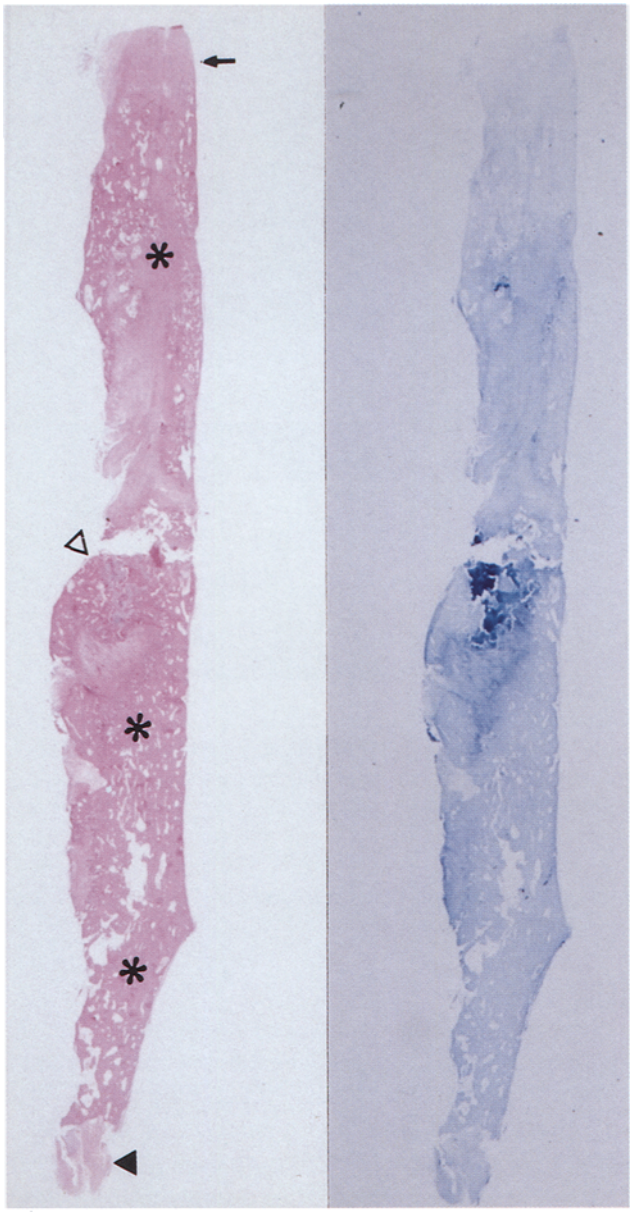

Fig. 2. Histology of the OPLL. Midsagittal sections of the surgical specimen (with the anterior side on the left and the cranial side on the top) obtained from the patient (case 3) were prepared and subjected to microscopical examination as described in Materials and Methods. (Left panel) H&E staining. Continuous bone mass is indicated by asterisks. Arrow, hypertrophic PLL; open arrowhead, fibrous tissue adjacent to the C3/4 intervertebral disc; closed arrowhead, PLL. (Right panel) The adjacent section stained with toluidine blue (pH 7.0). Dark blue staining was observed on the borders between cartilaginous and bone regions. Strong blue staining in the mid-portion of the section shows the degenerated disc material.

grown cells, which could more likely be the target of BMP in the case of the initiation of BMP-driven osteogenesis. These precursor-like fibroblastic cells, rather than the more osteoblast-like cells reported by others (Ishida and Kawai, 1993), may be responsible for the early evolution of osteogenesis in PLLs (Yamaura, 1993; Epstein, 1994).

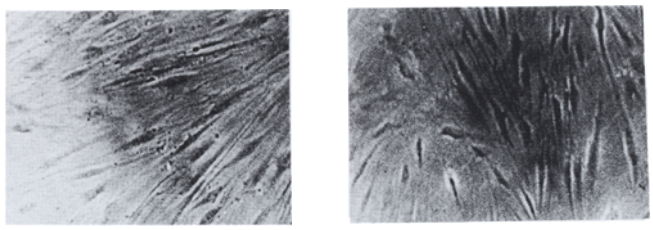

Fig. 3. Morphology of the cells derived from ossifying PLL. The cells were obtained from the ossified PLL tissue of the patient (case 3) whose roentogenogram was shown in Fig. 1. The cells were treated with 200 ng/mL BMP-2 (right panel) or vehicle alone (left panel) for 24 h. The culture condition is described in Materials and Methods.

In the same Northern blot analysis, we have also shown that type II transforming growth factor-β receptor (TβR) mRNA was expressed at an approximately similar level to that of the BMPR. In the process of embryonic development, as well as in osteogenesis in either normal or pathological bone formation, many cytokines appear to interact in the regulation of the osteoblastic function. Interestingly, we have observed moderate enhancement of BMP receptor mRNA levels in response to the treatment with BMP-2 in the cells from patients (cases 1 and 3). The same Northern blot filters did not show any effect of BMP-2 on TβR mRNA level at all, indicating the specificity of the BMP-2 effect on BMPR gene expression. On the other hand, the BMP-2 did not enhance the BMPR mRNA level in the cells from case 2. The reason for this lack of response is not clear. The tissues in PLL would consist of quite heterogeneous cell populations and therefore the diverse responses to BMP-2 of the cultures in this experiment may reflect the heterogeneity in these tissues. Although in vitro data indicated that these cells expressed mRNAs for the BMP and receptors, protein studies should be followed to verify that the mRNA levels do reflect the functional receptor protein levels in these cells and tissues. The observation on the BMP-2 effects on BMPR mRNA as well as that on α1(I) collagen mRNA suggested (but did not prove) the presence of functional BMPR in these cells. Furthermore, this in vitro observation may also suggest the positive feedback mechanism that could drive the ectopic osteogenesis in PLL in certain patients as a part of multiple factors involved in the pathogenesis of OPLL.

Ectopic hyperostosis has been suggested to associate with certain genetic traits (Tanikawa et al., 1986; Terayama, 1989; Hamanishi et al., 1995). If the BMP-BMPR system is involved in the pathogenesis of this hyperostotic disease in the PLL, there could be certain correlation between the activity or the abundance of the ligand and/or receptor versus the incidence of ossification in PLL. Recent twin studies with regard to osteoporosis suggested the association of certain polymorphism in human vitamin D receptor gene and the incidence of the disease (Morrison et al., 1994).

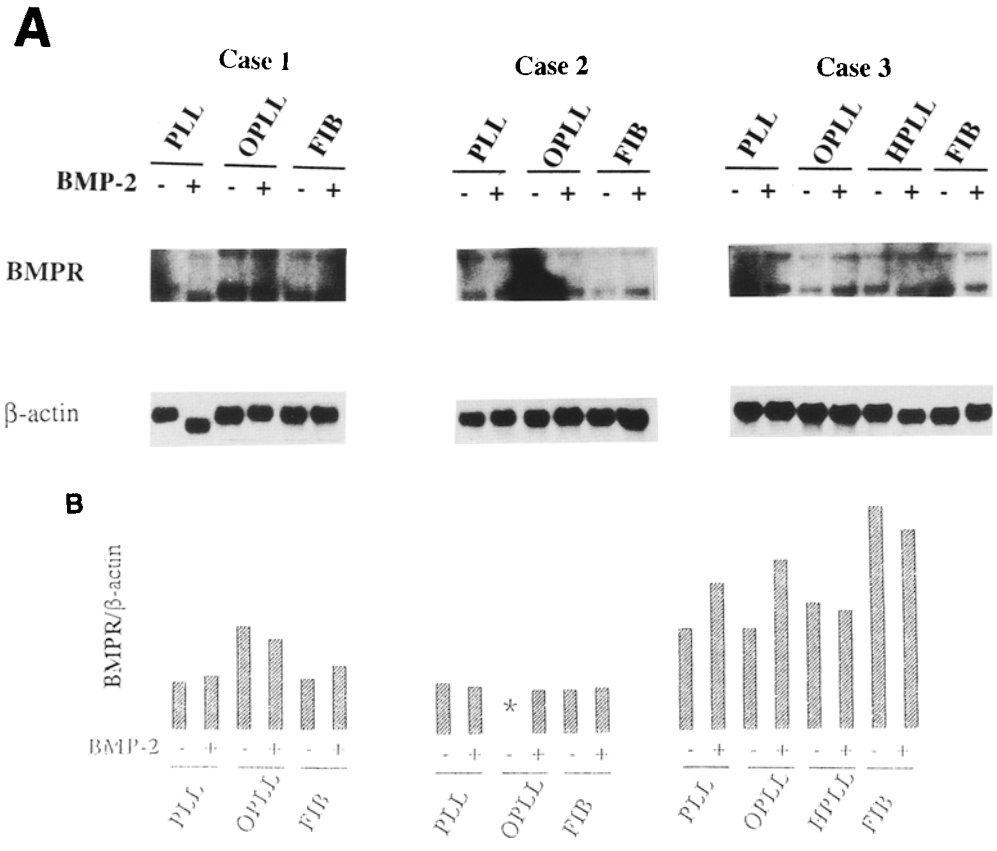

Fig. 4. Type I BMP receptor mRNA expression in the cells derived from OPLL. **(A)** Confluent cultures from the patients (cases 1, 2 and 3; corresponding to left, middle, and right sets of panels respectively) were treated for 24 h with either vehicle alone (–) or 200 ng/mL human recombinant BMP-2 (+). Total RNA was extracted and Northern blot analysis was carried out as described in Materials and Methods. Each lane was loaded with 10 μg of total RNA. Upper autoradiograms show 6 and 4 kb BMPR mRNA bands. The same filters were hybridized to actin cDNA (lower autoradiograms). **(B)** Relative ratios of BMPR mRNA abundance normalized against that of actin were calculated based on the quantification by a laser densitometer and shown in the panels. Because of the high background, quantitative data on the level of BMPR mRNA of the cells from OPLL of case 2 (middle panel*) was not available.

Further molecular linkage analysis, regarding the genomic polymorphisms of the genes encoding ligands of BMPRs and the incidence of ossification of the posterior longitudinal ligament, could contribute to give a clue to elucidate the pathogenesis of the disease.

Materials and Methods

Case Materials

Three patients with ossification of the posterior longitudinal ligament underwent anterior decompression surgery with removal of ossified PLL over a 3-mo period in 1993 at the Kudanzaka Hospital. The patients were two males and one female, ranging in age from 55 to 63 yr. The patients were evaluated at 23–26 mo postoperatively. Diagnostic procedures of these patients included magnetic resonance imaging (MRI) scans, computed tomographical myelography (CTM), myelography, and X-ray tomography. Clinical symptoms of these patients were listed in Table 1.

Materials

Recombinant human bone morphogenetic protein-2 (BMP-2) was purified from the supernatants of transfected COS cells. Murine type I BMP-2/4 receptor cDNA (TFR11) was recently cloned and reported elsewhere (Suzuki et al., 1994). Type II TGF-β receptor cDNA (TβR) was kindly provided by Dr. Massague. Cell culture reagents (media, serum, trypsin) were purchased from Gibco Life Technologies (Grand Island, NY). Tissue culture plastic wares were obtained from Costar Corporation (Cambridge, MA). [α-32P]-dCTP, with a specific activity of 111TBq/m*M* (3000Ci/m*M*), was purchased from NEN Research Products (Tokyo, Japan). Guanidinium thiocyanate and agarose were obtained from Sigma (St. Louis, MO). Other chemicals were purchased from Wako (Osaka, Japan).

Cell Cultures

En bloc resected ossifying PLLs were immediately subjected to cell culture. Ossifying PLL, PLL, and adjacent fibrous tissues were dissected under binocular microscope. The outgrown cells from each tissue were replated, grown to confluence in α-modification of Eagle's medium (α-MEM) supplemented with 10% fetal bovine serum. The cells used in this study were cultured in a humidified atmosphere of 5% CO_2 at 37°C. For the treatment with BMP-2, the medium was changed to a

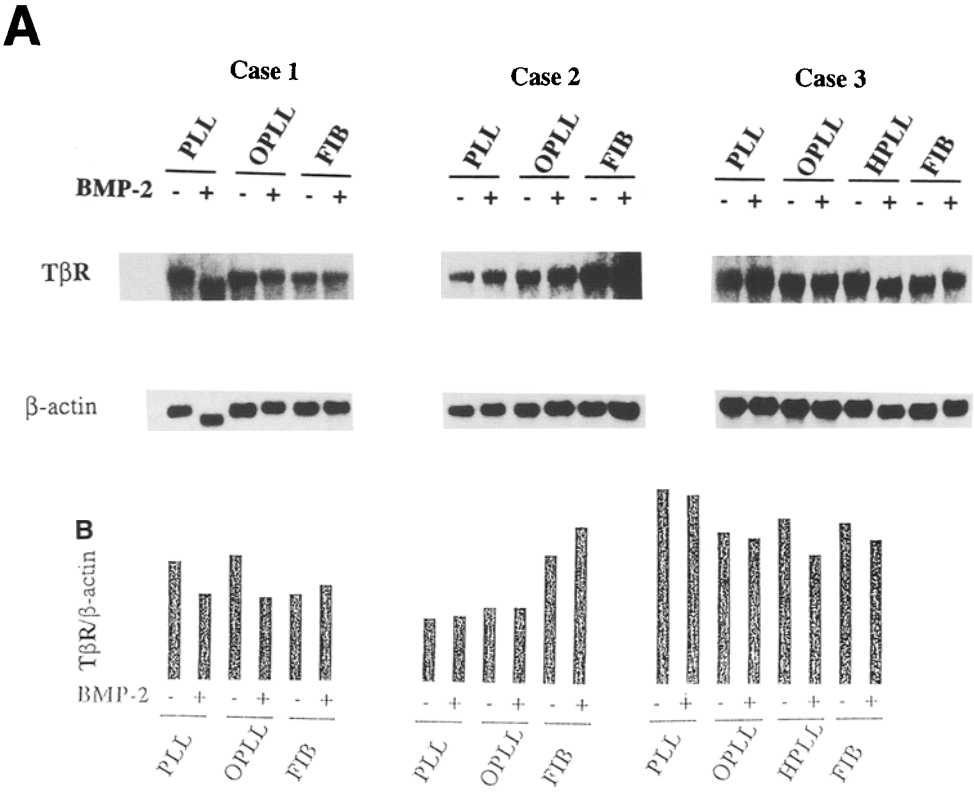

Fig. 5. Type II TGFβ receptor mRNA expression in the cells derived from OPLL. **(A)** Confluent cultures from the patients (cases 1, 2, and 3; corresponding to left, middle, and right panels, respectively) were treated for 24 h with either vehicle alone (–) or 200 ng/mL human recombinant BMP-2 (+). Total RNA was extracted and Northern blot analysis was carried out as described in Materials and Methods. Each lane was loaded with 10 μg of total RNA. Upper autoradiograms show 5 kb TβR mRNA bands. The same filters were hybridized to actin cDNA (lower autoradiograms). **(B)** Relative ratios of TβR mRNA abundance normalized against that of actin were calculated based on the quantification by a laser densitometer and shown in the panels.

fresh one a day before the initiation of the treatment, and the cells were exposed to BMP-2 (200 ng/mL) or vehicle for 24 h.

RNA Extraction

Total cellular RNA was extracted according to AGPC (acid guanidinium thiocyanate-phenol-chloroform) method (Chomczynski and Sacchi, 1987). Briefly, after discarding the medium, the cells were rinsed with PBS three times, and were lysed in 2 mL of solution D (4*M* guanidinium thiocyanate/0.1m*M*-mercaptoethanol). After shearing DNA by passing through 23-gage needles, the lysates were collected from each dish and transferred to polypropylene tubes. After addition of 0.2 mL of 2*M* sodium acetate, 2 mL water-saturated-phenol (pH 4.0), and 0.4 mL of chloroform:isoamyl alcohol (49:1), the lysates were mixed by shaking vigorously for 10 s, cooled on ice for 15 min, and centrifuged at 10,000*g* for 20 min at 4°C to separate aqueous phase, which was then mixed with 2 mL isopropanol, and kept at –20°C for 2 h followed by centrifugation at 10,000*g* for 20 min. The resulting RNA pellets were rinsed with 75% ethanol, vacuum dried (5 min), and dissolved in 30 μL TE (10m*M* Tris-Cl, pH 7.6/1 m*M* EDTA, pH 8.0).

Northern Blot Analysis

Total RNAs (10 μg) were fractionated by electrophoresis in 1% agarose gel containing 0.22*M* formaldehyde in 1X MOPS (20 m*M* 3-[*N*-morphilino] propanesulfonic acid/15 m*M* sodium acetate/1 m*M* EDTA). Direct staining with ethidium bromide (EtBr) was performed to monitor RNA integrity as well as uniformity. Overnight transfer of the RNAs to Hybond N nylon filters (Amersham, Arlington Heights, IL) were accomplished in 1X TAE (0.04*M* Tris-acetate / 0.001*M* EDTA). The filters were prehybridized overnight in 50% formamide/5X Denhardt's solution/5X SSC/0.1% sodium dodecyl sulfate (SDS)/50 μg/mL herring sperm DNA at room temperature. BMPR, TGF-β-R, human α1(I) collagen, and rat actin cDNAs were radiolabeled with [α-^{32}P]-dCTP according to the method described by Feinberg and Vogelstein (Feinberg and Vogelstein, 1983). The specific activities of the probes were approx 10^8 cpm/μg DNA. Overnight hybridization was performed at room temperature in a fresh buffer containing all the same ingredients in the prehybridization buffer in addition to the radiolabeled probe (approx 10^6 cpm/mL). Blots were washed three times for 5 min each at room temperature in 1X SSC/0.5% SDS, and once for 20 min at 60°C in 0.5X SSC/0.5% SDS, and were

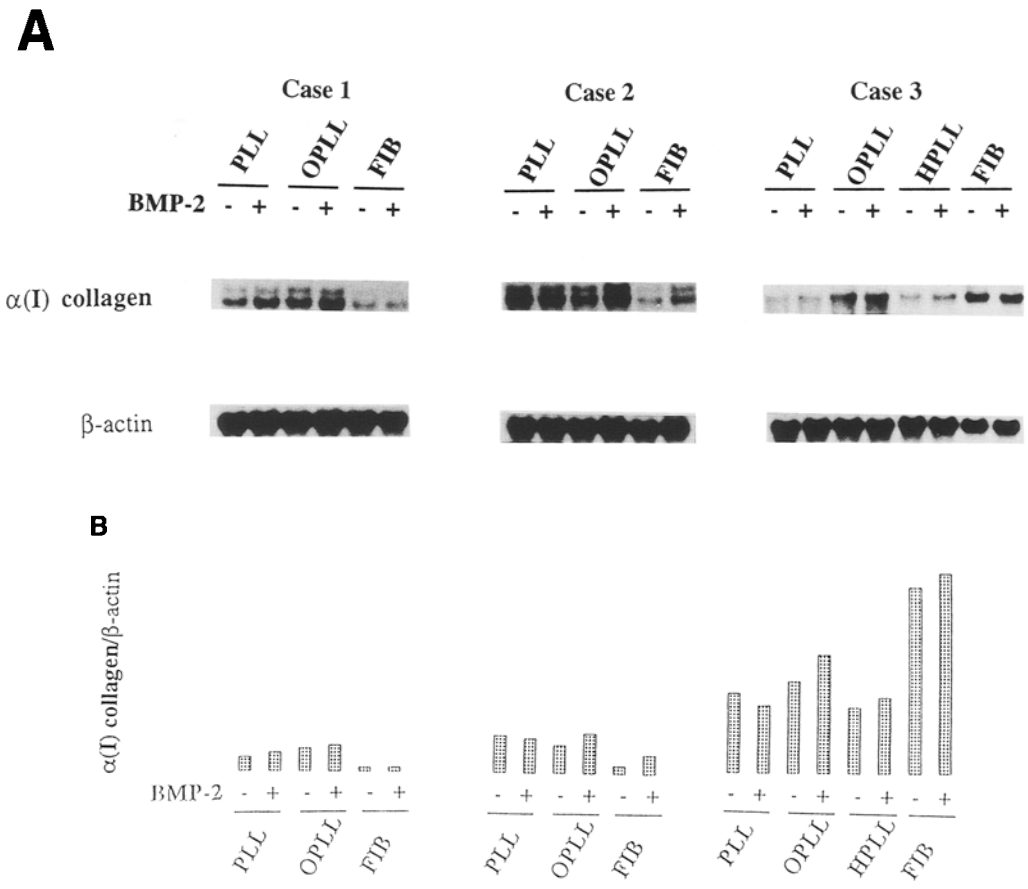

Fig. 6. α1(I) Collagen mRNA expression in the cells derived from OPLL. **(A)** Confluent cultures of the cells from the patients (cases 1, 2, and 3; corresponding to left, middle, and right panels, respectively) were treated for 24 h with either vehicle alone (–) or 200 ng/mL human recombinant BMP-2 (+). Total RNA was extracted and Northern blot analysis was carried out as described in Materials and Methods. Each lane was loaded with 10 μg of total RNA. Upper autoradiograms show 5.2 and 4.8 kb α(I) collagen mRNA bands. The same filters were hybridized to actin cDNA (lower autoradiograms). **(B)** Relative ratios of collagen mRNA abundance normalized against that of actin were calculated based on the quantification by a laser densitometer and shown in the panels.

Table 1
OPLL Cases

Case	Age	Sex	Duration of symptoms	Clinical symptoms	Decompression level	Follow-up, mo
1	63	M	6 yr	Gait disturbance Bilateral finger clumsiness Numbness of fingers and feet	C2-6	26
2	61	F	2 yr	Gait disturbance Left finger clumsiness Numbness of left fingers and right foot	C4-6	24
3	55	M	6 mo	Gait disturbance Bilateral finger clumsiness Numbness of fingers and feet	C2-6	23

M = male, F = female.

exposed to X-ray films for several days at –80°C using intensifying screens. Autoradiograms of the Northern blots were quantified using a laser densitometer Ultrascan XL (Pharmacia/LKB, Uppsala, Sweden) or BAS-2000 (Fuji, Tokyo, Japan).

Histological Preparation

Resected ossifying PLLs were cut longitudinally into halves; one half of each specimen was subjected to cell culture studies and the other half was fixed in formaldehyde and decalcified. The samples were dehydrated and embed-

ded in wax. Thin sections were prepared followed by staining with H&E or toluidine blue (pH 7.0).

Acknowledgments

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