Objective. To investigate the association between the polymorphism of TGF-[beta]1 and the radiologic characteristics of OPLL.

Summary of Background Data. Ossification of the posterior longitudinal ligament has a strong genetic background. Several genes contribute to the expression of OPLL. Transforming growth factor-[beta]1 is present in the ossified matrix and chondrocytes of cartilage adjacent to areas of OPLL.

Methods. The difference in the TGF–T[beta]1 allele distribution ("TT," "TC," and "CC") between 369 patients with OPLL and 258 control subjects was assessed. The relations between the allele frequency and radiologic features of OPLL involving the cervical, thoracic, and lumbar spine and the width of the ossification area were evaluated.

Results. There was no statistical difference with respect to the type of OPLL and the width of the ossification area for the TGF–T[beta]1 allele between the OPLL and the control groups. However, in the patients with "TC" or "CC" alleles, OPLL frequently was found in the cervical, thoracic, and/or lumbar spine.

Conclusions. Transforming growth factor-[beta]1 polymorphism is not a factor associated with the occurrence of OPLL, but rather a factor related to the area of the ossified lesion. The "C" allele might be a risk factor for patients with OPLL in other areas in addition to the cervical lesion.

Ossification of the posterior longitudinal ligament (OPLL) has a strong genetic component. [1–6] The segregation rate in the siblings of OPLL corresponds neither to an autosomal dominant nor to an autosomal recessive mode of inheritance. [4,5] Rather, multiple genetic and environmental components must contribute to the development of OPLL. Transforming growth factor-[beta] (TGF-[beta]) has been implicated as an important regulator of bone metabolism, [7–9] and is present in the ossified matrix and chondrocytes of cartilage adjacent to areas of OPLL. [10] It may play an important role in the development of OPLL. However, several polymorphisms of the TGF-[beta]1 gene have been identified. [11–13] One of the polymorphisms is a T->C transition at nucleotide 29 in the region encoding the signal sequence, resulting in a Leu->Pro substitution at amino acid position 10. This polymorphism brings about the change in the serum concentration of TGF-[beta]1. [14,15] The purpose of this study was to investigate the association between the polymorphism of TGF-[beta]1 and the radiologic characteristics of OPLL.

Patients and Methods

Participants.

The participants in this study were 369 patients with OPLL and 224 control subjects without OPLL (Table 1). The diagnosis of OPLL was based on radiologic findings including radiographs of the cervical, thoracic, and lumbar spine; tomogram; computed tomogram (CT); and magnetic resonance imaging (MRI). Ankylosing spondylitis and metabolic diseases associated with OPLL, such as hypophosphatemic rickets/osteomalacia and hyperparathyroidism were excluded on the basis of radiographic and biochemical examinations. Precise radiographic analysis of the whole spine was available in 95 OPLL patients. The types of OPLL, the maximum axial width of OPLL, and the levels of spinal involvement were evaluated. Axial OPLL width was measured on lateral radiographs or tomograms, and data were compared with the allele distribution of the TGF-[beta]1 polymorphism. None of the control patients exhibited OPLL.

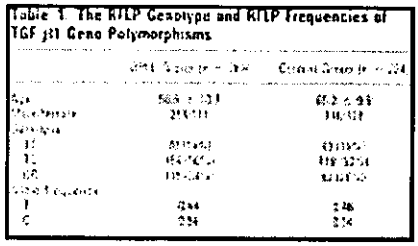

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Table 1. The RFLP Genotype and RFLP Frequencies of TGF-[beta]1 Gene Polymorphisms

Genotyping of the TGF-[beta]1 Gene.

Genotyping of the TGF-[beta]1 gene was performed using Yamada's method. 14,15 The genotype for the TGF-[beta]1 gene was determined using an allele-specific polymerase chain reaction (PCR) with two sense primers (sense primer 1,5'-CTCCGGGCTGCGGCTGCTGCT-3'; sense primer 2,5'-CTCCGGGCTGCGGCTGCTGCC-3') and an antisense primer (5'-GTTGTGGGTTTCCACCATTAG-3'). Reactions were performed in a total volume of 50 μL containing 0.5 μg of genomic DNA; 20 pmol of each primer; 0.2 mmol/L each of dCTP, dTTP, dGTP, and dATP; 1 U of Taq DNA polymerase (Amplitaq Gold; Perkin Elmer, Foster City, CA, USA); 50 mmol/L KCL,=; 1.5 mmol/L $MgCl_2$; 1.4% dimethyl sulfoxide; 0.01% gelatin; and 10 mmol/L Tris-HCl (pH 8.3). The thermocycling procedure consisted of initial denaturation at 94 C for 5 minutes; five cycles of denaturation at 94 C for 1 minute; annealing at 60 C for 1 minute and extension at 72 C for 1 minute; 30 cycles of 94 C for 30 seconds, 56 C for 30 seconds, and 72 C for 30 seconds; and a final extension at 72 C for 5 minutes. The PCR products were analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. The expected size of the specific amplification product was 346 bp.

Statistical Analysis.

Student's *t* test was used to assess the difference in the frequency of the alleles between the patients with OPLL and the control subjects. Regarding the maximum axial width of OPLL, Welch's *t* test was used between genotypes. Data are presented as the mean ± standard deviation. A *P* value less than 0.05 was considered statistically significant.

Results

The T/C polymorphisms at nucleotide 29 in exon 1 of the TGF-[beta]1 genome were accurately detected by the allele-specific PCR assay. The numbers of the "TT," "TC," and "CC" genotypes in this study population were 113, 302, and 178, and the frequencies were 19%, 51% and 30%, respectively. These results were similar to those in previous reports of Japanese subjects. The genomic distribution was in Hardy–Weinberg equilibrium.

The "TT," "TC," and "CC" alleles found in the OPLL group were 70 subjects, 184, and 115, and those in the control group were 43, 118, and 63, respectively (Table 1). Comparing the allele distributions, there was no statistical difference in the genotype and allele frequency for the TGF-[beta]1 polymorphism between the OPLL and control groups ($P = 0.72$).

All 94 patients with OPLL had ossification in the cervical spine, except for one patient who had OPLL only in the thoracic spine. There was no statistical difference in allele distribution with respect to the type of OPLL ($P = 0.31$). The maximum axial width of the ossification area in the cervical spine was 4.7 ± 2.0 mm in the "TT" allele group, 5.1 ± 2.3 mm in the "TC" allele group, and 5.5 ± 2.6 mm in the "CC" allele group. There was no

statistical difference among the three groups ($P = 0.7$).

The OPLL in 43 patients involved the cervical, thoracic, and/or lumbar spine. Cervical OPLL alone was found in 51 patients. Cervical and thoracic OPLL was found in 19 patients, and 8 patients had cervical and lumbar OPLL, whereas 16 had OPLL involving all three levels. There was a significant difference in the distribution of the TGF-[beta]1 polymorphism between the group with OPLL throughout the spine and the group with isolated cervical OPLL (Table 2). In the patients with "TC" or "CC" alleles of the TGF-[beta]1 polymorphism, OPLL was found frequently in all three levels (Figure 1). In contrast, only 2 of 15 patients with the "TT" allele had OPLL in the cervical, thoracic, and/or lumbar spine. The patient who had OPLL only in the thoracic spine had the "TC" allele.

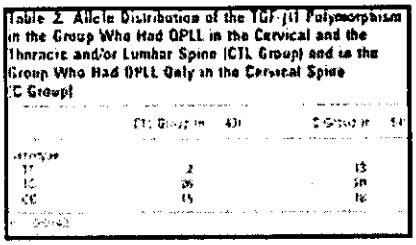

Table 2. Allele Distribution of the TGF-[beta]1 Polymorphism in the Group Who Had OPLL in the Cervical and the Thoracic and/or Lumbar Spine (CTL Group) and in the Group Who Had OPLL Only in the Cervical Spine (C Group)P = 0.0142.

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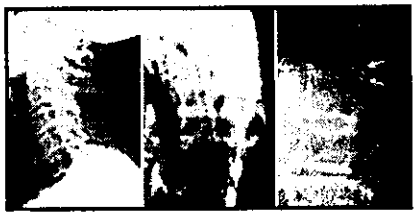

Figure 1. A 71-year-old man with "CC" allele of the transforming growth factor-[beta]1 (TGF-[beta]1) polymorphism had ossification of the posterior longitudinal ligament in the cervical spine (C3–C4,5,6 mixed type) (right), thoracic spine (T3–T8 continuous type) (middle), and lumbar spine (T12–L1) (arrows) (left). Patients with the "C" allele frequently have the ossification in the cervical, thoracic, and/or lumbar spine.

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Discussion

A previous study reported a significant association between the TGF-[beta]1 polymorphism and the prevalence of OPLL in the cervical spine. 16 The authors concluded that the T->C polymorphism of the TGF-[beta]1 gene is a genetic determinant of a predisposition to OPLL, with the "C" allele representing a risk factor for genetic susceptibility to OPLL. However, their study included only 46 patients with OPLL. The current study did not show a positive association between the TGF-[beta]1 polymorphism and OPLL. Transforming growth factor-[beta]1 plays an important role in bone metabolism, proving dominant among the isoform. 17,18 It has been shown to modulate bone development and fracture healing. 19–21 When TGF–T[beta]1 is applied locally or systemically *in vivo*, it acts as a potent stimulator of bone formation. In addition, TGF-[beta] is present in ossified matrix and in chondrocytes of cartilage adjacent to areas of OPLL, 10 and likely affects the development of OPLL. In the current study, there was a significant difference in the distribution of the TGF-[beta]1 polymorphism between the group with OPLL in the cervical, thoracic, and/or lumbar spine and the group with OPLL only in the cervical spine. The "C" allele is a risk factor for patients with OPLL in other areas in addition to the cervical spine. It has been reported that the "C" allele is a risk factor for genetic susceptibility to spinal osteophytosis. 14 Therefore, TGF-[beta]1 polymorphism is not a factor associated with the occurrence of OPLL, but a factor related to the area of the ossified lesion in the total spine in OPLL patients.

In conclusion, there was no association between the polymorphism of TGF-[beta]1 in the signal sequence and the prevalence of OPLL in a large number of subjects. However, there was a significant difference in the distribution of the TGF-[beta]1 polymorphism between the group with OPLL in the cervical, thoracic, and/or lumbar spine and the group with OPLL only in the cervical spine. Therefore, the "C" allele appears to be a risk factor for patients with OPLL involving any of the three spinal levels.

Key Points

* The relations between the TGF-[beta]1 polymorphism (T->C transition in the signal sequence) and the characteristics of OPLL were investigated.
* The difference in the TGF-[beta]1 allele distribution ("TT," "TC," and "CC") between 369 patients with OPLL and 258 control subjects was assessed.
* There was no statistical difference in the TGF-[beta]1 allele between the OPLL and control groups.
* In the patients with "TC" or "CC" alleles, OPLL was found frequently in the cervical, thoracic, and/or lumbar regions.

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[Key words: OPLL; PCR; TGF–T[beta]1 polymorphism] **Spine 2003;28:1424–1426**

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Clinical course of patients with ossification of the posterior longitudinal ligament: a minimum 10-year cohort study

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Object. Ossification of the posterior longitudinal ligament (OPLL) may produce quadriplegia. The course of future neurological deterioration in patients with radiographic evidence of OPLL, however, is not known. The authors conducted a long-term follow-up cohort study of more than 10 years to clarify the clinical course of this disease progression.

Methods. A total of 450 patients, including 304 managed conservatively and 146 treated by surgery, were enrolled in the study. All patients underwent neurological and radiographical follow-up examinations for a mean of 17.6 years. Myelopathy was graded using Nurick classification and the Japanese Orthopaedic Association scale.

Fifty-five (17%) of 323 patients without myelopathy evident at the first examination developed myelopathy during the follow-up period. Risk factors associated with the evolution of myelopathy included greater than 60% OPLL-induced stenotic compromise of the cervical canal, and increased range of motion of the cervical spine. Using Kaplan–Meier analysis, the myelopathy-free rate in patients without first-visit myelopathy was 71% after 30 years. A significant difference in final functional outcome was not observed between nonsurgical and surgical cases in which preoperative Nurick grades were 1 or 2. In patients with Nurick Grade 3 or 4 myelopathy, however, only 12% who underwent surgery eventually became wheelchair bound or bedridden compared with 89% of those managed conservatively. Surgery proved ineffective in the management of patients with Grade 5 disease.

Conclusions. Results of this long-term cohort study elucidated the clinical course of OPLL following conservative or surgical management. Surgery proved effective for the management of patients with Nurick Grades 3 and 4 myelopathy.

KEY WORDS • **ossification of the posterior longitudinal ligament** • **myelopathy** • **cervical spine stenosis** • **long-term follow-up study**

OSSIFICATION of the posterior longitudinal ligament, a subtype of diffuse idiopathic skeletal hyperostosis,[14] typically occurs in individuals age 50 years or older who initially present with myelopathy.[9,13,17] It is thought that Key[4] was the first to call attention to association of OPLL and myelopathy. Several papers on OPLL in caucasians have been published recently.[5,6,8] Thus, OPLL is more common in the aging population with myelopathy and may contribute to significant neurological disability. Its recognition and appropriate management is therefore critical. There are no long-term prospective studies of patients with OPLL, and little is understood of the natural history of this disease. To delineate better the rapidity of radiographic and symptomatic progression of the ossified PLL in the long term, we studied the clinical, radiographic, and conservative/surgical management data obtained in 450 patients treated since 1972.

Abbreviations used in this paper: JOA = Japanese Orthopaedic Association; OPLL = ossification of the posterior longitudinal ligament; ROM = range of motion.

Clinical Material and Methods

The current study is composed of 486 patients with OPLL treated at a single center. Of these 486 patients, 450 were available for the long-term cohort study; 36 died or moved and could not be followed. At the time of presentation, the mean patient age was 59.6 years (range 54–78 years), whereas at final follow up the mean age was 72.6 years (range 69–95 years). There were 319 men and 131 women. Clinical examinations and plain radiography were performed prospectively for a minimum 10 and maximum of 30 years (mean 17.6 years) since 1972. Patients were examined once a year for progression of disease on radiography, clinical myelopathic features, and their concomitant ability to perform the activities of daily living. Myelopathy was estimated using the Nurick[11] classification system and the JOA myelopathy scale.[18] The cumulative myelopathy-free rate from was calculated us-

ing the Kaplan–Meier method.[3] The radiographic state of disease was estimated using plain x-ray films.

Statistical Analysis

Parametric statistical analysis was performed using the Student t-test. Categorical variables were analyzed using chi-square analysis or Fisher exact test. All values are expressed as means with 95% confidence intervals.

Results

Clinical Course

Conservative therapy was undertaken in 304 patients and surgical therapy in 146 patients (Table 1). Conservative therapy was administered in 36 patients with myelopathy who refused to undergo surgery and in 268 patients who did not exhibit symptoms of myelopathy during the follow-up period (Fig. 1). An anterior decompression and fusion was performed in 54 patients and laminoplasty in 92 patients. This procedure was performed for ossification extending across two vertebrae, and laminoplasty was indicated for ossification extending more than two vertebrae. All patients who underwent surgery suffered myelopathy. Surgery was conducted after the initial examination in 91 patients and during follow up in 55 patients.

Myelopathy was originally recognized in 127 patients, 91 of whom were managed surgically. The remaining 36 myelopathic patients underwent conservative therapy, and increased myelopathy was observed in 23 (64%) of these individuals. Of the 323 patients without myelopathy at initial evaluation, 55 (17%) became myelopathic during the follow-up interval (Fig. 1). The Kaplan–Meier[3] estimates of the myelopathy-free rate in patients without myelopathy at presentation was 71% at 30-year follow-up examination (Fig. 2).

Risk Factors for Myelopathy

Forty-five patients in whom the spinal canal was compromised by OPLL-induced stenosis of greater than 60% suffered myelopathy. A correlation between the Nurick grade of stenosis and development of myelopathy was not found in the remaining 405 patients with less than 60%

TABLE 1

*Summary of demographic characteristics in 450 patients**

Characteristic	Treatment	
	Surgical	Conservative
no. of cases	146	304
sex		
male	104	215
female	42	89
age (yrs) at 1st exam		
mean	61.2	59.1
range	54–75	58–78
FU period (yrs)	18.2	17.2
mean	10–29	10–30
age (yrs) at last exam	73.4	71.5
mean	72–93	69–95

* FU = follow up.

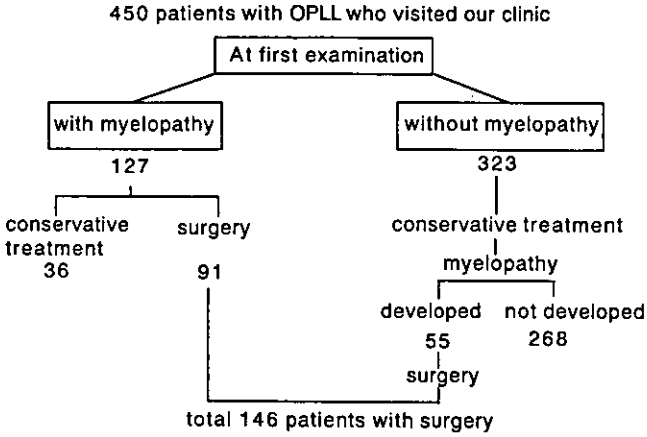

FIG. 1. Schematic diagram showing presentation and treatment data in 450 patients with OPLL.

OPLL-induced spinal canal stenosis. The longitudinal and coronal expansion of the ossified PLL occurred at a comparable rate in both surgically (65%) and nonsurgically treated cases (58%). A small amount of ossification seen on the initial examination rarely developed into greater than 60% ossification. As a dynamic factor, cervical ROM was calculated on lateral plain x-ray films with full extension and flexion; the angle between C-1 and the inferior margin of C-7 was measured, and the difference between maximal anteflexed and maximal retroflexed positions was obtained. The ROM was significantly ($p < 0.05$) greater in patients with myelopathy than those without myelopathy in patients with less than 60% spinal canal stenosis (Table 2). The sagittal curvatures of the cervical spine were classified, according to the method described by Toyama, et al.,[16] as the lordosis, straight, kyphosis, or swan-neck type based on the distance from straight line drawn from the posterior plane of the spinal axis to the C-7 posterior plane. Kyphosis or swan-neck type malalignment of the cervical spine was demonstrated in 54 patients during follow-up examination. Corelationship between the occurrence of myelopathy and malalignment of cervical spine, however, was not found.

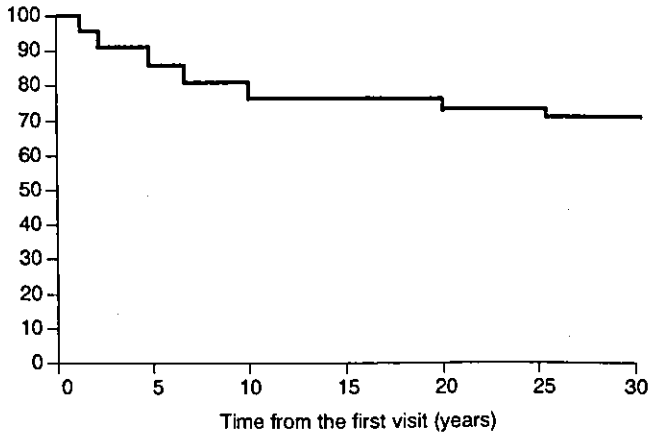

FIG. 2. Graph demonstrating results of Kaplan–Meier estimate regarding myelopathy-free rates in patients without myelopathy at initial examination.

TABLE 2

Cervical ROM in patients with and without myelopathy with less than 60% spinal canal stenosis

Factor	W/ Myelopathy*	W/Out Myelopathy*	p Value
no. of cases	137	268	
cervical ROM (°)	75.6 ± 18.3	36.5 ± 15.9	<0.05

* Values expressed as the mean ± standard deviation.

Surgical Treatment

In patients with myelopathy, the 146 patients who underwent surgery and the 36 patients who received conservative therapy, comparable clinical features were observed (Table 3). Of the 81 surgically treated patients in whom preoperative status was Nurick Grade 3 or 4 (ambulation with assistance and unable to work), only 12% (10 patients) suffered further deterioration, eventually becoming wheelchair bound or bedridden. Alternatively, 17 (89%) of 19 others with Grade 3 or 4 myelopathy managed conservatively became completely disabled. Surgery proved ineffective in all patients with Nurick Grade 5 myelopathy (wheelchair bound or bedridden). No significant differences in the final functional results were found between patients with Nurick Grade 1 status (mild myelopathy, normal gait) or Grade 2 status (mild gait impairment) who underwent surgery or conservative therapy (Table 4). Of note, no significant differences were noted with respect to surgical procedures. The same results were obtained when using the JOA classification.[18] Surgery proved significantly more effective in patients with a preoperative JOA score ranging from 5 to 11; in these patients moderate myelopathy was exhibited (Table 5).

Discussion

In the Japanese population of individuals older than 30 years of age, the incidence of OPLL is reportedly 1.9 to 4.3%.[7] Resnick, et al.,[14] reported that in 74 patients with diffuse idiopathic skeletal hyperostosis, OPLL was demonstrated in 50% on cervical radiographs. In 1994, Epstein[1] reported the high incidence of OPLL in caucasian patients with myelopathy (25%) and also proposed a new concept of early-stage OPLL (OPLL in evolution) characterized by hypertrophied PLL with punctuate calcification. Thus, OPLL is a more common disease in the aging population with myelopathy and may contribute to significant neurological disability.

TABLE 3

*Baseline characteristics in 182 patients with myelopathy**

Characteristic	Surgical	Conservative	p Value
no. of cases	146	36	
age (yrs) at onset of myelopathy†	62.9 ± 8.9	61.8 ± 9.5	NS
male/female ratio	2.31	2.46	NS
Nurick Grade			
1	16 (10.9)	5 (13.9)	NS
2	18 (12.3)	6 (16.7)	NS
3	42 (28.7)	9 (25.0)	NS
4	39 (26.7)	10 (27.8)	NS
5	31 (21.2)	6 (16.7)	NS

* NS = not significant.

† Values are expressed as the mean ± standard deviation.

TABLE 4

Summary of cases becoming wheelchair bound or bedridden at the end of the survey in each therapeutic category in 182 patients with myelopathy

Pretreatment Nurick Grade	No. Becoming Wheelchair or Bed Bound (%)		p Value
	Surgical	Conservative	
no. of cases	146	36	
grade			
1	0 (0) of 16	0 (0) of 5	NS
2	2 (11.1) of 18	1 (16.7) of 6	NS
3	4 (9.5) of 42	8 (88.9) of 9	<0.001
4	6 (15.3) of 39	9 (90) of 10	<0.001
5	26 (90.3) of 31	6(100) of 6	NS

During the follow-up period, the longitudinal and coronal expansion of the ossified PLL occurred at comparable rates in both surgically (65%) and nonsurgically treated populations (58%). Although myelopathy was recognized in all patients in whom greater than 60% of the spinal canal was compromised by the ossification, a small-sized ossified PLL observed at first examination rarely developed into a large-sized ossified PLL with more than 60% stenosis during the follow-up period. Therefore, one cannot simply say that myelopathy develops with progression of the ossification. Rather, dynamic factors such as ROM appear to be more important for the evolution of myelopathy in patients in whom less than 60% of the canal is compromised by the ossified mass. Generally, patients with OPLL exhibited restriction of ROM of cervical spine caused by ossification of spinal ligaments. Patients with OPLL in whom no restricted ROM of the cervical spine was demonstrated are susceptible to myelopathy caused by dynamic factors.

In the present study, we used the minimal diameter of the canal for evaluation of a static factor; however, recently the cross-sectional area of the spinal cord has been evaluated by computerized tomography or MR imaging as a static factor.[12,15,18] Okada, et al.,[12] have reported that this method yielded a higher correlation with myelopathy than minimal diameter as determined using plain radiography, and this method is gaining attention as a method for determining a static factor. Hereafter, evaluation of a cross section of the spinal cord may be necessary.

TABLE 5

Summary of cases in which assistance with activities of daily living was not required at the end of the survey in each therapeutic category in 182 patients with myelopathy

JOA Score	Assistance Not Required (%)		p Value
	Surgical Treatment	Conservative Treatment	
0–4	0 (0) of 19	0 (0) of 5	
5–11	63 (79.7) of 79	2 (16.7) of 12	<0.001
12–16	40 (83.3) of 48	14 (73.7) of 19	NS

Fairly good results have been reported for surgical treatments in patients with OPLL,[2,10] but the relation of surgery to prognosis is unclear because the natural course of clinical symptoms has not been fully detailed. There are no clear indications concerning the timing and extent of surgery. Laminectomy was previously performed for extensive ossification, but laminoplasty is now conducted to preserve posterior support for the spinal column and to prevent postoperative kyphosis. Anterior decompression and fusion are also performed in cases in which the ossification extends to two vertebrae. Analysis of findings in this long-term cohort study of patients with OPLL revealed that the cumulative myelopathy-free survival rate in patients without myelopathy at first presentation was 71% after 30 years. These data indicate that prophylactic surgery in patients with OPLL in the absence of myelopathy may not be necessary, particularly because the course of symptomatic progression appears more mild than anticipated. Surgery in patients with Nurick Grades 1 and 2 myelopathy appears to achieve the same clinical outcome as conservative therapy. On the other hand, surgery is definitely indicated for patients with Grade 3 and 4 disease because only 12% will suffer continued deterioration, compared with 89% of those managed nonsurgically who become wheel bound or bedridden. Finally, in patients with Nurick Grade 5 disease, severe myelopathy and poor outcomes appear to prevail with or without surgery.

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Possible Roles of CTGF/Hcs24 in the Initiation and Development of Ossification of the Posterior Longitudinal Ligament

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Study Design. A biochemical and histochemical study investigating the role of CTGF/Hcs24 in the ossification of the posterior longitudinal ligament (OPLL) was conducted.

Objective. To clarify the involvement of CTGF/Hcs24 in ectopic bone formation in OPLL through endochondral ossification using human tissue.

Summary of Background Data. Previous studies have shown that various cytokines are involved in the occurrence or development of ectopic bone formation in OPLL. Recently, the authors cloned an mRNA predominantly expressed in chondrocytes by differential display PCR and found that its gene, *hcs24,* is identical to that of connective tissue growth factor. It has been shown that CTGF/Hcs24 plays a major role in endochondral ossification.

Methods. Ossified ligament tissues were taken from seven male OPLL patients during surgery. Immunohistochemical staining was performed using an antibody specific for CTGF/Hcs24. Spinal ligament cells were isolated from five OPLL patients as well as five non-OPLL patients. The cells were incubated with recombinant human CTGF/Hcs24 or TGFβ. The expression of ALP was analyzed by RT-PCR. For the effects of TGFβ, the expression of CTGF/Hcs24 mRNA was analyzed.

Results. Immunohistochemical staining showed that chondrocytes in the transitional region from nonossified to ossified ligament were stained with an antibody against CTGF/Hcs24. It was found that CTGF/Hcs24 enhanced the expression ALP mRNA in OPLL cells, whereas the expression remained unchanged in non-OPLL cells. The expression of CTGF/Hcs24 mRNA in OPLL and non-OPLL cell lines was increased by TGFβ, and there was no significant difference between the two groups. However, TGFβ and CTGF/Hcs24 enhanced the expression of ALP mRNA only in OPLL cells.

Conclusions. According to the study results, CTGF/Hcs24 may not only be an important factor in the development of endochondral ossification in OPLL, but may also be responsible for initiating osteogenesis in spinal ligament cells. [Key words: CTGF/Hcs24, ectopic bone formation, endochondral ossification, ossification of the posterior longitudinal ligament] **Spine 2002;27:1852–1857**

Ossification of the posterior longitudinal ligament (OPLL) of the spine is a pathologic condition causing ectopic bone formation and resulting in various degrees of myelopathy. Ectopic bone formation, thought to occur through endochondral ossification,[12,13] is recognized as a common disorder among the Japanese and throughout Asia. Numasawa et al[11] reported that the genetic causality of OPLL lies within or near the retinoic X receptor β–collagen 11A2 locus. However, OPLL also seems to be influenced by multiple extrinsic factors including bone morphogenetic protein-2 (BMP-2),[6,7] transforming growth factor-β (TGF-β),[5,6] and insulin-like growth factor-1 (IGF-1).[3] The mechanism of ectopic ossification is still unknown.

The current study was conducted to clarify the involvement of CTGF/Hcs24 in ectopic bone formation in OPLL. The expression of CTGF/Hcs24 in OPLL tissues was examined immunohistochemically using an antibody specific for CTGF/Hcs24. The effects of adding recombinant human CTGF/Hcs24 (rhCTGF/Hcs24) to cultured spinal ligament cells were analyzed. The analysis showed that CTGF/Hcs24 is involved in the initiation of pathologic ossification.

■ Materials and Methods

The Ethics Committee of Hirosaki University School of Medicine approved this study.

Subjects. Ossified ligament tissues and posterior longitudinal ligaments (PLL) were taken from seven male OPLL patients during surgery to decompress the spinal cord for myelopathy (Table 1). As a control, cervical PLLs were taken from seven non-OPLL patients during surgery for cervical disc herniation and cervical spondylotic myelopathy. Tissue samples of ligamentum flavum at the point of insertion also were taken from two non-OPLL patients (cases 10 and 11) who had lumbar spinal canal stenosis and syringomyelia.

For cell culture, cervical PLLs were obtained aseptically from five OPLL patients and five non-OPLL patients during surgery (Table 1). The tissues from the OPLL patients were taken carefully more than 5 mm away from ossified sites to

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Table 1. List of Patients With Ossification of the Posterior Longitudinal Ligament (OPLL) and Those Without OPLL

Case	Diagnosis	Age	Gender	Tissue of Origin	Immunohistochemical Staining	Cell Culture No.
1	OPLL	48	M	PLL	Done	OPLL 1
2	OPLL	63	M	PLL	Done	OPLL 2
3	OPLL	65	M	PLL	Done	OPLL 3
4	OPLL	42	M	PLL	Done	
5	OPLL	48	M	PLL	Done	
6	OPLL	46	M	PLL		OPLL 4
7	OPLL	58	M	PLL		OPLL 5
8	CDH	49	M	PLL	Done	
9	CSM	47	F	PLL	Done	
10	LSCS	66	M	YL	Done	
11	Syringomyelia	63	F	YL	Done	
12	CDH	35	F	PLL		non-OPLL 1
13	CDH	49	F	PLL		non-OPLL 2
14	CSM	56	F	PLL		non-OPLL 3
15	CSM	64	F	PLL		non-OPLL 4
16	CSM	59	M	PLL		non-OPLL 5

PLL = posterior longitudinal ligament; CDH = cervical disc herniation; LSCS = lumbar spinal canal stenosis; YL = yellow ligament; CSM = cervical spondylotic myelopathy.

avoid contamination of osteogenic cells from ossified ligament.[3,7]

Tissue Preparation. Specimens were immediately fixed with 4% paraformaldehyde for 24 hours at 4° C. The specimens with ossified tissue were decalcified with 10% EDTA (pH 7.2) at 4° C, then bisected sagittally in the median plane. They were dehydrated with graded ethanol and embedded in paraffin. Paraffin sections 4 μm thick were prepared and subjected to staining by hematoxylin and eosin (H&E) and safranin O. Subsequently, immunohistochemical staining by antibodies against CTGF/Hcs24 and Type 2 collagen was performed.

Immunohistochemical Staining. Immunohistochemical staining was performed by the avidin–biotin complex method, using a HISTOFINE SAB-SPO kit (Nichirei, Tokyo, Japan). After the specimens were treated with 1 mg/mL of hyaluronidase (Sigma Chemical, St. Louis, MO) for 30 minutes at 37° C, endogenous peroxidase was blocked with methanol containing 3% hydrogen peroxide for 30 minutes at room temperature. After washing in tris-buffered saline containing 0.1% Tween 20 (TBST), they were incubated in TBST containing 10% normal goat serum for 15 minutes at room temperature to block nonspecific protein binding and then reacted with the primary antibody. Next, the specimens were reacted with anti-CTGF/Hcs24 antibodies or anti–Type 2 collagen polyclonal antibodies (Chemicon International, Temecula, CA) diluted 1:100 and 1:40, respectively, with TBST at 4 C overnight. Anti-CTGF/HCS24 polyclonal antibody was prepared as previously described.[14] The color reaction was performed with 3,3'-diaminobenzidine tetrahydrochloride containing hydrogen peroxidase. As a negative control, primary antibodies were replaced by preimmune rabbit serum at the same dilution.

Cell Cultures. After being washed several times with phosphate-buffered saline (PBS), the tissues were minced into approximately 1-mm^2 pieces, then placed in 100-mm-diameter culture dishes containing Dulbecco's Modified Eagle Medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS). The explants were incubated in a controlled 5% CO_2 atmosphere at 37° C. Cells migrating from the explants were harvested with 0.1% trypsin and replated in other 100-mm-diameter dishes. Cells after the fifth passage were seeded in 35-mm-diameter dishes at a density of 3×10^4 cells/mm^2. Cell lines obtained from OPLL and non-OPLL patients were named the OPLL and non-OPLL groups, respectively.

Stimulation of Spinal Ligament Cells. When the spinal ligament cells became confluent, the medium was replaced with DMEM having 1% FBS. After 24 hours, the cells were incubated with CTGF/Hcs24 (10 ng/mL) for 24 hours. We prepared rhCTGF/Hcs24 as previously described.[10] A control experiment was performed without rhCTGF/Hcs24 treatment. In the same way, the effects of human TGF-β1 (Wako Pure Chemical Industries, Osaka, Japan) were examined. Cells were incubated with TGF-β (10 ng/mL) for 12 hours. Subsequently, the expression of the osteoblastic marker, alkaline phosphatase (ALP) mRNA, was analyzed by reverse transcriptase–polymerase chain reaction (RT-PCR). For the effects of TGF-β, the expression of CTGF/Hcs24 mRNA was analyzed.

Isolation of RNA From Cultured Cells and RT-PCR Analysis. Total RNA was isolated from cultured ligament cells by use of the RNeasy Mini Kit (Qiagen, Hilden, Germany). The total RNA (0.5 μg) treated with DNase I (Gibco BRL, Gaithersburg, MD) was incubated with an oligo (dT) primer and then reverse transcribed to cDNA in a reaction mixture that contained 0.5 mmol/L of dNTP, 50 mmol/L of Tris-HCl (pH 8.3), 75 mmol/L of KCl, 3 mmol/L of $MgCl_2$, 20 mmol/L of DTT (Gibco BRL), 2 U of RNase inhibitor (Toyobo Biochemicals, Osaka, Japan), and 200 U of M-MLV reverse transcriptase (Gibco BRL) for 60 minutes at 37 C. Subsequent PCR amplification was performed using the *Taq* PCR Master Mix Kit (Qiagen). The primers used for amplification were as follows: CTGF/Hcs24: 5'-TTGTAGCTGATCAGTCTTTCCAC-3' (DD24) and 5'-CAACTAAAAAGGTGCAAACATGTAA-3' (DD24R2)[8]; ALP: 5'-ATCGCCTACCAGCTCATGCAT-3' and 5'-GTTCAGCTCGTACTGCATGTC-3'.[15]

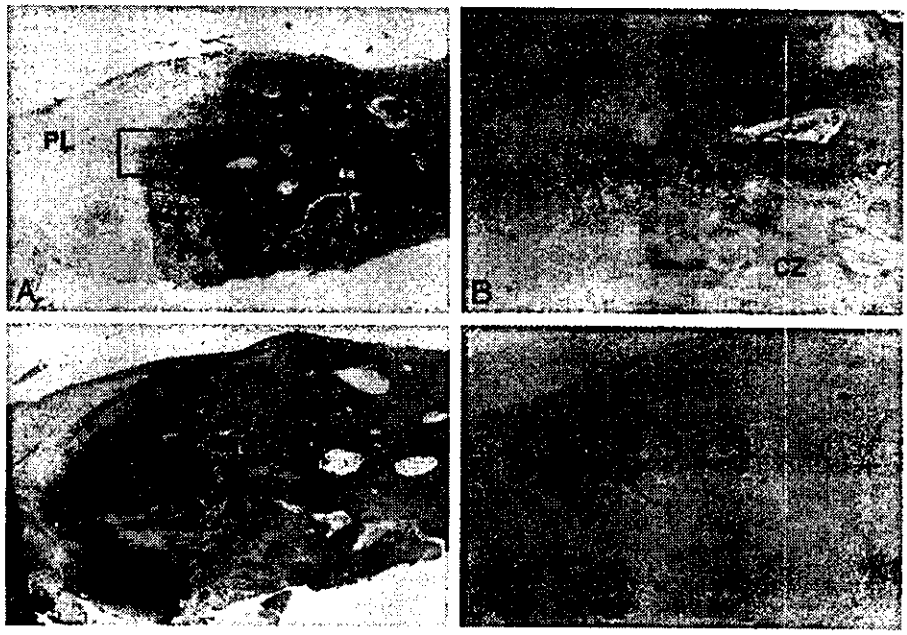

Figure 1. Histologic findings of ossified ligament. **A** and **B**, Photomicrographs of ossified ligament derived from an OPLL patient and stained with H&E. Safranin O (**C**) and anti-Type 2 collagen antibody (**D**). **B** is a higher magnification of the transitional region from nonossified to ossified ligament in **A**. Original magnification, ×40 (**A**, **C**, and **D**); ×200 (**B**). **PL**, posterior longitudinal ligament; **CZ**, calcified zone; **OL**, ossified ligament

For relative quantitative RT-PCR analysis, PCR was performed for an appropriate number of cycles in which the amount of PCR product increased exponentially. The PCR products were applied on agarose gels (2.5%) and then electrophoresed. Then the gels were stained with SYBR Green I (Molecular Probes, Eugene, OR) and analyzed with QuantiScan (BIOSOFT, Cambridge, UK).[1] In the densitometric analysis, the amount of specific PCR products was normalized by the number of PCR products of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) as an internal standard.

Statistical Analysis. All data were presented as means ± standard deviation. The differences were analyzed using an unpaired *t* test. The level of significance was set at a *P* value less than 0.05.

■ Results

CTGF/Hcs24 Expression in Ossified Ligament Tissues

Histologic examinations of ossified ligament tissues from OPLL patients showed that nonossified ligamentous tissue demonstrated a continuous transition to ossified tissue, and that cartilaginous tissue, including a calcified zone, was present around the ossified tissue (Figure 1A and 1B). Chondrocytes were observed in the transitional region from nonossified to ossified ligament. The extracelluar matrix of the transitional region was stained red with safranin O staining (Figure 1C). The matrix and surrounding chondrocytes were stained with anti-Type 2 collagen antibodies (Figure 1D). These findings demonstrated that cartilaginous tissue was present in the transitional region of OPLL.

Immunohistochemical staining showed that CTGF/Hcs24 was expressed in chondrocytes around the ossified tissue (Figure 2A and 2B). The chondrocytes, especially the matured and hypertrophic types in the calcified zone, showed an expression of CTGF/Hcs24 (Figure 2B). In the ligament remote from the ossified tissue, some fibroblasts also were weakly stained with anti-CTGF/Hcs24 antibody (data not shown). None of these areas or cells was stained with preimmune rabbit serum (data not shown). In the control cases, fibroblasts in the posterior longitudinal ligament tissues were not stained with anti-CTGF/Hcs24 antibody (data not shown). At the insertion of ligamentum flavum into bone, cartilaginous tissue was observed, and the chondrocytes were stained with anti-CTGF/Hcs24 antibody (data not shown).

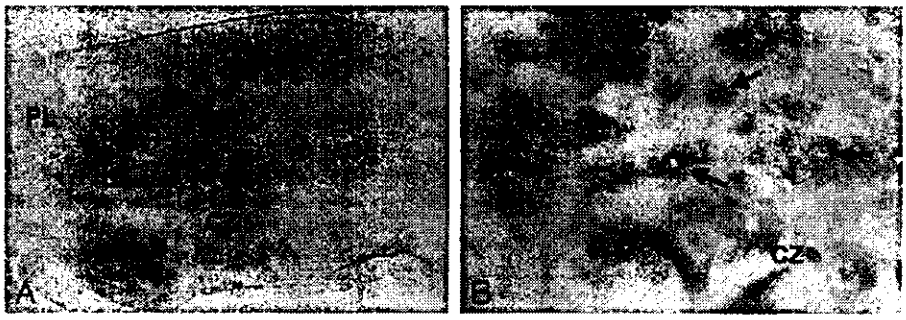

Figure 2. Immunohistochemical findings of CTGF/Hcs24 in ossified ligament derived from an OPLL patient. **A** and **B**, Photomicrographs of immunohistochemical staining with anti-CTGF/Hcs24 antibody. **B** is the transitional region from nonossified to ossified ligament. Original magnification, ×40 (**A**); ×200 (**B**). **PL**, posterior longitudinal ligament; **CZ**, calcified zone; **OL**, ossified ligament

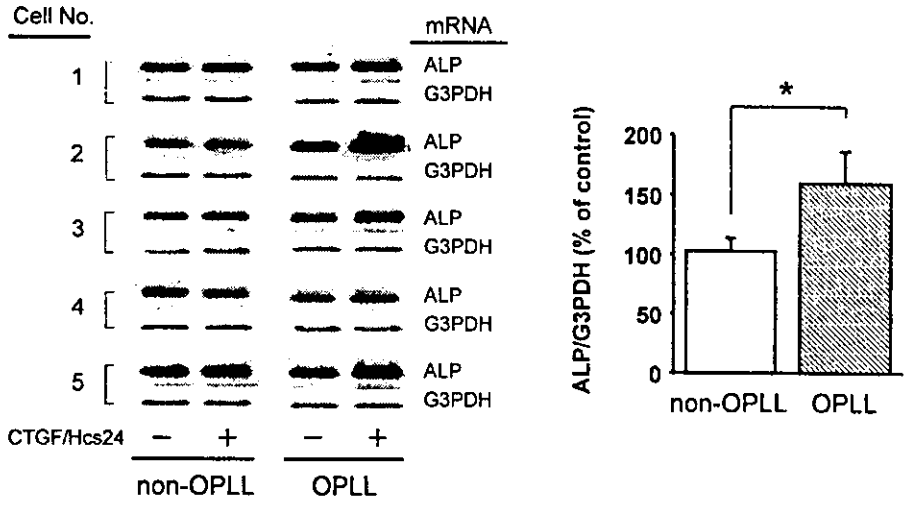

Figure 3. Effect of rhCTGF/Hcs24 on the expression of ALP mRNA in cultured spinal ligament cells. The total RNA (0.5 μg) derived from spinal ligament cell cultures treated with rhCTGF/Hcs24 (10 ng/mL) for 24 hours was reverse-transcribed and PCR amplified with ALP or G3PDH-specific primer. A control experiment was performed without rhCTGF/Hcs24. The bands for RT-PCR products appeared on agarose gel electrophoresis (left panel). The ALP mRNA levels were determined by quantitative measurement of bands using densitometric analysis and normalized by mRNA level for G3PDH from the same sample (right panel).

Effects of CTGF/Hcs24 on ALP mRNA in Spinal Ligament Cells

In spinal ligament cells from each of the five OPLL and non-OPLL patients, the effects of adding rhCTGF/Hcs24 to the ALP mRNA level were analyzed (Figure 3). The relative mRNA level (ALP/G3PDH) without CTGF/Hcs24 treatment varied depending on the cells. There was no significant difference between the two groups. However, CTGF/Hcs24 enhanced the expression of ALP mRNA in the cells from four of five OPLL patients, whereas it was not enhanced significantly in the non-OPLL patients. The relative mRNA level (ALP/G3PDH) without CTGF/Hcs24 treatment was expressed as 100%. The expression of ALP mRNA increased 155.5% ± 38.3% above the control level (without CTGF/Hcs24 treatment) in cells from the OPLL group, whereas that of the non-OPLL group was 102.1% ± 11.2%. The percentage of control was significantly different statistically between the OPLL and non-OPLL groups ($P < 0.05$) (Figure 3).

Effects of TGF-β on CTGF/Hcs24 mRNA in Spinal Ligament Cells

Cells from four OPLL and non-OPLL patients were incubated with TGF-β for 12 hours. As shown in Figure 4, TGF-β increased the expression of CTGF/Hcs24 mRNA to 207.5% ± 35.9% above the control level (without TGF-β treatment) in cells from the OPLL group, and to 187.0% ± 31.2% in the non-OPLL group, and there was no significant difference between the two groups. However, TGF-β enhanced differentially between the two groups on the expression of ALP mRNA. After TGF-β treatment, the expression of ALP mRNA in cells from the OPLL group increased to 170.9% ± 33.2% above the

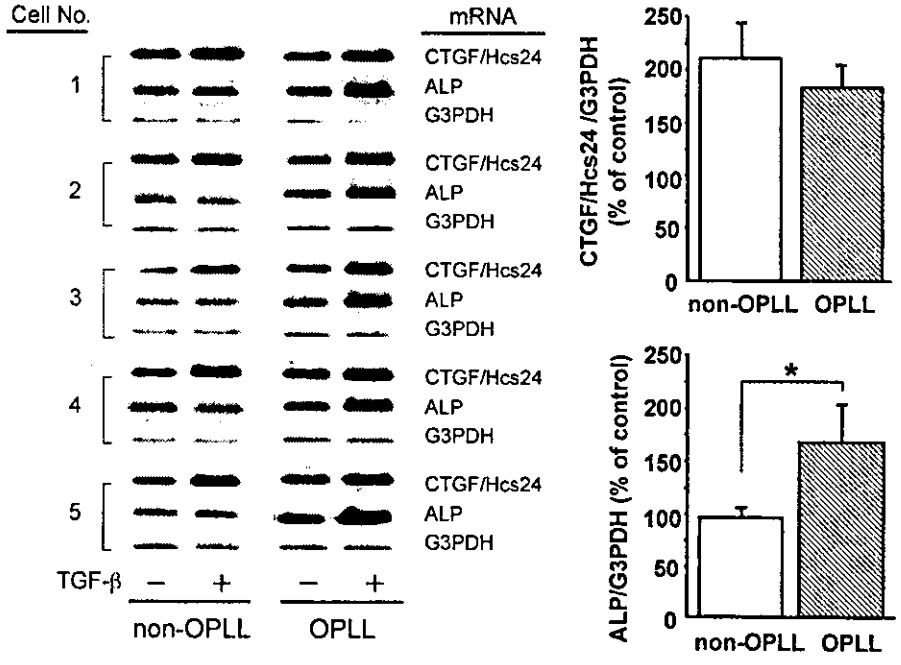

Figure 4. Effect of TGF-β on the expression of CTGF/Hcs24 and ALP mRNA in cultured spinal ligament cells. The total RNA (0.5 μg) derived from spinal ligament cell cultures treated with TGF-β (10 ng/mL) for 12 hours was reverse-transcribed and PCR amplified with CTGF/Hcs24, ALP, or G3PDH-specific primer. A control experiment was performed without TGF-β. The bands for RT-PCR products appeared on agarose gel electrophoresis (left panel). The ALP and CTGF/Hcs24 mRNA levels were determined by quantitative measurement of bands using densitometric analysis and normalized by mRNA level for G3PDH from the same sample (right panel).

control level, however, that of the non-OPLL group was unchanged (98.9% ± 9.2%). There was a significant difference between the two groups ($P < 0.01$), suggesting that TGF-β enhances ALP mRNA expression only in cells from the OPLL group.

■ Discussion

The current study demonstrated immunoreactivity to CTGF/Hcs24 in chondrocytes around the ossified tissues in OPLL patients, CTGF/Hcs24 enhancement of the ALP expression in spinal ligament cells from OPLL patients, and CTGF/Hcs24 expression upregulated by TGF-β in spinal ligament cells.

Initially, CTGF was identified from angioendothelial cells as a growth factor related to platelet-derived growth factor.[2] However, it promoted not only the proliferation and differentiation of chondrocytes, but also the proliferation and migration of vascular endothelial cells through an autocline or paracline mechanism.[8,9] Thus, CTGF/Hcs24 was redefined as a major factor that guides chondrocytes toward endochondral ossification.

The ectopic bone formation in OPLL is thought to occur through the endochondral ossification.[12,13] Therefore, the expression of CTGF/Hcs24 in the chondrocytes around the ossified tissue in OPLL suggests that CTGF/Hcs24 may play an important role in the development of OPLL. On the other hand, it has not been documented previously in ligaments other than those of the spine. These findings may be unique to spinal ligaments of OPLL patients.

Because CTGF/Hcs24 was found in some fibroblasts in the ligament remote from ossified tissue in OPLL, it also may be involved in the initiation of OPLL. Spinal ligament cells from OPLL patients may have a different character originally from those of non-OPLL patients. One of five cell cultures from OPLL patients was a nonresponder to CTGF/Hcs24. Sensitivity to CTGF/Hcs24 may reflect the future development of OPLL. Similar phenomena were observed in the case of BMP-2.[7] Progression of ossification of PLL is not constant in OPLL patients, but varies from case to case. In the OPLL patient whose ALP/mRNA level was not responsive to CTGF/Hcs24, progression of OPLL might have ended by the time of surgery.

Previous studies have shown that various cytokines, including BMP-2,[6,7] TGF-β,[5,6] and IGF-I,[3] were involved in the occurrence or development of ectopic bone formation in OPLL. In addition to BMP-2, the expression of CTGF/Hcs24 was induced by TGFβ in fibroblasts and a human chondrocytic cell line, HCS-2/8.[4,8] These results suggest that enhanced sensitivity to CTGF/Hcs24 of ligament cells in OPLL patients is involved in ectopic bone formation in combination with other growth factors.

In conclusion, CTGF/Hcs24 may not only be an important factor in the development of endochondral ossification in OPLL patients, but also may be responsible for guiding spinal ligament cells toward osteogenesis. It may be possible to stop the progression of ossification of PLL if the expression of CTGF/Hcs24 is blocked around the ossified ligament in OPLL patients. Future research is necessary, and will help to elucidate the precise etiology of OPLL.

■ Key Points

- The findings show that CTGF/Hcs24 is expressed in the chondrocytes of ossified tissue from OPLL patients.
- The sensitivity to CTGF/Hcs24 of spinal ligament cells from OPLL patients was higher than that of cells from non-OPLL patients.

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Calcified Tissue International

Laboratory Investigations

Uni-axial Cyclic Stretch Induces Cbfa1 Expression in Spinal Ligament Cells Derived from Patients with Ossification of the Posterior Longitudinal Ligament

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Abstract. Ossification of the posterior longitudinal ligament of the spine (OPLL) is characterized by ectopic bone formation in the spinal ligaments. Mechanical stress, which acts on the posterior ligaments, is thought to be an important factor in the progression of OPLL. To clarify this mechanism, we investigated the effects of *in vitro* cyclic stretch (120% peak to peak, at 0.5 Hz) on cultured spinal ligament cells derived from OPLL (OPLL cells) and non-OPLL (non-OPLL cells) patients. The mRNA expressions of Cbfa1 (an osteoblast-specific transcription factor), type I collagen, alkaline phosphatase (ALP), osteocalcin and integrin β1 (a mechanotransducer) were increased by cyclic stretch in OPLL cells, whereas no change was observed in non-OPLL cells. The effects of cyclic stretch on the spinal ligament tissues derived from OPLL and non-OPLL patients were also analyzed by immunohistochemistry using an antibody against Cbfa1. The expression of Cbfa1 was increased by cyclic stretch at the center of the spinal ligament tissues of OPLL patients, whereas no change was observed in the tissues of non-OPLL patients. Furthermore, U0126, a specific inhibitor of MAPK kinase (MEK), suppressed the stretch-induced mRNA expressions of Cbfa1, ALP and type I collagen in OPLL cells. These results suggest that in OPLL cells, mechanical stress is converted by integrin β1 into intracellular signaling and that Cbfa1 is activated through the MAP kinase pathway. Therefore, we propose that mechanical stress plays a key role in the progression of OPLL through an increase in Cbfa1 expression.

Key words: Posterior longitudinal ligament — Core-binding factor alpha 1 — Integrin β1 — Mechanical stress — Ossification — Immunohistochemistry

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Ossification of the posterior longitudinal ligament of the spine (OPLL) is a pathological condition causing ectopic bone formation. Sometimes ossification enlarges in the spinal canal and compresses the spinal cord, resulting in severe myelopathy or radiculopathy. Ossification of the ligaments occurs throughout the spine, frequently involving ossification of other spinal ligaments, such as the anterior longitudinal ligament, yellow ligament, supraspinous ligament and interspinous ligament. Consequently, it has been regarded as one of the manifestations of diffuse idiopathic skeletal hyperostosis (DISH), as described by Resnick et al. [1]. Although OPLL seems to occur and proceed through multiple extrinsic factors, systemic [2, 3] or regional factors [4–9] and genetic factors [10–12], it has been difficult to predict accurately what kind of factors are playing key roles in this disease.

Clinically, ossification progresses frequently after posterior decompressive surgery of the cervical spine, such as laminectomy or laminoplasty, which destroys the posterior supportive elements and results in various degrees of cervical instability [13–15]. The progression of OPLL was highly correlated with abnormal strain distribution in the intervertebral discs [16]. These observations suggest an important role for dynamic factors in the progression of OPLL.

In some reports, cultured cells obtained from the spinal ligaments of OPLL patients (OPLL cells) exhibited osteoblast-like properties. OPLL cells responded to various growth factors, such as transforming growth factor-β (TGF-β) [5, 17], bone morphogenetic protein-2 (BMP-2) [6], insulin-like growth factor-I (IGF-I) [7], and parathyroid hormone [8], and showed the ability of

Table 1. List of OPLL and non-OPLL patients

OPLL				Non-OPLL			
Case no.	Diagnosis/ossification type	Gender/age	Tissue	Case no.	Diagnosis	Gender/age	Tissue
1	OPLL/continuous	M/63	PLL	15	CDH	M/54	PLL
2	OPLL/segmental	M/46	PLL	16	CDH	M/73	PLL
3	OPLL+OYL/continuous	M/52	YL	17	CDH	F/65	YL
4	OPLL/segmental	F/63	YL	18	CDH	M/44	PLL
5	OPLL/segmental	M/70	PLL	19	CSM	M/46	YL
6	OPLL/segmental	M/59	YL	20	CDH	M/64	PLL
7	OPLL/segmental	M/69	PLL	21	CDH	M/68	PLL
8	OPLL/continuous	M/76	YL	22	CSM	M/60	PLL
9	OPLL+OYL/continuous	M/65	PLL	23	CSM	M/57	PLL
10	OPLL/continuous	M/65	YL	24	CDH	M/51	PLL
11	OPLL/continuous	M/47	YL	25	CDH	M/50	PLL
12	OPLL/continuous	M/51	YL	26	CSM	F/76	YL
13	OPLL/segmental	M/51	PLL	27	CDH	M/50	PLL
14	OPLL/continuous	M/44	PLL				

OPLL: ossification of the posterior longitudinal ligament, OYL: ossification of the yellow ligament, CDH: cervical disc herniation, CSM: cervical spondylotic myelopathy, PLL: posterior longitudinal ligament, YL: yellow ligament, M: male, F: female

calcification and high alkaline phosphatase (ALP) activity [4]. In addition, in the ossified ligament tissues of OPLL patients, osteogenic protein-1 (OP-1)/BMP-7 and its receptors (type-IA, -IB, and -II receptors) were expressed not only in chondrocytes around the calcified site but also in fibroblast-like cells at the sites of non-ossified ligament, while there was weak expression of the receptors at the ligamentous enthesis and no staining of (OP-1)/BMP-7 in the ligament tissues of non-OPLL patients [9]. These pieces of evidence indicate that OPLL development may result from the metaplasia of spinal ligament cells to osteogenic cells, such as osteoprogenitors, rather than the simple enthesopathy frequently observed in other cervical spine diseases.

Cbfa1, also known as RUNX2, OSF2, PEPB2αA or AML3, is a runt family transcription factor and plays a key role in osteoblast differentiation [18]. It binds to the osteoblast-specific *cis*-acting element 2 (OSE2) [19] found in the promoter regions of all the major osteoblast-specific genes, i.e., alkaline phosphatase [20], type I collagen [21], osteopontin [22], and osteocalcin [19], and controls their expression. In addition, Cbfa1 can bind to an OSE2 element in its own promoter, suggesting the existence of an autoregulatory feedback mechanism of transcriptional regulation [23]. Several lines of evidence have suggested that signaling through mitogen-activated protein kinases (MAPKs) is essential for the early stages of osteoblast differentiation [24–28]. Recently, it was reported that mechanical stress activated extracellular signal-regulated kinase (ERK) MAPK, resulting in phosphorylation of the endogenous Cbfa1 in osteoblasts. Furthermore, integrins are supposed to act as mechanotransducers [29–33] and it was reported that the application of mechanical stress to the integrin β1 subunit induced MAPK activation [32, 33].

We hypothesized that in OPLL, mechanical signals may be transmitted to induce the differentiation of spinal ligament cells into osteoblasts. To explore this possibility we investigated the effect of mechanical stress on OPLL development and the participation of Cbfa1 in ossification using cells derived from OPLL patients (OPLL cells). We examined the mRNA expression of Cbfa1 in cultured OPLL cells using quantitative RT-PCR and the distribution of Cbfa1 in spinal ligaments derived from OPLL patients using immunohistochemistry. Moreover, we also examined the mRNA expressions of type I collagen, osteocalcin and ALP, as osteoblast-specific genes, and the integrin β1 subunit, and the effect of a MAPK pathway inhibitor on the expression of these genes.

Materials and Methods

Informed consent was obtained from each patient and this study was approved by the Ethics Committee of Hirosaki University School of Medicine.

Clinical Diagnosis and Spinal Ligament Samples

The diagnosis of OPLL or non-OPLL (i.e., other cervical diseases) was confirmed by X-rays, computerized tomography, and magnetic resonance imaging of the cervical spine preoperatively. The clinical diagnoses and the spinal ligament tissues used in this study are shown in Table 1.

Cell Culture

The spinal ligaments were harvested aseptically from 14 OPLL patients during surgery to decompress the spinal cord for myelopathy. As a control, spinal ligaments were taken from 13 non-OPLL patients during surgery for cervical disc herniation and cervical spondylotic myelopathy. For cell culture, the ligaments were rinsed with phosphate-buffered saline (PBS),

after which the surrounding tissue was carefully removed under a dissecting microscope. The ligaments were extirpated carefully from a non-ossified site to avoid any possible contamination with osteogenic cells. The collected ligaments were minced into approximately 0.5 mm^3 pieces, washed twice with PBS, then plated on 100 mm culture dishes and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS), 1% L-glutamine, and 1% penicillin/streptomycin. The explants were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The fibroblast-like cells that migrated from the explants were released with 0.02% ethylenediamine tetraacetic acid (EDTA)/0.05% trypsin and replated in culture dishes for passage.

Stretch Apparatus

The cells (5^{th} passage) were placed in a 3.5 × 4.0 cm^2 silicon chamber (Scholertec Corp., Osaka, Japan) coated with 0.1% gelatin (Iwaki Glass, Tokyo, Japan) at a density of 10,000 cells/cm^2. After cultures reached confluence, the cells were incubated in DMEM supplemented with 1% FBS for 24 h and then subjected to motor-driven computer-controlled uni-axial cyclic stretch using a four-point bending apparatus (Scholertec Corp., Osaka, Japan) in 120% peak to peak, at 0.5 Hz in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

RNA Preparation and cDNA Synthesis

After different periods of cyclic stretch, total RNA was extracted simultaneously from the cell monolayers with an RNeasy Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's protocol. Total RNA was treated with RNase-free DNase I (Life Technologies Inc., Gaithersburg, USA) and reverse transcribed into cDNA using Oligo(dT)12-18 primer (Life Technologies Inc.). One μg of total RNA was heated at 70°C for 10 min in 10 μl of H_2O supplemented with 0.5 μg Oligo(dT)12-18 primer. The mixture was placed on ice, and cDNA synthesis was then performed by reverse transcription (RT) for 1 h at 37°C in a final volume of 20 μl of buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl and 3 mM $MgCl_2$) supplemented with 0.5 mM of dNTPs (Life Technologies Inc.), 2.5 mM of DTT (Life Technologies Inc.), 2 U of RNase inhibitor (TOYOBO, Osaka, Japan), and 200 U of M-MLV reverse transcriptase (Life Technologies Inc.). After the incubation, the cDNAs were heated to 72°C and stored at −20°C until use for amplification by the polymerase chain reaction (PCR).

PCR Analysis

For PCR amplification, specific oligonucleotide primers were designed as follows: glycerol 3-phosphate dehydrogenase (G3PDH): 5′-TCCACCACCCTGTTGCTGTA-3′ and5′-ACCACAGTCCATGCCATCAC-3′; Cbfa1 type II: 5′-ATGCTTCATTCGCCTCACAAAC-3′ and 5′-CCAAAAGAAGCTTTGCTGACATGG-3′ [35]; ALP: 5′-ATCGCCTACCAGCTCATGCAT-3′ and 5′-GTTCAGCTCGTACTGCATGTC-3′ [34]; type I collagen: 5′-TGACGAGACCAAGAACTG 3′ and 5′-CCATCCAAACCACTGAAACC-3′; osteocalcin: 5′-GATCCCCGCTTCCTCTTTAGAC-3′ and 5′-GGGCTATTTGGGGGTCATCC-3′; integrin β1 subunit: 5′-CTGGGCTTTACGGAGGAAGTAG-3′ and 5′-AGTCGTCAACATCCTTCTCCTTACA-3′. The primers for G3PDH, type I collagen and the integrin β1 subunit were designed on the basis of the sequences in the GenBank.

PCR amplification was carried out in a Perkin Elmer 9600 thermal cycler using a Taq PCR Master Mix Kit (Qiagen, CA, USA). The cycling conditions for G3PDH, Cbfa1, type I collagen and integrin β1 were 94°C for 2 min as an initial denaturation step, followed by cycles of 94°C for 20 sec, 60°C for 30 sec, 72°C for 90 sec for 17, 29, 22 and 23 cycles, respectively, and a final extension step at 72°C for 10 min. The cycling conditions for ALP were 94°C for 2 min as an initial denaturation step, followed by 23 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, and a final extension step at 72°C for 10 min. One μl of cDNA was used as the template in a 20 μl amplification mixture containing 1 U of Taq DNA polymerase, 0.5 μM of each of the 5′ and 3′ primers, and distilled water. All products were assayed in the exponential phase of the amplification curve and the PCR cycles were determined for each primer pair. The PCR products were separated in a 2.5% agarose gel and visualized by staining with SYBR Green-I (Molecular Dynamics, Sunnyvale, USA). The SYBR Green-I fluorescence was converted into a TIFF image by a CCD camera (C-900ZOOM, Olympus, Tokyo, Japan) and the intensity was quantified using the QuantiScan software (BIOSOFT, Ferguson, USA). All products were corrected for the G3PDH mRNA levels.

Tissue Preparation

The cervical interspinous ligaments were harvested *en bloc* during surgery from OPLL patients (cases 6, 8, 12) and non-OPLL patients (cases 17, 26, 28). In the OPLL patients, the ligaments at the intact level were used. The ligaments were anchored by suturing their ends to a silicon chamber (Scholertec Corp., Osaka, Japan) and incubated in DMEM supplemented with 1% FBS in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The silicon substrate was subjected to a uni-axial cyclic stretch of 120% peak to peak amplitude, at 0.5 Hz for 4 h. Because of the heterogeneous structure of the ligaments and the imperfect attachment to the substrate, the exact amount of stretch that the ligament was subjected to is not known although ligament stretch should be directly proportional to substrate stretch. As a control, the unstretched ligaments derived from the same OPLL and non-OPLL patients were incubated under the same conditions for the period of the stretch application.

Immunohistochemistry

After the cyclic stretch, the cervical interspinous ligaments (cases 6, 8, 12, 17, 26, 28) were fixed by immersion in 4% paraformaldehyde for 24 h and decalcified with 10% EDTA (pH 7.4) for 3–7 days at 4°C. The ligaments were then dehydrated through a graded ethanol series and embedded in paraffin. Sections of 4 μm thickness were prepared for immunohistochemical staining using a microtome.

Immunohistochemical staining was performed by an avidin-biotin complex method using a Histofine kit (Nichirei, Tokyo, Japan). After the specimens were treated with 1 mg/ml of hyaluronidase (Sigma Chemical Co., St. Louis, MO, USA) for 30 min at 37°C, endogenous peroxidase was blocked with methanol containing 0.3% hydrogen peroxide for 30 min at room temperature. After washing with PBS (pH 7.4), they were incubated in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 10% normal goat serum for 15 min at room temperature to block nonspecific protein binding. The specimens were reacted with an anti-Cbfa1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), diluted 1:150 with PBS, at 4°C overnight. They were then incubated with a biotinylated secondary antibody, and finally with an avidin-biotin peroxidase complex for 20 min at room temperature. Color was developed using 3,3′-diaminobenzidine tetrahydrochloride containing hydrogen peroxidase. The nuclei were lightly counterstained with hematoxylin. For the negative controls, PBS was used instead of the primary antibody.

Use of U0126

U0126 (Promega, Madison, WI, USA), a specific inhibitor of MAPK kinase (MEK), was dissolved in DMSO at a concentration of 100 mM, stored in aliquots at −80°C, and diluted in

fresh medium to 40 μM immediately prior to use. Cells were incubated with 40 μM U0126 or the same amount of DMSO as a control for 12 h before the cyclic stretch. The maximum concentration of DMSO in any experiment was 0.4% (v/v), which did not affect the activity of the cells.

Statistical Analysis

All data were expressed as the mean ± SEM. ANOVA with the Dunnett type test for multiple comparisons against a control was used in all experiments. $P < 0.05$ was considered significant.

Results

Effect of Cyclic Stretch on the Expression of Cbfa1 mRNA in OPLL Cells

To investigate the effect of cyclic stretch on osteoblast differentiation in OPLL cells, we performed RT-PCR with primers specific for Cbfa1 type II isoform which is the specific transcription factor to human osteoblast. In OPLL cells (cases 1–10), cyclic stretch significantly increased the mRNA expression of Cbfa1 about 200% ($P < 0.05$), 192% after stimulation for 6 and 9 h compared with the cells maintained in the resting state (0 h). However, in non-OPLL cells (cases 15–24) which were derived from non-OPLL patients, the mRNA expression of Cbfa1 was decreased about 72% ($P < 0.05$), 65% ($P < 0.01$) after stimulation for 3 and 6 h compared with the cells maintained in the resting state (0 h) (Fig. 1A, B).

Distribution of Cbfa1 in the Tissue of the Interspinous Ligaments from OPLL Patients

The surgically removed interspinous ligaments (Fig. 2A) from the OPLL and non-OPLL patients were stretched for 4 h or maintained in the resting state (control). To determine the quantity of the expression of Cbfa1 by immunohistochemistry, we investigated the location of the expressions of Cbfa1. In the OPLL patients, many fibroblasts tended to express Cbfa1 (arrows) at the center of the interspinous ligaments in the stretched group (Fig. 2B), whereas there were fewer in the unstretched group (Fig. 2C). With regard to the insertion of the ligament, chondrocytes weakly expressed Cbfa1 in both the stretched and control groups (Fig. 2D, E). In both stretched and non-stretched ligaments of non-OPLL patients, some chondrocytes were stained at the insertion of the ligament although no fibroblast nuclei were stained at the center of the ligament. There were no changes by stretch in non-OPLL ligaments (data not shown). None of these areas or cells were stained in negative controls (Fig. 2F, G).

Effects of Cyclic Stretch on Expressions of ALP, Type I Collagen and Osteocalcin mRNA in OPLL Cells

To investigate the effects of the cyclic stretch on osteoblast differentiation in OPLL cells, we performed RT-PCR with primers specific for ALP, type I collagen and osteocalcin as markers of osteoblast differentiation. In OPLL cells (cases 1–10), cyclic stretch significantly increased the mRNA expressions of ALP and type I collagen about 230% ($P < 0.05$) and 246% ($P < 0.05$), respectively, after stimulation for 9 h compared to 0 h (Fig. 3A, B). The expressions of osteocalcin mRNA tended to be increased about 165% by 9 h of cyclic stretch, but there was no significant difference. There were no changes in the non-OPLL cells (cases 15–24).

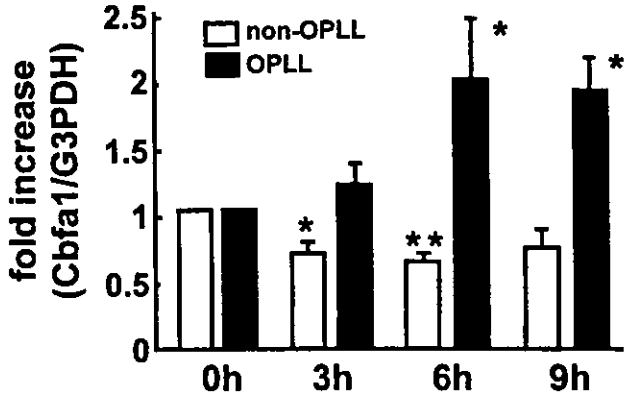

Fig. 1. RT-PCR analysis of Cbfa1 type II mRNA in spinal ligament cells from OPLL and non-OPLL patients (cases 1–10 and 15–24, respectively) following uni-axial cyclic stretch. The time courses of changes in the relative amounts of PCR products for the indicated time periods are shown. The densitometric quantification of the electrophoretic profiles of each gene was normalized to the corresponding G3PDH signal. The fold increases of Cbfa1 mRNA with respect to 0 h (Cbfa1/G3PDH ± SEM were non-OPLL; 1.24 ± 0.15 and OPLL; 1.12 ± 0.18) are shown. *Significantly different from 0 h, $P < 0.05$. **Significantly different from 0 h, $P < 0.01$.

mRNA Expression of the Integrin β1 Subunit in OPLL Cells

As mentioned above, the integrin β1 subunit is thought to act as a mechanotransducer, and mechanical stress applied to integrin β1 subunits induced MAPK activation which is important for osteoblast differentiation. Therefore, we investigated the expression of the integrin β1 subunit in OPLL and non-OPLL cells and the effect of mechanical stress (Fig. 4). In OPLL cells (cases 1–10), cyclic stretch significantly increased the mRNA expression of integrin β1 about 210% ($P < 0.05$) after stimulation for 9 h, whereas no change was observed in non-OPLL cells (cases 15–24).

Effects of a Specific Inhibitor of MAPK Kinase (MEK) on the Stretch-induced Expressions of Cbfa1, ALP, Type I Collagen, Osteocalcin, and the Integrin β1 Subunit

The MAPK pathway is an important transducer of integrin signals to the nucleus and phosphorylates and activates Cbfa1 in osteoblasts. U0126, a specific inhibitor of MEK, strongly binds to MEK1 and MEK2,

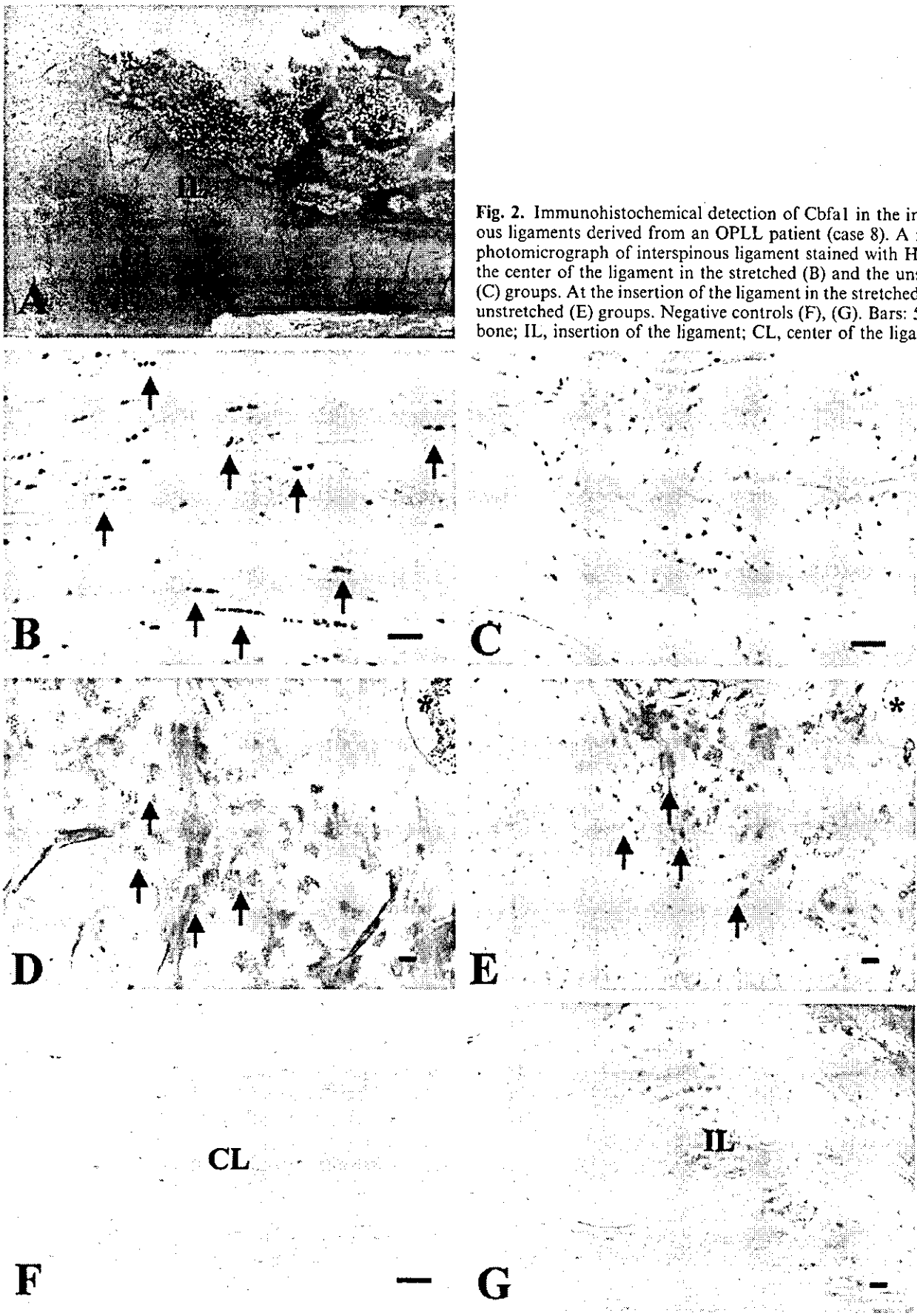

Fig. 2. Immunohistochemical detection of Cbfa1 in the interspinous ligaments derived from an OPLL patient (case 8). A is the photomicrograph of interspinous ligament stained with H&E. At the center of the ligament in the stretched (B) and the unstretched (C) groups. At the insertion of the ligament in the stretched (D) and unstretched (E) groups. Negative controls (F), (G). Bars: 50 μm. *, bone; IL, insertion of the ligament; CL, center of the ligament.