TABLE 2. CANDIDATE GENES INVOLVED IN BONE METABOLISM

Group	Ch		Candidate gene	Locus	AccessionNum
(1)	1	COL11A1	Collagen, type XI, alpha 1	1p21	NM_001854
	2	COL5A2	Collagen, type V, alpha 2	2q14	NM_000393
	3	CASR	Calcium-sensing receptor	3q21.1	NM_000388
	3	PTHR1	Parathyroid hormone receptor 1	3p22.3	NM_000316
	4	BMP3	Bone morphogenetic protein 3	4p14	NM_001201
	7	COL1A2	Collagen, type I, alpha 2	7q22.1	NM_000089
	8	BMP1	Bone morphogenetic protein 1	8p21	NM_001199
	12	IGF1	Insulin-like growth factor 1	12q22	NM_000618
	12	COL2A1	Collagen, type II, alpha 1	12q13.11	NM_001844
	12	VDR	Vitamin D receptor	12q12	NM_000376
	14	BMP4	Bone morphogenetic protein 4	14q21	NM_001202
	17	COL1A1	Collagen, type I, alpha 1	17q21.31	NM_000088
	17	SOX9	Sex-determining region Y-box 9	17q24.3	NM_000346
	17	ITGA3	Integrin, alpha 3	17p13.3	NM_002204
	20	BMP2	Bone morphogenetic protein 2	20p12	NM_001200
	20	BMP7	Bone morphogenetic protein 7	20q13.1	NM_001719
(2)	1	CSF1	Colony stimulating factor 1	1p21.3	NM_000757
	1	TGFb2	Transforming growth factor, beta 2	1q41	NM_003238
	1	IL6R	Interleukin 6 receptor	1q21	NM_000565
	1	BGLAP	Osteocalcin	1q25.1	NM_000711
	1	Smad	smad(serine protease-like protein isoform)	1	NM_004799
	1	VCAM1	Vascular cell adhesion molecule 1	1p32.3	NM_001078
	2	IL1A	Interleukin 1, alpha	2q14	X03833
	2	IL1B	Interleukin 1, beta	2q14	K02770
	2	PTHR2	Parathyroid hormone receptor 2	2q33	NM_005048
	2	ITGA4	Integrin, alpha 4	2q31.1	NM_000885
	3	ITGB5	Integrin, beta 5	3p26.3	NM_002213
	4	EGF	Epidermal growth factor	4q25	NM_001963
	4	OPN	Osteopontin	4q21.1	NM_000582
	5	CSF2	Colony stimulating factor 2	5q31.1	M13207
	5	IL4	Interleukin 4	5q23	XM_004053
	5	FGF1	Fibroblast growth factor 1	5q31	NM_000800
	6	ESR1	Estrogen receptor 1	6q25.1	NM_000125
	6	SRF	Serum response factor (c-fos)	6p12	NM_003131
	6	VEGF	Vascular endothelial growth factor	6p12	NM_003376
	6	CTGF	Connective tissue growth factor	6q23.1	NM_001901
	7	IL6	Interleukin 6	7p21	NM_000600
	7	CALCR	Calcitonin receptor	7q21.3	NM_001742
	7	ITGB8	Integrin, beta 8	7	NM_002214
	9	IL11RA	Interleukin 11 receptor, alpha	9p13	NM_004512
	9	INSL4	Insulin-like4	9p24	NM_002195
	10	PRG1	Proteoglycan 1	10q22.1	NM_002727
	11	MMP1	Matrix metalloproteinase 1	11q22.3	NM_002421
	11	PTH	Parathyroid hormone	11p15.3	NM_000315
	11	CALCA	Calcitonin	11p15.2	NM_001741
	12	MGP	Matrix Gla protein	12p13.1	NM_000900
	13	GAS6	Growth arrest-specific 6	13q34	NM_000820
	13	FGF9	Fibroblast growth factor 9	13q11.1	NM_002010
	14	ESR2	Estrogen receptor 2	14q22	NM_001437
	16	CDH13	Cadherin13	16q24.2	NM_001257
	19	IL11	Interleukin 11	19q13.31	NM_000641
	19	ICAM1	Intercellular adhesion molecule 1	19p13.3	NM_000201
	21	ITGB4BP	Integrin beta 4 binding protein	20q12	NM_002212
	22	LIF	Leukemia inhibitory factor	22q12.2	NM_002309
(3)	3	FETU[29]	Fetuin	3q27	XM_003292
	5	ANK[30]	Ankylosis	5p	AF274753
	6	NPPS[17]	Nucleotide pyrophosphatase	6q22	D12485
	7	IGFbP1	Insulin-like growth factor binding protein 1	7p13	NM_000596
	7	LEP	Leptin	7q31.3	NM_000230
	8	OPG[31]	Osteoprotegerin	8q24	NM_002546
	10	FGF8	Fibroblast growth factor 8	10q24	NM_006119
	14	TGFb3	Transforming growth factor, beta 3	14q24	NM_003239
	15	IGF1R	Insulin-like growth factor 1 receptor	15q25.1	NM_000875
	19	TGFb1	Transforming growth factor, beta 1	19q13.2	NM_000660

Group: candidate genes are selected according to the following criteria: (1) genes catalogued in the Skeletal Gene Database,[7] (2) genes that possibly affect bone formation, and (3) genes of which the gain or loss of function is related to bone formation according to cell or animal model studies. Ch, chromosome number; AccessionNum, Accession Number of GenBank Database (NCBI).

TABLE 3. LINKAGE RESULTS OF CANDIDATE GENES IDENTIFIED BY cDNA MICROARRAY PROFILE

Rank	Candidate gene	Marker	Dist	Het	Mean	p value	NPL score
1	Crystallin, alpha B	D11S4111	3.8	0.80	0.553	0.016*	1.834
2	Sortilin 1	D1S2726	2.7	0.72	0.500	0.505	−0.356
3	RNA helicase-related protein	D17S948	1.8	0.70	0.491	0.641	−0.368
4	Collagen, type VIII, alpha 1	D3S1271	1.2	0.60	0.491	0.653	−0.088
5	Leptin receptor	D1S230	3.8	0.61	0.502	0.474	0.269
6	Adrenomedullin	D11S902	3.0	0.84	0.504	0.453	0.279
7	Heat shock 105kD	D13S156	1.4	0.84	0.535	0.093	0.510
8	Phosphatidylinositol-(4,5)-bisphosphate 5-phosphatase	D17S849	1.4	0.84	0.496	0.557	0.242
9	Protein tyrosine kinase 2 beta	D8S505	2.9	0.84	0.522	0.179	0.846
10	Microseminoprotein, beta	D10S196	2.7	0.84	0.507	0.396	0.029
11	Prepropeptide specific to rod photoreceptor	D18S59	0.1	0.84	0.488	0.663	−0.806
12	Integrin, alpha 5	D12S83	4.2	0.84	0.503	0.447	−0.080
1	Tumor necrosis factor, alpha-induced protein 6	D2S335	0.1	0.84	0.539	0.052	0.903
2	Stromal cell-derived factor 1	D10S196	4.9	0.70	0.507	0.396	0.029
3	Methylene tetrahydrofolate dehydrogenase	D14S63	1.6	0.76	0.527	0.127	1.105
4	A disintegrin and metalloproteinase domain 12	D10S217	3.0	0.82	0.508	0.398	0.092
5	Insulin-like growth factor binding protein 3	D7S519	1.6	0.74	0.522	0.207	0.525
6	Human G0S2 protein gene	D1S425	3.3	0.61	0.476	0.812	−0.595
7	Plasminogen activator inhibitor, type II	D18S61	1.9	0.82	0.520	0.214	0.806
8	Plasminogen activator, urokinase	D10S537	3.1	0.82	0.464	0.873	−1.009
9	Insulin-like growth factor binding protein 5	D2S164	2.2	0.65	0.520	0.214	−0.546
10	P311 protein	D5S428	3.4	0.68	0.514	0.242	0.277
11	Antigen identified by monoclonal antibodies 4F2	D11S987	1.2	0.84	0.494	0.591	−0.102
12	Solute carrier family 21 (organic anion transporter)	D12S1617	2.7	0.84	0.493	0.595	0.230

Dist, distance (cM) between gene and marker; Het, heterozygosity of the marker. Mean proportion and p value were determined by SIBPAL. NPL score was calculated by GENEHUNTER.
*$p < 0.05$.

short induction period, typical osteoblast-related genes such as osteocalcin, osteonectin, osteopontin, and type I collagen were not detected. In a separate set of experiments, expressions of the four above-noted genes were increased on 4 days induction (data not shown). Twelve up-regulated (>3-fold increase) genes and 12 down-regulated (>3-fold decrease) genes were identified (Table 1). Among the 24 genes identified in cDNA microarray analysis, only one gene, αB crystallin, showed weak but significant evidence of linkage ($p = 0.016$, NPL = 1.83). αB crystallin is a heat-shock protein expressed in many nonlenticular tissues.[22,23] Because biological involvement of αB crystallin in bone metabolism is not clear at present, further investigation is needed to verify the involvement of the gene in OPLL.

The knowledge-based candidate gene approach is a standard protocol for identifying susceptibility genes of common diseases.[4] The criteria for knowledge-based candidate selection for OPLL included the genes catalogued in the skeletal gene database,[7] genes that possibly affect bone formation, and genes of which the gain or loss of function is related to bone formation according to cell or animal model studies. Sixty-four genes were examined, as summarized in Table 2. The marker (D14S276) for *BMP4* showed the best evidence of linkage by NPL analysis (NPL = 2.23), and reached "suggestive evidence of linkage."[19] Six other loci (*CDH13, PRG1, TGFb3, OPN, PTHR1,* and *IGF1*)

showed weak evidence of linkage by both SIBPAL and GENEHUNTER (Table 5). The functional role of BMP-4 in bone metabolism is well established, and BMP-4 can induce de novo cartilage and bone formation.[24] Immunohistochemical study of the ligamentum flavum of patients with ossification of the ligamentum flavum, a disorder closely related to OPLL, showed that BMP-4 expression is observed around the calcified zone at the insertion point of the ligamentum flavum to the bone.[25] These findings suggest that BMP-4 plays a crucial role in the pathogenesis of OPLL. Because molecular variants related to *BMP4* have not been found, further molecular screening for *BMP4* is needed to ascertain causality. Cadherin 13 (*CDH13*) is expressed in differentiated hMSC at a high level (data not shown). Although *CDH13* is not well recognized in bone metabolism, human osteoblast cells express a repertoire of cadherins, and cadherin-mediated cell–cell adhesion is essential for the commitment of cells to osteoblastic differentiation.[26–28] The functional role of *CDH13* in bone metabolism must also be determined.

There is a concept that the power of linkage analysis to locate susceptibility loci for complex diseases is much greater in animal models than that in humans. Thus, one strategy taken by human geneticists is first to localize a disease gene in an animal model and then to examine the homologous region in human families. However, the cau-

TABLE 4. LINKAGE RESULTS OF KNOWLEDGE-BASED CANDIDATE GENES

Ch*	Candidate gene	Marker	Dist	Het	Mean	p value
1	CSF1	D1S2726	2.7	0.72	0.500	0.505
1	COL11A1/VCAM1	D1S206	2.2/0.2	0.78	0.500	0.506
1	BGLAP	D1S498	5	0.73	0.438	0.968
1	IL6R	INT	0	0.81	0.473	0.802
1	Smad	D1S2797	1.4	0.78	0.497	0.556
1	TGFb2	D1S490	1.8	0.80	0.502	0.477
2	IL1A,B	INT	0	0.74	0.459	0.955
2	ITGA4/COL5A2	D2S364	1	0.78	0.455	0.940
2	PTHR2	D2S325	0.8	0.79	0.472	0.837
3	CASR	D3S1267	2.5	0.65	0.529	0.121
3	FETU	D3S1262	0	0.72	0.529	0.109
3	PTHR1	D3S1573	1.3	0.81	0.545	0.045*
3	ITGB5	D3S1267	0.5	0.67	0.529	0.121
4	OPN	D4S2964	2	0.70	0.541	0.044*
4	BMP3	D4S2947	0.5	0.65	0.509	0.366
4	EGF	D5S406	2.3	0.70	0.516	0.246
5	ANK	D5S1954	—	0.80	0.491	0.604
5	CSF2	D5S2115	1.3	0.74	0.518	0.228
5	FGF1	D5S436	4.6	0.75	0.533	0.097
5	IL4	INT	0	0.77	0.541	0.057
6	SRF	D6S1607	3.9	0.75	0.536	0.085
6	CTGF	D6S262	0.2	0.77	0.485	0.668
6	ESR1	D6S441	0.5	0.79	0.482	0.750
6	VEGF	D6S1604	0.1	0.78	0.501	0.481
7	COL1A2	INT	0	0.66	0.492	0.620
7	IGFbP1	D7S519	1.9	0.74	0.522	0.207
7	ITGB8	D7S507	3.2	0.82	0.512	0.353
7	IL6	INT	0	0.61	0.499	0.520
7	LEP	D7S530	2.3	0.72	0.484	0.711
7	CALCR	D7S657	2	0.77	0.469	0.834
8	BMP1	D8S1734	2.5	0.62	0.498	0.527
8	OPG	D8S514	1.5	0.77	0.499	0.519
9	IL11RA	D9S1817	2.1	0.86	0.518	0.289
9	INSL4	D9S288	3.1	0.81	0.523	0.225
10	FGF8	D10S192	2.1	0.84	0.535	0.084
10	PRG1	D10S1652	4.5	0.70	0.541	0.040*
11	MMP1	D11S1339	0.6	0.66	0.526	0.130
11	CALC	D11S902	0.4	0.84	0.504	0.453
11	PTH	D11S1307	0.3	0.78	0.500	0.498
12	COL2A1/VDR	D12S85	3	0.80	0.483	0.735
12	IGF1	INT	0	0.67	0.536	0.049*
12	MGP	INT	0	0.60	0.483	0.764
13	FGF9	D13S175	2	0.67	0.492	0.647
13	GAS6	D13S285	2.3	0.85	0.538	0.073
14	BMP4	D14S276	1.3	0.77	0.549	0.035*
14	ESR2	D14S1026	0.1	0.78	0.524	0.177
14	TGFb3	D14S74	1.9	0.80	0.545	0.041*
15	IGF1R	INT	0	0.67	0.492	0.673
16	CDH13	D16S3091	2.9	0.83	0.573	0.005[†]
17	COL1A1	D17S787	2.7	0.83	0.478	0.788
17	SOX9	D17S949	1.1	0.80	0.485	0.710
17	ITGA3	D17S787	0	0.83	0.478	0.788
19	IL11	D19S210	0.6	0.67	0.492	0.637
19	TGFb1	D19S220	0.1	0.87	0.497	0.549
19	ICAM1	D19S221	1.8	0.81	0.495	0.573
20	BMP2	INT	0	0.79	0.525	0.182
20	BMP7	D20S100	1.5	0.71	0.481	0.767
21	ITGB4BP	D21S266	0.5	0.82	0.540	0.061
22	LIF	D22S280	1.7	0.79	0.530	0.117

Ch, chromosome number; Dist, distance (cM) between gene and marker (average distance 1.7 cM); Het, heterozygosity of the marker; INT, intragenic microsatellite marker. Mean proportion and p value were determined by SIBPAL.
*, p < 0.05.
[†], p < 0.01.

TABLE 5. GENES WITH POSITIVE EVIDENCE OF LINKAGE

Candidate gene	Mean	p value	NPL score
CDH13 (cadherin13)	0.573	0.005	1.999
CRYAB (Crystallin, alpha B)	0.553	0.016	1.834
BMP4 (bone morphogenetic protein 4)	0.549	0.035	2.230
PRG1 (proteoglycan 1)	0.541	0.040	0.710
TGFb3 (transforming growth factor, beta 3)	0.545	0.041	1.304
OPN (osteopontin)	0.541	0.044	1.147
PTHR1 (parathyroid hormone receptor 1)	0.545	0.045	1.000
IGF1 (insulin-like growth factor 1)	0.536	0.049	0.878

Mean proportion and p value were determined by SIBPAL. NPL score was calculated by GENEHUNTER.

TABLE 6. AFFECTED SIB-PAIR ANALYSIS OF NPPS LOCUS

Locus 6q22	Marker name (heterozygosity)	Mean	p value
129.8 cM[a]	D6S262 (0.77)	0.485	0.670
130.3 cM	D6S457 (0.76)	0.521	0.240
131.5 cM	NPPS1 (0.67)	0.520	0.200
138.2 cM	D6S292 (0.85)	0.508	0.390

Mean and p values were determined by SIBPAL.
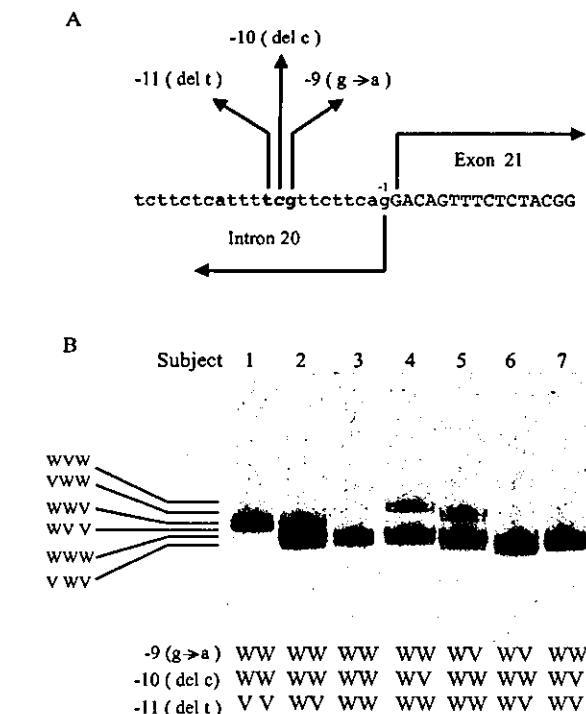[a] The distance from p-telomere of chromosome 6.

FIG. 2. Molecular variants at the intron 20/exon 21 junction of NPPS. (A) Three variations at positions −11(deletion), −10 (deletion), and −9 (substitution) of NPPS at intron 20 were identified. (B) PCR-SSCP screening of the intron 20/exon 21 junction of NPPS showed very complicated patterns revealing six distinct SSCP bands (W, wild type; V, variant). Samples were subjected to direct sequencing to verify variation, and each haplotype was determined.

sality of the animal model is not necessarily to be a human causality, especially in complex diseases. In an animal model for OPLL, the TWY mouse, the causal gene was identified as Npps, which encodes a membrane-bound enzyme that catalyzes the nucleotide phosphodiesterase reaction. The human counterpart, NPPS, reported as the causal gene in OPLL,[20] has been extensively studied by both linkage and allelic association studies. No evidence of linkage was obtained with the markers in the vicinity of NPPS (Table 6). An allelic association study with the polymorphism at intron 20, IVS20-11delT, was performed, and no allelic association between the polymorphism and OPLL was detected, which contradicts the earlier results of Nakamura et al.[20] This discrepancy could be attributable to typing difficulties in the region. In fact, by inspecting the allelic frequency results of Nakamura et al., a departure from Hardy–Weinberg equilibrium in the disease-associated allele was found in the control, indicating the typing uncertainty. Further investigations with increased sample size and different ethnic groups are required.

Although these linkage results may appear promising, it must be noted that none of them indicates "significant evidence of linkage,"[19] and so are only preliminary evidence for further investigations. However, further screening for mutations in each gene should be sufficient to clarify the molecular etiology of OPLL.

REFERENCES

1. Matsunaga S, Sakou T 1997 Epidemiology of ossification of the posterior longitudinal ligament. In: Yonenobu K, Sakou T, Ono K (eds.) Ossification of the Posterior Longitudinal Ligament. Springer-Verlag, Tokyo, Japan, pp. 3–7.
2. Terayama K 1989 Genetic studies on ossification of the posterior longitudinal ligament of the spine. Spine 14:1184–1191.

3. Terwilliger JD, Shannon WD, Lathrop GM, Nolan JP, Goldin LR, Chase GA, Weeks DE 1997 True and false positive peaks in genomewide scans: Applications of length-biased sampling to linkage mapping. Am J Hum Genet 61:430–438.

4. Duncan EL, Brown MA, Sinsheimer J, Bell J, Carr AJ, Wordsworth BP, Wass JA 1999 Suggestive linkage of the parathyroid receptor type 1 to osteoporosis. J Bone Miner Res 14:1993–1999.

5. Koga H, Sakou T, Taketomi E, Hayashi K, Numasawa T, Harata S, Yone K, Matsunaga S, Otterud B, Inoue I, Leppert M 1998 Genetic mapping of ossification of the posterior longitudinal ligament of the spine. Am J Hum Genet 62:1460–1467.

6. Maeda S, Ishidou Y, Koga H, Taketomi E, Ikari K, Komiya S, Takeda J, Sakou T, Inoue I 2000 Functional impact of human collagen α2(XI) gene polymorphism in pathogenesis of ossification of the posterior longitudinal ligament of the spine. J Bone Miner Res 16:948–957.

7. Ho NC, Jia L, Driscoll CC, Gutter EM, Francomano CA 2000 A Skeletal Gene Database. J Bone Miner Res 15:2095–2122.

8. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR 1999 Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

9. Keats BJ, Elston RC 1986 Determination of the order of loci on the short arm of chromosome 11 using two and three locus linkage analyses of pedigree and sib pair data. Genet Epidemiol Suppl 1:147–152.

10. Kruglyak L, Lander ES 1996 Parametric and nonparametric linkage analysis: A unified multipoint approach. Am J Hum Genet 58:1347–1363.

11. Cohen J 1988 Statistical Power Analysis for the Behavioral Science. Erlbaum, Hillsdale, NJ, USA, pp. 548–549.

12. Ono K, Yonenobu K, Miyamoto S, Okada K 1999 Pathology of ossification of the posterior longitudinal ligament and ligamentum flavum. Clin Orthop 359:18–26.

13. Yamazaki M, Moriya H, Goto S, Saitoh Y, Arai K, Nagai Y 1991 Increased type XI collagen expression in the spinal hyperostoic mouse (TWY/TWY). Calcif Tissue Int 48:182–189.

14. Furusawa N, Baba H, Imura S, Fukuda M 1996 Characteristics and mechanism of the ossification of posterior longitudinal ligament in the tip-toe walking Yoshimura (twy) mouse. Eur J Histochem 40:199–210.

15. Okano T, Ishidou Y, Kato M, Imamura T, Yonemori K, Origuchi N, Matsunaga S, Yoshida H, Ten Dijke P, Sakou T 1997 Orthotopic ossification of the spinal ligaments of Zucker fatty rats: A possible animal model for ossification of the human posterior longitudinal ligament. J Orthop Res 15:820–829.

16. Miura Y, Hikone R, Yamamoto K, Kawai K, Tanaka S, Imakiire A 1997 Pathological finding of ossification of the posterior longitudinal ligament and ossification of the spinal ligaments in an experimental animal model. In: Yonenobu K, Sakou T, Ono K (eds.) Ossification of the Posterior Longitudinal Ligament. Springer-Verlag, Tokyo, Japan, pp. 39–47.

17. Okawa A, Nakamura I, Goto S, Moriya H, Nakamura Y, Ikegawa S 1998 Mutation in Npps in a mouse model of ossification of the posterior longitudinal ligament of the spine. Nat Genet 19:271–273.

18. Phillips MS, Liu Q, Hammond HA, Dugan V, Hey PJ, Caskey CJ, Hess JF 1996 Leptin receptor missense mutation in the fatty Zucker rat. Nat Genet 13:18–19.

19. Lander E, Kruglyak L 1995 Genetic dissection of complex traits: Guidelines for interpreting and reporting linkage results. Nat Genet 11:241–247.

20. Nakamura I, Ikegawa S, Okawa A, Okuda S, Koshizuka Y, Kawaguchi H, Nakamura K, Koyama T, Goto S, Toguchida J, Matsushita M, Ochi T, Takaoka K, Nakamura Y 1999 Association of the human NPPS gene with ossification of the posterior longitudinal ligament of the spine (OPLL). Hum Genet 104:492–497.

21. Yamauchi T, Taketomi E, Matsunaga S, Sakou T 1999 Bone mineral density in patients with ossification of the posterior longitudinal ligament in the cervical spine. J Bone Miner Metab 17:296–300.

22. Vicart P, Caron A, Guicheney P, Li Z, Prevost MC, Faure A, Chateau D, Chapon F, Tome F, Dupret JM, Paulin D, Fardeau M 1998 A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. Nat Genet 20:92–95.

23. Klemenz R, Frohli E, Steiger RH, Schafer R, Aoyama A 1991 Alpha B-crystallin is a small heat shock protein. Proc Natl Acad Sci USA 88:3652–3656.

24. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA 1988 Novel regulators of bone formation: Molecular clones and activities. Science 242:1528–1534.

25. Hayashi K, Ishidou Y, Yonemori K, Nagamine T, Origuchi N, Maeda S, Imamura T, Kato M, Yoshida H, Sampath TK, Ten Dijke P, Sakou T 1997 Expression and localization of bone morphogenetic proteins (BMPs) and BMP receptors in ossification of the ligamentum flavum. Bone 21:23–30.

26. Lecanda F, Cheng SL, Shin CS, Davidson MK, Warlow P, Avioli LV, Civitelli R 2000 Differential regulation of cadherins by dexamethasone in human osteoblastic cells. J Cell Biochem 77:499–506.

27. Shin CS, Lecanda F, Sheikh S, Weitzmann L, Cheng SL, Civitelli R 2000 Relative abundance of different cadherins defines differentiation of mesenchymal precursors into osteogenic, myogenic, or adipogenic pathways. J Cell Biochem 78:566–577.

28. Cheng SL, Lecanda F, Davidson MK, Warlow PM, Zhang SF, Zhang L, Suzuki S, St. John T, Civitelli R 1998 Human osteoblasts express a repertoire of cadherins, which are critical for BMP-2-induced osteogenic differentiation. J Bone Miner Res 13:633–644.

29. Binkert C, Demetriou M, Sukhu B, Szweras M, Tenenbaum HC, Dennis JW 1999 Regulation of osteogenesis by fetuin. J Biol Chem 274:28514–28520.

30. Ho AM, Johnson MD, Kingsley DM 2000 Role of the mouse ank gene in control of tissue calcification and arthritis. Science 289:265–270.

31. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ 1998 Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176.

Address reprint requests to:
Ituro Inoue, M.D.
Division of Genetic Diagnosis
The Institute of Medical Science
The University of Tokyo
Shirokanedai 4-6-1
Minato-ku, Tokyo 108-8639, Japan

Trauma-induced myelopathy in patients with ossification of the posterior longitudinal ligament

SHUNJI MATSUNAGA, M.D., TAKASHI SAKOU, M.D., KYOJI HAYASHI, M.D., YASUHIRO ISHIDOU, M.D., MASATAKA HIROTSU, M.D., AND SETSURO KOMIYA, M.D.

Department of Orthopaedic Surgery, Faculty of Medicine, and Sakou Orthopaedic Surgery Clinic, Kagoshima University, Kagoshima, Japan

Object. In these prospective and retrospective studies the authors evaluated trauma-induced myelopathy in patients with ossification of the posterior longitudinal ligament (OPLL) to determine the effectiveness of preventive surgery for this disease.

Methods. The authors studied 552 patients with cervical OPLL, including 184 with myelopathy at the time of initial consultation and 368 patients without myelopathy at that time. In the former group of 184 patients retrospective analysis was performed using an interview survey to ascertain the relationship between onset of myelopathy and trauma. In the latter group of 368 patients prospective examination was conducted by assessing radiographic findings and noting changes in clinical symptoms apparent during regular physical examination. The follow-up period ranged from 10 to 32 years (mean 19.6 years).

In the retrospective investigation, 24 patients (13%) identified cervical trauma as the trigger of their myelopathy. In the prospective investigation, 70% of patients did not develop myelopathy over a follow-up period greater than 20 years (determined using the Kaplan–Meier method). Of the 368 patients without myelopathy at the time of initial consultation, only six patients (2%) subsequently developed trauma-induced myelopathy. Types of ossification in patients who developed trauma-induced myelopathy were primarily a mixed type. All patients in whom stenosis affected 60% or greater of the spinal canal developed myelopathy regardless of a history of trauma.

Conclusions. Preventive surgery prior to onset of myelopathy is unnecessary in most patients with OPLL.

KEY WORDS • myelopathy • ossification of the posterior longitudinal ligament • trauma

O SSIFICATION of the posterior longitudinal ligament, a subtype of diffuse idiopathic skeletal hyperostosis,[12,13] may contribute to the onset of and produce quadriplegia in older individuals.[17,19] A number of studies[1,6,8,11,14] of OPLL in the cervical spine have been performed since the publication of the autopsy report by Tsukimoto.[18] Ossification of the posterior longitudinal ligament may contribute to development of myelopathy in elderly individuals. Observation of the natural course of the disease has revealed that the development of ossification does not always lead to myelopathy.[7] It is difficult to predict the course of future neurological deterioration in patients in whom there is radiographic evidence of OPLL. Severe myelopathy can be induced by minor cervical trauma in patients with OPLL. Surgery-related results in patients with this condition are far from satisfactory. Some experts have recommended that preventive surgery be performed prior to the onset of myelopathy in patients with OPLL and potential spinal stenosis secondary to ossified ligaments. To justify the efficacy of preventive surgery in patients with potential spinal stenosis due to ossified ligaments, it should be demonstrated that trauma is a significant factor in the onset of myelopathy in cases of OPLL. In this study we reviewed data obtained in patients with OPLL treated in our clinic from 1967 by conducting retrospective and prospective investigations to assess the onset of trauma-induced myelopathy.

Clinical Material and Methods

Study Design

We used retrospective and prospective methods to examine patients with OPLL in an effort to assess the onset of cervical trauma-induced myelopathy. Patients in whom myelopathy had already developed by the time of initial consultation were included in the retrospective study in which we used an interview survey to ascertain the relationship between onset of myelopathy and trauma. Patients without myelopathy at the time of initial consultation were included in the prospective study (mean observation period 19.6 years) in which we noted radiographic findings and changes in clinical symptoms on regular physical examination twice a year. In all patients, we examined the relationships between ossification type and the presence/absence of trauma-induced myelopathy as well as the maximum degree of spinal canal stenosis caused by

172

the ossified ligament (Fig. 1) and the onset of myelopathy. Myelopathy was defined as disturbances in motor function associated with abnormal reflexes, muscle weakness, and sensory disturbance. Cervical trauma was determined when complaints such as cervical pain developed following whiplash injury and when medical treatment was required to treat symptoms such as cervical pain secondary to hyperflexion and hyperextension of the cervical spine after a fall.

Patient Population

Candidates in this study were 612 patients with cervical OPLL who had visited our clinic over a greater than 10-year period. A total of 552 patients were followed: 184 with and 368 without myelopathy at the time of initial consultation; the former group underwent surgery, and the latter was followed without surgery. In the 184 patients with myelopathy, age at presentation ranged from 39 to 75 years (mean 57 years); there were 123 men and 61 women. Based on criteria established by the Japanese Ministry of Public Health and Welfare,[19] the ossification type at presentation was determined to be continuous in 50 (27%), segmental in 86 (47%), mixed in 46 (25%), and other in two patients (1%). Continuous-type OPLL represents continuous ossification over several vertebral bodies; segmental type, segmental ossification of one or more vertebral bodies; and mixed type, continuous-type ossification associated with segmental type. Other-type OPLL represents the ossification of intervertebral disc level.

In the group of 368 patients without OPLL at presentation, age ranged from 29 to 79 years (mean 59 years); there were 286 men and 82 women. Ossification type at presentation was continuous in 108 (29%), segmental in 155 (42%), mixed in 98 (27%), and other in seven (2%). To patients in whom myelopathy had not developed by the time of initial consultation, we explained that cervical trauma should be avoided in daily life, and we observed changes in clinical symptoms thereafter. The follow-up period ranged from 10 to 32 years (mean 19.6 years). Age at the time of final examination ranged from 58 to 98 years (mean 79.6 years).

Results

Incidence of Trauma-Induced Myelopathy

Retrospective Study. In 24 (13%) of 184 patients who presented with myelopathy this condition had been triggered by cervical trauma: 13 suffered a fall and 11 were involved in MVAs. Fifty-two patients (28%) had a history of cervical trauma, and among these, 24 (46%) developed myelopathy due to trauma (Table 1).

Prospective Study. Of the 368 patients in whom no distinct myelopathy was present at the time of initial consultation, 70 (19%) developed myelopathy during the follow-up period. The Kaplan–Meier method[5] was used to determine the percentage of patients who did not develop myelopathy: 79% at 10-year follow up and 70% at longer than 20-year follow up (Fig. 2). Of the 70 patients in whom myelopathy occurred, six (9%) developed trauma-induced myelopathy, and in all of these patients there was a history of involvement in MVAs. Of these 368 patients, 14 (4%) suffered cervical trauma. Of these 14 patients, six
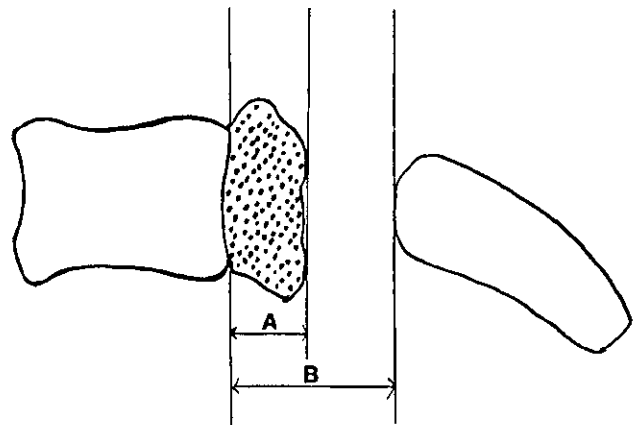

Fig. 1. Diagram showing measurement of maximum percentage of spinal canal stenosis based on the lateral cervical radiographs. *Dotted area* indicates the OPLL. Spinal canal stenosis rate (%) = A/B × 100.

(43%) developed trauma-induced myelopathy (Table 1). Characteristics observed in these six patients trauma-induced myelopathy and acute deterioration are presented in Table 2. In five of the six patients mixed-type OPLL was present. No myelopathic signs were detected in five of the six patients before trauma, and severe myelopathy (Nurick[9] Grade 4 or 5) developed after trauma.

Ossification Type and Maximum Percentage of Stenosis

Ossification types in the 30 patients in whom trauma-induced myelopathy developed were determined to be the following: mixed in 20 (67%); segmental in nine (30%); and continuous in one patient (3%). The risk of trauma-induced myelopathy is significantly higher in patients with mixed-type OPLL than in those with segmental- or continuous-type OPLL (Table 3). In all of the 45 patients with 60% or greater maximum stenosis of the spinal canal, myelopathy developed regardless of a history of cervical trauma. Analysis of the remaining 507 patients with maximum percentage spinal canal stenosis less than 60% revealed no significant difference in diameter of the remaining spinal canal between the groups of patients with and without myelopathy (Table 4).

Discussion

In our current investigation we found that in some pa-

TABLE 1

Summary of data obtained in the retrospective and prospective studies of myelopathy induced by trauma

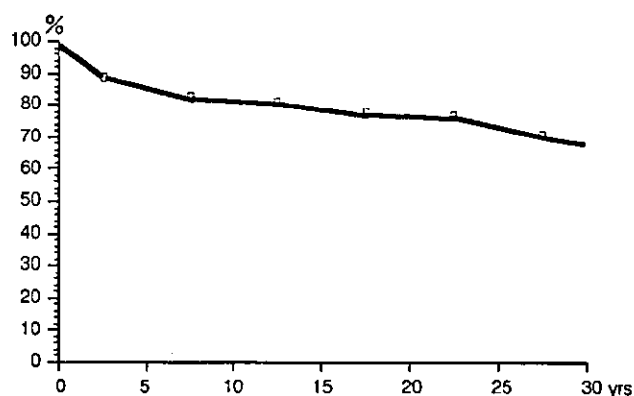| Factor	No. of Patients (%)		
	Retrospective Study	Prospective Study	p Value
no. w/ history of cervical trauma	52 (28)	14 (4)	0.001
no. w/ trauma-induced myelopathy	24 (13)	6 (2)	0.001
risk of myelopathy after trauma	24 (46)	6 (43)	0.93
total no. of patients	184	368	

Fig. 2. Graph showing Kaplan–Meier accumulated survival rate in 368 patients without myelopathy. All patients without myelopathy at the initial consultation underwent yearly examination.

TABLE 3
Risk of myelopathy after trauma stratified by type of OPLL

Type of OPLL	No. of Patients w/ Trauma	No. of Patients w/ Myelopathy (%)	p Value
continuous	13	1 (8)	0.19
segmental	28	9 (32)	< 0.001
mixed	25	20 (80)	

tients with OPLL, myelopathy clearly occurred in association with trauma. In one report the authors have suggested that some patients with cervical cord injury not accompanied by distinct bone injury may suffer OPLL.[15] A different group has noted that surgery-related results in patients with trauma-induced myelopathy are inferior to those in patients with nontrauma-related myelopathy.[4] As has been determined, results of treatment of trauma-associated myelopathy in patients with OPLL are far from satisfactory. Preventive surgery is often recommended prior to the onset of myelopathy in patients with OPLL and potential spinal stenosis due to ossified ligaments. There are no scientific data, however, to support the use of preventive surgery in patients with OPLL.

In the present study, follow-up prospective investigation was performed in individuals after explaining that they should avoid cervical trauma that might precipitate myelopathy. In this group, the frequency of cervical trauma decreased to 4%, compared with 28% in the retrospectively investigated patients. Almost no difference was noted in the incidence of myelopathy between retrospectively and prospectively studied patients who suffered trauma. A decrease in frequency of trauma-induced myelopathy in the prospective group may have been the result of efforts by patients to avoid trauma. Therefore, performing preventive surgery in patients in whom myelopathy has not developed is inappropriate. Informed consent concerning the possibil-

TABLE 2
Characteristics obtained in six patients with acute deterioration following trauma

Case No.	Age (yrs), Sex	Type of OPLL	Maximum % Stenosis	Nurick Grade*	
				Pretrauma	Posttrauma
1	65, M	mixed	65	0	4
2	59, M	mixed	60	0	5
3	62, M	mixed	58	0	4
4	72, M	mixed	56	0	4
5	68, F	mixed	52	1	4
6	76, M	continuous	68	0	5

* Nurick Grade: 0, no myelopathic sign; 4, myelopathy with gait disturbance; 5, bedridden condition.

ity of developing trauma-induced myelopathy may be important for patients with OPLL without myelopathy at the time of initial consultation. Instruction on how to avoid trauma is given to the individuals in the prospective investigation led to a low frequency of trauma-induced myelopathy; in 70% of patients myelopathy did not develop during long-term follow up. All episodes of trauma in patients who did develop trauma-induced myelopathy in the prospective investigation, however, were secondary to MVAs. Because driving a car is an essential element for functioning in modern society, complete eradication of MVA-related trauma-induced myelopathy may be impossible, despite careful efforts to avoid trauma in daily life.

Of those patients in whom trauma-induced myelopathy occurred, a large number developed mixed-type ossification with significant mobility at sites of noncontinuous-type ossification. This suggests that dynamic factors are important in the development of myelopathy in patients with preexisting OPLL. Currently, the degree of OPLL-induced stenosis in the spinal canal can be evaluated not only by plain radiography but also by CT scan[2,3,16] or magnetic resonance imaging.[10,20] Epstein[2,3] has recommended evaluation of this disease by CT scanning. In the future, evaluation based on the remaining area of spinal canal or volume may be necessary. A great number of researchers have used plain lateral radiography of the cervical spine to measure the diameter of the remaining spinal canal. In the present prospective study, CT evaluation was impossible because this study was begun in 1967. The maximum percentage of spinal canal stenosis was measured using plain lateral radiography, and in all cases in which 60% or more of the spinal canal was affected by stenosis, the patients developed myelopathy following cervical trauma. In patients in whom the maximum percentage of spinal canal stenosis was less than 60%, there was no significant difference in this maximum percentage of stenosis between those who developed trauma-induced myelopathy and those who did not. The reason for this may be as follows: even patients with segmental-type disease in whom there was a small maximum percentage of spinal canal stenosis may develop myelopathy; however, those with continuous-type disease in whom there is a large maximum percentage of spinal canal stenosis do not always develop trauma-induced myelopathy. For example, this is demonstrated when the cervical spine has limited mobility and little involvement of the dynamic factor. Preventive surgery in cases of OPLL should be limited to selected patients in whom mixed-type ossification and maximum (≥ 60%) spinal canal stenosis are present and whose lifestyle suggests that they may be at high risk of trauma secondary to MVAs.

TABLE 4

Maximum percentage of stenosis in patients with or without myelopathy *

	W/ Myelopathy (174 patients)	W O Myelopathy (333 patients)	p Value
mean maximum % of stenosis of spinal canal ± standard deviation	45 ± 19	41 ± 17	NS

* Calculation was performed for data obtained in 507 patients with less than 60% of the maximum percentage of spinal canal steno-sis. Abbreviation: NS = not significant.

Conclusions

Analysis of current results obtained prospectively in patients instructed on how to avoid trauma revealed a low incidence of trauma-induced myelopathy; in long-term follow up, 70% of the patients in this group did not develop myelopathy. In conclusion, preventive surgery prior to onset of myelopathy is unnecessary in most patients with OPLL.

References

1. Bakay LH, Cares HL, Smith RJ: Ossification in the region of the posterior longitudinal ligament as a cause of cervical my-elopathy. J Neurol Neurosurg Psychiatry 33:263–268, 1970
2. Epstein NE: Ossification of the posterior longitudinal ligament in evolution in 12 patients. Spine 19:673–681, 1994
3. Epstein NE: The surgical management of ossification of the posterior longitudinal ligament in 43 north americans. Spine 19:664–672, 1994
4. Fujimura Y, Nakamura M, Toyama Y. Influence of minor trauma on surgical results in patients with cervical OPLL. J Spinal Disord 11:16–20, 1998
5. Kaplan EL, Meier P: Nonparametric estimation from incomplete observation. J Amer Stat Assoc 53:457–481, 1958
6. Katoh S, Ikata T, Hirai N, et al: Influence of minor trauma to the neck on the neurological outcome in patients with ossification of the posterior longitudinal ligament (OPLL) of the cervical spine. Paraplegia 33:330–333, 1995
7. Matsunaga S, Sakou T, Taketomi E, et al: The natural course of myelopathy caused by ossification of the posterior longitudinal ligament in the cervical spine. Clin Orthop 305:168–177, 1994
8. Minagi H, Gronner AT: Calcification of the posterior longitudinal ligament: a cause of cervical myelopathy. Am J Roentgenol Radium Ther Nucl Med 105:365–369, 1969
9. Nurick S: The pathogenesis of the spinal cord disorder associated with cervical spondylosis. Brain 95:87–100, 1972
10. Okada Y, Ikata T, Yamada H, et al: Magnetic resonance imaging study on the results of surgery for cervical compression myelopathy. Spine 18:2024–2029, 1993
11. Onji Y, Akiyama H, Shimomura Y, et al: Posterior paraverte-bral ossification causing cervical myelopathy. A report of eighteen cases. J Bone Joint Surg (Am) 49:1314–1328, 1967
12. Resnick D, Guerra J Jr, Robinson CA, et al: Association of diffuse idiopathic skeletal hyperostosis (DISH) and calcification and ossification of the posterior longitudinal ligament. AJR Am J Roentgenol 131:1049–1053, 1978
13. Resnick D, Shaul SR, Robins JM: Diffuse idiopathic skeletal hyperostosis (DISH): Forestier's disease with extraspinal manifestations. Radiology 115:513–524, 1975
14. Sakou T, Taketomi E, Matsunaga S, et al: Genetic study of ossification of the posterior longitudinal ligament in the cervical spine with human leukocyte antigen haplotype. Spine 6:1249–1252, 1991
15. Saruhashi Y, Hukuda S, Katsuura A, et al: Clinical outcomes of cervical spinal cord injuries without radiographic evidence of trauma. Spinal Cord 36:567–573, 1998
16. Terada A, Taketomi E, Matsunaga S, et al: 3-dimensional computed tomography of ossification of the spinal ligament. Clin Orthop 336:137–142, 1997
17. Terayama K: Genetic studies on ossification of then posterior longitudinal ligament of the spine. Spine 14:1184–1191, 1989
18. Tsukimoto H: [On an autopsied case of compression myelopathy with a callus formation in the cervical spinal canal.] Nihon Geka Hokan 29:1003–1007, 1960 (Jpn)
19. Tsuyama N: Ossification of the posterior longitudinal ligament of the spine. Clin Orthop 184:71–84, 1984
20. Yone K, Sakou T, Yanase M, et al: Preoperative and postoperative magnetic resonance image evaluations of the spinal cord in cervical myelopathy. Spine 17:S388–S392, 1992

−70−

Role of Prostaglandin I_2 in the Gene Expression Induced by Mechanical Stress in Spinal Ligament Cells Derived from Patients with Ossification of the Posterior Longitudinal Ligament

HIROTAKA OHISHI, KEN-ICHI FURUKAWA, KOEI IWASAKI, KAZUMASA UEYAMA, AKIHIRO OKADA, SHIGERU MOTOMURA, SEIKO HARATA, and SATOSHI TOH

Departments of Orthopaedic Surgery (H.O., K.I., A.O., S.T.) and Pharmacology (K.-I.F., K.I., S.M.), Hirosaki University School of Medicine, Hirosaki, Japan; Hirosaki Memorial Hospital, Hirosaki, Japan (K.U.); and Aomori Central Hospital, Aomori, Japan (S.H.)

ABSTRACT

Ossification of the posterior longitudinal ligament of the spine (OPLL) is characterized by ectopic bone formation in the spinal ligaments, and mechanical stress has been suggested to play an important role in the progression of OPLL. To identify the genes that participate in OPLL, the differential display reverse transcription-polymerase chain reaction (RT-PCR) method was used. A 283-base pair cDNA fragment corresponding to prostaglandin I_2 (PGI_2) synthase was highly expressed in OPLL cells compared with non-OPLL cells. To examine the effect of mechanical stress on the expression of PGI_2 synthase, cells were subjected to uniaxial cyclic stretch (0.5 Hz, 20% stretch), and PGI_2 synthase mRNA expression was assessed by quantitative RT-PCR. Cyclic stretch induced an increase in PGI_2 synthase in OPLL cells in a time-dependent manner, whereas no change was observed in non-OPLL cells. Cyclic stretch for 9 h also induced a 2.86× increase in PGI_2 production. Beraprost (a stable PGI_2 analog) and dibutyryl cAMP (a membrane-permeable cAMP analog) increased the mRNA expression of alkaline phosphatase (ALP) as a marker for osteogenic differentiation up to 240 and 200%, respectively, in OPLL cells, whereas no change was observed in non-OPLL cells. The increases in ALP mRNA induced by beraprost and cyclic stretch were both inhibited by SQ22536, a potent adenylate cyclase inhibitor. These data suggest that the increase in PGI_2 synthase induced by mechanical stress plays a key role in the progression of OPLL, at least in part through the induction of osteogenic differentiation in spinal ligament cells via the PGI_2/cAMP system.

The posterior longitudinal ligament of the spine runs along the posterior aspect of the vertebral bodies. It is considered to be a part of the abnormal ossification of the spinal ligament. When this ligament becomes hypertrophic and ossified, it can compress the spinal cord and its roots, resulting in serious neurological deficiency. This condition is known as ossification of the posterior longitudinal ligament of the spine (OPLL). OPLL is often associated with concurrent ossification of other spinal ligaments, such as the anterior longitudinal ligament, ligamentum flavum, and supraspinatus ligaments. Consequently, it has been regarded as one of the

manifestations of diffuse idiopathic skeletal hyperostosis (Resnick et al., 1975). Therefore, systemic factors as well as local factors have been considered to play a role in the pathogenesis of this condition. The etiology of OPLL has been extensively investigated from various standpoints, however, its mechanism of development has not been clarified. The ossification process in OPLL is thought to occur through the endochondral mechanism (Hashizume, 1980; Yamamoto et al., 2002), but the involvement of multiple etiologic factors in the development of OPLL has been suggested, including genetic factors (Koga et al., 1998; Numasawa et al., 1999; Furushima et al., 2002), dietary habits (Wang et al., 1999), metabolic abnormalities (Baba et al., 1997), and some local factors. There are many studies on the progression of OPLL. OPLL often progresses after posterior decompressive surgery of the cervical spine, such as laminectomy or laminoplasty, which causes biological stimulation, defect of the dorsal ele-

---

ABBREVIATIONS: OPLL, ossification of the posterior longitudinal ligament of the spine; PGI_2, prostaglandin I_2; RT, reverse transcription; PCR, polymerase chain reaction; G3PDH, glycerol 3-phosphate dehydrogenase; ALP, alkaline phosphatase; BLAST, Basic Local Alignment Search Tool.

ment, and cervical instability (Matsunaga et al., 1994; Nakamura, 1994; Takatsu et al., 1999). These pieces of clinical evidence support the hypothesis that the mechanical stress that acts on the posterior ligaments plays an important role in the progression of OPLL.

Differences between OPLL cells and non-OPLL cells have been recognized histologically and morphologically (Goto et al., 1998; Ishida, 1998). For example, OPLL cells have several different phenotypic characteristics of osteoblasts. They are characterized by high alkaline phosphatase (ALP) activity, an increase in cAMP in response to parathyroid hormone (Ishida and Kawai, 1993b), and in vitro calcification. On the other hand, non-OPLL cells have a typical uniform fibroblast-like morphology, which is spindle shaped, and proliferate constantly (Goto et al., 1998; Ishida, 1998). The ALP activity is not high, and no bone-like calcification is observed in vitro (Ishida and Kawai, 1993a). Thus, these cells do not show any osteoblastic characters. Although differences have been noted between the two cell types, there are few reports that have compared the two types of cells at the gene expression level.

In this study, we compared OPLL cells with non-OPLL cells at the transcriptional level by differential display reverse transcription (RT)-polymerase chain reaction (PCR) and detected a difference in the expression of prostaglandin I_2 (PGI₂) synthase. Uniaxial cyclic stretch induced an increase in PGI₂ synthase mRNA. Furthermore, OPLL cells had a hyper-responsiveness to PGI₂ compared with non-OPLL cells. The possible role of PGI₂ in the development of OPLL and the influence of mechanical stress are discussed.

Materials and Methods

Materials. Specimens of the posterior longitudinal ligament were obtained from patients during spinal surgery. Six patients had cervical OPLL, and the other five had no ossification in any of the spinal ligaments. The subjects ranged from 28 to 56 years of age. 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 with no relation to OPLL) was confirmed on X-rays, computerized tomography, and magnetic resonance imaging of the cervical spine preoperatively. Although the ligament samples were not all from the same location in the cervical spine, we used all the samples for the experiments, because the cells from OPLL patients showed similar osteoprogenitor-like characteristics regardless of the location from which the tissue was extirpated in the cervical spine (Kon et al., 1997; Ishida, 1998).

Cell Culture. The ligaments were harvested aseptically from patients during surgery, rinsed with phosphate-buffered saline, and the surrounding tissue was carefully removed under a dissecting microscope. In all cases, the ligaments were extirpated carefully from nonossified sites to avoid any possible contamination with osteogenic cells. The collected ligaments were minced into approximately 0.5-mm³ pieces, washed twice with phosphate-buffered saline, and then plated on 100-mm culture dishes and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The cells derived from the explants were harvested from the dishes with 0.02% EDTA/0.05% trypsin for further passages.

Differential Display RT-PCR. After the cultures reached confluence, the total RNAs were extracted from the cell monolayers with

an RNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Each 1 μg of total RNA from OPLL and non-OPLL cells was reverse transcribed into cDNA using three 3'-anchored oligo(dT) primers. Three different arbitrary primers and three 3'-anchored oligo(dT) primers were used for the PCR amplification of the cDNA (1 μl). The PCR amplification was carried out in a volume of 20 μl using a Taq PCR Master Mix kit (QIAGEN), and the products were visualized by staining with SYBR Green-I (Amersham Biosciences Inc., Piscataway, NJ). The PCR cycling conditions were 95°C for 5 min, then 30 cycles of 95°C for 30 s, 42°C for 2 min, and 72°C for 30 s, and a final extension at 72°C for 5 min. The concrete pairs of primers were as shown in Tables 1 and 2. The reaction products were electrophoresed in a 6% SDS-polyacrylamide gel, and the differential bands were isolated from the gel. The cDNAs were eluted by boiling and then reamplified using the same primer pairs and PCR conditions.

DNA Sequencing. The reamplified bands were isolated from the gel, and the cDNAs were eluted by boiling again. The cDNAs were then refined with a Centricon YM-100 (Millipore Corp., Bedford, MA). cDNA sequencing was performed with a DNA Sequencing kit (Applied Biosystems, Foster City, CA) and the GeneAmp PCR system (PerkinElmer Life Sciences, Boston, MA) according to manufacturers' instructions. Gene database searches were made at the National Center for Biotechnology Information using the BLAST network service.

Uniaxial Cyclic Stretch. The cells (fifth passage) were placed on a 3.5 × 4.0-cm² silicon chamber coated with 0.1% gelatin (IWAKI GLASS, Tokyo, Japan) at a density of 10,000 cells/cm². After the cultures reached confluence, the cells were incubated in Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum for 24 h. The silicon chamber was attached to a four-point bending and stretching apparatus that was driven by a computer-controlled stepping motor (Scholertec Corp., Osaka, Japan) (Naruse et al., 1998). Uniaxial sinusoidal stretch (120% peak to peak, at 1 Hz) was applied 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, the total RNAs were extracted simultaneously from the cell monolayers with an RNeasy kit (QIAGEN) according to the manufacturer's protocol. The total RNA was treated with RNase-free DNase I (Invitrogen Corp., Carlsbad, CA) and reverse transcribed into cDNA using Oligo(dT)₁₂₋₁₈ primer (Invitrogen Corp.). One mi-

TABLE 1

Sequences of 3'-anchored oligo(dT) primers and arbitrary primers

Anchored primers
No. 1: 5'-GCGCAAGCTTTTTTTTTTTTTGG-3'
No. 2: 5'-GCGCAAGCTTTTTTTTTTTTTGC-3'
No. 3: 5'-GCGCAAGCTTTTTTTTTTTTTCG-3'

Arbitrary primers
No. 4: 5'-CGGGAAGCTTATCGACTCCAAG-3'
No. 5: 5'-CGGGAAGCTTTAGCTAGCATGG-3'
No. 6: 5'-CGGGAAGCTTGCTAAGACTAGC-3'

TABLE 2

Combination of primer pairs used for differential display RT-PCR in Fig. 2

Non-OPLL	Anchored Primers	Arbitrary Primers	OPLL
1	No. 1	No. 4	1*
2	No. 1	No. 5	2*
3	No. 1	No. 6	3*
4	No. 2	No. 4	4*
5	No. 2	No. 5	5*
6	No. 2	No. 6	6*
7	No. 3	No. 4	7*
8	No. 3	No. 5	8*
9	No. 3	No. 6	9*

crogram of total RNA was heated at 70°C for 10 min in 10 μl of H₂O supplemented with 0.5 μg of Oligo(dT)₁₂₋₁₈ primer. The mixture was placed on ice, and cDNA synthesis was then performed by 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 MgCl₂) supplemented with 0.5 mM of dNTPs (Invitrogen Corp.), 2.5 mM of DTT (Invitrogen Corp.), 2 U of RNase inhibitor (TOYOBO, Osaka, Japan), and 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp.). After the incubation, the cDNAs were heated to 72°C and then stored at −20°C until use for amplification by PCR.

PCR Analysis. For PCR amplification, specific oligonucleotide primers to human sequences were designed on the basis of the sequences in GenBank as follows: glycerol 3-phosphate dehydrogenase (G3PDH), 5'-TCCACCACCCTGTTGCTGTA-3' and 5'-ACCACAGTCCATGCCATCAC-3'; ALP, 5'-ATCGCCTACCAGCTCATG-CAT-3' and 5'-GTTCAGCTCGTACTGCATGTC-3'; PGI₂ synthase, 5'-GCCAAAAAAGAAGGTGCCGATTTC-3' and 5'-GAACTCCCGA-CCTCAAGTGATC-3'; and PGI₂ receptor, 5'-GTCCATGCTCATC-CTCTTTGCC-3' and 5'- GCGGAAAAGGATGAAGACCCA-3'.

The reaction was performed using a *Taq* PCR Master Mix kit as follows: 1 μl of cDNA was used as the template in a 20-μl amplification mixture containing 1 U of *Taq* DNA polymerase, 0.5 μM each of the 5' and 3' primers, and distilled water. All the products were assayed in the exponential phase of the amplification curve, and the PCR cycles were determined for each primer pair. PCR was performed in a PerkinElmer 9600 thermal cycler. The cycling conditions for G3PDH were 94°C for 20 s, 60°C for 30 s, 72°C for 5 s for 17 cycles, and a final extension at 72°C for 10 min. The cycling conditions for ALP were 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 23 cycles, and a final extension at 72°C for 10 min. The cycling conditions for PGI₂S and PGI₂ receptor were 94°C for 20 s, 61°C for 30 s, 72°C for 90 s for 24 and 36 cycles, respectively, and a final extension at 72°C for 10 min. The amplified products were resolved by electrophoresis in a 2.5% w/v agarose gel and visualized by staining with SYBR Green-I. The SYBR Green-I fluorescence was converted into a TIFF image by a charge-coupled device camera (C-900 ZOOM; OLYMPUS, Japan), and the intensity was quantified by QuantiScan software (BIOSOFT, Ferguson, MO). All the products were corrected for the G3PDH mRNA levels.

Enzyme-Linked Immunosorbent Assay of PGI₂. PGI₂ concentration in the medium after loading mechanical stress on OPLL cells was evaluated by measuring its stable metabolite 6-keto-PGF₁ₐ by enzyme-linked immunosorbent assay kit (Assay Designs, Inc., Ann Arbor, MI) according to the manufacturer's protocol.

Drugs. ONO-8713 and ONO-AE-248ONO were kindly provided by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). All other chemicals used in this study were of high-quality analytical grade.

Statistical Analysis. All data are expressed as the mean ± S.E.M. The Friedman test for a control was used in all experiments. $P < 0.05$ was considered significant. n means the number of ligament cell preparations obtained from different spinal ligament samples.

Results

Differential Display RT-PCR. To detect the differences in gene expression between OPLL cells and non-OPLL cells, we compared the two types of cells at the transcriptional level by differential display RT-PCR. At least five reproducible differences in the gene expression were detected (Fig. 1). The results were compared with the sequence databases in the BLAST network service, and one of them had 95% homology to human PGI₂ synthase (GI number: 14786310) (Fig. 2).

Effect of Mechanical Stress on PGI₂ Synthase Expression. It has been reported that mechanical stress plays an important role in the progression of OPLL (Kitajima et al., 2001). Furthermore, mechanical stress produces PGI₂ in osteoblasts (Rawlinson et al., 1993; Zaman et al., 1997). To
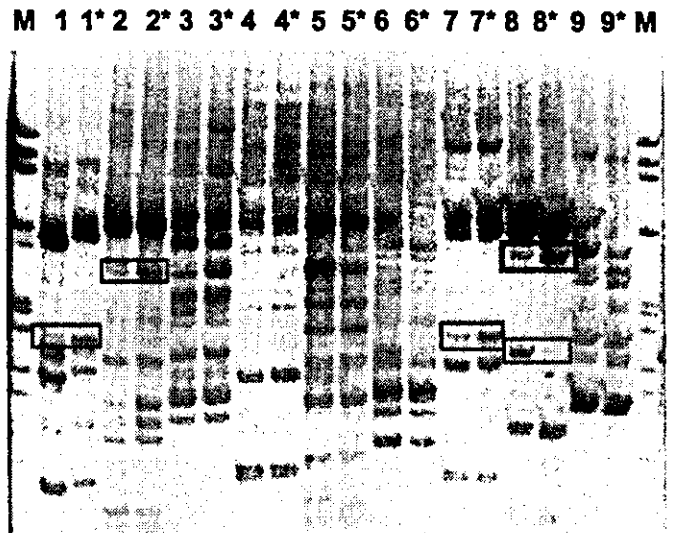

M 1 1* 2 2* 3 3* 4 4* 5 5* 6 6* 7 7* 8 8* 9 9* M

Fig. 1. Differential display analysis of gene expression in OPLL and non-OPLL cells. cDNA fragments amplified using pairs of 3'-anchored primers and arbitrary primers were separated in a 6% polyacrylamide gel. The concrete pairs of primer (1–9 for non-OPLL and 1*–9* for OPLL cells) used are shown in Tables 1 and 2. At least five reproducible differences in the gene expression, indicated with squares, were detected, and their DNA sequences were analyzed. M, DNA size marker.

investigate the relationship between PGI₂ signaling and mechanical stress, we performed RT-PCR with primers for PGI₂ synthase. The mRNA expression of PGI₂ synthase in OPLL cells was higher than that in non-OPLL cells maintained in the resting state (Fig. 3). In OPLL cells, cyclic stretch significantly increased the mRNA expression of PGI₂ synthase about 145 and 170% ($P < 0.05$) after stimulation for 6 and 9 h, respectively, compared with the cells maintained in the resting state (0 h) (Fig. 4). On the other hand, the expression level of PGI₂ synthase did not change in non-OPLL cells.

Effect of Mechanical Stress on PGI₂ Production. PGI₂ production during the mechanical loading was examined by enzyme-linked immunosorbent assay method. After loading the cyclic stretch on OPLL cells, medium was collected, and the concentration of 6-keto-PGF₁ₐ, the stable metabolite of PGI₂, was evaluated. PGI₂ released into the medium was significantly increased by cyclic stretch for 9 h ($P < 0.05$, 3128 ± 132 pg/ml, n = 4) compared with the medium of OPLL cells maintained in the resting state for 9 h (1091 ± 54 pg/ml, n = 4).

Effects of Beraprost and Dibutyryl-cAMP on ALP Expression. It has been reported that PGI₂ stimulates cAMP synthesis in osteoblasts (Partridge et al., 1982; Rawlinson et al., 1991; Khanin et al., 1999). To investigate the role of PGI₂ and cAMP in the osteogenic differentiation of OPLL cells, beraprost, a stable analog of PGI₂, and dibutyryl cAMP, a membrane-permeable cAMP analog, were added to the culture medium at final concentrations of 1 and 100 μM, respectively, and then the ALP mRNA expression level was assessed as a marker gene for osteogenic differentiation. Beraprost increased the mRNA expression of ALP about 150 and 240% ($P < 0.05$) after addition for 6 and 9 h, respectively, and dibutyryl cAMP increased it about 150 and 200% ($P < 0.05$) after addition for 6 and 9 h, respectively, compared with the cells with no drugs (Figs. 5 and 6). No change was observed in non-OPLL cells.

```
ccDNA:      1 aaccaaaggagtcccttagaagatctgcttccgtggg-ccccatttgccagattgccccc 59
              |||||||||||||||||||||||||||||||||||| |||| ||||||||||||||||||||
PGI₂S : 2054 aaccaaaggagtcccttagaagatctgcttccctggggccccatttgccagattgcccca 2113

ccDNA:     60 atgctcacactacttgagaaaatgcagtagagcttcccccaaggctgatgcattcccggt 119
              |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
PGI₂S : 2114 ttgctcacactacttgagaaaatgcaggagagcttcccccaaggctgatgcattcccggt 2173

ccDNA:    120 gcagaacaggggcaccctccaaacactgggctctgaggagtggagttctctg-tctagag 178
              |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
PGI₂S : 2174 gcagaacaggggcaccctccaaacactgggctctgaggagtggagttctctgttctagag 2233

ccDNA:    179 tgacaggcaccagatgggatgggctttctcagtgtcagctctcaggtagggagctaagga 238
              |||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||
PGI₂S : 2234 tgacaggcaccagatgggatgggctttctcagtgtcagcactcaggtagggagctaagga 2293

ccDNA:    239 agacacagcccagacaagatggctggaagttgcctgccaggactc 283
              ||||||||||||||||||||||||||||||||| ||| ||||||||
PGI₂S : 2294 agacacagcccagacaagatggctggaaggagccagccaggactc 2338
```

Fig. 2. The base sequence of the clone identified by differential display. The clone highly expressed in OPLL cells, indicated by the thick line box in Fig. 1, had a DNA sequence with 95% homology to *Homo sapiens* PGI₂ synthase (GI number: 14786310). ccDNA, the sequence of the cDNA fragment cloned by differential display RT-PCR; PGI₂S, the sequence of PGI₂ synthase.
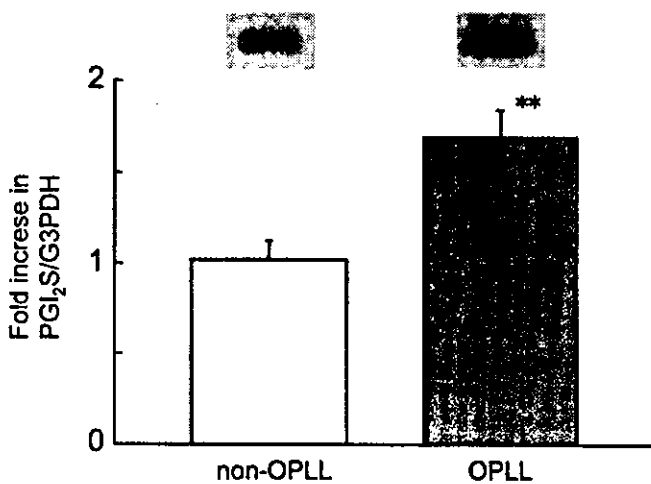

Fig. 3. RT-PCR analysis of the expression of PGI₂ synthase mRNA in cells maintained in the resting state. The total RNAs from OPLL (n = 6) and non-OPLL (n = 5) cells were examined for the expression of PGI₂ synthase by RT-PCR. The RT-PCR products were analyzed by 2.5% agarose gel electrophoresis. The densitometric quantification of the electrophoretic profiles of PGI₂ synthase was normalized to the corresponding G3PDH level. **, significantly different from 0 h, P < 0.01.
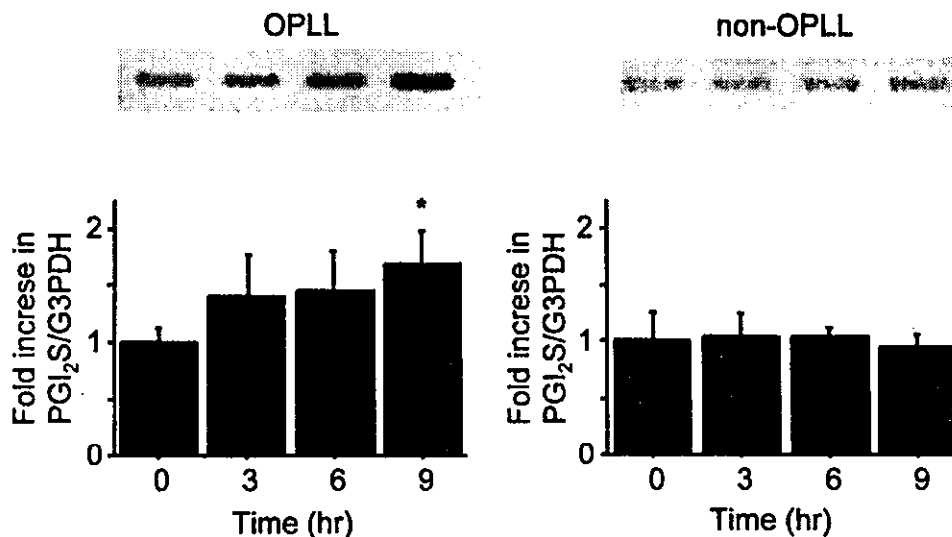
Effects of Adenylate Cyclase Inhibition on the Gene Expression Induced by Beraprost.

To investigate the involvement of adenylate cyclase in the signal transduction in OPLL cells stimulated by PGI₂, the cells were incubated with 100 μM SQ22536 (Schilling et al., 1998), a potent inhibitor of adenylate cyclase, for 30 min and then incubated with beraprost in the presence of SQ22536 for 9 h. The increase in ALP mRNA expression induced by beraprost was diminished by the addition of 100 μM SQ22536 (Fig. 7).

Effects of Adenylate Cyclase Inhibition on the Gene Expression Induced by Mechanical Stress.

To investigate the involvement of adenylate cyclase in the signal transduction in OPLL cells stimulated by mechanical stress, the cells were incubated with 100 μM SQ22536 for 30 min and then subjected to uniaxial cyclic stretch in the presence of SQ22536 for 9 h. The expressions of ALP were significantly increased by stretch (P < 0.05, ALP/G3PDH = 0.99 $\pm$ 0.45, n = 4) compared with the group without stretch (ALP/G3PDH = 0.52 $\pm$ 0.26, n = 4), and these stretch-induced expressions of ALP were almost completely suppressed by SQ22536 (ALP/G3PDH = 0.54 $\pm$ 0.27, n = 4).

The Presence of PGI₂ Receptor. The presence of PGI₂ receptor was confirmed in OPLL and non-OPLL cells by RT-PCR using specific primers for PGI₂ receptor (Fig. 8). There was no significant difference in the expression of PGI₂

Fig. 4. RT-PCR analysis of the expression of PGI₂ synthase mRNA in cells subjected to cyclic stretch. OPLL (n = 6) and non-OPLL (n = 5) cells were subjected to uniaxial cyclic stretch for the indicated time periods. The total RNAs from both cell types were examined for the expression of PGI₂ synthase by RT-PCR. The RT-PCR products were analyzed by 2.5% agarose gel electrophoresis. The densitometric quantification of the electrophoretic profiles of PGI₂ synthase was normalized to the corresponding G3PDH level. Each value is expressed as the ratio versus time 0 h. *, significantly different from 0 h, P < 0.05. PGI₂ synthase/G3PDH at 0 h was 1.40 $\pm$ 0.68 in OPLL cells and 0.97 $\pm$ 0.30 in non-OPLL cells.

OPLL

non-OPLL
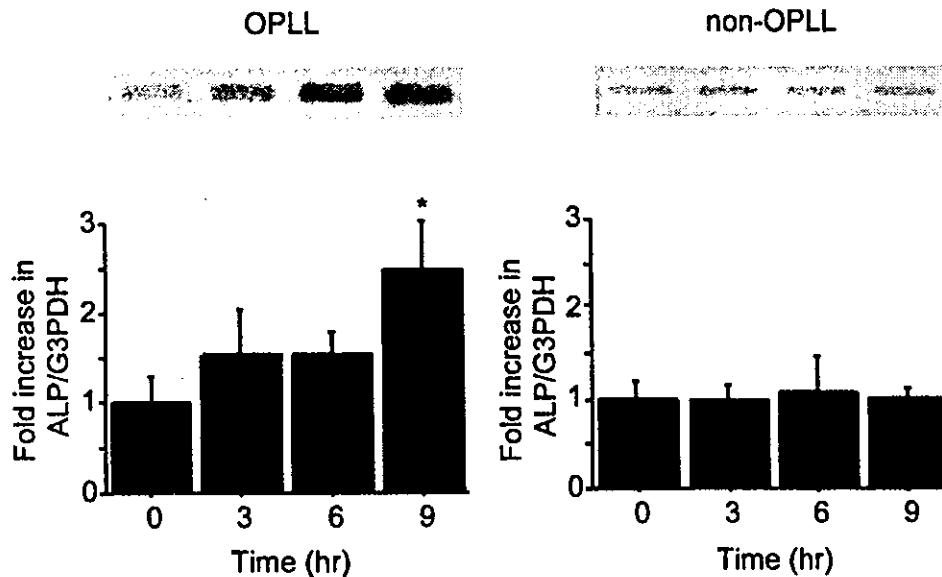

Fig. 5. Effects of beraprost on ALP expression. OPLL ($n = 6$) and non-OPLL ($n = 5$) cells were treated with 1 μM of beraprost for the indicated time periods, and then the expression levels of ALP mRNA were analyzed by RT-PCR. The densitometric quantification of the electrophoretic profiles of each gene was normalized to the corresponding G3PDH level. Each value is expressed as the ratio versus time 0 h. *, significantly different from 0 h, $P < 0.05$. ALP/G3PDH at 0 h was 0.25 ± 0.08 in OPLL cells and 0.21 ± 0.02 in non-OPLL cells.

receptor between the two types of cells. This result suggests that the difference in sensitivity to PGI_2 between the two types of cells may be due to differences in the intracellular signal transduction.

Effects of Prostaglandin E_2 Receptor Agonist and Antagonists on the ALP Expression. To investigate the involvement of prostaglandin E_2 receptors in the signal transduction in OPLL cells stimulated by PGI_2, the cells were incubated with 1 μM ONO-8713 (an EP1 antagonist), 100 μM AH6809 (an EP1 and EP2 antagonist), 10 μM ONO-AE-248 (an EP3 agonist), and 100 μM AH23848B (an EP4 antagonist) for 30 min and were followed by the incubation with 1 μM beraprost for 9 h except for the case of the EP3 agonist. The expressions of ALP were increased by beraprost about 199% ($P < 0.05$, ALP/G3PDH = 1.06 ± 0.15, $n = 4$) compared with the group without beraprost (ALP/G3PDH = 0.59 ± 0.18, $n = 4$). The expressions of ALP in the presence of prostaglandin E_2 receptor antagonists were 150% ($P < 0.05$, ALP/G3PDH = 0.86 ± 0.16, $n = 4$), 171% ($P < 0.05$, ALP/G3PDH = 0.94 ± 0.08, $n = 4$), and 145% ($P < 0.05$,

ALP/G3PDH = 0.76 ± 0.11, $n = 4$), respectively. On the other hand, the EP_3 agonist (ONO-AE-248) failed to increase the ALP expression (ALP/G3PDH = 0.61 ± 0.08, $n = 4$).

Discussion

Among the natural prostaglandins, PGI_2 is the major component produced in bone metabolism and the most potent inhibitor of bone resorption (Fortier et al., 2001). PGI_2 is converted from prostaglandin H_2 by PGI_2 synthase, the key enzyme in PGI_2 production. Differential display RT-PCR revealed higher expression of this enzyme in OPLL cells compared with non-OPLL cells. This result suggests that OPLL cells have an osteoblastic phenotype rather than a fibroblastic phenotype and that the PGI_2-signaling system plays some role in the progression of OPLL. Several lines of evidence support the view that OPLL cells have an osteoblastic phenotype and the metaplasia of OPLL cells into osteoprogenitor cells had already occurred in OPLL (Ishida and Kawai, 1993a; Kon et al., 1997).
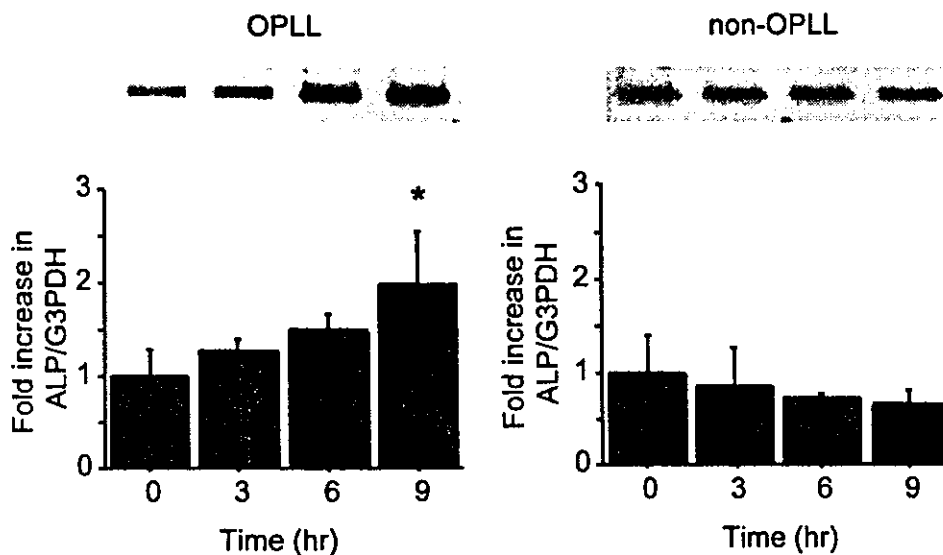
OPLL

non-OPLL

Fig. 6. Effects of dibutyryl cAMP on ALP expression. OPLL ($n = 6$) and non-OPLL ($n = 5$) cells were treated with 100 μM of dibutyryl cAMP for the indicated time periods, and then the expression levels of ALP mRNA were analyzed by RT-PCR. The densitometric quantification of the electrophoretic profiles of each gene was normalized to the corresponding G3PDH level. Each value is expressed as the ratio versus time 0 h. *, significantly different from 0 h, $P < 0.05$. ALP/G3PDH at 0 h was 0.44 ± 0.12 in OPLL cells and 0.56 ± 0.17 in non-OPLL cells.
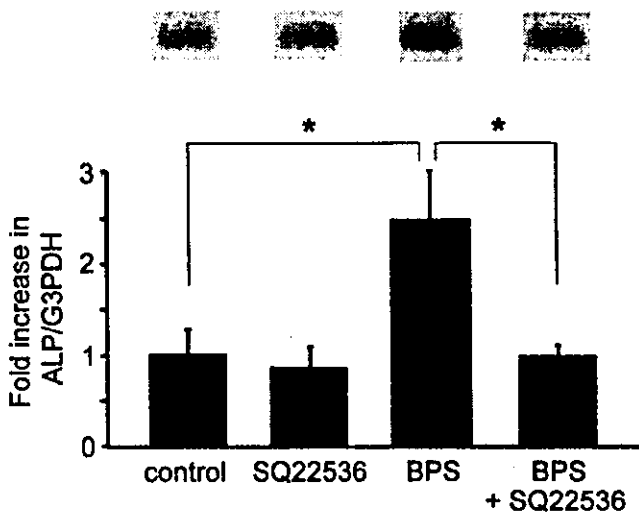
Fig. 7. Effects of adenylate cyclase inhibition on the gene expression induced by beraprost. OPLL cells (n = 6) were incubated with or without 100 μM of SQ22536 for 30 min and then stimulated by beraprost for 9 h. The total RNA prepared from these cells was subjected to analysis for the expression levels of ALP mRNA using primers specific for ALP. The densitometric quantification of the electrophoretic profiles of the PCR product from the ALP mRNA was normalized to the corresponding G3PDH level. Each value is expressed as the ratio versus the control. *, significantly different from the control; $P < 0.05$. ALP/G3PDH of the control was 0.25 ± 0.08.
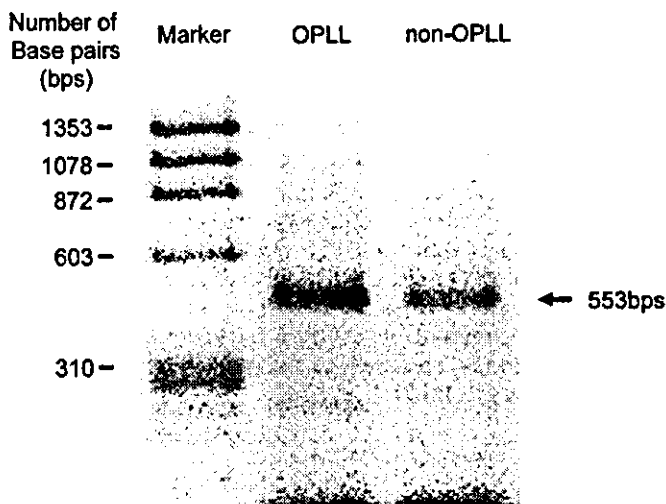

Fig. 8. Expression of PGI₂ receptor in OPLL and non-OPLL cells. Total RNAs from confluent cultures of both cell types were examined for expression of PGI₂ receptor by RT-PCR, and the PCR products were analyzed by agarose gel electrophoresis. The data are representative of three experiments. An arrow indicates the position of the PCR products corresponding to the PGI₂ receptor (553-base pairs).

Mechanical stress is known as a regulator of bone remodeling, which increases not only the osteoblast cell number but also the expressions of various osteogenic marker genes, such as alkaline phosphatase, type I collagen, osteopontin, and osteocalcin (Harter et al., 1995). OPLL often progresses after posterior decompressive surgery of the cervical spine, such as laminectomy or laminoplasty, which causes cervical instability (Matsunaga et al., 1994; Nakamura, 1994; Takatsu et al., 1999). These clinical observations suggest that the mechanical stress that acts on the posterior ligaments is an important factor in the progression of OPLL. In fact, uniaxial cyclic

stretch increases the expressions of osteogenic marker genes in both OPLL cells (Tanno et al., unpublished observations) and ligament tissues (Iwasaki et al., unpublished observations). On the other hand, mechanical loading induces PGI₂ production in osteocytes and osteoblasts, resulting in an induction of bone remodeling (Rawlinson et al., 1993; Zaman et al., 1997). Furthermore, exogenous PGI₂ stimulates an increase in ALP activity in osteocytes and osteoblasts (Rawlinson et al., 1993). The present study revealed that uniaxial cyclic stretch enhanced the expression of PGI₂ synthase and also PGI₂ production. Beraprost, a stable PGI₂ analog, induced an increase in ALP mRNA in OPLL cells but not in non-OPLL cells. These observations suggest that mechanical stress affects the progression of OPLL through the activation of the PGI₂-signaling system.

PGI₂ interacts with a specific receptor, IP, which is a G protein-coupled cell surface receptor. The IP receptor was detected in fetal bone and osteoblasts (Fortier et al., 2001). Although the IP receptor was expressed in both OPLL cells and non-OPLL cells, beraprost only increased the ALP mRNA expression in OPLL cells. Ligament cells have been reported to express other prostagrandin receptors (i.e., EPs). However, antagonists for EP receptors, including EP1, EP2, and EP4, did not affect on the ALP expression induced by beraprost, and an agonist for the EP3 receptor by itself failed to induce the ALP expression. These results allow us to speculate that the cellular effects of PGI₂ are mediated through the PGI₂ receptor but there are differences between OPLL cells and non-OPLL cells with regard to the response to mechanical stress and intracellular signal transduction.

PGI₂ enhances cAMP synthesis in the osteoblastic-like cell line UMR-106 (Khanin et al., 1999) and osteoblasts (Partridge et al., 1981), and this is mediated by adenylate cyclase. Dibutyryl cAMP mimics the effect of PGI₂ in osteoblasts (Partridge et al., 1982; Rawlinson et al., 1991; Khanin et al., 1999). The present study demonstrated that beraprost and dibutyryl cAMP only increased the ALP mRNA expression in OPLL cells and that SQ22536, a potent adenylate cyclase inhibitor, diminished the stimulatory effect of beraprost. These results suggest that the PGI₂/cAMP system plays a pivotal role in the osteogenic differentiation of OPLL cells.

In conclusion, PGI₂ synthase was expressed more highly in OPLL cells than in non-OPLL cells. Uniaxial cyclic stretch, as a mechanical stress, further increased PGI₂ synthase expression only in OPLL cells, resulting in the stimulation of ALP expression via an increase in the intracellular cAMP level. We propose that the increase in PGI₂ synthase induced by mechanical stress plays a key role in the progression of OPLL, at least in part through the induction of osteogenic differentiation in spinal ligament cells through the PGI₂/cAMP system.

References

Baba H, Furusawa N, Fukuda M, Maezawa Y, Imura S, Kawahara N, Nakahashi K, and Tomita K (1997) Potential role of streptozotocin in enhancing ossification of the posterior longitudinal ligament of the cervical spine in the hereditary spinal-hyperostotic mouse (twy/twy). Eur J Histochem 41:191-202.

Fortier I, Patry C, Lora M, Samadfan R, and de Brum-Fernandes AJ (2001) Immu-

nohistochemical localization of the prostacyclin receptor (IP) human bone. *Prostaglandins Leukotrienes Essent Fatty Acids* **65**:79–83.

Furushima K, Shimo-Onoda K, Maeda S, Nobukuni T, Ikari K, Koga H, Komiya S, Nakajima T, Harata S, and Inoue I (2002) Large-scale screening for candidate genes of ossification of the posterior longitudinal ligament of the spine. *J Bone Miner Res* **17**:128–137.

Goto K, Yamazaki M, Tagawa M, Goto S, Kon T, Moriya H, and Fujimura S (1998) Involvement of insulin-like growth factor I in development of ossification of the posterior longitudinal ligament of the spine. *Calcif Tissue Int* **62**:158–165.

Harter LV, Hruska KA, and Duncan RL (1995) Human osteoblast-like cells respond to mechanical strain with increased bone matrix protein production independent of hormonal regulation. *Endocrinology* **136**:528–535.

Hashizume Y (1980) Pathological studies on the ossification of the posterior longitudinal ligament (OPLL). *Acta Pathol Jpn* **30**:255–273.

Ishida Y (1998) Studies on induction mechanism of ossification of the posterior longitudinal ligament of the spine–especially on the cultured cells from the human spinal ligament. *Nippon Seikeigeka Gakkai Zasshi* **62**:1019–1027.

Ishida Y and Kawai S (1993a) Characterization of cultured cells derived from ossification of the posterior longitudinal ligament of the spine. *Bone* **14**:85–91.

Ishida Y and Kawai S (1993b) Effects of bone-seeking hormones on DNA synthesis, cyclic AMP level and alkaline phosphatase activity in cultured cells from human posterior longitudinal ligament of the spine. *J Bone Miner Res* **8**:1291–1300.

Khanin M, Liel Y, and Rimon G (1999) Differential effect of TPA on PGE2 and cicaprost-induced cAMP synthesis in UMR-106 cells. *Cell Signal* **11**:165–169.

Kitajima I, Tachibana S, Mikami Y, Hirota Y, and Nakamichi K (2001) Development of ossification of the posterior longitudinal ligament of the cervical spine after atlanto-axial fusion. *J Orthop Sci* **6**:591–594.

Koga H, Sakou T, Taketomi E, Hayashi K, Numasawa T, Harata S, Yone K, Matsunaga S, Otterud B, Inoue I, et al. (1998) Genetic mapping of ossification of the posterior longitudinal ligament of the spine. *Am J Hum Genet* **62**:1460–1467.

Kon T, Yamazaki M, Tagawa M, Goto S, Terakado A, Moriya H, and Fujimura S (1997) Bone morphogenetic protein-2 stimulates differentiation of cultured spinal ligament cells from patients with ossification of the posterior longitudinal ligament. *Calcif Tissue Int* **60**:291–296.

Matsunaga S, Sakou T, Taketomi E, Yamaguchi M, and Okano T (1994) The natural course of myelopathy caused by ossification of the posterior longitudinal ligament in the cervical spine. *Clin Orthop* **305**:168–177.

Nakamura H (1994) A radiographic study of the progression of ossification of the cervical posterior longitudinal ligament: the correlation between the ossification of the posterior longitudinal ligament and that of the anterior longitudinal ligament. *Nippon Seikeigeka Gakkai Zasshi* **68**:725–730.

Naruse K, Yamada T, and Sokabe M (1998) Involvement of SA channels in orienting

response of cultured endothelial cells to cyclic stretch. *Am J Physiol* **274**:H1532–H1538.

Numasawa T, Koga H, Ueyama K, Maeda S, Sakou T, Harata S, Leppert M, and Inoue I (1999) Human retinoic X receptor beta: complete genomic sequence and mutation search for ossification of posterior longitudinal ligament of the spine. *J Bone Miner Res* **14**:500–508.

Partridge NC, Kemp BE, Livesey SA, and Martin TJ (1982) Activity ratio measurements reflect intracellular activation of adenosine 3', 5'-monophosphate-dependent protein kinase in osteoblasts. *Endocrinology* **111**:178–183.

Partridge NC, Kemp BE, Veroni MC, and Martin TJ (1981) Activation of adenosine 3', 5'-monophosphate-dependent protein kinase in normal and malignant bone cells by parathyroid hormone, prostaglandin E2 and prostacyclin. *Endocrinology* **108**:220–225.

Rawlinson SC, el-Haj AJ, Minter SL, Tavares IA, Bennett A, and Lanyon LE (1991) Loading-related increases in prostaglandin production in cores of adult canine cancellous bone in vitro: a role for prostacyclin in adaptive bone remodeling? *J Bone Miner Res* **6**:1345–1351.

Rawlinson SC, Mohan S, Baylink DJ, and Lanyon LE (1993) Exogenous prostacyclin, but not prostaglandin E2, produces similar responses in both G6PD activity and RNA production as mechanical loading and increases IGF-II release, in adult cancellous bone in culture. *Calcif Tissue Int* **53**:324–329.

Resnick D, Shaul SR, and Robins JM (1975) Diffuse idiopathic skeletal hyperostosis (DISH): Forestier's disease with extraspinal manifestations. *Radiology* **115**:513–524.

Schilling L, Kanzler C, Schmiedek P, and Ehrenreich H (1998) Characterization of the relaxant action of urocortin, a new peptide related to corticotropin-releasing factor in the rat isolated basilar artery. *Br J Pharmacol* **125**:1164–1171.

Takatsu T, Ishida Y, Suzuki K, and Inoue H (1999) Radiological study of cervical ossification of the posterior longitudinal ligament. *J Spinal Disord* **12**:271–273.

Wang PN, Chen SS, Liu HC, Fuh JL, Kuo BI, and Wang SJ (1999) Ossification of the posterior longitudinal ligament of the spine. A case-control risk factor study. *Spine* **24**:142–144.

Yamamoto Y, Furukawa KI, Ueyama K, Nakanishi T, Takigawa M, and Harata S (2002) Possible roles of CTGF/Hcs24 in the initiation and development of ossification of the posterior longitudinal ligament. *Spine* **27**:1852–1857.

Zaman G, Suswillo RF, Cheng MZ, Tavares IA, and Lanyon LE (1997) Early responses to dynamic strain change and prostaglandins in bone-derived cells in culture. *J Bone Miner Res* **12**:769–777.

Address correspondence to: Ken-Ichi Furukawa, Department of Pharmacology, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan. E-mail: furukawa@cc.hirosaki-u.ac.jp

ELSEVIER

Uniaxial cyclic stretch induces osteogenic differentiation and synthesis of bone morphogenetic proteins of spinal ligament cells derived from patients with ossification of the posterior longitudinal ligaments

M. Tanno,[a] K.-I. Furukawa,[b,*] K. Ueyama,[a] S. Harata,[a] and S. Motomura[b]

[a] Department of Orthopaedic Surgery, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, 036-8562, Japan
[b] Department of Pharmacology, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, 036-8562, Japan

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 elucidate this mechanism, we investigated the effects of in vitro sinusoidal cyclic stretch (120% peak to peak, at 1 Hz) on cultured spinal ligament cells derived from OPLL and non-OPLL patients. The mRNA expressions of alkaline phosphatase (ALP), osteopontin, bone morphogenetic protein (BMP)-2, BMP-4, and BMP receptors as well as ALP activity in cell layers and production of BMPs into the conditioned medium were significantly increased by cyclic stretch in OPLL cells, whereas no change was observed in non-OPLL cells. A stretch-activated Ca^{2+} channel blocker, Gd^{3+}, the voltage-dependent L-type Ca^{2+} channel blockers diltiazem and nifedipine, and Ca^{2+}-free medium suppressed stretch-induced ALP activity, which suggests a role of Ca^{2+} influx in the signal transduction of mechanical stress to the osteogenic response of OPLL cells. Our study provides first evidences that mechanical stress plays a key role in the progression of OPLL through the induction of osteogenic differentiation in spinal ligament cells and the promotion of the autocrine/paracrine mechanism of BMPs in this lesion. © 2003 Elsevier Inc. All rights reserved.

Keywords: Ossification of the posterior longitudinal ligament; Mechanical stress; Bone morphogenetic protein; Calcium channel; Alkaline phosphatase

Introduction

Ossification of the posterior longitudinal ligament of the spine (OPLL) is characterized by ectopic bone formation in the spinal ligament. In some cases ossification enlarges in the spinal canal and compresses the spinal cord, resulting in serious neurological damage. Ossification of the posterior longitudinal ligament occurs throughout the spine, frequently involving ossification of other spinal ligaments, and it is assumed to be related to diffuse idiopathic skeletal hyperostosis [1] or ankylosing spinal hyperostosis [2].

As an established therapy, surgical treatment is often applied to OPLL patients with neurological symptoms,

though there have still been some problems, such as a higher risk of neurological complications and, in almost all cases of posterior decompression surgery, postoperative neck pain which has a negative impact on the patient's activities of daily living. On the other hand, several drugs for pharmacological suppression of the progression of ossification have been studied in several animal models [3,4]; however, the effectiveness of these agents on OPLL patients has not yet been made clear. It is important to elucidate the mechanisms responsible for producing the ossification of spinal ligaments, because this may provide the information needed for developing future therapeutic interventions for OPLL patients.

The ossification process in OPLL is thought to occur through the endochondral mechanism [5,6] based on characteristic abnormalities of spinal ligament cells that promote their differentiation into osteogenic cells. And the involve-

* Corresponding author. Department of Pharmacology, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan. Fax: +81-172-39-5023.
E-mail address: furukawa@cc.hirosaki-u.ac.jp (K.-I. Furukawa).

ment of multiple etiologic factors in the development of OPLL has been suggested, including genetic factors [7,8], dietary habits [9], metabolic abnormalities [10], and some local factors [6,11–16]. Recently, when compared with normal spinal ligament cells, spinal ligament cells derived from OPLL patients (OPLL cells) have been shown to have several phenotypes of osteoblasts i.e., in vitro calcification and high alkaline phosphate (ALP) activity in culture [13]. Also, OPLL cells exhibiting osteoblastic properties were also shown to respond to transforming growth factor-β (TGF-β) [12], bone morphogenetic protein-2 (BMP-2) [15], insulin-like growth factor-I (IGF-I) [11], and parathyroid hormone [14]. However, the nature of OPLL cells was thought to be close to that of osteoprogenitor cells rather than osteoblasts, and this population of cells very small in spinal ligament tissue; therefore, its identity was not clear. In an immunohistochemical study of ossified ligament tissues of OPLL patients, the expressions of osteogenic protein-1 (OP-1)/BMP-7 and its receptors (type-IA, -IB, and -II receptor) were enhanced and widely detected even in fibroblast-like cells at the sites of nonossified fibrous tissue, whereas in control cases (i.e., ligament tissue of non-OPLL patients) showed only weak expression of these receptors in ligamentous enthesis, where ligamentous ossification was surrounded by cartilaginous tissue, and no staining by anti OP-1/BMP 7 [16]. Accumulating evidence indicates 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.

There are numerous clinical studies about the progression of OPLL. OPLL often progresses after posterior decompressive surgery of the cervical spine, such as laminectomy or laminoplasty, which causes a significant defect in the structural integrity of the dorsal element and results in various degrees of cervical instability, especially in the former [17–19]. The intervertebral disc is subjected to a variety of forces and stresses during physiologic spinal motions of flexion, extension, and lateral bending. The posterior longitudinal ligaments lie on the posterior surfaces of the intervertebral discs and vertebral bodies; therefore, as a mechanical stress, the instability between two adjacent vertebrae, or bulging of the intervertebral disc, is directly transmitted to this ligament [20]. In an analysis of dynamic lateral X-ray films of the cervical spine in OPLL patients by a digitizer, the progression of OPLL was highly correlated with abnormal strain distribution in the intervertebral discs, and progression was frequently observed when strain in the tensile direction was distributed over the disc, which caused tensile force in the posterior longitudinal ligament [21]. These clinical evidences support the notion that the mechanical stress which acts on the posterior ligaments or ligamentous enthesis of vertebral bodies is thought to be an important factor in the progression of OPLL.

We hypothesized in OPLL that mechanical signals may be transmitted to induce spinal ligament cell differentiation into osteoblasts or may stimulate the release of some growth factors, which participate in the ossification processes of spinal ligaments. To examine these possibilities, we investigated whether osteogenic responses in OPLL cells were induced by uniaxial cycle stretch, and if so, what kind of pathways were required for transmitting mechanical signals in these processes. Here, we describe, to our knowledge, the first report of the biochemical effects of mechanical stress on OPLL cells by determining the response of the following to uniaxial cyclic stretch: the mRNA expression of ALP and osteopontin, the level of BMP-2 and BMP-4 and its receptors, ALP activity in cell assay, and the production of BMP2/4 in medium. We also determined that the $\alpha 1C$ subunit of the voltage-dependent L-type Ca^{2+} channel was expressed in OPLL cells and demonstrated its involvement in the pathway of mechanically induced osteogenic differentiation.

Materials and methods

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-ray, 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. Although the ligament samples were not all from the same locations in the cervical spine, we used all samples for experiments, because the cells from OPLL patients showed similar osteoprogenic characteristics independent of the location from which the tissue was extirpated in the cervical spine [11,15].

Cell culture

Ligaments harvested aseptically from patients during surgery were rinsed with PBS, after which surrounding tissue was carefully removed under a dissecting microscope. In all cases the ligaments were extirpated carefully from nonossified sites to avoid any possible contamination of osteogenic cells. Collected ligaments were minced into about 0.5-mm^3 pieces and washed twice with PBS, then plated in 100-mm culture dishes, and maintained in DMEM (10% FBS, 1% L-glutamine, 100 units/ml of penicillin G sodium, 100 μg of streptomycin sulfate) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The cells derived from explants were removed from the dish with 0.02% EDTA/0.05% trypsin for passage.

Table 1
Tissue samples used in this study, showing clinical diagnosis, patient's sex and age, and tissue derived

OPLL				non-OPLL			
Code	Diagnosis	Sex/age	Tissue	Code	Diagnosis	Sex/age	Tissue
O-1	OPLL	M/ 45	PLL	N-1	CDH	M/ 46	PLL
O-2	OPLL	M/ 46	PLL	N-2	CDH	M/ 60	PLL
O-3	OPLL	M/ 57	PLL	N-3	CDH	M/ 60	PLL
O-4	OPLL	M/ 63	PLL	N-4	CDH	F/ 46	PLL
O-5	OPLL	M/ 50	LF	N-5	CDH	F/ 49	PLL
O-6	OPLL	M/ 53	LF	N-6	CDH	M/ 59	LF
O-7	OPLL	F/ 58	LF	N-7	CSM	M/ 44	PLL
O-8	OPLL	F/ 50	LF	N-8	CSM	M/ 59	PLL
O-9	OPLL	F/ 64	LF	N-9	CSM	M/ 58	LF
O-10	OPLL	M/ 60	ISL	N-10	CSM	F/ 58	ISL

OPLL, ossification of the posterior longitudinal ligament; CDH, cervical disc herniation; CSM, cervical spondylotic myelopathy; PLL, posterior longitudinal ligament; LF, ligamentum flavum; ISL, interspinous ligament; M, male; F, female.

Mineralization assay

Cells from OPLL (OPLL cells) and non-OPLL patients (non-OPLL cells) were plated at 40,000 cells/35-mm gelatin-coated dish and maintained in DMEM supplemented with 10% FBS. On confluence, designated day 0, cells were then exposed to osteogenic medium containing DMEM supplemented with 10% FBS, 50 μg/ml of ascorbic acid, and 5 mM β-glycerophosphate, replacing medium for 3–4 days. Samples were processed 8 weeks and alizarin red assay (Sigma Chemical, St. Louis, MO, USA) was performed to determine mineralization. Briefly, cells were washed with PBS and water and fixed with ice-cold 100% ethanol for 60 min at 4°C. Fixed cultures were incubated with 1% alizarin red for 30 min and washed with distilled water several times. Extracellular matrix mineral-bound stain was photographed under microscopy.

Stretch apparatus

The cells (fifth passage) were placed on a 3.5 × 4.0 cm² silicon chamber coated with 0.1% gelatin (IWAKI GLASS, Tokyo, Japan) at a density of 10,000 cells/cm². After cultures reached confluence, cells were incubated in DMEM supplemented with 1% FBS for 24 h and then subjected to motor-driven computer-controlled uniaxial sinusoidal cycle stretch by using a four-point bending apparatus (Scholertec Corp., Osaka, Japan) [44], in 120% peak to peak, at 1 Hz in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

RNA preparation and cDNA synthesis

After different time 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 treated with RNase-free DNase 1 (Life Technologies, Inc., Rockville, MD, USA) was reverse transcribed into cDNA using Oligo(dT)12–18 primer (Life Technologies, Inc.). One micro-

gram of total RNA was heated at 70°C for 10 min in 10 μl of H_2O supplemented with 0.5 μg of 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 20 μl of buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, and 3 mM MgCl$_2$) supplemented with 0.5 mM dNTPs (Life Technologies, Inc.), 2.5 mM DTT (Life Technologies, Inc.), 2 U of RNase inhibitor (TOYOBO, Osaka, Japan), and 200 U of M-MLV reverse transcriptase (Life Technologies, Inc.). After incubation, the cDNAs were heated to 72°C and stored at −20°C until used for amplification by polymerase chain reaction (PCR).

PCR analysis

For PCR amplification, specific oligonucleotide primers to human sequences were designed on the basis of sequences in GenBank (Table 2). Reaction was performed using the Taq PCR Master Mix Kit (Qiagen, Inc.) as follows: 1 μl of cDNA was used as the template in a 20-μl amplification mixture consisting of 1 U of *Taq* DNA polymerase, 0.5 μM each of the 5′ and 3′ primers, and distilled water. For quantification of gene expression, all products were assayed in the exponential phase of the amplification curve and the PCR cycles were determined for each primer pair. PCR was performed in a Perkin–Elmer 9600 thermal cycler, with an initial denaturation at 94°C for 2 min, followed by each cycle of 20 s at 94°C, 30 s at the temperature indicated in Table 2, and extension at 72°C for 90 s and additional extension step at 72°C for 10 min. Amplification products were resolved by electrophoresis on a 2.5% w/v agarose gel and were visualized by staining with SYBR Green-I (Molecular Dynamics, Sunnyvale, CA, USA). The SYBR Green-I luminescence was converted to a TIFF image by CCD camera (C-900ZOOM, OLYMPUS, Tokyo, Japan) and the intensity was quantitated by QuantiScan software (BIOSOFT, Ferguson, MO, USA). All products were corrected for glycerol 3-phosphate dehydrogenase (G3PDH) mRNA levels.

Table 2
PCR primers

Target gene	S/AS	Primer sequence	T_m (°C)	Cycle	Product size (bp)
ALP	S	ATCGCCTACCAGCTCATGCAT	55	28	291
	AS	GTTCAGCTCGTACTGCATGTC			
Osteopontin	S	AGCCGTGGGAAGGACAGTTATG	60	30	475
	AS	GGAGTTTCCATGAAGCCACAAAC			
BMP-2	S	AGCAGAGCTTCAGGTTTTCCG	60	29	433
	AS	GCGTTTCCGCTGTTTGTGTT			
BMP-4	S	CTGGTCAATTCTGTCAATTCC	60	29	444
	AS	GGTCAAGGTGAATGTTTAGG			
BMPR-IB	S	GCAGCACAGACGGATATTGT	60	30	632
	AS	TTTCATGCCTCATCAACACT			
BMPR-II	S	TGACCTCACTGCCAGGCTATT	60	26	447
	AS	CCTGCAATTTCCCATCGAGAT			
α1C subunit	S	ATACCCAATGCTCTCCCTCTATGAA	60	28	831
	AS	GCTCCTCATTGGCTTGTTCAGGTT			
Osteocalcin	S	GATCCCCGCTTCCTCTTTAGAC	60	27	268
	AS	GGGCTATTTGGGGGTCATCC			
G3PDH	S	ACCACAGTCCATGCCATCAC	60	19	450
	AS	TCCACCACCCTGTTGGTGTA			

S, sense; AS, antisense.

Alkaline phosphatase assay

The ALP activity was measured after rinsing the cells twice with PBS and then adding 0.6 ml of alkaline lysis buffer (0.75 M 2-amino-2-methyl-1-propanol, pH 10.3) containing p-nitrophenol phosphate substrate (2 mg/ml). For the generation of a standard curve, serial dilutions of a p-nitrophenol standard solution were prepared and 100 μl of each concentration was included in each microtiter plate. Following incubation at 37°C for 30 min, 0.6 ml of 50 mM NaOH was added to each chamber to stop the reaction. The absorbance was read at 410 nm [22]. Concentrations of protein were determined with a Bradford protein assay, with BSA as the standard. Results are expressed as micrograms of p-nitrophenol per milliliter of cellular protein.

Western blot analysis

Samples from the conditioned medium of cells were concentrated 20-fold with Centricon YM-10 (Millipore Corp., Bedford, MA, USA). Equal amounts of total protein (40 μg/ml) were treated with 1× sample buffer, loaded onto an SDS–polyacrylamide gel on 10% acrylamide gels according to Laemmli [45], and electrophoresed. Proteins were electrotransferred to PVDF membranes (Millipore Corp.) at 300 V for 1 h. They were blocked using 5% milk in TBS (20 mM Tris at pH 7.5, 500 mM NaCl, and 0.05% Tween 20) and then hybridized for 1 h with antihuman BMP-2/4 polyclonal antibody (R & D Systems, Inc., Minneapolis, MN, USA) diluted 1:1000 in TBS with 5% milk. This was followed by 1 h with ALP-conjugated rabbit antigoat IgG (Vector Laboratories, Inc., Burlingame, CA, USA). Antibody binding was detected by exposure to enhanced chemiluminescence using Immun-Star Enhancer (Bio-Rad Laboratories, Hercules, CA, USA) and then visu-

alized on a BioImage Analyzer (Bio-Rad Laboratories). For immunodetection of β-actin protein, the cells were washed twice with ice-cold phosphate-buffered saline. Cell lysis buffer (150 mM NaCl, 1% $C_{12}E_8$, 50 mM Tris–HCl (pH 8.0), 2 μg/ml of aprotinin, 2 μg/ml of leupeptin, 1 μg/ml of pepstatin A, 100 μg/ml of PMSF, 1 mM EDTA) was added and cell lysate was collected. The supernatant was obtained by a centrifugation for 10 min at 100,000g and subjected to SDS–PAGE. Immunoblotting was performed as described above by using anti-β-actin monoclonal antibody (Sigma Chemical).

Statistical analysis

All data are expressed as means ± SEM. ANOVA with Dunnett type test for multiple comparisons against a control and Student's t test was used. $P < 0.05$ was considered significant.

Results

Mineralization

To confirm the characteristic differences between OPLL cells and non-OPLL cells, we first tested whether these cells exhibited mineralization when maintained in osteogenic medium. On confluence, both cells exhibited a fibroblast-like, spindle-shaped appearance. Morphological change was seen in some non-OPLL cells, but none of these cells exhibited mineralization (data are not shown). On the other hand, the matrix began to mineralize and crystals appeared on collagen fibers at 4 to 8 weeks after confluence in 6 of 10 OPLL cell cultures (Figs. 1A and B). Although all of 10 OPLL cells were used in the following experiments, they