T. Nakase
K. Ariga
K. Yonenobu
N. Tsumaki
F. P. Luyten
Y. Mukai
I. Sato
H. Yoshikawa

Activation and localization of cartilage-derived morphogenetic protein-1 at the site of ossification of the ligamentum flavum

Received: 29 May 2001
Accepted: 6 June 2001
Published online: 23 June 2001
© Springer-Verlag 2001

Supported in part by a grant from the Japanese Ministry of Education and the Ministry of Health and Welfare's Research Grant for Specific Diseases in Japan.

T. Nakase (✉) · K. Ariga · K. Yonenobu
Y. Mukai · I. Sato · H. Yoshikawa
Department of Orthopaedic Surgery,
Osaka University Medical School,
2-2, Yamadaoka, Suita, 565-0871, Japan
e-mail: nakase@ort.med.osaka-u.ac.jp,
Tel.: +81-6-6879-3552, Fax: +81-6-6879-3559

N. Tsumaki
Department of Orthopaedic Surgery,
Osaka Police Hospital, Osaka, Japan

F.P. Luyten
Department of Rheumatology,
University Hospitals, Leuven, Belgium

Abstract Localization and expression of cartilage-derived morphogenetic protein (CDMP)-1 in tissues at the site of ossification of the ligamentum flavum (OLF) were examined by immunohistochemistry and in situ hybridization. The CDMP-1 protein and messenger ribonucleic acid (mRNA) were localized in spindle-shaped cells and chondrocytes in the OLF tissues. CDMP-1 was not detected in cells in non-ossified sites. These data indicate that CDMP-1 is locally activated and localized in spindle-shaped cells and chondrocytes at the site of OLF. Given the previously reported promoting action of CDMP-1 for chondrogenesis, the current results suggest that CDMP-1 may be involved in the progression of OLF, leading to the narrowing of

spinal canal and thus causing severe clinical manifestations.

Keywords Ossification · Ligamentum flavum · Cartilage-derived morphogenetic protein-1 · In situ hybridization · Immunohistochemistry

Introduction

Ossification of the ligamentum flavum (OLF) is a pathologic condition leading to compression of the spinal cord and nerve roots, thus sometimes causing severe myeloradiculopathy [18]. Many cases of ossification of spinal ligaments have been reported, mainly in Japan [18]. However, ossification of spinal ligaments is now recognized as an important disease not only in the Japanese but also in peoples in Europe and the United States [5, 7].

The cause of this disease is not known, but recent etiological studies have indicated that the onset of ossification of spinal ligaments is associated with patients' genetic background [15]. Many patients have subclinical OLF [14]; symptomatology depends on the degree of the nar-

rowing of the spinal canal that is caused by the increased mass of OLF [17]. In addition to the etiological studies about the patients' genetic background, such as the search for mutations, researchers should therefore also investigate the local regulators involved in the progression of OLF after the onset of this condition. The process of OLF has been reported to be a novel form of endochondral ossification [8], in which cartilaginous template determines the shape and volume of the skeleton [13]. Recently, a key molecule regulating cartilage formation has been identified. The molecule is called cartilage-derived morphogenetic protein (CDMP)-1 and has been identified as a member of the transforming growth factor (TGF)- β superfamily [1]. Mutations of the CDMP-1 gene result in abnormal formation of the skeleton, such as seen in acromesomelic chondrodysplasia [6]. Thus CDMP-1 appears to

be a key molecule in physiologic chondrogenesis in humans. Furthermore, we recently reported that transgenic mice with overexpression of the CDMP-1 gene in chondrocytes in cartilaginous template exhibited apparent expansion of cartilage [16]. This reported activity of CDMP-1 suggests that the molecule plays a role in the expansion of the OLF mass via promotion of chondrogenesis. However, involvement or localization of CDMP-1 in OLF has not yet been reported.

The purpose of this study is to examine the activation and localization of CDMP-1 at the site of OLF. In situ hybridization and immunohistochemical analysis were performed with a complementary ribonucleic acid (cRNA) probe and an antibody against human CDMP-1.

Materials and methods

Specimens of OLF tissues were obtained from three patients during surgery. Control specimens of ligamentum flavum without ossification were obtained from two patients with lumbar canal stenosis during surgery. Informed written consent was obtained from all patients. The characteristics of these five patients are summarized in Table 1. The tissues were fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) in phosphate-buffered solution (PBS; pH 7.4; Sigma Chemical, St. Louis, MO, USA). These tissues were decalcified in 20% ethylenediaminetetraacetic acid (EDTA; Nakalai Tesque, Kyoto, Japan) and embedded in paraffin as described [11]. Histological sections of 4- μ m thickness were prepared. These sections were stained with Masson trichrome stain to reveal the elastic fibers and with safranin O/fast green stain to distinguish the cartilaginous matrix.

In situ hybridization

Digoxigenin-11 uridine triphosphate (UTP)-labeled single-strand cRNA was prepared as described. A human complementary deoxyribonucleic acid (cDNA) specific for CDMP-1 (an ApaI fragment, residue 470–1155) [1] was subcloned into pBluescript SKII⁺ plasmid and was either linearized with *Eco*RI and transcribed by T7 RNA polymerase to generate an antisense probe or linearized with *Hind*III and transcribed by T3 to generate a sense probe. In situ hybridization was carried out as described [11]. Hybridized signals were detected using a Nucleic Acid Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions.

Table 1 Characteristics of patients with ossification of the ligamentum flavum (OLF) and controls

Patient	Age (years)	Sex	Disease	Site
1	61	Male	OLF	Th10
2	69	Male	OLF	Th7/8
3	57	Male	OLF	Th2/3
4	57	Male	LCS	L4/5
5	74	Male	LCS	L4/5

OLF, ossification of the ligamentum flavum; LCS, lumbar canal stenosis.

Immunohistochemistry

Immunohistochemistry was performed as described [10]. Goat polyclonal antibody against human CDMP-1 (purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a primary antibody at a 1:100 dilution. Immunoreactions were detected using the streptavidin–peroxidase method with Histofine SAB-PO kits (Nichirei, Tokyo, Japan) according to the manufacturer's instructions.

The number of positive cells detected by immunohistochemistry and in situ hybridization was counted using a microscope as described [9].

Results

In the non-ossified lesion (*NO* in Fig. 1a) in tissues of OLF patients, the ligament fibers were dense, regularly organized, and attached to the lamina (Fig. 1b). Neither CDMP-1 protein nor messenger RNA (mRNA) was detected in these locations (Fig. 2a,b).

In the OLF lesion (*OLF* in Fig. 1a), there were fewer ligament fibers and they were severely disorganized. In the portion distant from the ossification front (*D* in Fig. 1a), there were numerous spindle-shaped cells (Fig. 1c). In the intermediate portion close to the ossification front (*IM* in Fig. 1a), a few chondrocytes were found among the disorganized ligament fibers (Fig. 1d,e). Numerous chondrocytes were seen in the calcified ossification front (*OS* in Fig. 1a,f,g). CDMP-1 was immunolocalized in spindle-shaped cells distant from the ossification front (Fig. 2c). Chondrocytes in the intermediate zone (Fig. 2e) and ossification front (Fig. 2g) also showed positive immunoreactivity for anti-CDMP-1 antibody. In situ hybridization with antisense cRNA probe for human CDMP-1 showed results similar to those of the immunohistochemical analysis (Fig. 2d,f,h). Furthermore, CDMP-1 protein and mRNA were not detected in any lesion in ligamentum flavum obtained from patients without OLF (data not shown). The percentage of positive cells based on the results of immunohistochemistry and in situ hybridization assays is summarized in Table 2.

Immunohistochemical analysis using the normal serum of the same species as the primary antibody showed no immunoreactivity. In situ hybridization using a sense probe showed no signals. Positive control studies were performed using human neonatal articular cartilage. Articular chondrocytes were positive for both the anti-CDMP-1 antibody and the antisense CDMP-1 probe.

Discussion

This study is the first to describe the localization of CDMP-1 in the ossification of spinal ligaments. Both CDMP-1 protein and mRNA were activated and localized specifically in spindle-shaped cells and chondrocytes in OLF tissues.

Fig. 1a–g Photomicrograms showing histology of tissue at the site of ossification of the ligamentum flavum (OLF) from patient 1. **a** OLF is observed beside the lamina (L) under macroscopic magnification, with Masson's trichrome staining. **b** Higher magnification ($\times 200$) of a non-ossified (NO) area. Ligament fibers (*asterisk*) besides the lamina are regularly organized. Masson's trichrome staining. **c** Higher magnification ($\times 200$) of an area distant from an ossification front (D). Ligament fibers are fewer and severely disorganized (*asterisk*). Numerous spindle-shaped cells are observed (*arrows*). Masson's trichrome staining. **d** Higher magnification ($\times 200$) of an intermediate portion close to the ossification front (IM). Ligament fibers are few and disorganized. Masson's trichrome staining. **e** Higher magnification ($\times 200$) of IM area. There are a few chondrocytes (*arrows*) between the ligament fibers; their territorial matrix is stained red by safranin-O. Safranin-O/fast green staining. **f** Higher magnification ($\times 200$) of an ossification front (OS). The number of ligament fibers is very small, and a newly formed bone (*asterisk*) is visualized. Masson's trichrome staining. **g** Higher magnification ($\times 200$) of an OS area. There are numerous chondrocytes (*arrows*). Safranin-O/fast green staining.

CDMP-1 is reportedly localized in undifferentiated mesenchymal cells and chondrocytes in the growth plate of human fetuses [1]. Distribution of CDMP-1 in undifferentiated spindle-shaped cells and chondrocytes in OLF tissues shown in the current study do not conflict with this previous report.

Receptors for CDMP-1, such as bone morphogenetic receptors types IB and II [12], are reportedly activated and localized in spindle-shaped cells and chondrocytes in OLF tissues. In view of this previous study, the current findings indicated the involvement of local signaling of CDMP-1 in cells in OLF tissues [3]. The reported role of

◀ **Fig. 2a–h** Immunohistochemistry (IH) and in situ hybridization (ISH) of tissue at the site of ossification of ligamentum flavum (OLF) from patient 1. **a** In the non-ossified (NO) area, no cells are positive for anti-cartilage-derived morphogenetic protein (CDMP)-1 antibody by IH. **b** In the NO area, no cells are positive for antisense cRNA probe for CDMP-1 by ISH. **c** In the area distant from an ossification front (D), spindle-shaped cells (arrows) are positive for anti-CDMP-1 antibody by IH. **d** In the D area, spindle-shaped cells (arrows) are positive for antisense cRNA probe for CDMP-1 by ISH. **e** In the intermediate portion close to the ossification front (IM), chondrocytes (arrow) are positive for anti-CDMP-1 antibody by IH. **f** In the IM area, chondrocytes (arrow) are positive for antisense cRNA probe for CDMP-1 by ISH. **g** In an ossification front (OS) area, chondrocytes (arrow) are positive for anti-CDMP-1 antibody by IH. **h** In an OS area, chondrocytes (arrow) are positive for antisense cRNA probe for CDMP-1 by ISH. Original magnification, $\times 400$

Table 2 Protein and mRNA localization of cartilage-derived morphogenetic protein (CDMP)-1 in tissue at the site of ossification of the ligamentum flavum (OLF)

Site and cell	CDMP-1 localization	
	Protein	mRNA
Cells in NO	–	–
Spindle-shaped cells in D	++	++
Chondrocytes in IM	++	++
Chondrocytes in OS	+	+

NO, non-ossified site; D, area distant from ossification front; IM, intermediate portion close to ossification front; OS, ossification front; –, 0% of cells stained; +, 0–25% cells stained; ++, 25–50% cells stained.

CDMP-1 is to induce and promote chondrocyte proliferation and differentiation [6]. In addition, we previously reported that transgenic mice with overexpression of

CDMP-1 apparently exhibited expansion of the cartilage template, which led to the increase of the mass of the skeleton [16]. Given the findings of this previous study, local activation of the CDMP-1 in spindle-shaped cells and chondrocytes at the site of OLF may cause the the OLF lesion to expand, leading to the narrowing of the spinal canal. If CDMP-1 is one of the molecules which regulates the chondrogenic process of OLF, targeting the local regulation of CDMP-1 may control the progress of OLF and the associated clinical severity of this disease.

Other growth factors, such as TGF- β [4], bone morphogenetic protein (BMP) [3, 4], and insulin-like growth factor (IGF)-1 [2], are reportedly involved and localized in the process of ossification of spinal ligaments. These molecules may play synergistic and/or different roles in the process of OLF, and further studies are needed to elucidate the precise actions of CDMP-1 and its relationship to other local growth factors in the local regulation of the pathogenesis of ossification of spinal ligaments.

Conclusion

Immunohistochemical and in situ hybridization analyses of CDMP-1 in surgical specimens of OLF revealed that CDMP-1 was activated and localized in spindle-shaped cells and chondrocytes in OLF tissues. CDMP-1 reportedly regulates and promotes expansion of cartilage formation; the current findings suggest that CDMP-1 may be involved in the progression of OLF, which causes severe clinical deterioration.

Acknowledgement We would like to thank Kaori Izumi and Kanae Asai for their technical assistance in preparing tissue sections. Our thanks also go to Technical Scientific and Corporate Communications for helping us with the English.

References

- Chang SC, Hoang B, Thomas JT et al (1994) Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development. *J Biol Chem* 269:28227–28234
- Goto K, Yamazaki M, Tagawa M et al (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
- Hayashi K, Ishidou Y, Yonemori K et al (1997) Expression and localization of bone morphogenetic proteins (BMPs) and BMP receptors in ossification of the ligamentum flavum. *Bone* 21:23–30
- Kawaguchi H, Kurokawa T, Hoshino Y et al (1992) Immunohistochemical demonstration of bone morphogenetic protein-2 and transforming growth factor-beta in the ossification of the posterior longitudinal ligament of the cervical spine. *Spine* 17:S33–36
- Kruse JJ, Awasthi D, Harris M, Waguespack A (2000) Ossification of the ligamentum flavum as a cause of myelopathy in North America: report of three cases. *J Spinal Disord* 13:22–25
- Luyten FP (1997) Cartilage-derived morphogenetic protein-1. *Int J Biochem Cell Biol* 29:1241–1244
- Maiuri F, Iaconetta G, Gambardella A, Buonamassa S (2000) Cervical spine stenosis due to ossification of the posterior longitudinal ligament in Italian patients: surgical treatment and outcome. *Arch Orthop Trauma Surg* 120:441–444
- Miyasaka K, Kaneda K, Sato S et al (1983) Myelopathy due to ossification or calcification of the ligamentum flavum: radiologic and histologic evaluations. *Am J Neuroradiol* 4:629–632
- Nakase T, Ariga K, Miyamoto S et al (2001) Distribution of genes for bone morphogenetic protein-4, -6, growth differentiation factor-5, and bone morphogenetic protein receptors in the process of experimental spondylosis in mice. *J Neurosurg* 94 [suppl 1]:68–75
- Nakase T, Kaneko M, Tomita T et al (2000) Immunohistochemical detection of cathepsin D, K, and L in the process of endochondral ossification in the human. *Histochem Cell Biol* 114:21–27
- Nakase T, Nomura S, Yoshikawa H et al (1994) Transient and localized expression of bone morphogenetic protein 4 messenger RNA during fracture healing. *J Bone Miner Res* 9:651–659

12. Nishitoh H, Ichijo H, Kimura M et al (1996) Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5. *J Biol Chem* 271:21345–21352
13. Olsen BR (1999) Bone morphogenesis and embryonic development. In: Favus MJ (ed) *Primer on the metabolic bone diseases and disorders of mineral metabolism*, 4th edn. Lippincott Williams & Wilkins, Philadelphia, pp 11–14
14. Ono M, Russell WJ, Kudo S et al (1982) Ossification of the thoracic posterior longitudinal ligament in a fixed population. Radiological and neurological manifestations. *Radiology* 143: 469–474
15. Sakou T, Matsunaga S, Koga H (2000) Recent progress in the study of pathogenesis of ossification of the posterior longitudinal ligament. *J Orthop Sci* 5: 310–315
16. Tsumaki N, Tanaka K, Arikawa-Hirasawa E et al (1999) Role of CDMP-1 in skeletal morphogenesis: promotion of mesenchymal cell recruitment and chondrocyte differentiation. *J Cell Biol* 144:161–173
17. Yanagi T (1997) Clinical manifestation of thoracic ossification of the posterior longitudinal ligament and ossification of the yellow ligament. In: Yonenobu K, Sakou T, Ono K (eds) *OPLL: ossification of the posterior longitudinal ligament*. Springer, Berlin Heidelberg New York, pp 95–98
18. Yonenobu K, Ebara S, Fujiwara K et al (1987) Thoracic myelopathy secondary to ossification of the spinal ligament. *J Neurosurg* 66:511–518