Pathogenesis of calcium crystal deposition in the ligamentum flavum correlates with lumbar spinal canal stenosis

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Abstract

Objective
To investigate the histological and immunohistochemical properties of degenerative changes and calcium crystal deposition in the lumbar ligamentum flavum.

Methods
We examined the ligamentum flavum harvested from 119 surgical cases with symptomatic lumbar spinal stenosis. Sections of the ligament were examined by scanning electron microscopy (SEM), energy dispersive X-ray micro-analysis, and were immunostained for S-100 protein, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and CD34. The results were compared with those of ligament tissue harvested from 10 cases of lumbar disc herniation.

Result
The elastic fibres of the ligamentum flavum showed regular, or sometimes irregular, and fragmented fibre bundles. Large areas of fibrosis with reduced elastic component and increased collagenous tissue were frequently seen in the degenerated ligaments. Calcium crystal deposits were observed in these fibrous ligaments, associated with many hypertrophic chondrocytes, and with small blood vessel formation. These chondrocytes stained positively for S-100 protein, VEGF and bFGF. Calcium pyrophosphate dihydrate crystals were identified in the calcium deposit area.

Conclusion
We believe that rupture of elastic fibre bundles is the first change to occur in degeneration of the ligamentum flavum. Calcium crystal deposition was seen within these fibrous and chondrometaplastic areas. Hypertrophic chondrocytes regulate crystal formation and tissue reconstruction by secreting cytokines.

Key words
Ligamentum flavum, elastic fibre, calcium crystal deposition.
Calcification of ligamentum flavum / T. Yayama et al.

Introduction
McCarty and colleagues (1, 2) were the first group to report in 1962 arthritis induced by calcium pyrophosphate dihydrate (CPPD) crystals and termed it “CPPD crystal deposition disease.” This pathological condition is currently known as “articular chondrocalcinosis” or the “pseudogout,” and acute or chronic inflammation caused by CPPD crystal deposition can occur in knee, hip, shoulder, spine, and other large joints (3,4). As for the pathomechanism of this disease, Schumacher et al. (5), Gester et al. (6) and Lagier et al. (7) reported that debris derived from injured cytoplasm was observed in areas of CPPD crystal deposition, and that proliferation of degenerated collagen fibres was observed in the surrounding matrix. Reginato et al. (8) proposed that proteoglycans mediate CPPD crystal formation. By using a scanning electron microscope (SEM), Ishikawa et al. (9) speculated that hypertrophic chondrocytes, containing amorphous materials and proteoglycan, play an important role in crystal formation, and always appear around crystal deposits. According to recent reports, extracellular inorganic pyrophosphate released from hypertrophic chondrocytes is overproduced due to a metabolic disorder, which is assumed to be associated with CPPD crystal formation (10-14).

CPPD crystal deposition has been observed in normal asymptomatic elderly individuals (10,15). CPPD crystal deposition is observed in the supraspinous ligaments and intervertebral discs in the spine, and is especially abundant in the ligamentum flavum (16), and may cause nerve compression. Since Elsberg (17) first reported hypertrophic changes of the ligamentum flavum in 1913, it has been reported in the process of ligamentum flavum degeneration. Yoshida et al. (18) reported, in their histological study of the ligamentum flavum, that elastic fibres displayed an irregular arrangement associated with the degeneration process. Kashiwagi et al. (19) quantitatively measured the decrease of elastic fibres and the increase of collagen fibres in degenerated ligaments. Quantitative determinations have also been made for expanding chondrometaplastic areas, the regularity of elastic fibres, and the intraligamentous content associated with degeneration of ligaments (20, 21).

Although a variety of studies of degeneration of the ligamentum flavum or CPPD crystal formation have been published, the histology of crystal deposition in the ligamentum flavum has not been elucidated. It has been thought that CPPD crystal deposition is accompanied by degeneration of ligaments, and that metabolic disorders or rheumatoid factor were involved in this process (22-24). We have already reported that in CPPD crystal deposition in the lumbar ligamentum flavum, proteases such as elastase and chymotrypsin are involved in the degeneration of elastic fibres, and that crystal formation is regulated by sex hormones and TGF in chondrocytes (25-27). The purpose of the present study was to investigate, histologically and immunohistochemically, the role of chondrocytes in the crystal formation process in the ligamentum flavum by immunohistochemistry.

Materials and methods

Patients
119 patients (43 men, 76 women; average age 69.7 years, range 43-85 years) underwent decompressive surgery of degenerative spinal stenosis. All patients presented with lumbar radiculopathy or cauda equina syndrome, but no patient had clinical symptoms of the “marked pain attack” which is infrequently associated with so-called chondrocalcinosis. The indication for surgery was based on clinical, neurophysiological and radiological findings. Radiological examination showed that all patients had central or lateral type lumbar spine stenosis, which was not associated with inflammatory disease of the spine (spondyloarthropathy), pyogenic spondylitis, or a collagen disease. The diagnosis of lumbar canal stenosis was degenerative in 93 cases (148 interlaminar spaces) and was associated with degenerative spondylolisthesis in 26 cases (32 interlaminar spaces). Ligamentum flavum tissue was harvested at L1-L2 in 3 patients, at L2-L3 in 11 patients, at L3-L4 in 44 patients, at L4-L5 in 106 patients, and at
L5-S1 in 16 patients (Table I). Control samples were ligamentum flavum tissues of 10 patients (5 men, 5 women; average age 25.9 years, range 15-35 years) who underwent decompressive surgery for lumbar disc herniation. The study protocol was approved by the Human Ethics Review Committee of our University and a signed consent form was obtained from each subject.

### Histopathological analyses
The harvested ligaments were fixed with 10% buffered formaldehyde at 4°C for 48 hours and were further embedded with paraffin. Serial 4-µm thick sections were examined after being deparaffinized with xylene and replaced by ethanol, followed by staining with haematoxylin and eosin or elastica van Gieson staining using standards methods. All sections were examined under a light microscope.

### Immunohistochemical analyses
For immunohistochemical examination, serial 4-µm thick sections were prepared from paraffin-embedded specimens, deparaffinized with xylene and replaced by ethanol. After washing with water, the intrinsic peroxidase was blocked with 0.3% H2O2 solution dissolved in absolute methanol at 20°C for 10 minutes, and washed again with phosphate-buffered saline (PBS, pH 7.4). The sections were irradiated three times in a polypropylene slide-holder with a cap filled with PBS (pH 7.4), for periods of 5 minutes (total 15 minutes), using a microwave oven (200 w; ER-245; Toshiba, Tokyo) for antigen retrieval. The sections were then coated with 2% skim milk (Yukijirushi, Sapporo, Japan) dissolved in PBS (pH 7.4) at 37°C for 30 minutes to block background adsorption of antiseraum. Then they were reacted with a blocking solution (PBS containing carrier protein and 15 mM sodium azide; LSAB kit; Dako, Glostrup, Denmark) at 20°C for 10 minutes. This was followed by reaction at 4°C overnight with the following primary antibodies: polyclonal anti-S-100 protein (Dako Corporation, Carpinteria, CA; 10 µg dissolved in 2.5 ml PBS at pH 7.4), monoclonal anti-vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology, Santa Cruz, CA; 200 µg dissolved in 1 ml of PBS, containing 0.1% sodium azide and 0.2% gelatin), monoclonal anti-basic fibroblast growth factor (bFGF) (Wako Pure Chemicals, Osaka, Japan; 200 µg dissolved in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] solution, containing 0.05% sodium azide and 4% gelatine), and monoclonal endothelial cell marker (CD34) (Novocastra Laboratories, Newcastle Upon Tyne, UK; dissolved in lyophilised tissue culture supernatant containing 15 mM sodium azide). The sections were further reacted with LINK (biotinylated anti-mouse and anti-rabbit immunoglobulins in PBS, containing carrier protein and 15 mM sodium azide, LSAB kit, Dako) at 20°C for 60 minutes and rinsed with PBS at pH 7.4, and allowed to react with streptavidin solution (streptavidin conjugated to horseradish peroxidase in Tris-HCl buffer, LSAB kit, Dako) at 20°C for 30 minutes, and rinsed with PBS at pH 7.4.

In order to visualise the peroxidase co-our reaction, the sections were incubated with 3, 3-diaminobenzidine tetrahydrochloride (DAB) solution (Dojin, Kumamoto, Japan; 50 mg dissolved in 100 ml of 0.05 M Tris-HCl buffer at pH 7.4) at 20°C for 10 minutes, and washed in water. Nuclear counterstaining was carried out with haematoxylin. Specimens stained by DAB were mounted with Biolite (Oken, Tokyo) and examined under a light microscope.

### Scanning electron microscopy and energy dispersive X-ray microanalysis
Sections obtained from each specimen were examined by scanning electron microscopy (SEM) and energy dispersive X-ray microanalysis. Sections were fixed with 2.5% glutaraldehyde (Wako) water solution at 4°C for 2 hours, and then immersed in 1% osmium tetroxide (Merck, Darmstadt, Germany) at 4°C for 2 hours. After dehydration with serial concentrations of ethanol followed by absolute isoamyl acetate solution (Nakalai, Kyoto, Japan), the specimen was allowed to dry in a critical point dryer (HCP-2; Hitachi, Tokyo), and then coated with gold-palladium (Eiko Engineering, Ibaraki, Japan) by evaporation using an ion coater (IB-3; Eiko engineering). In the final stage, SEM (S-450; Hitachi) and an energy dispersive X-ray microanalyzer (EMAX-2000; Horiba, Kyoto, Japan) were used to identify calcium crystals.

### Results
**Ligamentum flavum degeneration**
The most superficial layer of the control ligamentum flavum tissue obtained from lumbar disc herniation had a uniform appearance, except for the area in close proximity to the bony attachment. The elastic fibres were in a regular arrangement, oriented parallel to the major axis of the ligament, and branched with other fibres. The collagen fibres were mostly oriented parallel to the elastic fibres. There were few chondrocytic or expanding chondrometaplastic areas (Fig. 1a, d).

In the lumbar spinal canal stenosis cases, elastic fibres were in an irregular arrangement and the fibres were of smaller diameter than in the lumbar disc herniation cases (Fig. 1b, e). In other areas, elastic fibres with abnormally small diameters were fragmented or separated by thick bundles of collagen fibres. Large fibrotic areas with a de-

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**Table I. Level of spinal surgery and harvested ligamentum flavum.**

<table>
<thead>
<tr>
<th>Level</th>
<th>Spondylosis</th>
<th>Spondylolisthesis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1-2</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>L2-3</td>
<td>11</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>L3-4</td>
<td>37</td>
<td>7</td>
<td>44</td>
</tr>
<tr>
<td>L4-5</td>
<td>83</td>
<td>23</td>
<td>106</td>
</tr>
<tr>
<td>L5-S1</td>
<td>14</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>148</td>
<td>32</td>
<td>180</td>
</tr>
</tbody>
</table>

Spondylosis: degenerative spondylosis. Spondylolisthesis: degenerative spondylolisthesis.
increased elastic component and increased collagenous tissue were frequently observed. In other areas, few, if any, elastic fibres were visible (Fig. 1c, f). There were hypertrophic chondrocytes in these areas.

A total of 148 ligamentum flavum specimens were studied from degenerative spondylosis cases; in 7 of these, the elastic fibres showed a regular arrangement, 63 showed an irregular arrangement and 78 showed fragmentation of elastic fibres. Thirty of 32 ligamentum flavum specimens in degenerative spondylolisthesis showed fragmented elastic fibres (Table II). Calcium deposits were seen in 56 of 148 (38%) degenerative spondylosis cases, and in 17 of 32 (53%) degenerative spondylolisthesis cases. All ligaments with calcium deposits showed fragmented elastic fibres and expanded fibrous areas (Table II).

**CPPD crystal deposition**

Macroscopically, calcium crystal deposition in the ligamentum flavum was observed as a granulated white chalky mass (Fig. 2). These crystal deposits were oval shaped, in various sizes, and found in the interlaminar and capsular portions of the ligamentum flavum. The area of calcium deposition was frequently seen on the dorsal side of the ligament, and gradually invaded towards the dural side of the ligament. The structures of the deep layers of the ligament were comparatively unchanged. The deposited calcium crystals were morphologically various, appearing as pin-like, rod-like, or rectangular crystals when observed under SEM (Fig. 3a). The diameter of these crystals was approximately 4 µm or more. The ratio

<table>
<thead>
<tr>
<th>Elastic fibre</th>
<th>LDH</th>
<th>Spondylosis</th>
<th>Spondylolisthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular</td>
<td>10 (0)</td>
<td>7 (0)</td>
<td>-</td>
</tr>
<tr>
<td>Irregular</td>
<td>-</td>
<td>63 (0)</td>
<td>2 (0)</td>
</tr>
<tr>
<td>Fragmentation</td>
<td>-</td>
<td>78 (56)</td>
<td>30 (17)</td>
</tr>
<tr>
<td>Total</td>
<td>10 (0)</td>
<td>148 (56)</td>
<td>32 (17)</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent number of cases with calcium deposits.

Regular: elastic fibres showed regular arrangement; irregular: elastic fibres showed irregular arrangement and ruptured; fragmentation: ruptured elastic fibres with fragmentation and assimilation; LDH: lumbar disc herniation.

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**Fig. 1.** Photomicrographs of the ligamentum flavum. Bundles of elastic fibres showed a regular arrangement (a: HE, x 10, d: elastica van Gieson, x 10). Fibre bundles were irregular in arrangement (b: HE, x 10, e: elastica van Gieson, x 10). Elastic fibre bundles showed fragmentation and the collagenous area was expanded (c: HE, x 10, f: elastica van Gieson, x 10).
of calcium to phosphate in these crystals, as measured on X-ray microanalysis, was approximately 1:1, indicating that the crystals consisted of CPPD (Fig. 3b).

Histologically, in the ligamentum flavum around the nodules, a number of hypertrophic chondrocytes and neutrophils were found, together with many small blood vessels (Fig. 4a). These chondrocytes often showed cluster formation and exhibited hypertrophic changes. The elastic fibres appeared as ruptured and fragmented, and abundant fibrocartilaginous tissue was present around the areas of crystal formation (Fig. 4b). In the tissue of the calcium deposits, extracellular matrix away from calcium deposit formations showed various degenerative changes. The elastic fibres were irregular, but not fragmented, and few hypertrophic chondrocytes were found.

**Immunohistological studies**

In cases of lumbar spinal canal stenosis, small blood vessel formations were seen on the dorsal side of the ligamentum flavum following the rupture of elastic fibres (Fig. 5c). These blood vessel formations, which immunostained positively for CD34, tended to occur in ruptured or fragmented elastic fibres and in expanded fibrous areas. Intense immunostaining with VEGF or bFGF was noted in the hypertrophic chondrocytes in degenerated ligamentum flavum tissue. A large number of VEGF-positive or bFGF-positive hypertrophic chondrocytes was seen around the calcium crystal deposits (Fig. 5a, b). In the ligamentous enthesis, chondrocytes showed no hypertrophic changes and had no immunoreactivity for VEGF or bFGF. In the lumbar disc herniation cases, there were no small blood vessel formations, and staining for VEGF, bFGF, or CD34 was negative. Immunostaining for S-100 protein was also positive in hypertrophic chondrocytes in the degenerated ligaments. It was strongly positive in the cytoplasm of these chondrocytes. The S-100-positive cells were predominantly abundant in the extracellular matrix around the calcium deposits (Fig. 5d).

**Discussion**

The ligamentum flavum is normally a two-layer structure, divided into a superficial layer and a deep layer when viewed from the dorsal side of the ligament (28). The ligamentum flavum is regularly arranged, except for the ligamentum enthesis, and bundles of elastic fibres run parallel to each other, with collagen fibres and fibroblast-like cells among them. The deep layer of the ligamentum flavum has a laminar structure as if the layer lined the dura mater. In ligamentum flavum tissue, elastic fibres account for 60-70% of the dry weight, and it is speculated that little metabolic activity is required (29).

Our present study suggests that the first change in the degeneration and hypertrophy of the ligamentum flavum is rupture of these elastic fibres. In general, the cross-sectional area and viscoelasticity of elastic fibres vary according to age-related regressive change and systemic hormone balance (30, 31). It is thought that the resistance of elastic fibres to stress, and the elasticity of fibre itself, are decreased and that the fibres are ruptured by chronic microstress due to spinal movement. This explains why there are so many spinal degenerative diseases seen among the elderly and in women.

We reported in our previous studies that elastic fibres ruptured by microtrauma are predisposed to degradation by proteases such as elastase and chymotrypsin (26). In areas with decreased numbers of elastic fibres, collagen fibres increase, as if in compensation, to
induce fibrosis of the matrix. We suspected that such fibrosis is induced by bFGF. This cytokine, secreted by hypertrophic chondrocytes, prompts collagen fibre production from fibroblasts (32, 33). It has been speculated that the increase of collagen fibres occurs focally at first, and that the areas progressively expand by merging with one another.

Chondrocytes in the normal enthesis include cells differentiated from fibrocartilage cells. These chondrocytes are known to be highly active biologically, mesenchymal cells maintaining their activity (34). In the control group, no chondrocytes were seen in the ligament except at the ligamentous enthesis.

However, in the stenosis group, abnormal hypertrophic chondrocytes were seen in areas other than the enthesis, accompanied by chondroid metaplastic background matrix. There is a theory that chondrocytes in the enthesis change into these abnormal chondrocytes, whereas Yahia et al. (35) reported that the metaplastic chondrocytes are derived from fibroblasts in normal ligaments. In our study, chondrocytes appeared only in areas where elastic fibres had ruptured and collagen fibres had increased, and there was no continuity between chondrocytes in ligaments and those in the enthesis, which leads us to believe that these chondrocytes were induced from fibroblasts. We assume that TGF-β is involved in this induction of chondrocytes (27), because TGF-β was a major cytokine inducing chondrocytes to the matrix.

The spinal ligamentum flavum is thought to have little blood flow, and little vasculature was seen in the control ligamentum flavum. However, formation of small blood vessels was seen in cases with rupture of elastic fibres and increase of collagen fibres. This angiogenesis initiated from the dorsal side of the ligament and gradually extended to the dura mater side, which suggested that those blood vessels were induced from the venous plexus in the dorsal side of the lumbar ligamentum flavum. It was speculated that the induction was due to cytokines such as VEGF and bFGF, which are powerful blood vessel inducers (32, 36, 37), originating from the chondrocytes appearing in the degenerated ligaments. This angiogenesis may further accelerate ligamentous fibrosis and expansion of the chondroid metaplastic area.

Crystal deposition in the ligamentum flavum occurs in ligaments where advanced fibrosis and chondrometaplasia exist, and the crystals are surrounded by chondrocytes, which are thought to be important for crystal formation and deposition. We have reported the expression of proteases such as neutrophil elastase and chymotrypsin, or crystal formation via TGF-β, as being roles of chondrocytes (26). The results of our present study indicate that the initial change that occurs in crystal de-

Fig. 4. (a) Note the presence of chondrocytes around calcium deposits with small blood vessel formations (HE, x 10), in particular, (b) fragmentation of elastic fibre bundles around calcium deposits (elastica van Gieson, x 10, Ca: calcium).

Fig. 5. Photomicrographs of immunohistochemical staining. (a) Note the hypertrophic chondrocytes of VEGF staining around the calcium deposits (x 20, Ca: calcium). (b) A number of cells positively stained for bFGF are present within the calcified ligament (b: x 20). (c) Small blood vessel formations, staining positive for CD 34, were seen beside the calcium deposition (x 20). (d) Immunostaining for S-100 protein; a number of chondrocytes were present (d: x 20).
position in the ligamentum flavum is rupture of elastic fibres in the ligamentum flavum. Collagen fibres then increase as if replenishing the sites where the elastic fibres ruptured, and chondrocytes are induced from fibroblasts. Chondrocytes induce growth of blood vessels by VEGF and bFGF, further fibrosis of the matrix by bFGF, and the expansion of chondrometaplastic areas. This matrix becomes the site of crystal deposition. The cytoplasm of hypertrophic chondrocytes was S-100 protein-positive. S-100 protein has been reported to increase PPi concentration, leading to phosphorylation of chondrocytes, and to further investigations are required. S-100 protein is one factor promoting crystal formation mediated by secretions of TGF-β and subsequent calcium influx accompanied by angiogenesis and phosphate influx with increased PI concentration, leading to mineralization of the matrix.

We speculate that hypertrophic chondrocytes play an important role in CPPD crystal formation. With respect to other factors involved in crystal deposition in ligaments, genetic factors, metabolic disorders, and systemic chondrocalcinosis are likely contributors (40, 41). We currently have limited knowledge of factors that significantly affect the function of chondrocytes, and further investigations are required.

Acknowledgements

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References