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Microfilamentous type VI collagen in the hyalinized stroma of the hypertrophied ligamentum flavum

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Summary. Thickened ligamenta flava obtained from 14 patients with spinal canal stenosis were examined with special reference to type VI collagen. The characteristic histological finding in the thickened area was rupture of normal elastic fibre meshwork with resultant fibrosis which usually appeared hyaline. Using an immunohistological method, collagen types VI, I and III were found to be present in the hyaline matrix. Ultrastructural study revealed many microfilamentous structures of type VI collagen admixed in loosely packed, banded collagen fibres. With differential salt precipitation of pepsin-extracted collagen the existence of type VI collagen was confirmed by SDS-polyacrylamide gel electrophoresis analysis and Western blotting analysis using anti-type VI collagen antibody. Quantification of type VI collagen in pepsin-extracted crude collagen samples by an inhibition enzyme-linked immunosorbent assay showed an increasing amount of type VI collagen in the thickened ligamenta flava compared to the normal ligaments. Thus, increase of type VI collagen is the main contribution to the thickening of the ligamentum flavum. This may represent an adaptational and reparative process associated with disruption of elastic fibres.

Key words: Ligamentum flavum – Type VI collagen – Hyalinization – Spinal canal stenosis

Introduction

The thickening of the ligamentum flavum which was documented in relation to low back pain and intermittent claudication (Naffziger et al. 1938; Mensor and Fender 1941) is known to be one of the causative lesions of spinal canal stenosis. With the progression of such a lesion there is additional calcification (Drouillard and Mrstik 1988) or ossification (Kurihara et al. 1988) which aggravates the lumbar spinal stenosis.

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The histopathology of the hypertrophied ligamentum flavum is characterized by substitution fibrosis secondary to the rupture of elastic fibres with resultant hyalinization (Mensor and Fender 1941). Such a fibrosing lesion is generally composed of type I, III, V and VI collagen molecules. These have been demonstrated, by immunohistological and biochemical methods, in the course of tissue repair or in a variety of pathological conditions and type VI collagen in particular appears in a chronic phase in the course of tissue repair with type I and III collagens (Kawahara et al. 1990). It is suggested that type VI collagen plays an important role in connecting cells and fibrous proteins (Keene et al. 1988; Aumailey et al. 1989) not only in the dense fibrous connective tissue such as a tendon and dermis but also in the elastic tissue of the aortic media and the ligamentum nuchae (Gibson and Clearly 1982). In the present study, we have paid particular attention to an increase of type VI collagen in the diseased ligamentum flavum as demonstrated by morphological and quantitative methods. We discuss the hyalinization of the lesion probably resulting from an accumulation of type VI collagen in the elastic tissue.

Materials and methods

Eleven cases of thickened ligamentum flavum causing spinal canal stenosis were selected from surgical pathology files of Fukui Hospital in 1988. The clinical and gross findings of those cases in terms of the degree of the thickening of the ligamentum flavum, bony hypertrophy and bulging of intervertebral discs thought to be related to spinal canal stenosis are described in Table 1. Sections of formalin-fixed and paraffin-embedded tissue specimens were stained with haematoxylin and eosin (H&E), elastica van Gieson, and alcian blue. Immunolocalization of collagen types I, III, V and VI and fibronectin was detected by an avidin-biotin peroxidase method. Anti-human collagen types I, III, V and fibronectin polyclonal antibodies were raised in rats. Anti-human collagen type VI polyclonal antibodies were raised in rabbits. Type-specific polyclonal anti-collagen antibodies were purified by cross-adsorption of affinity columns consisting of heterogeneous collagen types as previously described (Minamoto et al. 1988; Oda et al. 1988). Anti-

Table 1. Clinical findings of specimens for light microscopic study

Case no.	Age (years)	Sex	Location	Gross findings		
				Lig.[a]	Bone[b]	Disc[c]
1	67	M	L4/5	++	−	−
2	73	M	L4/5	+++	+	−
3	63	M	L5/S1	+	−	++
4	72	F	L4/5	++	+	−
5	59	F	L4/5, L5/S1	+	−	−
6	61	M	L4/5	+	++	−
7	71	M	L4/5, L5/S1	++	++	−
8	71	M	L3/4	+	+	−
9	74	M	L3/4, L4/5	+++	−	−
10	73	M	L4/5, L5/S1	++	+	−
11	54	M	L4/5	++	−	+

[a] Thickening of the ligamentum flavum
[b] Bony hypertrophy
[c] Bulging of the intervertebral disc
−, Negative; +, mild; ++, moderate; +++, severe degree

Table 2. Clinical findings of specimens for ultrastructural and biochemical analysis

Case no.	Age (years)	Sex	Location	Gross findings		
				Lig.[a]	Bone[b]	Disc[c]
12	79	M	L2/3, 3/4	−	−	−
13	60	M	L2/3, 3/4	−	−	−
14	37	M	L4/5	++	−	+
15	55	M	L4/5	+	−	−
16	54	M	L4/5	++	+	−

[a] Thickening of the ligamentum flavum
[b] Bony hypertrophy
[c] Bulging of the intervertebral disc

fibronectin antibody was affinity-purified as previously described (Kawahara et al. 1989). Anti-human type IV collagen monoclonal antibody was purchased from Shiseido (Japan).

For immunostaining paraffin sections were pre-treated by 0.05% protease (type XXIV; Sigma, St. Louis, Mo.) for 30 min at 37° C for collagen types except for type IV, and 0.4% pepsin (Sigma) for 2 h at 37° C for type IV collagen and fibronectin. Primary antibodies were incubated overnight at 4° C. The working dilutions of antibodies to collagen types I, III, IV, V and VI and fibronectin were 40, 40, 20, 1000, 200, and 400, respectively. Normal rabbit, rat or mouse serum diluted at 1:1000 was incubated in spite of primary antibodies on a section as a negative control.

Three fresh cases of the thickened ligamenta flava were obtained from Kanazawa University Hospital. Two autopsy cases used as controls showed no gross abnormalities in the spinal canal (Table 2). Each tissue specimen was sliced into small fragments and submitted for electron microscopic, immunoelectron microscopic and biochemical studies. Tissue for electron microscopy was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. It was post-fixed in 2% osmium tetroxide, dehydrated with graded alcohols and embedded in Epon 812. Ultra-thin sections were stained with uranyl acetate mixed with equal volumes of tannic acid solution for the elastic fibre stain described by Kajikawa et al. (1975) and lead citrate. Tissue for immunoelectron microscopy were used en bloc according to a protocol described by Sakai et al. (1986). In brief, the freshly excised tissue specimens were cut into pieces, washed in phosphate-buffered saline (PBS), pH 7.4 for 2 h and then incubated with antibodies against type VI collagen, diluted 1:10, in PBS overnight. Following an extensive rinse in PBS, the samples were fixed with Karnovsky's fixative for 1 h. They were then reacted with goat anti-rabbit IgG antibody with 10 nm gold conjugate (Janssen, Olen, Belgium) diluted 1:2 in PBS overnight followed by an extensive rinse in PBS. The specimens were rinsed briefly in 0.1 M sodium cacodylate buffer, pH 7.4 and fixed with 2% osmium tetroxide. All the procedures were carried out at 4° C. Epon-embedded materials were prepared and ultra-thin sections were stained with uranyl acetate and lead citrate. Normal rabbit serum diluted 1:100 was incubated instead of anti-type VI collagen antibody as a negative control.

Type VI collagen from the tissue fragments was extracted by limited pepsin digestion and differential salt precipitation as follows (Furuto and Miller 1980). The sliced tissues were homogenized by a Dounce homogenizer in a 0.4 M sodium chloride solution, pH 7.6, containing a cocktail of proteinase inhibitors. After extensive rinses in this buffer, collagens were extracted with limited pepsin digestion, 2.5 mg pepsin/g wet tissue weight in 0.5 M acetic acid, with stirring at 4° C for 24 h, and then centrifuged at 15000 rpm at 4° C for 1 h. The extracted collagens were then precipitated by 1.2 M sodium chloride in 0.5 M acetic acid. The supernatants were subsequently adjusted to a concentration of 2.0 M sodium chloride, and the resulting precipitate containing type VI collagen was redissolved in 0.5 M acetic acid. The proteins in the fractions were denatured and identified by electrophoresis on 10% polyacrylamide slab gels containing 0.1% sodium dodecyl sulphate (SDS-PAGE) followed by Coomassie blue staining. The protein fraction transferred on nitrocellulose membranes from polyacrylamide gels was detected by auro-dye (Janssen), and by Western blotting using anti-type VI collagen antibodies.

Type VI collagen extracted in the supernatants after limited pepsin digestion was quantified by an inhibition enzyme-linked immunosorbent assay (ELISA) described by Rennard et al. (1980). Polyvinyl microtitre plates (200 µl/well; Nunc, Naperville, IL, USA) were coated by adding to the wells 100 µl of 10 µg/ml purified human placental type VI collagen (Oda et al. 1988) that was prepared from acetic acid solutions into 20 mM carbonate buffer, pH 9.6. Plates coated were stored overnight at 4° C in a humidified atmosphere. For pre-treatment of inhibition study, standard type VI collagen and each crude sample diluted serially and adjusted to pH 7.0 were mixed with equal volumes of anti-type VI collagen antibody diluted at 1:100 in plastic tubes, and incubated overnight at 4° C. After allowing the antibody to bind antigen, those samples were transferred to the washed type VI collagen coated wells. After incubation for 2 h at 37° C and washing, alkaline phosphatase-conjugated anti-rabbit IgG antibody (Cappel, Malvern, PA, USA) was added to each well and incubated for 90 min. After washing the plates, enzyme substrate was added and incubated for 30 min. An absorbance at 405 nm was measured. The amount of type VI collagen was calculated by 50% inhibition.

Results

The degree of hypertrophy of the ligamenta flava examined is listed in Tables 1 and 2. Macroscopically it was easy to detect the area of hypertrophy since the colour changed to white from yellow.

Microscopically, the framework of the ligamentum flavum consisted of elastic fibres with interspersing thin collagenous matrix. Elastic fibres comprised a branching network of fibres (Fig. 1a).

By H&E and elastica van Gieson staining, the thickened ligamenta flava were characterized by rupture of elastic fibres and hyalinization. In the injured portion elastic fibres were lost or fragmented and the defect was filled with fibrous tissue (Fig. 2a–c) and rarely with

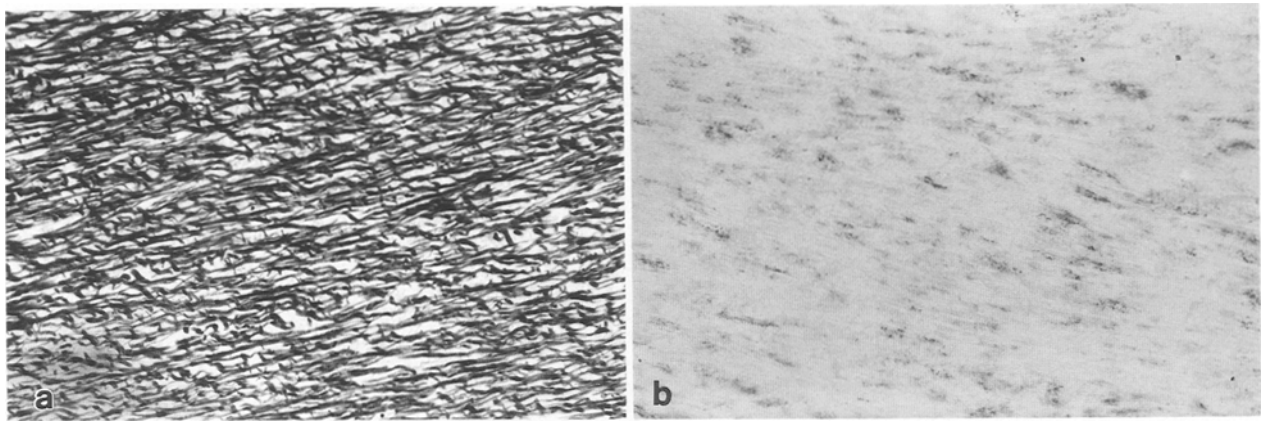

Fig. 1a, b. Ligamentum flavum of uninjured portion. **a** Elastic fibres are a main component which runs parallel each other with interspersing collagenous matrix. Elastica van Gieson, ×50. **b** Part of collagenous matrix was faintly positive for anti-type VI collagen antibody. Immunoperoxidase, ×120

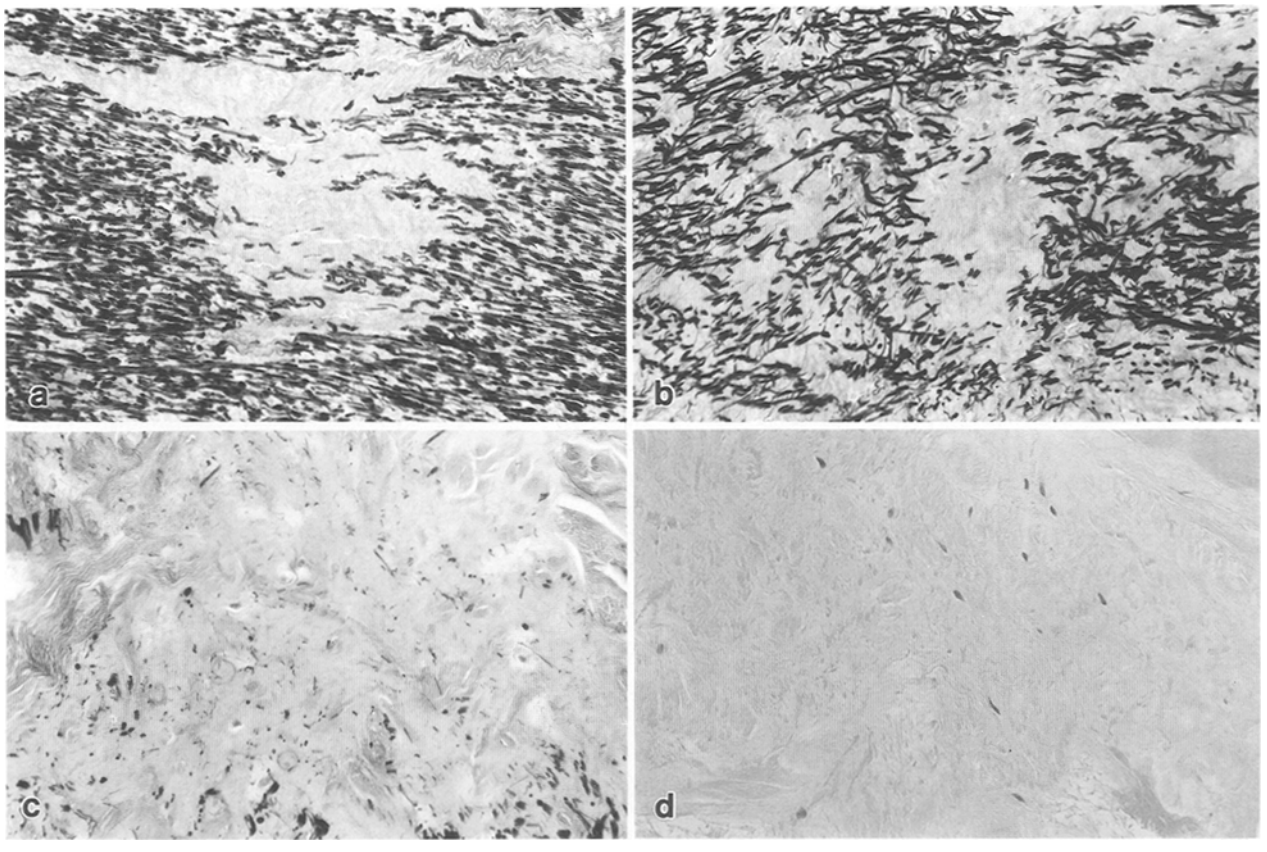

Fig. 2a–d. Thickened ligamenta flava. **a** Small area of ruptured elastic fibres. The lesion is replaced by collagenous fibrous matrix. Elastica van Gieson, ×50. **b** In the margin of substitutional collagenous fibrous matrix, elastic fibres are partly fragmentous. Elastica van Gieson, ×120. **c** A small amount of fragmented and granular elastic fibres scattered in the broad hyaline matrix. Elastica van Gieson, ×120. **d** Hyalinous tissue. Pink and amorphous matrix is admixed with fibrous matrix. H&E, ×120

granulation tissue. In many cases those fibrous tissues appeared hyaline. At the margin of hypertrophy, elastic fibres were swollen and degenerated and the collagenous matrix was increased and hyaline (Fig. 2b). In the severe cases (cases 2 and 9), the hyaline area tended to be wider, and intersecting collagenous fibres became blurred with the ample amorphous eosinophilic material and granular elastic fibres with a few cells (Fig. 2c, d). Alcian blue staining showed only a small amount of proteoglycan. Ossification or calcification in the thickened ligamenta flava was not included in the specimens examined.

Immunohistochemically, in controls, collagen types I and III were present in the collagen among elastic fibres, and type VI collagen was also faintly decorated

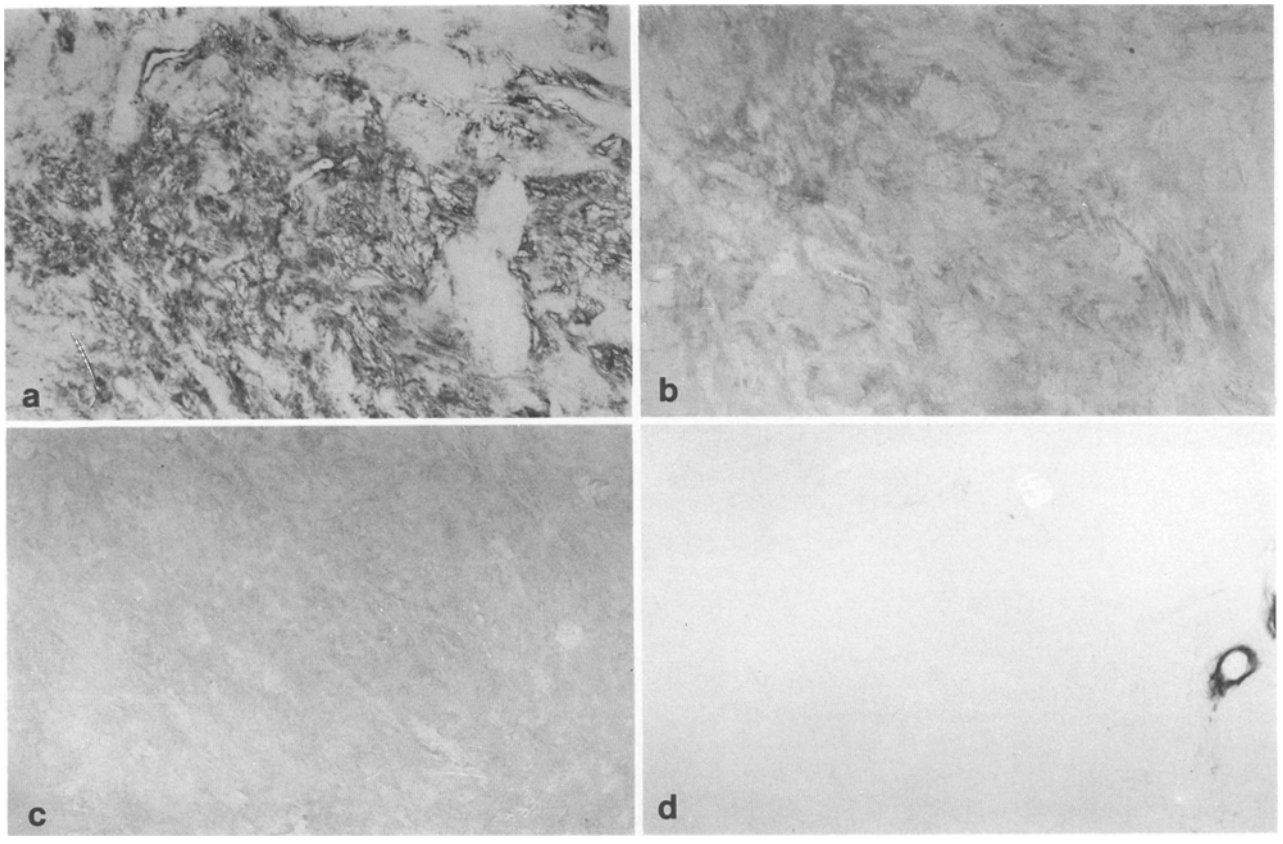

Fig. 3a–d. Immunostaining of the thickened ligamentum flava. **a** Type VI collagen-positive stroma in the hyalinous matrix. Thick collagen fibres are negative for type VI collagen. ×120. **b** Type I collagen is diffusely positive. ×120. **c** Type III collagen is positive faintly and diffusely. **d** Type IV collagen is negative other than vascular basement membranes

(Fig. 1b). In the thickened area type VI collagen was intensely and widely decorated in hyalinized fibrous tissue (Fig. 3a). Collagen types I and III were also positive (Fig. 3b, c). The type V collagen-positive area was scattered in the hyalinized stroma. Under polarized microscopy type VI collagen was positively detected among white birefringent thick collagen fibres. Fibronectin was usually negative. Type IV collagen was negative other than in vascular basement membranes (Fig. 3d). Thus, disruption of elastic fibres and hyalinization seems to be closely associated with intense positivity for type VI collagen in the thickened ligamenta flava.

Ultrastructural study revealed that bundles of elastic fibres in the uninjured portion were regularly arranged, and collagen fibre bundles which were composed of fibres 200–300 nm in diameter ran parallel with elastic fibres and a small amount of collagen fibres arranged perpendicular to elastic fibres (Fig. 4). In the thickened and hyaline region, banded collagen fibres were loosely arranged or focally packed with a small amount of small and granular elastic fibres which is composed of central amorphous elastin aggregates and peripheral elastin microfibrils about 10 nm in diameter. It was noted that among loosely packed thin collagen fibres there were a lot of microfilamentous structures somewhat different from elastin microfibrils (Fig. 5).

In immunoelectron microscopy only microfilamentous structures smaller than 10 nm in diameter were positive for anti-type VI collagen antibodies, and elastic fibre-associated microfibrils were completely negative for the antibody. Type VI collagen-positive microfilaments were localized predominantly among loosely arranged thin collagen fibres (Fig. 6) and around granular elastic fibrils. Among a package of thick collagen fibre bundles there were scanty anti-type VI collagen antibody-positive microfilaments. The microfilaments were scattered singly, or formed a complex microfilamentous network and sometimes dense aggregates of microfilaments. In a control block, microfilaments were not labelled with colloidal gold.

The reduced samples of the precipitated fractions with 2.0 M sodium chloride on pepsin-extracted collagens were identified by SDS-PAGE as shown in Fig. 7 three peptide bands at molecular weights of approximately 50000–70000. These bands completely corresponded to α_1, α_2 and α_3 bands of type VI collagens that we previously extracted from human placenta. Western blotting analysis using anti-type VI collagen antibody which reacts specifically with α_1 and α_2 chains of type VI collagen showed α_1 and α_2 chains of type VI collagen in reduced conditions and only one band at a macromolecular weight in non-reduced conditions (Fig. 7).

The amount of type VI collagen in the pepsin-extracted crude collagen samples was quantified by an inhibition ELISA and is shown in Table 3. There was a signifi-

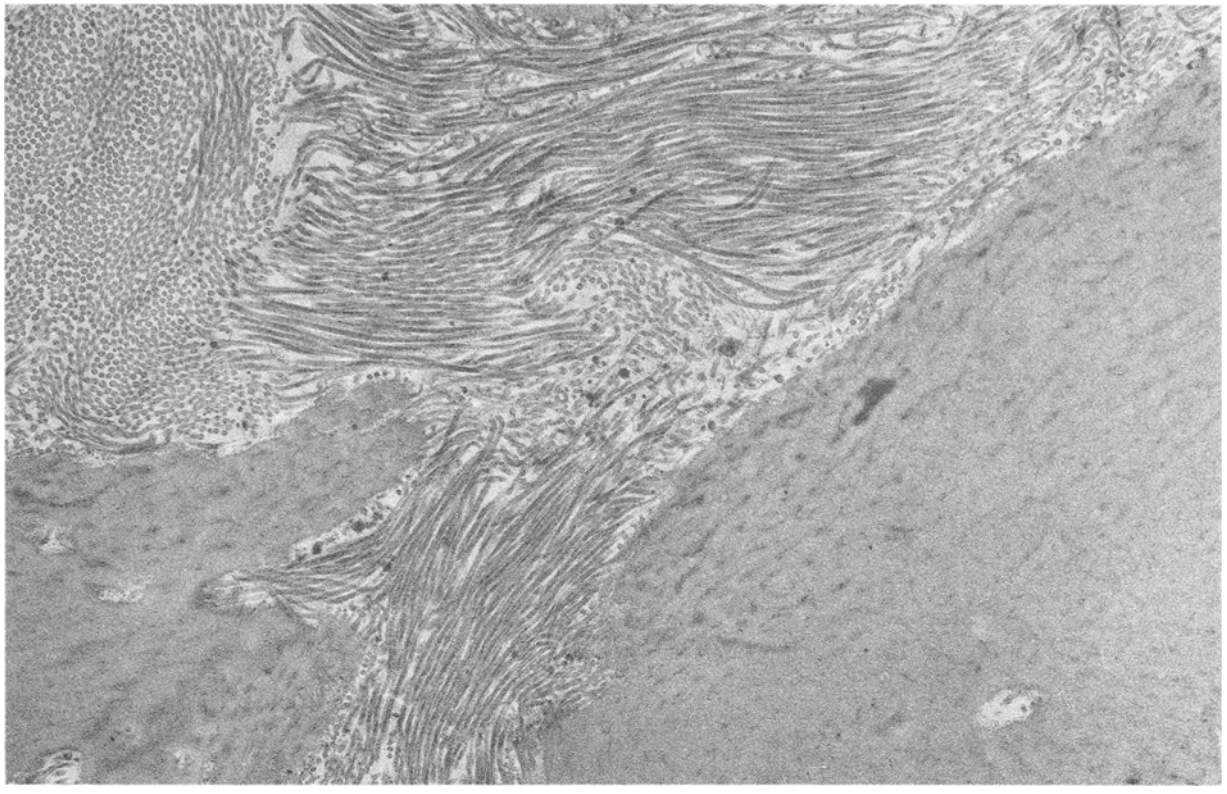

Fig. 4. Electron micrograph of a normal ligamentum flavum. Elastic fibres of amorphous elastin aggregates without elastin microfibrils were thick. Collagen fibres were tightly packed and run parallel each other. Tannic acid, uranyl acetate and lead acetate. ×17000

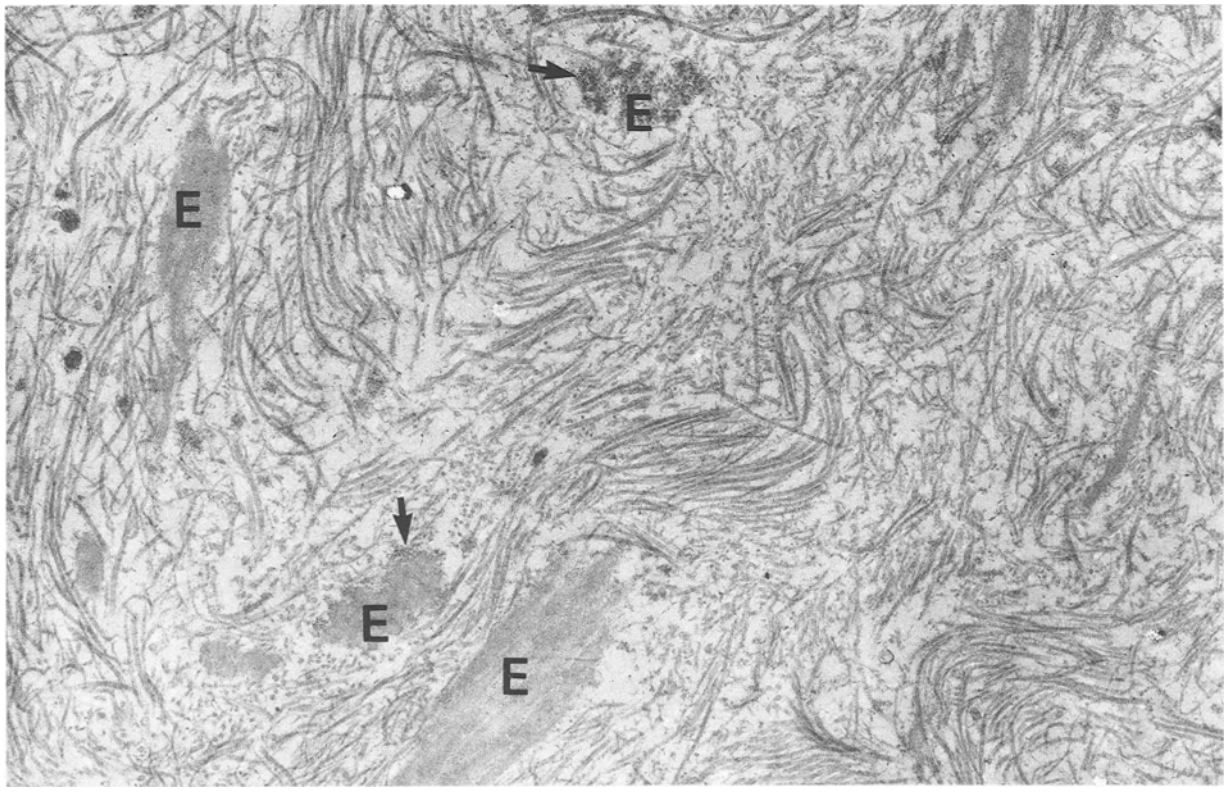

Fig. 5. Electron micrograph of hyalinized matrix. Collagen fibres run in a random fashion and among the loosely packed collagen fibres filamentous components less than 10 nm in diameter were dispersed. Elastic fibres (*E*) were fragmentous with elastin microfibrils (*arrows*). Tannic acid, uranyl acetate and lead acetate. ×17000

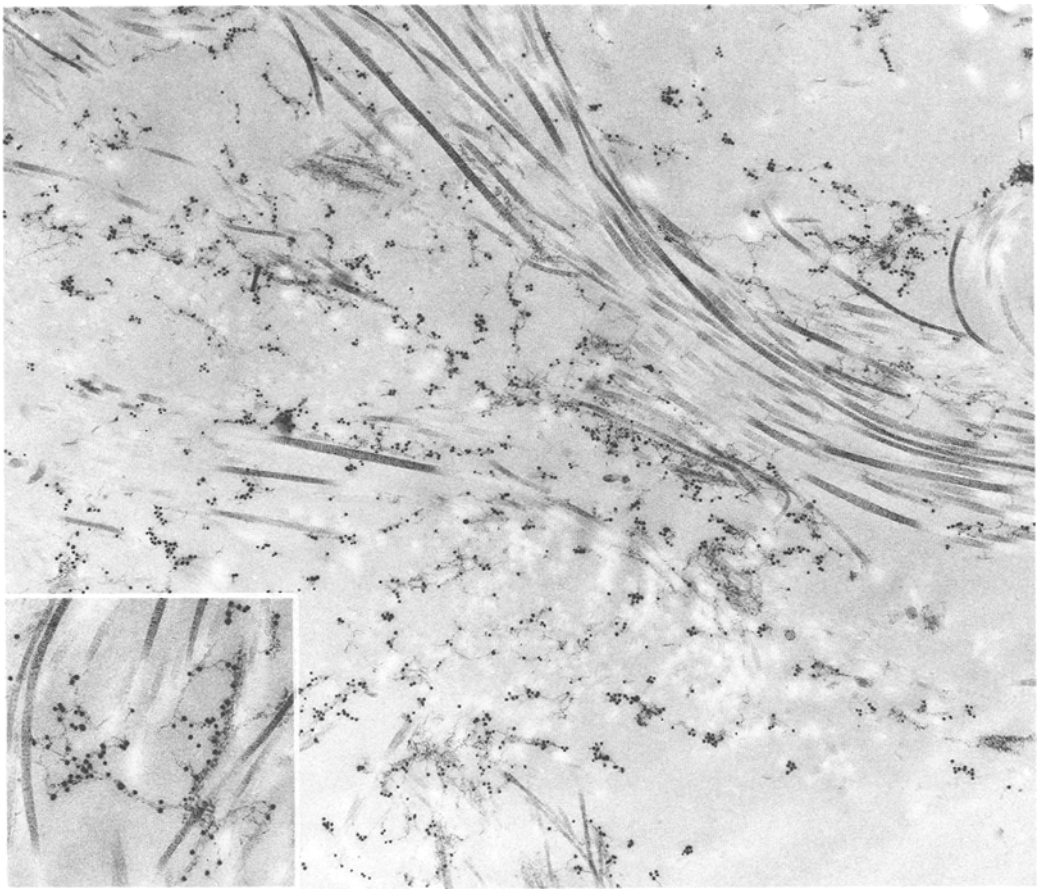

Fig. 6. Distribution of type VI collagen recognized by anti-type VI collagen antibodies by immunogold labelling. Gold particles are seen only on microfilaments thinner than 10 nm in diameter. Microfilaments were abundant among loosely packed collagen fibres and among loosely packed collagen fibre bundles. Microfilaments show filamentous network and filamentous aggregates. ×12000. *Inset:* Higher magnification of microfilamentous network of type VI collagen by immunogold labelling. ×40000

Table 3. Amount of type VI collagen (μg/g wet weight of the ligamentum)

	Control		Thickened group		
Case no.	12	13	14	15	16
	178	325	718	830	1080

cant difference between the non-thickened group and the thickened group by *t*-test ($p = 0.0243$).

Discussion

We have demonstrated that type VI collagen occurring together with types I and III is a major component of cicatricial and hyaline thickening of the ligamenta flava after rupture of the normal elastic fibre-framework. Previous immunoelectron microscopical studies have indicated that type VI collagen is localized along a filamentous network (Gibson and Clearly 1983; von der Mark et al. 1984), which is quite similar to the colloidal gold-labelled filamentous structures seen in the present study. As loosely packed banded-fibres in the thickened ligamentum flavum seem to represent collagen types I, III and V as demonstrated by Birk et al. (1988) it seems probable that microfilamentous type VI collagen increases more than fibrous interstitial collagen in the reparative process in the injured ligamenta flava.

In considering the anatomical situation of the ligamentum flavum, it is evident that its functional repertoire, in association with the intervertebral joint, is to limit excessive flexion of the spine, and to protect the disc from the background shearing stress of one vertebra relative to its caudal neighbour. Thus, the ligamentum flavum should be an elasto-fibrotic tissue in which, in the normal condition, there is a regular arrangement of abundant elastic fibre bundles intersected by thick collagen fibres. The disruption of elastic fibres and resultant replacement by rigid collagenous tissue may lead to complete loss of elasticity in the ligament. Such a reparative process appears to be unfavourable. In reality, a microfilamentous structure mainly consisting of type VI collagen seems to be important in maintaining some elasticity in the scarred ligament. The microfilamentous network of type VI collagen may act substitutively as a scaffold for the elastic tissue when elastic fibres are disrupted and lost. This may be the reason why in elastic

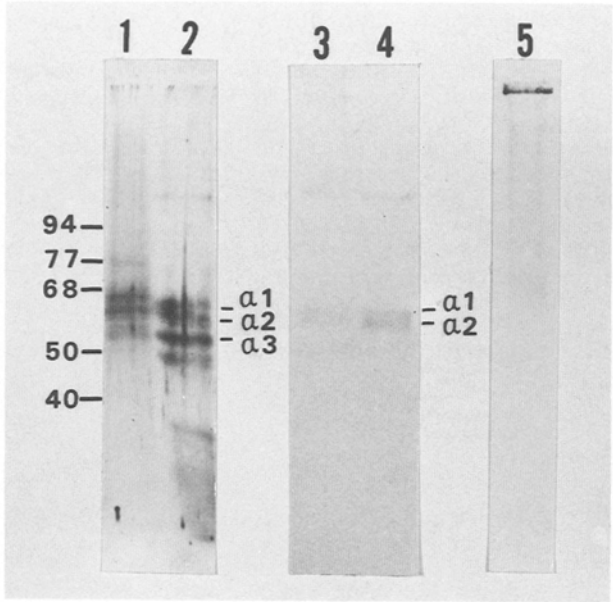

Fig. 7. SDS-polyacrylamide gel electrophoresis (*lanes 1 and 2*) and Western blotting (*lanes 3–5*) under reduced conditions (*lanes 1–4*) and non-reduced conditions (*lane 5*) using anti-type VI collagen antibody. *Lanes 1 and 2* are stained by auro-dye on a nitrocellulose membrane transferred from an SDS polyacrylamide gel. Collagen samples of lanes 1, 3 and 5 are 2.0 M sodium chloride precipitates following fractionation in 1.2 M sodium chloride solution of pepsin-extracted crude collagens. *Lanes 2 and 4* are pepsin-extracted type VI collagen prepared from placenta for comparison

fibre-rich organs type VI collagen is extractable and increases in amount in pathological conditions (Gibson and Clearly 1982).

The term hyalinization is a widely used descriptive histological term and hyaline refers to any alteration in the extracellular space which gives a homogeneous glassy, pink appearance in routine histological sections stained with H&E (Robbins et al. 1984). For example, hyaline of renal glomeruli represents a conglomeration of plasma proteins, basement membrane material and mesangial matrix. Hyaline in arteriosclerosis is composed of precipitated plasma proteins and duplication of the basement membrane (Biava et al. 1964). The plasma proteins which contribute to vascular hyaline are mainly composed of immunoglobulin, complement and fibrin (Krawczynski 1971). Electron microscopically, the deposits of plasma protein in vascular walls and renal glomeruli appear to be in the form of dense granules about 20 nm in diameter (Biava et al. 1964). In hyaline other than vascular hyaline, plasma protein does not represent a major constituent. Electron microscopical studies of this type of hyaline have shown a complex of heterogeneous structures, including a filamentous network in hyalinosis cutis (Rodermund and Klingmüller 1970) and aggregation of collagen fibres in the pleura and splenic capsule (Stolpmann 1967); Rodermund and Klingmüller (1970) reported that the diameters of hyaline filaments in such lesions were 5–10 nm. This is consistent with the type VI collagen microfilamentous structure in this study. Recently, it was reported that hyalinized glomeruli were also positive for type VI collagen (Karkavelas et al. 1988) and type VI collagen was abundant in schwannoma showing a hyalinized appearance (Oda et al. 1988). Thus, an accumulation of microfilamentous type VI collagen may be a major contribution for hyalinosis by conventional histology not only in the fibro-elastic tissue but also in the ubiquitous interstitial tissue. Irregular arrangement of banded collagen fibres composed of collagen types I, III and V would be another constituent of extravascular hyaline as shown in the present thickened ligamenta flava.

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