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Basic Science

Myofibroblasts are increased in the dorsal layer of the hypertrophic ligamentum flavum in lumbar spinal canal stenosis

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Abstract

BACKGROUND CONTEXT: Hypertrophy of the ligamentum flavum (LF) is a major contributor to the development of lumbar spinal canal stenosis (LSS). Although previous studies have identified some factors related to hypertrophy of the LF, the etiology remains unclear. It is well known that myofibroblasts have a key role in the pathology of fibrosis in other tissues, including the skin, liver, kidney, and lung. We hypothesized that myofibroblasts were also important players in the pathology of fibrosis in the LF.

PURPOSE: To elucidate the distribution and role of myofibroblasts in the hypertrophic LF.

STUDY DESIGN: A histological, immunohistochemical, and gene expression analysis of the LF in the human lumbar spine.

PATIENT SAMPLE: Hypertrophic LF tissue samples were collected from patients with LSS.

OUTCOME MEASURES: Histology, immunohistochemistry, microarray, reverse transcription-quantitative polymerase chain reaction, western blotting, and enzyme-linked immunosorbent assay.

METHODS: The degree of fibrosis in the dural and dorsal layers of the LF was evaluated by Masson's trichrome tissue staining. Collagen gene expression was evaluated by quantitative reverse transcription polymerase chain reaction. Immunostaining of αSMA was performed to evaluate localization of myofibroblasts in LF tissue. The association between gene expression of alpha-smooth muscle actin (αSMA) and that of several types of collagen was investigated. The signal activated on the dorsal side of LF was examined by gene set enrichment analysis using microarray data. Expression levels of αSMA and several types of collagen in LF fibroblasts were investigated under hypoxic conditions.

RESULTS: In the histological study using Masson's trichrome staining, the fibrosis score was significantly higher in the dorsal layer than in the dural layer. Gene expression levels for several types of collagen (COL1A1, COL1A2, COL3A1, COL5A1, COL6A1, and COL11A1) and heat shock protein 47 (a collagen-specific chaperone) were significantly higher in the dorsal layer. Furthermore, immunohistochemistry revealed a significantly greater number of αSMA-stained cells in the dorsal layer. There was a strong correlation of αSMA mRNA expression with COL1A-1 in LF

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fibroblasts. Gene set enrichment analysis showed that the set of fibrosis-related gene signals, including those for epithelial-mesenchymal transition, hypoxia, and inflammation, were significantly upregulated in the dorsal layer compared with the dural layer. Under hypoxic stimulation, expression of αSMA and several types of collagen was increased in LF fibroblasts.

CONCLUSIONS: This study is the first to reveal that myofibroblast expression levels are higher in the dorsal layer of the LF than in the dural layer. We confirmed that hypertrophy of the LF in LSS is associated with increased expression of myofibroblasts in the dorsal layer. Hypoxia could be a cause of expression of myofibroblasts leading to fibrosis and finally to hypertrophy of the LF.

CLINICAL SIGNIFICANCE: The results of this study partially elucidate the molecular mechanisms of LF hypertrophy and suggest that myofibroblasts may be involved in age-related degeneration of the LF.

Keywords: Collagen; Fibrosis; Gene analysis; Histology; Ligamentum flavum; Lumbar spinal canal stenosis; Myofibroblasts

Introduction

Lumbar spinal canal stenosis (LSS) is one of the main causes of lower leg pain, sciatica, and intermittent claudication in the elderly, and the main etiology in patients with this spinal disorder is hypertrophy of the ligamentum flavum (LF) [1]. Histopathological investigations have been performed to understand the pathological mechanism of hypertrophy of the LF. In the non-hypertrophic ligament, 60% to 70% of the extracellular matrix is composed of elastic fibers [2]. However, in the hypertrophic ligament, the proportion of elastic fibers is decreased, the proportion of collagen fibers is increased, and fibrotic changes are evident [3]. Several studies have investigated the mechanism underlying hypertrophy of the LF and found that the expression levels of several growth factors, namely, connective tissue growth factor, basic fibroblast growth factor, and transforming growth factor-beta (TGF-β), inflammatory cytokines (interleukin-1 and interleukin-6), collagenases (matrix metalloproteinases-1, -3, and -9), angiogenesis factors (vascular endothelial growth factor and platelet-derived growth factor), and fibroblast growth factor 9 are increased in the hypertrophic LF associated with LSS when compared with non-hypertrophic ligaments associated with lumbar disc herniation [4−6]. Sairyo et al [7−9] found that these fibrotic changes were more common in the dorsal layer than in the dural layer. Mechanical stress was considered as a cause of fibrosis when three-dimensional finite element modeling revealed that the mechanical stress was greater on the dorsal layer than on the dural layer [7]. However, the cause of fibrosis in the dorsal layer has not been fully elucidated.

It is well known that myofibroblasts have a key role in fibrosis in other body tissues, including the skin, lung, liver, heart, and kidney [10−14]. Tissue damage caused by several stimuli triggers an acute reaction and initiates the wound healing process. Inflammation and immunocytes stimulate conversion of fibroblasts to myofibroblasts, which promote expression of smooth muscle actin, synthesis of collagen, and matrix deposition. We hypothesized that myofibroblasts would have an important role in the pathology of fibrosis in the dorsal layer of the LF.

Therefore, the aim of this study was to clarify whether myofibroblasts are present in the hypertrophic LF, and if so, to investigate the role of myofibroblasts in this ligament.

Materials and methods

Patients

Fifty-one LF specimens were collected during surgery from 37 patients with LSS or degenerative spondylolisthesis (16 men, 21 women; 24 underwent one-level surgery, 12 underwent two-level surgery, and 1 underwent three-level surgery) with LSS or degenerative spondylolisthesis during surgery. Mean age at the time of surgery was 69.3 (range, 51−81) years. Exclusion criteria were systemic inflammatory disease, rheumatoid arthritis, spondylolytic spondylolisthesis, scoliosis (Cobb angle >10°), and lumbar revision surgery for the following reasons. In the case of systemic inflammatory disease and rheumatoid arthritis, the signals that normally occur may be suppressed because steroids and immunosuppressive agents are taken orally. In the case of scoliosis, because the mechanical stress applied to the right side and the left side is different, it is not clear which to evaluate. In the case of spondylolytic spondylolisthesis, the mechanical stress normally applied does not apply to the ligamentum flavum because of the fracture. In the case of reoperation, it is not possible to evaluate whether the ligamentum flavum remains. Patients with LSS and calcification of the LF detected on computed tomography were also excluded. The study protocol was approved by the institutional review board of each institution involved in the investigation.

Isolation and culture of cells

We harvested 14 LF specimens (1 at L1/2, 2 at L2/3, 4 at L3/4, and 7 at L4/5) from 12 patients (8 men, 4 women; mean age, 58.7 [range, 51−81] years) during surgery. The LF tissue samples were washed in phosphate-buffered saline, minced, and incubated for 1 h at 37°C in Dulbecco's modified Eagle's medium containing 0.2% type 1 collagenase (Fujifilm Wako Pure Chemical Corporation, Osaka,

Japan) and 1% penicillin-streptomycin (Fujifilm Wako Pure Chemical Corporation). The LF cells were filtered through a sterile 70-μm-pore nylon mesh and centrifuged to collect the cells. Subsequent experiments were performed using cells from the first and second passages. The LF cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA)

Histological analysis

Twenty-one of the 51 LF specimens (1 at level L2/3, 5 at L3/4 5, 13 at L4/5, and 2 at L5/S1) from 16 patients (5 men, 11 women); mean age 72.6 [range, 58−79] years) were used in this study. The specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and sagittally sectioned. The sections were stained using Masson's trichrome to assess the degree of fibrosis. Fibrosis was graded on a five-point scale according to the method devised by Sairyo et al [8] (grade 0, normal tissue showing no regions of fibrosis; grade 1, fibrosis occupying <25% of the entire area; grade 2, fibrosis occupying 25%−50% of the entire area; grade 3, fibrosis occupying 50%−75% of the entire area; grade 4, fibrosis occupying >75% of the entire area).

Immunohistochemistry

Immunohistochemistry was performed to detect alpha-smooth muscle actin (α-SMA), a myofibroblast marker, using standard protocols in 7 LF samples harvested from 7 patients (4 men, 3 women) of mean age 74.0 (range, 63−79) years. Briefly, the LF samples were fixed in 4% paraformaldehyde. After defatting using 70% ethanol extraction, the decalcified tissues were embedded in paraffin and sectioned into 4-μm slices. The deparaffinized sections were stained with rabbit anti-α-SMA (1:100, Abcam, Cambridge, UK). The slides were counterstained with hematoxylin. It is known that α-SMA is also expressed in vascular smooth muscle cells, and thus these cells were determined to be myofibroblasts if the cytoplasm was α-SMA-positive on immunohistochemistry and they did not form a vascular lumen. The proportion of α-SMA-positive cells was counted in five areas of the dorsal layer and in five areas of the dural layer.

RNA analysis

Twenty-four LF samples (8 at level L3/4, 12 at L4/5, and 4 at L5/S) from 22 patients (10 men, 12 women; mean age, 71.8 [age range, 61−79] years) were used in this study. The samples were separated into a dorsal layer and a dural layer and frozen in liquid nitrogen. Next, the samples were crushed, and total RNA was extracted using the RNeasy Lipid Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The total RNA was also extracted from the cultured cells.

The total RNA from the tissue and cells was used as a template for synthesis of cDNA with ReverTra Ace qPCR RT Master Mix and gDNA Remover (Toyobo, Osaka, Japan). A Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with Thunderbird qPCR Mix (Toyobo) or FastStart Universal SYBR Green Master (Roche Diagnostics, Basel, Switzerland) were used for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses. 18S ribosomal RNA was used for the internal controls. The primers used are listed in Supplemental Table 1.

Selection of LF samples

LF samples that could be removed en mass were used for RNA analysis and tissue analysis in this study: the first 24 specimens were used for RNA analysis and the next 21 specimens were used for tissue analysis. The 14 LF samples that could not be removed en mass were used for the in vitro assay.

Immunoblot analysis

The LF fibroblast lysates were prepared in RIPA buffer (50-mM Tris-HCl, 150-mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate) containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and phosphatase inhibitor cocktail (Biotools Inc., Jupiter, FL, USA) using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The protein concentrations in the lysates were measured using the bicinchoninic acid method, and immunoblot analyses were performed as described previously [12]. Proteins were detected using anti-hypoxia inducible factor (HIF)-1α and α-tubulin (Cell Signaling Technology, Danvers, MA, USA). Bands were detected using WesternSure ECL Substrate (Li-Cor Biosciences, Lincoln, NE, USA), and images were acquired using an EZ-Capture II Cooled CCD Camera System (ATTO Corp., Tokyo, Japan).

Hypoxic treatments

The LF fibroblasts were cultured under hypoxic conditions in 1% oxygen for 24 hours, 36 hours or 48 hours using a BIONIX-1 hypoxic culture kit (Sugiyamagen, Tokyo, Japan) according to the manufacturer's instructions.

Measurement of procollagen type I in the medium

The concentrations of procollagen type I in the medium were determined using a commercially available enzyme-linked immunosorbent assay kit (Procollagen Type I C-Peptide EIA Kit; Takara Bio, Kusatsu, Japan) according to the manufacturer's instructions. Each sample was analyzed in duplicate.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed on 4 LF samples (1 at level L3/4, 3 at L4/5) from 4 patients (2 men, 2 women; mean age, 67.8 [range, 52−77] years). We

divided the samples into a dorsal layer and a dural layer, giving a total of 8 samples. Before performing the microarray analysis, a Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA) was used to label total RNA purified with the RNeasy MinElute Cleanup Kit (Qiagen). The labeled RNA was then used to probe a SurePrint G3 Human Gene Expression 8 × 60K microarray, and the signals were scanned with a G2565 microarray scanner (both from Agilent Technologies).

The microarray data were extracted from the scanned images using Feature Extraction 10.7 (Agilent Technologies). Gene expression pathway analysis was performed using GSEA software V3.0 (Broad Institute, MIT, Cambridge, MA, USA) [15]. GSEA performs a competitive analysis of predefined gene sets, so is suitable for examining relatively heterogeneous biological samples. In brief, GSEA first ranks all genes analyzed by expression arrays according to their differential expression between two categories of samples; in this study, these categories were the dural and dorsal layers.

Next, "h.all.v6.0.symbols.gmt" was chosen as the gene set used for the enrichment analysis. GSEA calculates a pathway enrichment score that reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes. Default settings were used, except for the maximum size of the gene sets, which was set to 1,500 in order to include all predefined gene sets for analysis. Thresholds for significance of the enrichment score were determined by analysis of 1,000 permutations. A p-value of <.05 and a false discovery rate of <0.25 were considered statistically significant.

Statistical analysis

Pearson's correlation test was used to analyze the cells in the LF. All quantitative data are reported as the mean ± standard error of the mean. The Student's *t* test was used for normally distributed data. The statistical analysis was performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA). All tests were two-tailed. A p-value <.05 was considered statistically significant.

Results

Fibrosis was aggravated in the dorsal layer of the ligament

Fibrosis of the LF was evaluated by Masson's trichrome staining. The tissue in the dorsal layer stained blue well and the fibrosis score was significantly higher in the dorsal layer compared with the dural layer (3.5±0.2 vs. 0.9±0.2, p<.05; Fig. 1A, B). The 24 LF tissue samples were divided into

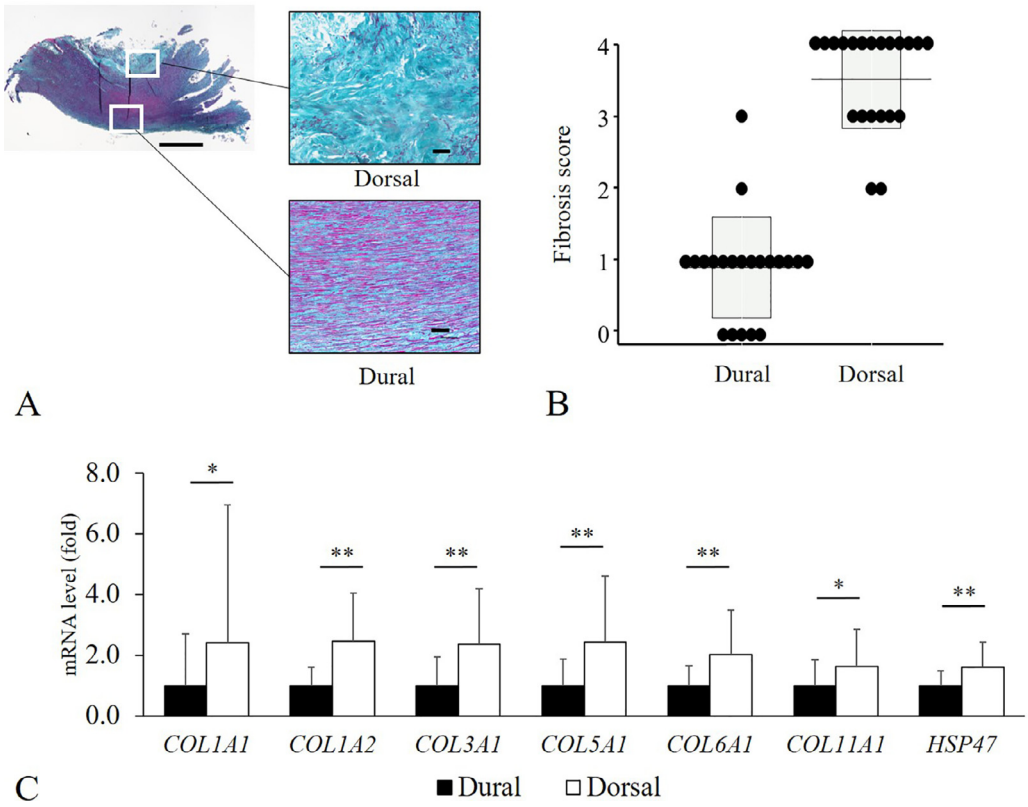

Fig. 1. Fibrosis was aggravated in the dorsal layer of LF tissue. (A) Representative histopathology of the LF fibrosis score by Masson's trichrome staining. Whole image. Bar, 1 mm. Blue indicates collagen fibers and red indicates elastic fibers. Bar, 50 μm. (B). Fibrosis scores for the dorsal and dural layers of the LF (n=21). (C) The LF was divided into a dorsal layer and a dural layer. Comparison of mRNA expression of *COL1A-1, COL1A-2, COL3A-1, COL5A-1, COL6A-1, COL11A-1*, and *HSP47* mRNA in the dorsal and dural layers of the LF (n=24). Data are presented as the mean ± standard error of the mean. *p<.05, **p<.01. LF, ligamentum flavum.

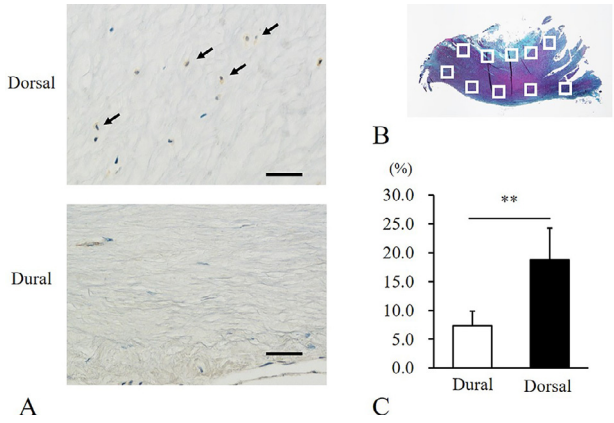

Fig. 2. The number of α-SMA-positive cells was increased in the dorsal layer of the LF tissue. (A) Representative immunohistochemistry of α-SMA expression in the LF tissues. Arrows indicate α-SMA-positive cells. Bar, 50 μm. (B, C) Comparison of the average in 5 points of the ratio of α-SMA-positive cells in the dural layer and the dorsal layer of the LF (n=7). Data are presented as the mean ± standard error of the mean. **p<.05. LF, ligamentum flavum; α-SMA, alpha-smooth muscle actin.

dural and dorsal layers, and the mRNA expression was evaluated. Expression of several types of collagen mRNA was significantly greater in the dorsal layer compared with the dural layer (collagen (*COL)1A1*: 1.0±1.7 vs. 2.4±4.5, *COL1A2*: 1.0±0.6 vs. 2.4±1.6, *COL3A1*: 1.0±1.0 vs. 2.4± 1.8, *COL5A1*: 1.0±0.7 vs. 2.4±2.2, *COL6A1*: 1.0±0.7 vs. 2.0±1.5, *COL11A1*: 1.0±0.8 vs. 1.6 ± 1.2; Fig. 1C). Moreover, the expression of heat shock protein (HSP) 47, which is a collagen-specific chaperone required for normal collagen synthesis, was also increased in the dorsal layer (1.0± 0.5 vs. 1.6±0.8). The fibrosis was found to be aggravated in the dorsal layer both on histology (Fig. 1A, B) and gene expression analysis (Fig. 1C).

Myofibroblasts increased in the dorsal layer of the ligament

Immunohistochemistry with antibodies against α-SMA was used to evaluate the localization of myofibroblasts in 7 LF tissue samples. There was a significantly greater number of α-SMA-stained cells in the dorsal layer (16.8%±5.5%) compared with the dural layer (7.4%± 2.5%), indicating that there were more fibroblasts in the dorsal layer (Fig. 2A–C).

mRNA expression of α-SMA was positively associated with expression of COL1A1

To clarify whether α-SMA-expressing cells contribute to production of collagen, the correlation between expression of α-SMA and that of various types of collagen was investigated in vitro. There was a strong correlation between expression of α-SMA mRNA and expression of COL1A1 mRNA (r=0.665, p=.009) in LF cells (Fig. 3). Expression levels of α-SMA mRNA and COL3A1 mRNA tended to correlate (r=0.505, p=.066). There was no significant correlation between expression of α-SMA mRNA and that of COL5A1 mRNA (r=0.428, p=.127).

Hypoxic stimulation increased α-SMA gene expression and production of collagen

To investigate the signal pathway activated in the dorsal layer, we performed a microarray analysis of four samples each in the dorsal and dural layers. We performed GSEA using the microarray results and found that the set of fibrosis-related gene signals, including epithelial-mesenchymal transition, hypoxia, and inflammation, was strongly associated with fibrosis in the dorsal layer of the LF (Table).

We investigated whether hypoxic stimulation also increased α-SMA gene expression and collagen production in LF fibroblasts. HIF-1α was apparently induced in LF fibroblasts when cultured under hypoxic conditions (1% oxygen) for 24 h, 36h or 48 h, and LF fibroblasts were appropriately stimulated under hypoxic condition (Fig. 4A). We found that mRNA expression of α-SMA was increased under hypoxic conditions (1.0±0.1 vs. 2.1±0.2). Moreover, under these conditions, there was an increase in mRNA expression of several types of collagen (*COL1A1*: 1.0 ± 0.1 vs. 2.9±0.2, *COL1A2*: 1.0±0.1 vs. 1.8±0.1, *COL3A1*: 1.0±0.2 vs. 2.1±0.3, *COL5A1*: 1.0±0.1 vs. 4.0± 0.3, *COL6A1*: 1.0±0.1 vs. 2.4±0.1, *COL11A1*: 1.0±0.1 vs .2.1±0.1), fibronectin (1.0±0.0 vs. 1.8±0.1), and HSP47 (1.0±0.1 vs. 1.6±0.2). Given that collagen is a secretory

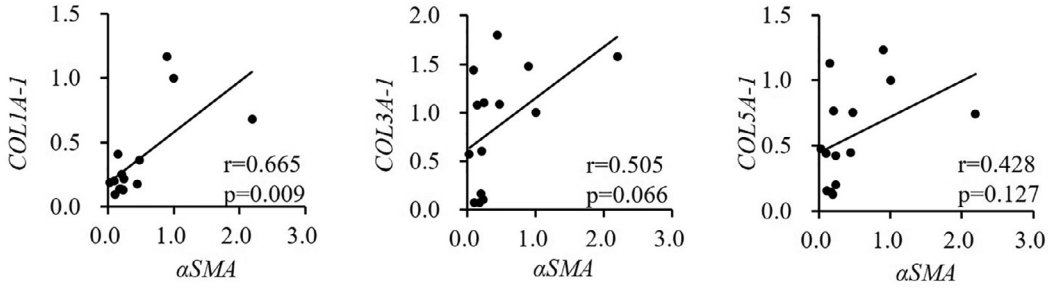

Fig. 3. Association between mRNA expression of *α-SMA* and *COL1A1, COL3A1*, and *COL5A1* in fibroblasts in the ligamentum flavum (n=14).

Table
Top 14 sets of upregulated genes in the dorsal and dural layers of the ligamentum flavum identified by gene set enrichment analysis.

Name	NOM p-val	FDR q-val
G2M checkpoint	.000	0.001
Epithelial mesenchymal transition	.000	0.000
E2F targets	.000	0.004
Heme metabolism	.000	0.015
Hypoxia	.001	0.020
MTORC1 signaling	.001	0.030
Glycolysis	.005	0.017
MYC targets V1	.007	0.042
Oxidative phosphorylation	.022	0.107
Unfolded protein response	.028	0.088
Angiogenesis	.030	0.043
DNA repair	.032	0.113
Estrogen response late	.038	0.128
Inflammatory response	.085	0.218

protein, its production in culture medium was evaluated by enzyme-linked immunosorbent assay. Under hypoxic conditions, the production of procollagen type 1 was increased at 24, 36, and 48 hours.

Discussion

The three main findings of this study were as follows: (1) the amount of myofibroblasts, indicated by α-SMA-positive cells, was increased in the dorsal layer of the LF compared to with the dural layer; (2) there was a positive correlation between the expression of α-SMA and that of COL1A-1 in fibroblasts from LF tissue; and (3) hypoxia induced fibrosis via the increased amount of myofibroblasts.

It is well known that myofibroblasts have a key role in tissue fibrosis. After tissue injury, fibroblasts differentiate into myofibroblasts that express α-SMA as a result of stimulation by mechanical stress and TGF-β and synthesize components of the extracellular matrix, such as collagen types I and III, which contribute to tissue repair during wound healing [3,11−14,16−18]. Furthermore, myofibroblasts have contractile properties that can compensate for tissue defects [13,16]. Myofibroblasts normally disappear from granulation tissue via apoptosis after closure of a wound [19]. However, in some circumstances, myofibroblasts persist and may contribute to formation of pathological scar tissue. Secretion of protein from the extracellular

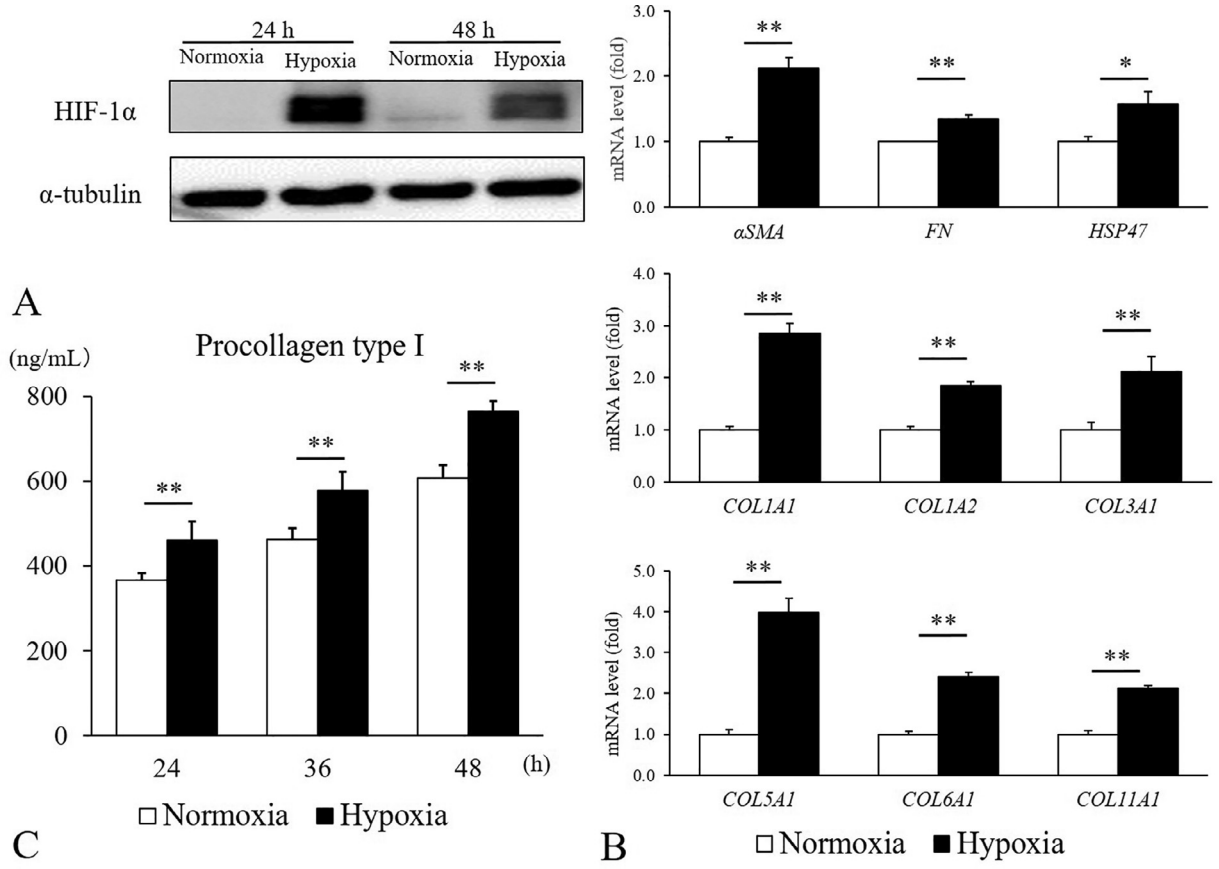

Fig. 4. Hypoxic stimulation increased α-SMA gene expression and production of collagen. (A) Expression of HIF-1α in LF fibroblasts under normoxic conditions (21% oxygen) and hypoxia (1% oxygen) by Western blotting at 24 and 48 hours. (B) Comparison of mRNA expression of *α-SMA, FN, HSP47*, and *COL1A-1, COL1A-2, COL3A-1, COL5A-1, COL6A-1*, and *COL11A-1* in LF fibroblasts under normoxic conditions (21% oxygen) and hypoxia (1% oxygen) by quantitative reverse transcription polymerase chain reaction at 24 hours (n=4). Data are presented as the mean±standard error of the mean. (C) Concentrations of procollagen type I in the medium were determined by enzyme-linked immunosorbent assay. Data are presented as the mean ± standard error of the mean. *p<.05, **p<.01. HIF-1α, hypoxia-inducible factor 1-alpha; LF, ligamentum flavum.

matrix in the tissues and organs may be excessive, as in fibroproliferative disorders (FPDs), such as scleroderma, Dupuytren's disease, some heart, hepatic, and kidney diseases, and pulmonary fibrosis [20–22]. It has been reported that fibroblasts and myofibroblasts are key players in FPDs [21], and drug treatment targeted to myofibroblasts is being investigated as a strategy for prevention of fibrosis [23]. Therefore, it is important to clarify the role of myofibroblasts in the LF.

Recently, Hur et al [24] reported that myofibroblasts were present among fibroblasts obtained from LF and that there was a positive correlation between the expression of α-SMA and that of TGF-β in these cells. However, in that study, it was not clear where in the ligament the myofibroblast became localized. It was also uncertain whether production of the collagen that causes fibrosis was actually increased in α-SMA-expressing cells because only the relationship between α-SMA and TGF-β was investigated. Delen et al [25] subsequently reported increased expression of α-SMA in the dorsal layer of the LF. However, they evaluated homogenized LF tissue, which contains a mixture of various types of cells. Other cells, including vascular smooth muscle cells, also express α-SMA, so it was not clear in that study whether or not there was an actual increase in numbers of myofibroblasts in the LF. Therefore, we evaluated the localization of α-SMA-positive cells by immunohistochemistry and found more α-SMA-positive cells in the dorsal layer of the ligament than in the dural layer, indicating that in patients with LSS, the hypertrophic LF contains more myofibroblasts, especially in the dorsal layer. Moreover, we found a positive correlation between the expression of α-SMA and that of COL1A-1 in fibroblasts from the LF. Consequently, myofibroblasts can be assumed to contribute to fibrosis in LF as in other FPDs.

Next, we performed a microarray analysis of the dorsal and dural layers of the LF to determine the reason for the increased amount of myofibroblasts in the dorsal layer of the LF. Some microarray studies have used the LF from patients with lumbar disc herniation as the control [26,27]. In this study, we used the dural layer in the same LF sample as the control in an attempt to identify the precise pathological mechanism, independent from individual differences. We attempted to identify the predominant signal pathway that becomes activated using GSEA, and found that fibrosis-related signals, including epithelial-mesenchymal transition, hypoxia, and inflammation, were activated more in the dorsal layer than in the dural layer, indicating that these signals are involved in the expression of myofibroblasts.

Many researchers have investigated the relationship between hypertrophy of the LF and inflammation. However, our microarray results showed that gene expression in the dorsal layer of the LF was more positively associated with hypoxic stimulation than with inflammation signaling pathways. Therefore, in this study, we evaluated the relationship between hypoxia and hypertrophy of the LF.

In addition to direct enhancement of the ability to produce collagen, enhanced production of extracellular matrix, and suppression of degradation via TGF-β, TIPM1 and TIMP-2 have been reported as a further mechanism of hypoxia-induced fibrosis [28]. Higgins et al [29] reported that proximal tubular epithelial cells changed to a fibroblast-like morphology when cultured under hypoxic conditions. Moreover, expression of ZO-1, which is a tight junction protein of epithelial cells, was found to disappear, and the expression of α-SMA was confirmed. Until now, the role of hypoxia in hypertrophic LF was not known. In this study, we found that hypoxic stimulation increased α-SMA gene expression and collagen production, leading to fibrosis. Jezek et al [1] reported supportive findings. They evaluated the microvascular density of LF tissue morphometrically as the length density and found that vascular density decreased up to the age of 50. It is possible that this decrease in vascular density leads to hypoxia.

Although the exact reason why hypoxic stimulation increased the amount of fibroblasts in the dorsal layer of the LF is not known, we suspect that mechanical stress was involved. Sairyo et al [7,8] reported that tissue damage induced by mechanical stress, especially in the dorsal layer of the LF, would be the initial triggering event for fibrosis of the LF. Nakashima et al [30] reported that when mechanical stress was reduced after indirect decompression using lateral lumbar interbody fusion, the LF became thinner . Moreover, Wang et al [31] found increased expression of HIF-1a in fibroblasts in the anterior cruciate ligament and increased hypoxia signaling after mechanical stretching. From these reports, we believe that hypoxia signaling is activated in the dorsal layer of the LF due to mechanical stress and leads to fibrosis.

This study has several limitations. First, expression of α-SMA in the LF was not evaluated by qRT-PCR. We anticipated that vascular smooth muscle cells rather than myofibroblasts would be expressed α-SMA in the dural layer, even in samples from a normal LF. Therefore, we decided that use of qRT-PCR would be inappropriate and hence we evaluated α-SMA-positive cells, including their morphology, by immunohistochemistry. Second, we cannot explain why the hypoxia signaling pathways were activated in the dorsal layer. We suspect that these pathways may have been activated by tissue damage as a result of mechanical stress [7]. Accordingly, further investigations in an animal model of hypertrophic LF are needed [9,32].

Conclusion

This study is the first to reveal that there are more myofibroblasts in the dorsal layer of the LF than in the dural layer in patients with LSS. Hypoxia might trigger expression of myofibroblasts, and lead to fibrosis and hypertrophy of the LF. If a pharmacological agent can be developed that specifically targets the signal for proliferation of myofibroblasts in the LF,

fibrosis at this site could be decreased and the symptoms of LSS attenuated.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.spinee.2021.11.003.

References

[1] Jezek J, Sepitka J, Daniel M, Kujal P, Blankova A, Waldauf P, et al. The role of vascularization on changes in ligamentum flavum mechanical properties and development of hypertrophy in patients with lumbar spinal stenosis. Spine J 2020;20(7):1125–33.
[2] Evans JH, Nachemson AL. Biomechanical study of human lumbar ligamentum flavum. J Anat 1969;105(Pt 1):188–9.
[3] Yoshiiwa T, Miyazaki M, Kawano M, Ikeda S, Tsumura H. Analysis of the relationship between hypertrophy of the ligamentum flavum and lumbar segmental motion with aging process. Asian Spine J 2016;10:528–35.
[4] Amudong A, Muheremu A, Abudourexiti T. Hypertrophy of the ligamentum flavum and expression of transforming growth factor beta. J Int Med Res 2017;45:2036–41.
[5] Lakemeier S, Schofer MD, Foltz L, Schmid R, Efe T, Rohlfs J, et al. Expression of hypoxia-inducible factor-1alpha, vascular endothelial growth factor, and matrix metalloproteinases 1, 3, and 9 in hypertrophied ligamentum flavum. J Spinal Disord Tech 2013;26:400–6.
[6] Habibi H, Suzuki A, Hayashi K, Salimi H, Terai H, Hori Y, et al. Expression and function of FGF9 in the hypertrophied ligamentum flavum of lumbar spinal stenosis patients. Spine J 2021. https://doi.org/10.1016/j.spinee.2021.02.004. Online ahead of print.
[7] Sairyo K, Biyani A, Goel V, Leaman D, Booth Jr, Thomas J, et al. Pathomechanism of ligamentum flavum hypertrophy: a multidisciplinary investigation based on clinical, biomechanical, histologic, and biologic assessments. Spine (Phila Pa 1976) 2005;30:2649–56.
[8] Sairyo K, Biyani A, Goel VK, Leaman DW, Booth Rjr, Thomas J, et al. Lumbar ligamentum flavum hypertrophy is due to accumulation of inflammation-related scar tissue. Spine (Phila Pa 1976) 2007;32:E340–7.
[9] Sato N, Taniguchi T, Goda Y, Kosaka H, Higashino K, Sakai T, et al. Proteomic analysis of human tendon and ligament: solubilization and analysis of insoluble extracellular matrix in connective tissues. J Proteome Res 2016;15:4709–21.
[10] Valatas V, Filidou E, Drygiannakis I, Kolios G. Stromal and immune cells in gut fibrosis: the myofibroblast and the scarface. Ann Gastroenterol 2017;30:393–404.
[11] Baum J, Duffy HS. Fibroblasts and myofibroblasts: what are we talking about? J Cardiovasc Pharmacol 2011;57:376–9.
[12] Brown RD, Ambler SK, Mitchell MD, Long CS. The cardiac fibroblast: therapeutic target in myocardial remodeling and failure. Annu Rev Pharmacol Toxicol 2005;45:657–87.
[13] Desmouliere A, Darby IA, Gabbiani G. Normal and pathologic soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. Lab Invest 2003;83(12):1689–707.
[14] Wynn TA. Integrating mechanisms of pulmonary fibrosis. J Exp Med 2011;208:1339–50.
[15] Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545–50.
[16] Hinz B. Formation and function of the myofibroblast during tissue repair. J Invest Dermatol 2007;127:526–37.
[17] Musumeci M, Vadala G, Russo F, Pelacchi F, Lanotte A, Denaro V. Dupuytren's disease therapy: targeting the vicious cycle of myofibroblasts? Expert Opin Ther Targets 2015;19:1677–87.
[18] Rockey DC, Bell PD, Hill JA. Fibrosis−a common pathway to organ injury and failure. N Engl J Med 2015;372:1138–49.
[19] Sarrazy V, Billet F, Micallef L, Coulomb B, Desmouliere A. Mechanisms of pathological scarring: role of myofibroblasts and current developments. Wound Repair Regen 2011;19(Suppl 1):s10–5.
[20] Hertz MI, Henke CA, Nakhleh RE, Harmon KR, Marinelli WA, Fox JM, et al. Obliterative bronchiolitis after lung transplantation: a fibroproliferative disorder associated with platelet-derived growth factor. Proc Natl Acad Sci U S A 1992;89:10385–9.
[21] Huang C, Ogawa R. Fibroproliferative disorders and their mechanobiology. Connect Tissue Res 2012;53:187–96.
[22] Kis K, Liu X, Hagood JS. Myofibroblast differentiation and survival in fibrotic disease. Expert Rev Mol Med 2011;13:e27.
[23] Prakash J, Pinzani M. Fibroblasts and extracellular matrix: targeting and therapeutic tools in fibrosis and cancer. Adv Drug Deliv Rev 2017;121:1–2.
[24] Hur JW, Bae T, Ye S, Kim JH, Lee S, Kim K, et al. Myofibroblast in the ligamentum flavum hypertrophic activity. Eur Spine J 2017;26:2021–30.
[25] Delen E, Doganlar O, Delen O, Doganlar ZB, Kilincer C. The role of JAK-STAT signaling activation in hypertrophied ligamentum flavum. World Neurosurg 2020;137:e506–16.
[26] Xu YQ, Zhang ZH, Zheng YF, Feng SQ. MicroRNA-221 regulates hypertrophy of ligamentum flavum in lumbar spinal stenosis by targeting TIMP-2. Spine (Phila Pa 1976) 2016;41:275–82.
[27] Yabe Y, Hagiwara Y, Ando A, Tsuchiya M, Minowa T, Takemura T, et al. Chondrogenic and fibrotic process in the ligamentum flavum of patients with lumbar spinal canal stenosis. Spine (Phila Pa 1976) 2015;40(7):429–35.
[28] Orphanides C, Fine LG, Norman JT. Hypoxia stimulates proximal tubular cell matrix production via a TGF-beta1-independent mechanism. Kidney Int 1997;52:637–47.
[29] Higgins DF, Kimura K, Bernhardt WM, Shrimanker N, Akai Y, Hohenstein B, et al. Hypoxia promotes fibrogenesis in vivo via HIF-1 stimulation of epithelial-to-mesenchymal transition. J Clin Invest 2007;117:3810–20.
[30] Nakashima H, Kanemura T, Satake K, Ishikawa Y, Ouchida J, Segi N, et al. Indirect decompression on MRI chronologically progresses after immediate postlateral lumbar interbody fusion: the results from a minimum of 2 years follow-up. Spine (Phila Pa 1976) 2019;44:E1411–8.
[31] Wang Y, Dang Z, Cui W, Yang L. Mechanical stretch and hypoxia inducible factor-1 alpha affect the vascular endothelial growth factor and the connective tissue growth factor in cultured ACL fibroblasts. Connect Tissue Res 2017;58:407–13.
[32] Saito T, Hara M, Kumamaru H, Kobayakawa K, Yokota K, Kijima K, et al. Macrophage infiltration is a causative factor for ligamentum flavum hypertrophy through the activation of collagen production in fibroblasts. Am J Pathol 2017;187:2831–40.