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# HYPERTROPHY OF LIGAMENTUM FLAVUM IN LUMBAR SPINAL STENOSIS ASSOCIATED WITH INCREASED PROTEINASE INHIBITOR CONCENTRATION

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**Background:** It is well known that age-related fibrosis, or decreases in the elastin-to-collagen ratio of the ligamentum flavum, along with hypertrophy of the ligamentum flavum, are associated with lumbar spinal stenosis. However, the molecular mechanism by which this fibrosis and hypertrophy develop is unknown. Tissue inhibitors of matrix metalloproteinase (TIMPs) are proteinase inhibitors that suppress extracellular matrix degradation. Elevated TIMP-1 and TIMP-2 expression has been implicated in various fibrotic diseases of the liver, kidney, lung, and heart. These TIMPs can also induce cellular proliferation and inhibit apoptosis in a wide range of cell types. These findings led us to postulate that TIMP-1 and TIMP-2 might also be associated with hypertrophy and fibrosis of the ligamentum flavum in lumbar spinal stenosis.

**Methods:** We quantified and localized TIMP expression in ligamentum flavum tissues that had been obtained during surgery from thirty patients with spinal stenosis and from thirty gender-matched control patients with disc herniation. The thickness of the ligamentum flavum at the level of the facet joint was measured on axial T1-weighted magnetic resonance images. In addition, we examined ligamentum flavum tissues for the expression of markers of cellular proliferation and apoptosis.

**Results:** The ligamentum flavum was significantly thicker in the patients with spinal stenosis (mean, 5.68 mm) than in the patients with disc herniation (mean, 2.70 mm) ( $p < 0.001$ ). The concentration of TIMP-2 in the ligamentum flavum was significantly higher in the patients with spinal stenosis (mean, 12.62 ng/mL) than in those with disc herniation (mean, 8.85 ng/mL) ( $p = 0.028$ ). TIMP-1 and TIMP-2 were detected in the cytoplasm of ligamentum flavum fibroblasts. TIMP-1 and TIMP-2 concentrations were associated with hypertrophy of the ligamentum flavum ( $p = 0.015$  and  $p = 0.003$ , respectively). None of the samples from the patients with stenosis had evidence of proliferation of ligamentum flavum fibroblasts. The expression of markers for apoptosis was significantly higher in the patients with spinal stenosis (58.8%) than in those with disc herniation (26.6%) ( $p < 0.001$ ).

**Conclusions:** Increased TIMP expression has been implicated in fibrosis and hypertrophy of the extracellular matrix of several organs. Our results suggest that increased expression of TIMP-2 in ligamentum flavum fibroblasts is associated with fibrosis and hypertrophy of the ligamentum flavum in patients with spinal stenosis.

**Clinical Relevance:** Gaining insights into the molecular pathogenesis of hypertrophy and fibrosis of the ligamentum flavum may eventually result in new therapeutic and potentially noninvasive alternatives to the treatment of spinal stenosis.

The ligamentum flavum is composed of elastin and collagen fibers in a 2:1 ratio. The elastin fibers impart a yellow color to the structure and provide the elasticity, while the collagen provides the stiffness and stability. With age, the elastin-to-collagen ratio decreases, resulting in decreased elastic-

ity and increased stiffness or fibrosis. In addition, the ligamentum flavum hypertrophies, and the combination sometimes results in lumbar spinal stenosis<sup>1-5</sup>. Although the hypertrophy and fibrosis have been postulated to be due to degenerative changes secondary to the aging process and mechanical stress sec-

ondary to instability, the pathogenesis remains undetermined.

Matrix metalloproteinases (MMPs) are a family of more than twenty enzymes that digest proteins in the extracellular matrix<sup>6-9</sup>. There are also inhibitors of MMPs, called tissue inhibitors of matrix metalloproteinase (TIMPs), and it is believed that, together, MMPs and TIMPs regulate the integrity and homeostasis of the extracellular matrix. There are four known TIMPs (TIMP-1 through 4) that suppress extracellular matrix degradation by forming an inhibitory 1:1 complex with the MMPs<sup>10-18</sup>. Elevated expressions of TIMP-1 and TIMP-2 have been implicated in the increased fibrosis found in a wide variety of human organs, including the liver, kidney, lung, and heart. TIMPs are also known to increase cellular proliferation<sup>19-21</sup> and inhibit programmed cell death (apoptosis) in a wide range of cell types<sup>22-26</sup>.

On the basis of what is known about TIMPs, we hypothesized that they might also play a role in the hypertrophy of the ligamentum flavum seen in lumbar spinal stenosis. We postulated that TIMPs might alter the ligamentum flavum in three ways: by increasing the density of the extracellular matrix and promoting fibrosis by inhibiting MMPs, by increasing fibroblast proliferation, and by reducing the rate of fibroblast apoptosis. In the present study, our goals were to determine (1) whether there is an increased concentration of TIMPs in the ligamentum flavum of patients with spinal stenosis compared with that in patients with disc herniation, and (2) whether the ligamentum flavum in spinal stenosis has increased fibroblast proliferation and/or decreased apoptosis, either of which would result in increased cellularity. To our knowledge, no one has previously investigated the association between hypertrophy of the ligamentum flavum and concentrations of TIMP-1 and TIMP-2.

## Materials and Methods

Thirty samples of ligamentum flavum tissue were obtained from thirty patients who were undergoing decompression because of neurogenic claudication due to lumbar spinal stenosis that had been unresponsive to conservative measures for at least three months. None of these patients received epidural or selective nerve-root blocks. Twenty-one patients were female, and nine were male. The mean age of the patients at the time of surgery was 63.7 years (range, fifty-two to seventy-eight years). Patients with degenerative spondylolisthesis were excluded from the study. Seventeen patients underwent a one-level operation; nine, a two-level operation; and four, a three-level operation. We tried to obtain the entire layer of the central portion of the ligamentum flavum, and we removed the epidural fat from the ligamentum flavum tissues. Half of each ligamentum flavum specimen was fixed in 4% neutral formalin, decalcified with 20% EDTA for six weeks, and embedded in paraffin; the other half was kept in a freezer at  $-70^{\circ}\text{C}$  for subsequent enzyme-linked immunosorbent assay (ELISA).

We randomly selected thirty gender-matched control patients with lumbar disc herniation from a group of 124 patients who were being operatively managed for that disorder. The mean age of the control patients was 31.7 years (range, nineteen

to forty-three years), which was significantly younger than the mean age of the patients with spinal stenosis ( $p < 0.001$ ).

### Measurement of the Thickness of the Ligamentum Flavum

An axial T1-weighted magnetic resonance image (repetition time, 600 msec; echo time, 30 msec) was made, with a 1.5-T unit (Somatom Plus; Siemens, Erlangen, Germany), at the facet joint level of the lesion for each patient. The maximum thickness of the ligamentum flavum was traced with use of the manual cursor technique and was computed automatically by the installed software in the magnetic resonance imaging scanner. All of the radiographic analyses were independently performed by two experienced spine surgeons who were not involved in the care of the patients. Each observer independently measured the thickness of the ligamentum flavum twice, and the average of the four measurements was used as the final thickness.

### Quantification of TIMP-1 and TIMP-2 Concentrations by ELISA

After thawing, 100 mg of the ligamentum flavum tissue was homogenized with phosphate-buffered saline solution at 3000 rpm in a tissue homogenizer (model 985-370, Tissue-Tearor; Bio-Spec Products, Racine, Wisconsin). Centrifugation to 15,000 rpm was then performed at  $4^{\circ}\text{C}$  for thirty minutes, and the supernatant was obtained. Quantification of protein was performed according to Bradford's method<sup>27</sup> with use of a protein assay kit (catalogue number 500-0006; Bio-Rad Laboratories, Hercules, California), and optical density was measured at 595 nm with a spectrophotometer (Ultraspec 3000; Pharmacia Biotech, Cambridge, United Kingdom) and adjusted equally to levels of 0.5 mg/mL (total protein concentration) for each sample.

TIMP-1 and TIMP-2 concentrations were measured twice in each sample with use of an ELISA kit with antibodies that recognize human TIMP-1 and TIMP-2 (R and D Systems, Minneapolis, Minnesota); the kit was used according to the manufacturer's instructions. The average of the two measurements was considered to be the final concentration. For calibration, we used human recombinant TIMP-1 and TIMP-2 provided by the supplier to construct a standard curve and to obtain absolute values.

### Apoptosis: Analysis with In Situ Nick End-Labeling (TUNEL) Assay

In situ terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) reaction was performed with use of a TACS 2 TdT DAB In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, Maryland) according to the manufacturer's instructions. One section (4  $\mu\text{m}$  thick) was treated with proteinase K. Endogenous peroxidase was removed with 2%  $\text{H}_2\text{O}_2$ . The 3'-OH ends of the fragmented DNA were marked with TdT dNTP and divalent cation by adding TdT. Two pathologists, who were unaware of the clinical data, were responsible for counting total and TUNEL-positive ligamentum flavum fibroblasts under ten randomly selected high-power fields ( $\times 400$ ). The apoptosis index was calculated as TUNEL-

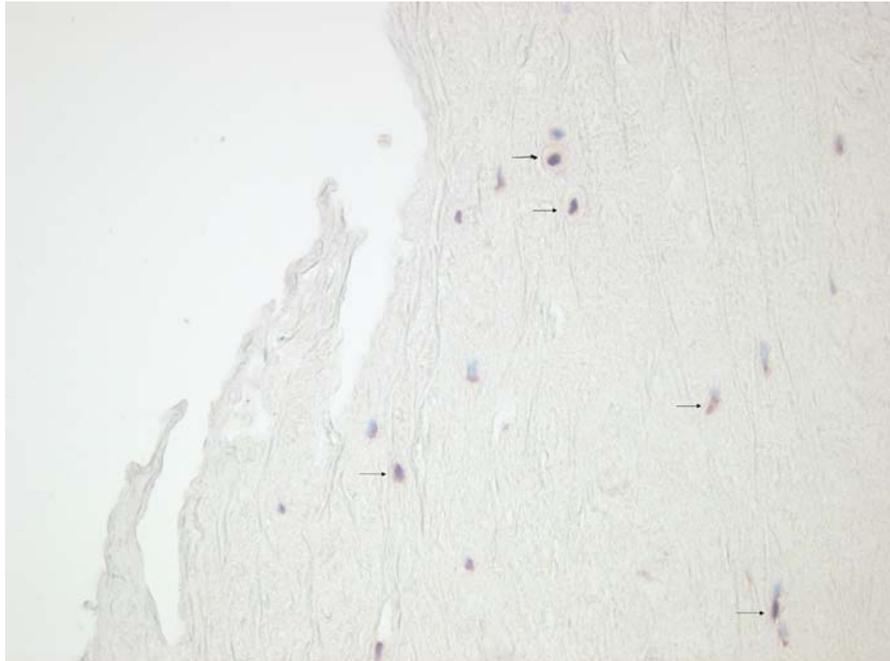


Fig. 1-A

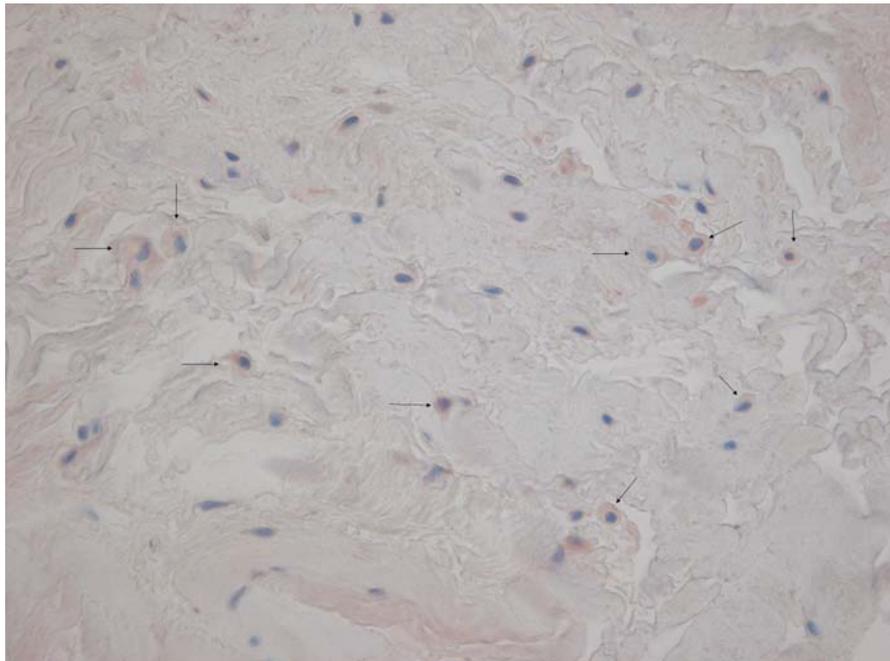


Fig. 1-B

Immunohistochemical studies showing fibroblasts within the ligamentum flavum that are positively stained (arrows) for TIMP-1 (Fig. 1-A) and TIMP-2 (Fig. 1-B) ( $\times 400$ ).

positive cells/total cells  $\times 100\%$ . Human tonsil tissue was used as a positive control.

**Cellular Proliferation: Analysis with Immunohistochemistry for TIMPs and Ki-67**

Ki-67, a cellular proliferation marker, was assayed to determine

if there was evidence of proliferation of ligamentum flavum fibroblasts. Three consecutive 4- $\mu\text{m}$ -thick sections were cut on a microtome, deparaffinized in xylene, and rehydrated. To determine the expression of TIMP-1, TIMP-2, and Ki-67, the avidin-biotin-peroxidase complex method and a Histostain-plus SP kit (Zymed Laboratories, South San Francisco, California)



Fig. 2-A  
TUNEL-positive fibroblasts (arrows) were identified in samples of ligamentum flavum from patients with spinal stenosis ( $\times 400$ ).

were used according to the manufacturer's instructions. Purified mouse monoclonal antibodies to TIMP-1 and TIMP-2 (NeoMarkers, Fremont, California) and Ki-67 (DakoCytomation, Glostrup, Denmark) were used for this study at an optimum dilution of 1:20, 1:50, and 1:75, respectively. A human breast carcinoma and a human colon carcinoma were used as

positive controls for TIMP-1 and TIMP-2, and tonsil tissue was used for Ki-67.

#### *Statistical Analysis*

The independent-sample t test was used to compare the patients with lumbar spinal stenosis and those with disc herniation in

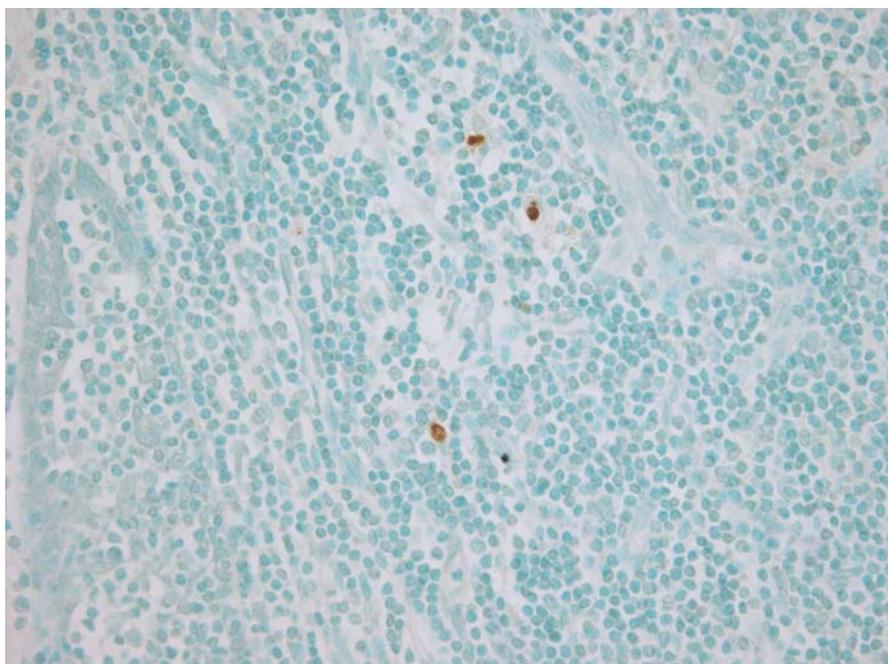


Fig. 2-B  
Human tonsil tissue was used as a positive control.



Fig. 3-A  
Immunohistochemical study showing that the fibroblasts within the ligamentum flavum are not stained with Ki-67 ( $\times 400$ ).

terms of the patients' age, TIMP-1 and TIMP-2 concentrations, ligamentum flavum thickness, and apoptosis index. The relationship between the TIMP concentration and the thickness of the ligamentum flavum was determined with the Pearson correlation test. A p value of  $<0.05$  was considered to be significant.

## Results

### *Thickness of the Ligamentum Flavum*

The mean thickness of the ligamentum flavum (and standard deviation) was  $5.68 \pm 1.02$  mm (range, 4.5 to 7.2 mm) in the patients with lumbar spinal stenosis and  $2.70 \pm 0.35$  mm

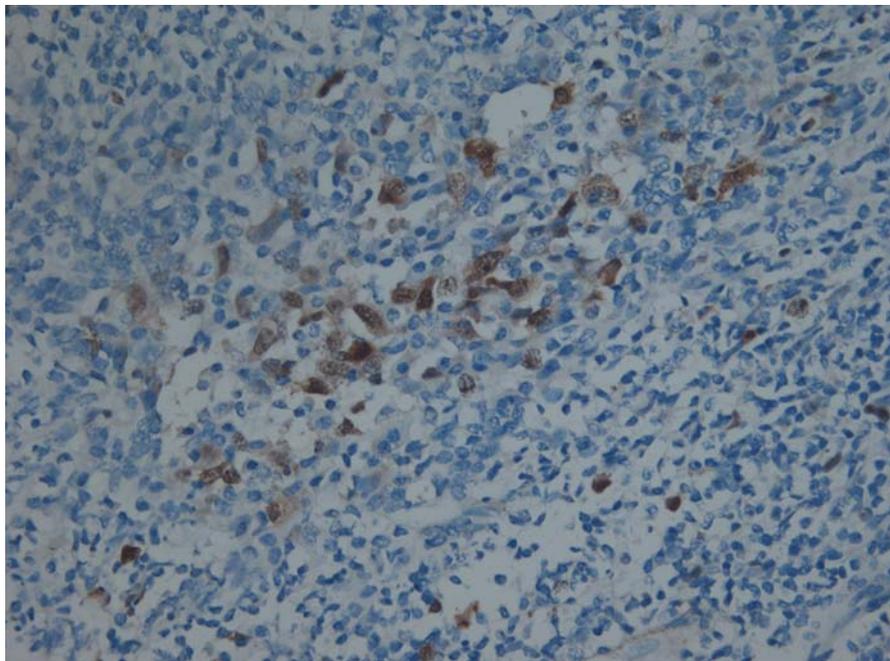


Fig. 3-B  
Human tonsil tissue was used as a positive control.

**TABLE I Comparison of the Thickness of Ligamentum Flavum and the Concentrations of TIMP-1 and TIMP-2 Between the Patients with Lumbar Spinal Stenosis and Those with Disc Herniation**

	Spinal Stenosis* (N = 30)	Lumbar Disc Herniation* (N = 30)	P Value
Ligamentum flavum thickness	5.68 ± 1.02 mm	2.70 ± 0.35 mm	<0.001†
Concentration of TIMP-1	6.33 ± 2.81 ng/mL	4.55 ± 2.06 ng/mL	0.081
Concentration of TIMP-2	12.62 ± 3.47 ng/mL	8.85 ± 4.33 ng/mL	0.028†

\*The values are given as the mean and standard deviation. †The value was determined to be significant with the independent-sample t test.

(range, 2.2 to 3.3 mm) in those with lumbar disc herniation. The difference was significant ( $p < 0.001$ ) (Table I).

#### *TIMP-1 and 2 Concentrations*

With the number of samples available, there was no significant difference in the mean concentration of TIMP-1 between the patients with lumbar spinal stenosis ( $6.33 \pm 2.81$  ng/mL) and those with lumbar disc herniation ( $4.55 \pm 2.06$  ng/mL) ( $p = 0.08$ ) (Table I). In contrast, the difference between the mean concentration of TIMP-2 in the patients with lumbar spinal stenosis ( $12.62 \pm 3.47$  ng/mL; range, 6.9 to 19.1 ng/mL) and that in the patients with disc herniation ( $8.85 \pm 4.33$  ng/mL; range, 4.3 to 12.6 ng/mL) was significant ( $p = 0.028$ ) (Table I). Fibroblasts in the ligamentum flavum of both groups of patients showed positive cytoplasmic staining for TIMP-1 and TIMP-2 (Figs. 1-A and 1-B).

#### *Correlation Between TIMP Concentration and Ligamentum Thickness*

The Pearson correlation test showed a positive correlation between the TIMP-2 concentration and the thickness of the ligamentum flavum (correlation coefficient = 0.631,  $p = 0.003$ ) and between the TIMP-1 concentration and the thickness of the ligamentum flavum (correlation coefficient = 0.534,  $p = 0.015$ ).

#### *Apoptosis of Ligamentum Flavum Cells*

There was a mean of  $35.7 \pm 18.4$  total ligamentum flavum fibroblasts and  $21.0 \pm 10.7$  TUNEL-positive ligamentum flavum fibroblasts per ten high-power fields ( $\times 400$ ) in the specimens from the patients with spinal stenosis; the apoptosis index was  $58.8\% \pm 18.3\%$  (Figs. 2-A and 2-B). The specimens from the patients with disc herniation showed a mean of  $53.1 \pm 17.7$  total and  $14.1 \pm 5.63$  TUNEL-positive ligamentum flavum fibroblasts per ten high-power fields, and the apoptosis index was  $26.6\% \pm 21.3\%$ . The apoptosis index was significantly higher for the group with lumbar spinal stenosis than it was for the group with disc herniation ( $p < 0.001$ ), which disproved our theory that ligamentum flavum hypertrophy was due to a decrease in cell death.

#### *Fibroblast Proliferation in the Ligamentum Flavum*

None of the fibroblasts within the ligamentum flavum samples from the patients with spinal stenosis or from those with disc herniation stained positively with Ki-67 (cellular prolifer-

ation marker), suggesting that fibroblasts were not proliferating in any of the samples (Figs. 3-A and 3-B).

#### **Discussion**

There are four types of tissue inhibitors of matrix metalloproteinase (TIMP-1 through 4). They bind strongly but noncovalently to activated MMPs (matrix metalloproteinases), enzymes that digest proteins in the extracellular matrix. TIMPs are co-expressed with the MMPs and contribute to the regulation of their local activity so that increases in TIMP levels reduce MMP activity. Decreased MMP activity impairs matrix degradation, which has been demonstrated to be associated with fibrotic diseases. As the activity of MMPs is regulated by specific TIMPs, the imbalance between MMPs and TIMPs is thought to be an important determinant of extracellular matrix deposition and breakdown. TIMPs also have other important biological functions, including promotion of cellular proliferation and inhibition of apoptosis (programmed cell death) in a wide range of cell types.

On the basis of this information, we postulated that TIMPs may also play a role in the ligamentum flavum hypertrophy associated with lumbar stenosis, which is characterized by increased extracellular matrix and fibrosis in the ligamentum. We also hypothesized that TIMPs might influence ligamentum hypertrophy by increasing proliferation and decreasing apoptosis of the ligamentum fibroblasts. To test our hypotheses, we measured the concentrations of TIMP-1 and TIMP-2 in ligamentum flavum tissues from patients with lumbar spinal stenosis and compared them with those from a gender-matched group of patients who had lumbar disc herniation.

We found that the TIMP-2 concentration was significantly (1.43-fold) higher in the patients with spinal stenosis than it was in those with disc herniation ( $p = 0.028$ ), and a significant association was observed between the TIMP-2 concentration and the thickness of the ligamentum flavum ( $p = 0.003$ ). The TIMP-1 concentration in the patients with spinal stenosis was also positively associated with the thickness of the ligamentum flavum ( $p = 0.015$ ). In addition, TIMP-1 and 2 were positively stained in the cytoplasm of ligamentum flavum fibroblasts. These results suggest that increased expression of TIMPs, especially TIMP-2, in ligamentum flavum fibroblasts may be associated with hypertrophy of the ligamentum flavum in spinal stenosis.

We had theorized that TIMPs might play a role in steno-

sis by decreasing the rate at which fibroblasts undergo programmed cell death, resulting in increased cellularity. We found that the expression of intracellular TIMP-2 in the ligamentum flavum fibroblasts was higher in the patients with spinal stenosis than in the patients with disc herniation. However, contrary to our hypothesis, the programmed death rate of fibroblasts was significantly higher and the number of total cells was lower in the patients with spinal stenosis than they were in those with disc herniation. These findings suggest that the ligamentum flavum hypertrophy in spinal stenosis is not caused by an increase in cell number resulting from a delay in cell death. Furthermore, these results suggest that TIMPs do not have the biologic function of inhibiting apoptosis of ligamentum flavum fibroblasts.

We had also theorized that TIMPs might play a role in ligamentum flavum hypertrophy not just by decreasing cell death, but also by inducing fibroblastic cell proliferation. We utilized the Ki-67 antigen, a nuclear protein, which is preferentially expressed during all active phases of the cell cycles ( $G_1$ , S,  $G_2$ , and M-phase) as well as mitosis but is absent in resting cells ( $G_0$ -phase). The antigen is rapidly degraded as the cell enters the nonproliferative state. Therefore, the Ki-67 antigen is accepted as an excellent marker for cellular proliferation. Again, contrary to our hypothesis, the ligamentum flavum fibroblasts from the patients with spinal stenosis and those with disc herniation tested negative for the Ki-67 antigen, suggesting that they were not proliferating. These results suggest that TIMPs do not have the biologic function of inducing proliferation of ligamentum flavum fibroblasts.

Few authors have investigated the biochemical etiology of ligamentum flavum hypertrophy. We previously demonstrated the expression of transforming growth factor-beta 1 (TGF- $\beta$ 1) in ligamentum flavum fibroblasts and found that the concentration was significantly higher in patients with spinal stenosis than it was in patients with disc herniation<sup>28</sup>. In an *in vitro* study, Nakatani et al. found that mechanical stretching force promotes collagen synthesis by cultured cells from human ligamentum flavum tissues through increased TGF- $\beta$ 1 production<sup>29</sup>. These studies support our finding that TGF- $\beta$ 1 can influence the expression of both MMPs and TIMPs<sup>30,31</sup>. Together, these studies suggest that biochemical as well as mechanical factors should be considered as possible etiologies of ligamentum flavum hypertrophy in lumbar spinal stenosis.

As with any investigation, our study had limitations. First, the patients with spinal stenosis were significantly older than those with disc herniation. Therefore, we cannot exclude the possibility that the natural aging process had an impact on the

hypertrophy of the ligamentum flavum or on the other variables that we measured in this study. In other words, some (perhaps all) of the changes that were noted in our study could have simply been related to age. Second, it is not known whether the increased concentration of TIMP-2 in the patients with spinal stenosis was a local or systemic phenomenon. To address this issue, we are currently in the process of comparing serum TIMP-1 and TIMP-2 concentrations in patients with lumbar spinal stenosis with those concentrations in normal healthy volunteers.

In conclusion, the increased expression of TIMPs, especially TIMP-2, in ligamentum flavum fibroblasts might result in fibrosis and hypertrophy of the ligamentum flavum in spinal stenosis by inhibiting MMP activity, but not by causing proliferation of ligamentum flavum fibroblasts or inhibiting apoptosis of ligamentum flavum fibroblasts. Gaining insights into the biochemical pathophysiology of ligamentum flavum hypertrophy might elucidate novel noninvasive therapeutic approaches to the treatment of spinal stenosis. ■

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