Hypertrophy of the ligamentum flavum in lumbar spinal canal stenosis is associated with increased bFGF expression

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Abstract

Purpose A prospective study was undertaken to investigate basic fibroblast growth factor (bFGF) expression in hypertrophic ligamentum flavum (LF) from patients with lumbar spinal canal stenosis (LSCS) and to determine whether there was a correlation of bFGF expression with LF thickness.

Methods Twenty patients with lumbar spinal canal stenosis were enrolled in this study. bFGF mRNA and protein expressions in LF were analyzed using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA), respectively. The thickness of LF was measured by axial T1-weighted magnetic resonance imaging.

Results Expression of bFGF was substantially higher in the hypertrophic LF group than in the control group ($P<0.001$)

as quantified by quantitative real-time PCR. In immunohistochemical study, bFGF was positively stained on the fibroblasts within hypertrophic LF compared to nonpathologic LF of controls. Subsequent ELISA analysis revealed that bFGF concentration in the hypertrophic LF group was remarkably higher than that in the control group ($P=0.003$). The thickness of LF in the hypertrophic LF was significantly greater than that in the control group ($P<0.001$). LSCS patients with greater severity of LF hypertrophy had significantly higher bFGF levels in the LF tissues ($P<0.001$). Furthermore, the bFGF concentration exhibited a positive correlation with the LF thickness ($r=0.974$, $P<0.001$).

Conclusions These findings suggest that the increased expression of bFGF is associated with the hypertrophy of ligamentum flavum in patients with LSCS.

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Introduction

Lumbar spinal canal stenosis (LSCS) is one of the most common spinal disorders in the elderly and is secondary to the growth of osteophytes, redundancy of the ligamentum flavum (LF), and posterior bulging of the intervertebral discs [1]. Lumbar stenosis may be located in the centre of the canal, the lateral recess, or the intervertebral foramen, and may occur at single or multiple levels. Hypertrophy of LF commonly is a main contributing factor for the narrowing of the lumbar spinal canal [2, 3]. With central canal stenosis, pain in one or both legs occurs with walking. Surgical decompression is indicated for patients who are totally incapacitated by pain. Several environmental, mechanical, and biochemical factors have been recognized as playing a crucial role in LSCS development. Although mechanical stress affecting the ligament is proposed to be the most important aetiological factor, the precise reasons for the hypertrophy of the LF remain poorly understood.

Fibroblast growth factors comprise a large cytokine family of structurally related multifunctional polypeptide mitogens of widespread tissue distribution [4]. They employ mitogenic activity towards a variety of cells and play crucial roles in cell proliferation, differentiation, angiogenesis, and tissue repair. Nine members of the fibroblast growth factor family have been identified. Basic fibroblast growth factor (bFGF, also known as FGF-2) is one of the most well-characterized members of the family and one of the most powerful angiogenic polypeptide with a molecular weight of 18 kD [5]. bFGF secreted by fibroblasts, platelets or other cells is responsible for connective tissue formation and wound healing [6–8]. A previous study has demonstrated that bFGF expression was evident in LF degeneration and was involved with calcium pyrophosphate crystal deposition in the LF of degenerated lumbar spine [9].

We hypothesized that hypertrophy of LF could be associated with bFGF expression in LSCS patients. To prove this hypothesis, we have examined the transcript and protein expressions of bFGF in LF of LSCS patients. The objective of the present study was to investigate basic fibroblast growth factor expression in hypertrophic LF from patients with lumbar spinal canal stenosis and to determine whether there was a correlation of bFGF expression with LF thickness. To our knowledge, there has been no published data regarding the correlation between hypertrophy of LF and expression of bFGF.

Patients and methods

Patients

This study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University. The present study was conducted in compliance with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from the patients prior to their participation in the study.

Twenty patients aged 50 to 80 years diagnosed with one-level lumbar spinal canal stenosis at L4/L5 necessitating decompressive surgery (12 females and eight males; mean age 62.1 ± 11.1 years) were prospectively recruited in the present study. Patients with degenerative spondylolisthesis and concurrent lumbar disc herniation were excluded from this study. Specimens of hypertrophic LF for a pathologic LF group were obtained at surgery from patients with LSCS, whose pre-operative magnetic resonance images (MRI) showed marked hypertrophy of LF with single level at L4/L5. Nonpathologic LF specimens from L3/L4 selected from the same patients served as a control group. The entire layers of the central portion of the LF were carefully obtained for the appropriate specimen. All tissue samples were stored in liquid nitrogen immediately after harvesting

and kept at -80°C until analysis. Nine samples of each group were utilized for RT-PCR reactions.

Laboratory methods

Real-time reverse transcriptase-polymerase chain reaction

Quantitative measurement of mRNA expression in LF was investigated using real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Frozen LF tissue samples were crushed with mortar and pestle under liquid nitrogen and total RNA was extracted with RNeasy mini kit (Qiagen, Valencia, USA). Real-time RT-PCR was performed using SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, USA) in a Corbett research Rotor-Gene 3000 according to the manufacturer's instruction. Human gene-specific primer sequences were as follows:

bFGF forward primer: 5'-TAC AAC TTC AAG CAG AAG AG-3'

bFGF reverse primer: 5'-CAG CTC TTA GCA GAC ATT GG-3'

GAPDH forward primer: 5'-GTG AAG GTC GGA GTC AAC GG-3'

GAPDH reverse primer: 5'-TCA ATG AAG GGG TCA TTG ATG G-3'

The thermal cycling protocols were as follows: 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds for 30 cycles. The PCR products were visualized on 2.0 % agarose gel with ethidium bromide staining, and analyzed by the gel imaging system. Subsequent real time RT-PCR was utilized to obtain quantitative expression data. Three-step amplification was carried out in the presence of SYBR green dye to determine gene expression levels. The bFGF mRNA was normalized to the GAPDH mRNA in each sample. Gene expression was quantified using the $\Delta\Delta\text{CT}$ method [10] calculating the difference between the threshold cycle (CT) values of the target and reference gene of each sample, and comparing the resulting between different samples.

Determination of bFGF protein expression from LF

Frozen LF tissue samples (30 mg) were crushed and homogenized with phosphate-buffered saline at 3,000 rpm and then this was lysed in lysis buffer. The supernatant was obtained following centrifugation at 15,000 rpm at 4°C for 30 minutes. Quantification of protein was performed according to Bradford's method with using the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Company, Rockford, USA) and using bovine serum albumin as a standard. The optical density was measured at 570 nm by spectrophotometer. LF specimen extracts containing 100 μg of protein were utilized

for quantitative analysis of bFGF. Protein extracts were analyzed for bFGF using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D Systems, Minneapolis, USA) according to manufacturer's protocols. Recombinant human bFGF provided by the supplier was utilized for calibration. bFGF values were normalized by the protein content in each well.

Immunohistochemistry of the bFGF stained LF specimens

The LF specimens were immediately fixed in 10 % formaldehyde and embedded in paraffin. Serial sections of paraffin-embedded tissue were cut in 5- μ m thickness and processed for bFGF staining. Sections were deparaffinized and rehydrated in Tris-buffered saline. Endogenous peroxidase activity was blocked with 0.3 % H_2O_2 for ten minutes. For antigen retrieval, tissue sections were microwave heated in 10 mmol/L citrate buffer for five minutes. Nonspecific binding was blocked for ten minutes with 5 % goat serum (DAKO, Glostrup, Denmark), followed by incubation with antibody to bFGF (1:500; Santa Cruz Biotech, Santa Cruz, USA) in Tris-buffered saline containing 2 % rabbit serum and 1 % bovine serum albumin for two hours. Tissues were incubated with the same buffer without the antibody to serve as negative controls. Sections were subsequently stained with biotinylated goat antirabbit immunoglobulins (1:400; DAKO) and streptavidin/horseradish peroxidase complex (1:400; DAKO) and incubated at room temperature for 45 minutes. Reaction products were visualized using diaminobenzidine (Sigma, St. Louis, USA) as the chromogen. The sections were subsequently counterstained with Mayer's hematoxylin and mounted onto microscope slides using a permanent medium.

Measurement of LF thickness

The LF thickness was assessed in the patient's pre-operative MRI as previously described [11]. On the axial T1-weighted image through the facet joint, the maximum thickness of LF was traced by the manual cursor technique and measured automatically using a picture analyzing system (PACS, Agfa-Gevaert group, Mortsels, Belgium). Thickness at the middle portion of LF was measured [12]. The measurements of LF were repeated at least three times and their average value was recorded as the final LF thickness.

Statistical analysis

Statistical analysis was performed using the statistical package for social sciences (SPSS) software, version 16.0 for Windows. All values are expressed as mean $\pm$ standard deviation (SD). Student's *t*-test was employed to compare the means of two independent groups, and one-way analysis of

variance (ANOVA) was used to compare the means of more than two independent groups. Comparisons between groups were made using Mann–Whitney *U*-test (for two groups) or Kruskal–Wallis test (for more than two groups) when the variances were not equal among the groups. The correlation between bFGF expression and LF thickness was analyzed using Pearson's correlation coefficient (*r*). A *P*-value <0.05 was considered to indicate statistical significance.

Results

Reverse transcriptase-polymerase chain reaction analysis

bFGF gene expression was identified by the presence of the 283 bp product (Fig. 1). Standardization was performed using the GAPDH housekeeping gene as an internal control. Band density was expressed relative to the density of the GAPDH band. There was higher bFGF gene expression in the hypertrophic LF group as compared with the control group. In quantitative real-time PCR, the expression of bFGF was substantially higher in the hypertrophic LF group than in the control group ($P<0.001$) (Fig. 2).

bFGF expression by immunohistochemical analysis

Immunohistochemical study showed that bFGF was positively stained on the fibroblasts within hypertrophic LF compared to nonpathologic LF of controls (Fig. 3). The bFGF positive cell ratio in hypertrophic LF was significantly higher than that in nonpathologic LF of controls ($P=0.02$). Marked bFGF expression was evident in the fibroblasts

Fig. 1 RT-PCR amplification of bFGF in human LF. The densitometric band intensity of PCR products was displayed after normalization by GAPDH. M = DNA standard marker. **a** Hypertrophic LF. **b** Control

Fig. 2 Comparison of relative amount of bFGF mRNA normalized by GAPDH between hypertrophic LF and control using quantitative real-time PCR

and inflammatory cells and slight expression was noted in the extracellular matrix of hypertrophic LF. In contrast, slight bFGF expression was observed the fibroblasts in nonpathologic LF of controls (Fig. 3).

Correlation between bFGF measurement and LF thickness

In the ELISA study, the average level of bFGF in the hypertrophic LF (256.7 ± 205.9 pg/mg protein) was remarkably

elevated compared with that of controls (75.7 ± 36.6 pg/mg protein, $P=0.003$). The mean thickness of LF was 4.0 ± 1.1 mm in the hypertrophic LF and 2.5 ± 0.4 mm in the controls. A significant difference was observed in the LF thickness between the two groups ($P<0.001$). In order to compare bFGF level in LF based on the severity of LF hypertrophy, the pathologic LF tissue samples of LSCS patients were divided into three categories as follows: mild (<3 mm LF thickness, $n=7$), moderate (3–5 mm LF thickness, $n=8$), and severe (>5 mm LF thickness, $n=5$). The bFGF levels in LF of mild, moderate, and severe hypertrophy groups were 65.7 ± 41.8 , 245.5 ± 44.6 , and 573.0 ± 107.3 pg/mg protein, respectively. As illustrated in Fig. 4, LSCS patients with greater severity of LF hypertrophy had significantly higher bFGF levels in the LF tissues ($P<0.001$). Furthermore, the bFGF concentration exhibited a positive correlation with the LF thickness ($r=0.974$, $P<0.001$).

Discussion

Ligamentum flavum is also called the yellow ligament since the normal LF is yellow due to its greater content of elastic fiber than collagen fibers. The LF covers most of the posterior and lateral part of the lumbar spinal canal [13]. Thickening of the LF can reduce the diameter of the spinal canal, compressing the dural sac and nerve roots, resulting in low back pain and sciatica [14]. Degenerative changes secondary to the aging process injuries and mechanical stress such as instability attribute to hypertrophy of the ligamentum flavum and to lumbar spinal stenosis [11, 15–17].

Fig. 3 Representative immunohistochemistry of bFGF expression in the LF tissues. **a, b** Pictures shown are representative of the LF tissues with severe hypertrophy. Note the brown, positively stained fibroblasts within hypertrophic LF are strong positive for bFGF. **c, d** Pictures shown are representative of the LF tissues from control. bFGF expression is slightly detected in the fibroblasts of nonpathologic LF. (**a** and **c** X100, **b** and **d** X400)

Fig. 4 Comparison of bFGF levels in LF classified according to the severity of LF hypertrophy (LF thickness) and control

In the recent years, several cytokines and growth factors have been studied in patients with LSCS. TGF- β has been documented to play a significant part for the hypertrophy of LF [18]. Zhang and coworkers reported that the increased expression of platelet-derived growth factor-BB was observed in the hypertrophic lumbar LF [19]. Furthermore, connective tissue growth factor expression was upregulated and correlated with the LF thickness [20]. However, the role of bFGF in the hypertrophy of LF has not been studied.

In this study, the mean thickness of hypertrophic LF group was significantly greater than that in the control group. In addition, there was greater bFGF expression in the hypertrophic LF of LSCS patients as quantified by RT-PCR and real-time PCR analysis. Furthermore, immunohistochemical study showed significantly higher numbers of bFGF positive cells in the hypertrophic LF group than in the control group. To the best of our knowledge, the present study is the first to document the correlation of bFGF expression and hypertrophic LF and illustrate the involvement of bFGF in hypertrophy of the LF in LSCS patients.

bFGF is a profibrotic factor which plays an essential role in the production and maintenance of fibrotic lesion [21–23]. Therefore, we postulate that bFGF could be a possible candidate involved in the hypertrophy of LF in lumbar spinal stenosis. To prove our hypothesis, we performed the quantitative real-time PCR, immunohistochemical, and ELISA studies using surgical specimens. In immunohistochemical study, intense staining was evident in the fibroblasts, infiltrated inflammatory cells, and extracellular matrix of hypertrophic LF, but weak staining in the LF of controls, indicating that bFGF is principally synthesized by those cells and responsible for the elevated expression of bFGF in the hypertrophic LF of LSCS. Subsequent ELISA analysis showed there was an approximate 3.5-fold increase of bFGF level in the hypertrophic LF group compared

with that in the control group. Moreover, there was a strongly positive correlation between the bFGF protein expression and the LF thickness. These findings suggested that the enhanced expression of bFGF by fibroblasts could be responsible for the development of hypertrophy of the LF via autocrine or paracrine mechanisms in lumbar spinal stenosis.

It should be mentioned, however, that this study inevitably has some limitations. First, since this was an association study, it might not establish the causal relationship between bFGF and the LF thickness. It is not certain which factors directly contribute to the hypertrophy of the LF. Second, our study was based on a single-center trial. Prospective study conducted on a random sample of multiple centers with larger sample size will be needed. Third, it is not precisely known whether the upregulated expression of bFGF in hypertrophic LF was a local or systemic phenomenon. The mechanisms underlying increased bFGF expression in the hypertrophic LF remain to be further investigated. Correlation of bFGF levels and patient age of some LSCS subjects has been studied recently [24]. We have not previously reported bFGF expression using immunohistochemistry or the relationship between the severity of LF hypertrophy (LF thickness) and bFGF in LSCS.

In summary, the current study demonstrates that bFGF expression is increased in hypertrophic LF in LSCS and bFGF protein expression is positively correlated with the LF thickness. These findings suggest that the increased expression of bFGF is associated with hypertrophy of the LF in patients with lumbar spinal canal stenosis. Gaining insights into the role of bFGF in pathogenesis of LF hypertrophy may highlight possible therapeutic alternatives for the treatment of lumbar spinal stenosis.

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