Basic fibroblast growth factor expression in hypertrophic ligamentum flavum of lumbar spinal stenosis

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Background: Lumbar spinal canal stenosis is the most common spinal disorder in elderly patients. Canal narrowing is partly the result from hyperthrophy of ligamentum flavum (LF), which mechanically compresses nerve roots. Basic fibroblast growth factor (bFGF) is a potent regulator of many cellular functions including proliferation, differentiation, wound healing, and angiogenesis.

Objective: We examined the pattern of bFGF expression in the ligamentum flavum of lumbar spinal stenosis patients.

Methods: We quantified and localized bFGF expression in LF tissues obtained during surgery from nineteen patients with lumbar spinal stenosis. bFGF expression was determined using quantitative real-time polymerase chain reaction (real-time PCR). The concentration of bFGF was analyzed by enzyme-linked immunosorbent assay.

Results: The bFGF expression was significantly higher in hypertrophic LF of spinal stenosis than that in non-pathologic LF of controls. In real-time PCR, the expression of bFGF was substantially higher in the hypertrophic LF than in controls (P<0.001). The average concentration of bFGF in the hypertrophic LF (mean, 256.7 pg/mg protein) was markedly elevated compared with that of controls (mean, 75.7 pg/mg protein) (P=0.003). There was greater bFGF expression in lumbar spinal stenosis patients as quantified by real-time PCR and enzyme-linked immunosorbent assay.

Conclusion: Our study reveals that increased bFGF expression may be associated with degenerative changes of hypertrophic LF. This suggests that bFGF may contribute to pathogenesis of lumbar spinal stenosis.

Keywords: Basic fibroblast growth factor, ligamentum flavum, lumbar spinal stenosis
of the ligamentum flavum and was associated with calcium crystal deposition in the ligamentum flavum of lumbar spinal stenosis patients [9].

In the present study, we postulate that degenerative changes in LF result in fibroblast proliferation and that these changes are accompanied by an increase in bFGF synthesis. Therefore, LF degeneration and high bFGF expression suggest cellular and molecular events which contribute to the pathogenesis of spinal stenosis. To test this hypothesis, we investigated the bFGF expression in LF specimens from lumbar spinal stenosis patients. The purpose of this study was to determine transcript and protein levels of bFGF in LF of patients with lumbar spinal stenosis. The secondary objective was to correlate the expression of bFGF to the age of patients.

Patients and methods

Ethical approval of this study was obtained from the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University. The present study was conducted according to the guidelines of the Declaration of Helsinki. Written informed consent was received from every participant before entering the study.

Ligamentum flavum specimens were derived from 19 patients (8 male and 11 female) who had undergone decompressive surgery for treatment of symptomatic lumbar tenosis (such as neurogenic claudication or leg pain). Mean age of the patients at the time of surgery was 61.8±11.0 years (range, 45-78 years). Ligamentum flavum specimens were harvested from L4-L5 of each patient. All tissues were stored in liquid nitrogen immediately and kept at -80°C until further analysis.

Quantitative real-time polymerase chain reaction

Ligamentum flavum specimens were homogenized and total RNA samples were isolated using the Rneasy Mini Kit (Qiagen, Valencia, USA) according to the manufacturer’s recommendations. The purity and amount of isolated RNA was analyzed by spectrophotometric measurement at 260 and 280 nm. Total RNA 1 μg was reverse transcribed into cDNA with 0.5 μg of oligo dT primer, 10 mmol/L of each of the 4 dNTP, 25 mmol/L of MgCl₂, 10 U of RNA inhibitor and 50 U of superscript II reverse transcriptase (Invitrogen, Carlsbad, USA) according to the manufacturer’s instruction. The final solution was used directly for PCR amplification. Each cDNA reaction was diluted 5x, along with a calibrator sample containing the transcript of interest, and run in 25 μL of Bio-Rad SYBR Green reactions using 10 pmol of primers. Human gene-specific oligonucleotide sequences were as follows: bFGF, forward primer-5’-TAC AAC TTC AAG CAG AAG AG-3’, reverse primer-5’- CAG CTC TTA GCA GAC ATT GG-3’, GAPDH, forward primer-5’-GTG AAG GTC GGA GTC AAC GG-3’, reverse primer-5’-TCA ATG AAG GGG TCA TTG ATG G-3’. Reactions were processed using one initial denaturation cycle (5 minutes at 94°C), then 30 cycles of denaturation (30 s at 95°C), annealing (45 s at variable for gene), and amplification (1 minute at 72°C) followed by melt curve determination consisting of one denaturation cycle (1 minute at 95°C), annealing (one cycle for 1 minute at 55°C), and then 80 cycles (5 s each at 55°C-95°C). The GAPDH ribosomal RNA primer pair reaction was run on every sample for template content normalization purpose. A calibrator RT sample containing cDNA of interest was run to yield a standard curve for each primer set, and individual primer reaction efficiencies were calculated from this curve using the iCycler software. The iCycler software calculated a threshold cycler for each sample; threshold cycles and primer pair efficiencies were used in the ΔΔCT method to yield differences in each mRNA expression as normalized to the GAPDH housekeeping gene.

Protein extraction and quantification of bFGF from ligamentum flavum

A total of 30 mg of ligamentum flavum tissue was 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 bichinchoninic acid (BCA) protein assay kit (Pierce Chemical Company, Rockford, IL, USA) and using bovine serum albumin as a standard. The optical density was measured at 570 nm by spectrophotometer. Ligamentum flavum 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 instructions. Concisely, standards of recombinant human bFGF and protein extract samples were added to 96-well microtiter
plates precoated with mouse monoclonal antibody against human bFGF and incubated for two hours at room temperature. The wells were then washed four times with washing buffer and incubated for two hours at room temperature with a horseradish peroxidase-conjugated monoclonal antibody against bFGF. After four washes, substrate solution was added to each well, and the plate was incubated for 30 minutes at room temperature in the dark. Finally, the reaction was stopped with the stop solution, and then absorbance was measured at 450 nm using automated microplate reader. The bFGF level was determined from a standard curve generated for each set of samples assayed.

Statistical analysis
Statistical analyses were performed using the SPSS version 16.0 software package (SPSS Inc., Chicago, IL, USA). Data are expressed as means±standard deviation (SD). Comparisons between groups were analyzed by the Mann-Whitney U test and unpaired t-test. Correlation between bFGF in protein extracts and the patient age was assessed using Pearson’s correlation coefficient (r). P-values <0.05 were considered to be statistically significant for differences and correlations.

Results
The mean age of patients at time of surgery was 61.8±11.0 years (range, 45-78 years). In quantitative real-time PCR, we observed bFGF expression in the ligamentum flavum of both pathologic LF and non-pathologic LF groups. The mean of relative bFGF mRNA expression ratios were 1.14x10^5 for the pathologic LF group and 26.12 for the non-pathologic LF group, respectively (P<0.001). The bFGF mRNA expression in the pathologic LF group was remarkably higher than in the non-pathologic LF group (Figure 1).

The mean concentration of bFGF in protein extract of LF tissues was 256.7 pg/mg protein in the pathologic LF group and 75.7 pg/mg protein in the non-pathologic LF group (P=0.003). bFGF level in protein extracts was significantly greater in the pathologic LF groups when compared to the non-pathologic LF groups (Figure 2). Moreover, there was a positive correlation between the tissue bFGF expression of the pathologic LF group and patient age in spinal stenosis patients (r=0.63, P<0.001) (Figure 3).

![Figure 1](image_url). Comparison of relative amount of bFGF mRNA normalized by GAPDH between pathologic and non-pathologic LF using quantitative real-time PCR.
Discussion

Lumbar spinal canal stenosis is a common age-related disorder resulting from degenerative processes in the posterior structures of the lumbar spine, such as disc bulging, bony proliferation of facet joints, as well as hypertrophy of ligamentum flavum [10-12]. A number of patients with lumbar spinal stenosis relieve symptoms after surgical treatment [13, 14]. Removal of pathologic ligamentum flavum is an essential goal of the surgical treatment. Although several

Figure 2. Comparison of concentration of bFGF in ligamentum flavum between pathologic and nonpathologic LF using ELISA.

Figure 3. Correlation of bFGF levels in pathologic ligamentum flavum and patient age.
investigations studied the mechanism of ligamentum flavum hypertrophy from anatomy, histology, and biology, the pathogenesis of ligamentum flavum hypertrophy remains unknown. Etiologies of ligamentum flavum hypertrophy are multifactorial including age, mechanical stress, and growth factors. The fibroblast growth factors comprise a large cytokine family of structurally related multifunctional polypeptide mitogens of widespread tissue distribution [15]. Nine members of the fibroblast growth factor family have been identified. Basic fibroblast growth factor (bFGF) is one of the first growth factors identified (also known FGF-2) and is responsible for the formation of connective tissue and wound healing [16, 17]. Excessive bFGF accumulation is associated with pathologic tissue fibrosis including degeneration of ligamentum flavum [9]. Fibrosis is the major contributor of ligamentum flavum hypertrophy, and fibrosis results from the accumulation of mechanical stress with the aging process and accumulation of inflammation-related scar tissue [18], particularly ligamentum flavum.

An interesting finding of this study was the significant positive correlation between tissue bFGF expression and patient age. The precise underlying relationship remains unclear. A recent study has illustrated a positive correlation between the thickness of ligamentum flavum and patient age in spinal stenosis [19, 20], and bFGF expression was significantly higher in the pathologic LF groups when compared to the nonpathologic LF groups. It was postulated that bFGF might play a potential role in the pathogenesis of ligamentum flavum hypertrophy in lumbar spinal stenosis patients. Further investigation is essential to clarify the relationship between patient age and tissue bFGF expression in spinal stenosis patients.

Our study had some limitations. It was designed as a cross-sectional examination of degenerative changes and definite cause and effect relationships may not be concluded. Another potential limitation is its small sample size for providing representative statistical analysis. It is not clear which factors directly contribute to the hypertrophy of ligamentum flavum in lumbar spinal stenosis. Moreover, it is not exactly known whether the elevation of bFGF expression in lumbar spinal stenosis was a local or systemic phenomenon. To address this issue, circulating and tissue bFGF expression could render more valuable information on the pathogenic role of bFGF in spinal stenosis. Additional studies are required to definitively resolve these problems.

In summary, the current study demonstrated higher bFGF expression in the pathologic LF group as compared with non-pathologic LF group. The increased expression of bFGF in ligamentum flavum could lead to fibrosis and hypertrophy of the ligamentum flavum in lumbar spinal stenosis. To our knowledge, this study is the first to investigate bFGF expression in the ligamentum flavum of lumbar spinal stenosis patients and demonstrate the relationship between bFGF expression in ligamentum flavum hypertrophy and patient age. Further researches on the biochemical pathophysiology of ligamentum flavum hypertrophy are warranted to gain insights into the potential noninvasive treatment of degenerative spinal diseases.

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References


