Original Article
Transforming growth factor β/Smad pathway participates in lumbar ligamentum flavum hypertrophy

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Received January 18, 2017; Accepted March 15, 2017; Epub May 1, 2017; Published May 15, 2017

Abstract: Aims: Hypertrophy of ligamentum flavum (LF) contributes to lumbar spinal stenosis (LSS) and is caused mainly by fibrosis. This study aimed to investigate the involvement of TGFβ/Smad pathway in human lumbar LF hypertrophy. Methods: Six patients with LSS were enrolled in this study. The control group included six patients with lumbar disc herniation (LDH). LF samples were collected during surgery for immunohistochemistry, real-time PCR and Western blot analysis. Human LF cells were cultured and treated with TGFβ1, and type I collagen and Smad expression were detected. Results: Hypertrophic LF was characterized by a considerable distortion of the elastic matrix and fibrotic transformation by extracellular collagen deposition. Immunohistochemical analysis detected TGFβ1, p-Smad 2/3, Smad 4 and Smad 7 in all LF samples. TGFβ1, p-Smad 2/3 and Smad 4 levels were significantly higher in LSS group than in LDH group, while Smad 7 level was lower in LSS group than in LDH group. The levels of Smad 2, 3 and 4 were slightly upregulated 1 h after treatment with TGFβ1. However, Smad 2/3 levels significantly decreased 24 h after TGFβ1 treatment. In contrast, Smad 7 showed dynamic change: expression was markedly increased 1 h after TGFβ1 stimulation but decreased at 24 h. The expression of type I collagen was increased at both 1 h and 24 h after TGFβ1 stimulation. Conclusion: During LF hypertrophy, the expression of TGFβ/Smad pathway components is changed. TGFβ/Smad pathway may contribute to LF hypertrophy.

Keywords: TGF-β/Smad pathway, ligamentum flavum, hypertrophy, fibrosis

Introduction
Lumbar spinal stenosis (LSS) is a common condition in elderly patients and can be caused by various factors related to the thickened ligamentum flavum (LF) [1, 2]. Also known as LF hypertrophy, LF thickness is a typical degenerative process that involves a decrease in the elastin-to-collagen ratio, resulting in decreased elasticity and increased stiffness or fibrosis [3-8]. The pathogenesis of LF thickness remains unclear.

Transforming growth factor β (TGFβ) plays diverse role in regulating growth, differentiation, immune response and tissue fibrosis [9-14]. It has been reported that TGFβ enhances wound healing and induces scar formation and tissue remodeling [15, 16]. TGFβ contributes to LF hypertrophy by enhancing matrix synthesis, especially collagen production [17-20]. A number of genes that are associated with tissue growth and fibrosis have been shown to be directly regulated by TGFβ/SMAD pathway [21, 22].

In this study we aimed to investigate the involvement of TGFβ/Smad pathway in LF hypertrophy. We compared TGFβ1, p-Smad 2/3, Smad 4, 7 and collagen levels in hypertrophied LF tissues and control tissues. In addition, we analyzed temporal changes of p-Smad 2/3 and Smad 4, 7 levels in LF cells after treatment with TGFβ1.

Material and methods
Specimen collection
LF samples were obtained from 12 patients (7 males, 5 females, and average 68.7 years old, range 63-73 years) who underwent decompressive laminectomy due to symptomatic degenerative lumbar spinal stenosis. As the control, LF samples were obtained from 12 patients (8 males, 4 females, and average 32.5 years old,
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range 15-38 years) with lumbar disc herniation who were operatively treated for this disorder. LF samples were from L4/5 and subjected to histological staining, immunohistochemical analysis and biological evaluation. The study was approved by the institutional ethics review board with written informed consent obtained from each patient.

Histological and immunohistochemical analysis

Specimens were cut sagittally, fixed in 10% formalin for 48 h and embedded in a paraffin block. Thin-sliced sections (4 μm) were prepared and stained by hematoxylin and eosin (H&E) staining by an experienced pathologist. For immunohistochemistry, sections were incubated with rabbit polyclonal antibody specific to p-Smad 2/3, Smad 4 (Abcam), rat polyclonal antibody specific to Smad-7 (Abcam) and rabbit polyclonal antibody to TGF-β (Abcam), and then incubated with secondary antibody and 3,3-diaminobenzidine tetrahydrochloride.

Real-time PCR

Total RNA was isolated by standard techniques using TRIzol (Invitrogen Corp., Carlsbad, CA). RNA was converted into cDNA and subjected to standard PCR analysis (40 cycles, 94°C for 30 seconds, 60°C for 20 seconds, 72°C for 15 seconds) using SYBR green dye on a thermal cycler. The following primers were used: β-Actin forward 5'-ATCTGGCACCACCTTCTACAATGCTGCG-3', reverse 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3', TGF-β1 forward 5'-CAACATTTCTCTGGCAGGATACTCA-3', reverse 5'-GTAGTAACCCCGTTGATGTC-3', Smad 2 forward: 5'-TTGATGGCCGTCTTCAGGTT-3', reverse: 5'-AGAGCCGGCAATATAACATGTG-3', Smad 3 forward: 5'-TGGGCCTACTGTCCAATGTCA-3', reverse: 5'-TCCCAATGTGTCGCCTTGTA-3', Smad 4 forward: 5'-TGGTGTTCCATTGCTTACTTTG-3', reverse: 5'-TCCCCATGCTGTTGCTGTA-3', Smad 5 forward: 5'-TTGATGCGCCTCTCAAGTTTA-3', reverse: 5'-CGTCATACTCCTGCTTGCGTATCCACATCTGC-3', reverse: 5'-GTAGTAACCCCGTTGATGTC-3', Smad 7 forward: 5'-TTGATGCGCCTCTCAAGTTTA-3', reverse: 5'-CGTCATACTCCTGCTTGCGTATCCACATCTGC-3', reverse: 5'-GTAGTAACCCCGTTGATGTC-3'. All the primers were synthesized by Shenggong, Inc. ΔΔCT method was used to calculate the difference between the threshold cycle (CT) values of the target and reference gene of each sample.

Primary culture of LF fibroblast cells

LF samples were obtained aseptically from six young patients undergoing spinal surgery. The dissected specimens were minced into small pieces and digested in serum-free medium (Gibco) containing 250 U/ml type I collagenase (Sigma) at 37°C in an atmosphere containing 5% CO₂. The digested specimens were washed with serum-containing medium to inhibit colla-
serum-starved overnight before treatment with human TGFβ1. The concentration of TGFβ1 was 10 ng/mL based on previous studies [23, 24]. To inhibit de novo RNA synthesis, cells were incubated with actinomycin D (2.5 μg/mL) for 24 h before stimulation with TGFβ1.

**Western blot analysis**

Tissue specimens or cells in culture were lysed in RIPA buffer supplemented with protease inhibitor cocktail. Equivalent amounts of protein were electrophoresed and transferred onto Immobilon-P membranes (Millipore). The membranes were blocked with 5% non-fat milk in Tris-buffered saline and then incubated with p-Smad 2/3, Smad 4, Smad 7, TGFβ1 and β-actin monoclonal antibody (Abcam, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibody (Abcam, USA). The signals were detected with an Immobilon Western chemiluminescent HRP substrate.

**Immunocytochemical staining**

Cells were seeded on 8-well chamber slides (Lab-Tek Chamber Slide, Nalge Nunc International, Rochester, NY, USA) at 1×10⁶ cells/slide in RPMI-1640 medium. Serum-starved subconfluent cells were treated with or without TGFβ1 and then fixed with 4% paraformaldehyde in PBS. Immunostaining was performed with similar procedures to those used for immunohistochemical staining. Slides were viewed under a fluorescent microscope and the data were analyzed with ImageJ 1.42.

**Statistical analysis**

Data were evaluated by a one-way ANOVA test. *P* value of less than 0.05 was considered statistically significant.
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Results

Histologic findings in hypertrophied ligamentum flavum

Histological analysis showed that the elastic fiber area decreased, and the collagen area increased in LF from LSS group compared to LDH group. In LDH group, rich elastic fibers were arrayed in a regular pattern, and the fibers were oriented parallel to the major axis of the ligamenta flava (Figure 1A-C). However, in LSS group, the elastic fibers were fragmented, disorganized, and focally lost. In areas with marked degeneration, the elastic fibers frequently showed disorganization of the fiber bundle arrangement, thinning of the elastic fibers, accompanied by the proliferation of collagen fibers in the matrix (Figure 1D-F).

Expression of TGFβ1, p-Smad 2/3, Smad 4 and 7 in LF tissues

Immunohistochemical analysis demonstrated that TGFβ1, p-Smad 2/3, Smad 4 and 7 were positively stained on LF fibroblasts of the patients with spinal stenosis and disc herniation (Figure 2).

Western blot analysis showed that the expression of p-Smad 2/3, Smad 4 and TGFβ1 in LF samples of LSS group was significantly higher than in LDH group. However, the expression of Smad 7 in LF samples of LSS group was lower than that of LDH group (Figure 3A, 3B).

Morphological changes of LF fibroblast cells stimulated by TGFβ

Next we examined the association between LF fibroblast cell differentiation and the activation of TGFβ1/Smad in human LF fibroblast cells. Compared with unstimulated cells (Figure 4A), TGFβ1 stimulated cells showed higher percentages of elongated and spindle-shaped cells after 1 h of treatment (Figure 4B). Twenty-four hours after TGF-β1 stimulation, cultured cells showed hypertrophy with a greater percentage of cells expressing type I collagen (Figure 4C). The percentages of cells positive for type I collagen in the untreated group and 1 hour and 24 hours after treatment groups were 15%, 25% and 33%, respectively, showing a significant increase following TGF-β1 stimulation.

Double immunostaining for type I collagen and p-Smad 2/3 showed, a large number of cells with nuclear reactivity for p-Smad 2/3 1 h after TGFβ1 stimulation. However, less than 10% of p-Smad 2/3 positive cells were positive for type I collagen (Figure 5A). Twenty-four hours after TGF-β1 stimulation, the percentage of cells with nuclear reactivity for p-Smad 2/3 was markedly decreased and more than 50% of stimulated cells were positive for type I collagen (Figure 5B).

Expression of Smad 2/3 and 4, 7 in LF fibroblast cells stimulated by TGFβ

Real-time PCR showed that mRNA levels of Smad 2, 3 and Smad 4 were slightly upregulated 1 hour after treatment with TGFβ1. However, at 24 hours after TGFβ1 treatment, Smad 2/3 expression was significantly inhibited. In contrast, Smad 7 mRNA level showed a dynamic change: markedly increased 1 hour after TGFβ1 treatment but then significantly decreased at 24 hours after treatment. The mRNA expres-
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Nakatani et al. showed that TGFβ1 induced collagen synthesis, especially type I collagen [26]. TGFβ1 has been widely implicated in the fibrotic process and has been shown to upregulate the production of several ECM proteins. Smad 2 and 3 are well-known downstream signaling molecules of TGFβ signaling and mediate transcriptional activation of collagen [9-12]. Because the phosphorylation of Smad 2/3 is a major step in the initiation of TGFβ signal transduction, changes in the phosphorylation of Smad 2/3 in the lumbar ligamentum flavum were investigated in this study. While normal ligamentum flavum showed the accumulation of phosphorylated Smad 2/3, the phosphorylation of Smad 2/3 was significantly increased in hypertrophic ligamentum flavum. These data suggest that p-Smad 2/3 levels change during inflammation and hypertrophy of the ligamentum flavum.

In present study, we detected the activation of TGFβ/Smad pathway in LF cells. TGFβ1 stimulation increased p-Smad 2/3 levels in LF cells at 1 hour after treatment and further increased p-Smad 2/3 levels at 24 hours after treatment.

Discussion

Hypertrophy of LF is a significant cause of lumbar spine stenosis, which is a common cause of low back and lower extremity pain, particularly in elderly patients [1, 2]. However, the molecular mechanisms underlying hypertrophic process remain unclear. Recent studies suggest that high expression of TGFβ1 by fibroblasts might be related to the development of LF hypertrophy [19, 21, 25-28]. In this study, we investigated the expression of TGFβ1 and agonistic (Smad 2, Smad 3, and Smad 4) and antagonistic (Smad 7) Smad proteins in LF samples from patients with lumbar spinal stenosis and disc herniation.

Some studies demonstrated that macrophages, fibroblasts and endothelial cells considerably contributed to TGFβ1 expression during the advanced stage of ligamentum flavum degeneration and may stimulate subsequent fibrosis and hypertrophy [25]. TGFβ1 increases collagen synthesis by fibroblasts in vitro [29-31].

Western blot analysis showed that type I collagen, p-Smad 2/3 and Smad 4 protein levels increased at 1 hour and further increased at 24 hours after TGFβ1 treatment. In contrast, Smad 7 protein level markedly increased 1 hour after TGFβ1 treatment but then significantly decreased at 24 hours after treatment (Figure 6B, 6C).

Figure 4. Florescent microscope analysis of LF fibroblasts. A. Untreated cells. B. 1 hour after TGFβ1 treatment (10 ng/mL). C. 24 hours after TGFβ1 treatment (10 ng/mL). The nuclei were stained blue (×100).

Figure 5. Double immunostaining for type I collagen and p-Smad 2/3 in LF fibroblasts. A. 1 hour after TGFβ1 treatment (10 ng/mL). B. 24 hours after TGFβ1 treatment (10 ng/mL). p-Smad 2/3 was stained green while type I collagen was stained red. Yellow indicated co-staining of p-Smad 2/3 and type I collagen (×100).
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Notably, increased p-Smad 2/3 levels were correlated with increased expression of type I collagen. These findings suggest that collagen deposition in the ligamentum flavum is dependent on TGFβ/Smad pathway [32]. In addition, our results showed that Smad 4 levels increased during inflammatory and hypertrophic phases of ligamentum flavum injury. These observations suggest that Smad 4 may play an important role in the induction of ECM components, such as collagen I, and thus contribute to the hypertrophy of ligamentum flavum tissue. As previously reported, increased Smad 4 expression and accumulation have been shown in various fibrotic tissues [33-36].

TGFβ/Smad signaling is well-known for an auto-inhibitory loop that involves Smad 7 [11, 18]. In this study we found that Smad 7 protein levels decreased in LSS group compared with control tissues, consistent with a causal relationship between decreased Smad 7 and fibrosis [37-39]. Nakao et al. demonstrated that Smad 7 suppressed type I collagen mRNA in lung fibrotic lesions [40]. Moreover, inhibition of endogenous Smad 7 function in normal fibroblasts resulted in enhanced collagen synthesis [41]. These observations strengthen the emerging paradigm that decreased Smad 7 expression may be an important mechanism underlying enhanced TGF-β activity in the hypertrophy of the ligamentum flavum.

There are several limitations to our present study. First, our sample size was limited by the ethics review board, and we could not collect normal LF. Therefore, we used control specimens from patients with disc herniation whose average age was significantly younger than that of the patients with spinal stenosis. Therefore, we cannot exclude the possibility that aging may have an impact on the status of TGFβ/Smad pathway. Second, we only used 10 ng/mL concentration of TGFβ1 to treat LF fibroblast cells and observed the changes at 1 hour and 24 hours. Further investigations using a range of stimulation responses over a range of time points are needed.

In conclusion, we reported the expression of Smad in hypertrophic LF samples and changes in Smad expression in cultured LF cells after TGFβ1 stimulation of. Our results provide strong evidence that TGFβ/Smad pathway participate in the hypertrophy of LF.

Acknowledgements

This study was supported by Medical-Engineering Joint Fund of Shanghai Jiao Tong University (No. YG2015MS50).
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Disclosure of conflict of interest

None.

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References

Löhr M, Hampl JA, Lee JY, Ernestus RI, Deckert M, Stenzel W. Hypertrophy of the lumbar ligma-


Park JB, Chang H, Lee JK. Quantitative analysis of transforming growth factor-beta1 in liga-

Kim HJ, Park JB, Won HY, Chang H. Serum lev-
els of TGF-beta1, TIMP-1 and TIMP-2 in pa-

Rdghow K, Postlehwaite AE, Keskı-Oja J, Mo-
es HL, Kang AH. Transforming growth factor-beta increases steady state levels of type I procollagen and fibronectin messenger RNAs post-
transcriptionally in cultured human dermal fi-


Rosendahl A, Checchin D, Fehniger TE, ten Di-
jke P, Heldin CH, Sideras P. Activation of the TGF-beta/activin-Smad 2 pathway during aller-

Dong C, Zhu S, Wang T, Yoon W, Li Z, Alvarez RJ, ten Dijke P, White B, Wigley FM, Gold-schmidt-Clermont PJ. Deficient Smad 7 expres-
sion: a putative molecular defect in scleroder-

Takagawa S, Lakos G, Mori Y, Yamamoto T, Nishioka K, Varga J. Sustained activation of fibroblast transforming growth factor-beta/Smad signaling in a murine model of sclero-


Nakao A, Fujii M, Matsumura R, Kurokawa T, Nishio K, Varga J. Sustained activation of fibroblast transforming growth factor-beta/Smad signaling in a murine model of sclero-

Chen SJ, Yuan W, Lo S, Trojanowska M, Varga J. Interaction of Smad 3 with a proximal smad-
binding element of the human alpha2(I) pro-
collagen gene promoter required for transcrip-