Angiopoietin-like Protein 2 Contributes To The Degeneration And Hypertrophy Of Ligamentum Flavum In Lumbar Spinal Canal Stenosis

Takayuki Nakamura1, Toru Fujimoto1, Takaumi Nakamura2, Hiroshi Mizuta1.
1Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan, 2Nakamura Orthopaedic Clinic, Kumamoto, Japan.


Introduction: Chronic inflammation and subsequent fibrosis induced by mechanical stress play an important role in ligamentum flavum (LF) hypertrophy and degeneration in patients with lumbar spinal canal stenosis (LSCS), and several molecules, such as transforming growth factor (TGF)-β1, inflammatory cytokines, and matrix metalloproteinase (MMPs) participate in the pathological processes. However, the mechanisms underlying the induction of these molecules have not been fully elucidated. Angiopoietin-like protein 2 (Angptl2), a tissue remodeling factor, is induced by various stress conditions and regulates TGF-β1, inflammatory cytokines such as interleukin(IL)-6, and MMPs. In this study, we investigated whether Angptl2 contributes to the pathogenesis of LSCS by analyzing Angptl2 expression and function in LF tissue and LF fibroblasts obtained from LSCS patients.

Methods: This study was conducted after approval was obtained from the Kumamoto University Ethics Committee and written informed consent was received from each patient. LF samples (58) for this study were provided by 31 patients (21 male and 10 female) who underwent lumbar surgery. LFs from the stenotic intervertebral levels comprised the samples for the LSCS group (n = 43; mean age, 66.8 years). LFs from the intervertebral levels of patients with diseases other than LSCS, such as lumbar disc herniation and cauda equina tumors, comprised the samples for the non-LSCS group (n = 15; mean age, 61.9 years). LF thickness was measured at the facet joint level by T1-weighted magnetic resonance (MR) imaging. Total RNA was extracted from LF tissue, and Angptl2 mRNA expression was evaluated by real-time polymerase chain reaction (PCR). Anti-human Angptl2 antibody was used for immunohistochemistry. For in vitro experiments, LF fibroblasts were isolated from LF tissue and cultured. LF fibroblasts were applied to stretching stimulation (10% elongation, 10 cycles/min) for 12 h, and Angptl2 expression was investigated by PCR and Western blot analysis. Recombinant Angptl2 protein (5 μg/ml) was added to the cells, followed by 6 h incubation, after which the RNA was extracted, and TGF-β1, IL-6, and MMP2 mRNA expression was evaluated by PCR.

Results: LF thickness was significantly increased in the LSCS group relative to the non-LSCS group (P < 0.01; Figure 1-A). Angptl2 mRNA expression in hypertrophied LF tissue from the LSCS group was also significantly increased relative to that in LF tissue from the non-LSCS group (P < 0.01; Figure 1-B), and it was positively correlated with LF thickness (R = 0.60, P < 0.01; Figure 1-C). In immunohistochemistry, we found a markedly increased number of Angptl2-expressing fibroblasts in hypertrophied LF tissue from the LSCS patient group relative to normal LF tissue from non-LSCS control subjects (Figure 1-D). These findings suggest that Angptl2 produced by fibroblasts could contribute to LF hypertrophy in LSCS pathogenesis. In vitro experiments, we examined whether Angptl2 is induced by mechanical stress in LF fibroblasts. Angptl2 mRNA and Angptl2 protein expression in LF fibroblasts were elevated after stretching stimulation (Figure 2), suggesting that mechanical stress induced Angptl2 expression in LF...
tissue. Next, we examined whether Angptl2 regulates TGF-β1, IL-6, and MMP2 mRNA expression. These gene expression in LF fibroblasts were elevated after treatment with Angptl2 recombinant protein (Figure 3). These results suggest that Angptl2 could upregulate TGF-β1, IL-6, and MMP2 expression in LF tissue.

**Discussion:** This study shows that Angptl2 was abundantly expressed in hypertrophied LF tissue, and Angptl2 expression was induced by mechanical stress, in addition, Angptl2 activated TGF-β1, IL-6, and MMP2 expression in LF fibroblasts. In conclusion, Angptl2 induced by mechanical stress in LF fibroblasts promotes LF tissue degeneration by activating the TGF-β1, IL-6, and MMP2 expression, thus resulting in LF hypertrophy in patients with LSCS.

**Significance:** Our findings suggest that Angptl2 could play an important role in the mechanism underlying LF degeneration and hypertrophy, and might lead to detail elucidation of the mechanism underlying LSCS pathogenesis.
Figure 1. Angpt2 expression is positively correlated with the thickness of the LF and Immunohistochemistry for Angpt2 in hypertrophied LF tissue from LSCS patients.

A, B: Comparison of ligamentum flavum (LF) thickness (A) and Angpt2 mRNA expression in the LF (B) in the LSCS group (n = 43) and the non-LSCS group (n = 12). The value in the non-LSCS group was set to 1. C: Correlation between LF thickness and Angpt2 mRNA expression. The minimum value of Angpt2 expression in the sample analyzed was set to 1. D: Immunohistochemical analysis of Angpt2 in LF tissue from the LSCS group (left) and the non-LSCS group (right). The inset in the left panel shows higher magnification of the area surrounded by a dashed line. Arrowheads indicate Angpt2-positive cells. Scale bar represents 50 μm in each panel. Data are presented as the mean ± SEM. **p < 0.01 vs. non-LSCS group.
Figure 2. Mechanical stretching stress induces Angpt2 expression in LF fibroblasts.

A: Comparison of Angpt2 mRNA expression in LF fibroblasts ($n=3$) after stretching stimulation (elongation ratio of 10%, 10 cycles m). Angpt2 expression in LF fibroblasts without stretching stimulation was set to 1. B: Representative data of western blot analysis of Angpt2 expression (upper) and Hsc70 (lower) in non-stretched (NS) cells or stretched cells 12 h of stretching. Data represent the mean ± SEM. *$P<0.05$, **$P<0.01$. 

Relative expression of Angpt2 mRNA

- Non-Stretching
- Stretching

(fold)
Figure 3. Angpt12 induces TGF-β1, IL-6, and MMP2 expression in LF fibroblasts. Changes in each mRNA expression in LF fibroblasts (n=3) in response to 5 μg/ml Angpt12 protein for 6 h. Each mRNA expression in LF fibroblasts without Angpt12 stimulation was set to 1. Data represent the mean ± SEM. **P < 0.01 versus control (without Angpt12 stimulation).