Cyclic stretch enhances apoptosis in human lumbar ligamentum flavum cells via the induction of reactive oxygen species generation

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Objective: The lumbar ligamentum flavum (LF) is an important part of the spine to maintain the stability of the spine. In this study we aimed to examine whether mechanical force by cyclic stretch could induce apoptosis in human LF cells and investigate the underlying mechanism.

Methods: LF cells were isolated from six young patients undergoing spinal surgery and then cultured in vitro. LF cells were subjected to cyclic stretch and the apoptosis was detected by flow cytometry. The level of intracellular reactive oxygen species (ROS) and caspase-9 activity were measured.

Results: Cyclic stretch at a frequency of 0.5 Hz with 20% elongation induced the apoptosis of human LF cells in vitro, and this was correlated with increased ROS generation and activation of caspase-9.

Conclusion: Our study suggests that cyclic stretch-induced apoptosis in human LF cells may be mediated by ROS generation and the activation of caspase-9.

Keywords: Cyclic stretch, Ligamentum flavum, Apoptosis, Reactive oxygen species, Caspase-9

Introduction

The lumbar ligamentum flavum (LF), as the cover of the posterolateral part of the lumbar spinal canal, is an important part of the spine and its main role is to limit excessive flexion and maintain the stability of the spine.¹ Degeneration and hypertrophy of LF are the main causes of stenosis which could lead to low back pain.² The pathological mechanism of LF degeneration and hypertrophy are unknown, but may involve age-related degeneration, mechanical (flexion, extension, axial loading) stretch, and activities.³⁻⁵

Cyclic stretch plays role in the growth, maintenance, remodeling and disease onset in the viscoelastic tissues of the spine.⁶ As a risk factor for low back disorder, cyclic stretch causes the hypertrophy of LF, leading to degenerative spinal canal stenosis. LF is continuously

subjected to a variety of stretch, and the mechanism by which LF cells respond to mechanical forces is not completely understood. Mechanical stretch force could promote transforming growth factor-1 (TGF- β 1) production and collagen synthesis by LF cells and result in LF hypertrophy.⁷ The apoptosis of ligament cells has been described in previous studies.^{8,9} However, the relationship of cyclic stretch and LF cell apoptosis remains largely unknown.

Mechanical stretch has been reported to enhance the generation of reactive oxygen species (ROS).^{10,11} ROS are reactive chemical entities that broadly participate in cellular signaling, metabolism, survival and apoptosis. ROS modulate many physiological and pathological processes including cell growth, fibrosis, contraction/dilation, and inflammation.

Therefore, we hypothesized that cyclic stretch may trigger apoptotic process in LF cells and stretch-induced ROS generation is a key regulator of LF cell apoptosis. In this study we evaluated apoptotic changes of human

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lumbar LF cells subjected to cyclic stretch in vitro. In addition, we investigated the mechanism underlying cyclic stretch induced apoptosis in LF cells by examining ROS levels in LF cells.

Methods

Cell culture

Primary LF cells were isolated and cultured as described previously.¹ Briefly, LF samples were obtained aseptically from six young patients undergoing spinal surgery. The dissected specimens were minced and digested in serum-free medium (Gibco) supplemented with 250 U/mL type I collagenase (Sigma) at 37 °C in humid atmosphere with 5% CO₂. The digested specimens were washed with serum-containing medium to inhibit collagenase activity and then placed in 35 mm dishes in Dulbecco's Modified Eagle Medium and Ham's F-12 medium (DMEM/F12, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). The medium was changed every two days. About two weeks later, the cells migrated from the ligament chips to form a monolayer. The cells were maintained for two to three weeks in DMEM/F12 supplemented with 10% FBS, 1% v/v penicillin, and streptomycin (Sigma) in humidified atmosphere with 5% CO₂.

Cyclic stretch treatment

Cultured primary LF cells were seeded on elastic silicone membrane coated with collagen I (Flexercell, McKeesport, PA, USA) at 1.0×10^6 cells/well. At 80–90% confluence, the cells were serum starved in DMEM/F-12 for 24 hours for synchronization and then stretched using a Flexercell Tension Plus system at 37 °C in a 5% CO₂ incubator in DMEM/F-12 supplemented with 10% FBS. 20% stretch at a frequency of 0.5 Hz was delivered for 12 and 72 h. Other cells were cultured under the same conditions in the absence of cyclic stretch force to serve as controls. To evaluate cellular injury after mechanical stretch, the cell viability was monitored by cell count after trypan blue staining as described previously.^{12,13}

Flow cytometry analysis of apoptosis

Apoptosis was detected by using Annexin V-FITC kit I (BD Pharmingen) following the manufacturer's instructions. LF cells were washed with ice-cold PBS, and then resuspended in 100 µl binding buffer at 1×10^6 cells/ml. LF cells were then incubated with 10 µl annexin V-FITC solution and 5 µl PI (propidium iodide, 1 mg/ml) for 30 minutes. The samples were then immediately analyzed by flow cytometry.

Measurement of intracellular ROS

After stretch, LF cells were incubated with 10 µM 2,7-dichlorofluorescein diacetate (DCFDA, Fluka, Buchs, Switzerland) in DMEM at 37 °C for 30 min. The cells were washed with ice-cold phosphate buffered saline (PBS) and sonicated on the ice in lysis buffer (10 mM Tris, 150 mM NaCl, 0.1 mM EDTA, 0.5% Triton X-100, pH7.5). After centrifugation at 15,000 g for 10 min at 4 °C, the supernatants were collected and intracellular ROS level was determined using DCFDA. The DCF fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm using flow cytometer.

Measurement of caspase-9 activity

The activity of caspase-9 in LF cells was determined using a fluorometric immunosorbent enzyme assay kit. Briefly, the supernatants of lysed cells were incubated with dithiothreitol, caspase-9 substrates, leucine-glutamic acid-histidine-aspartic acid-(7-amino-4-trifluoromethyl coumarin) (LEHD-AFC) for 1 hour at 37 °C. The fluorescence intensity was measured at an excitation wavelength of 405 nm using a fluorescence microplate reader.

Statistical analysis

Data were presented as mean $\pm$ SD from 3 independent experiments and analyzed by one-way analysis of variance using SPSS 10.0 software (SPSS, Chicago, IL, USA). Quantitative data is expressed as mean $\pm$ SD and analyzed by one-way ANOVA followed by post hoc Tukey's test. $P < 0.05$ is considered to be significant.

Results

Morphological changes of LF cells after cyclic mechanic stretch

In control cells, most LF cells exhibited a spindle shape, while some appeared to have a polygonal shape and were arranged disorderly (Fig. 1A). After cyclic mechanic stretch, LF cells changed direction perpendicular to applied stress,¹⁴ with the abundance of polygonal cells (Figs. 1B and 1C). The phenotypic characteristics did not differ in LF cells untreated or treated with cyclic stretch.

The viability of LF cells after cyclic mechanic stretch

LF cells showed a viability of $94 \pm 5\%$ and $94 \pm 3\%$ in 12-hour stretched and static groups, respectively, and $90 \pm 4\%$ and $91 \pm 2\%$ in 72-hour stretched and static groups, respectively. The viability of stretched LF cells showed no significant difference from non-stretched cells.

Figure 1 Cyclic stretch-induced morphological changes in human LF cells. **A** Cells cultured in the absence of cyclic stretch were aligned multi-directionally and arranged disorderly. **B** After cyclic stretch of 20% at a frequency of 0.5 Hz for 12 hours, LF cells changed direction perpendicular to applied stress. **C** After cyclic stretch of 20% at a frequency of 0.5 Hz for 72 hours, LF cells changed direction more obviously. Bar = 200 μm .

Cyclic mechanic stretch induced the apoptosis of LF cells

As shown in Figure 2A, the lower left quadrant indicated viable cells (V-FITC-/PI-); the lower right quadrant indicated cells in early apoptosis (V-FITC+/PI-); the upper right quadrant indicated cells in late apoptosis (V-FITC+/PI+); and the upper left quadrant indicated necrotic cells (FITC-/PI+). After stretch for 12 hours, the rate of apoptosis (counting cells in early and late stages of apoptosis) increased slightly by $1.7 \pm 0.2\%$, compared to control cells ($1.4 \pm 0.1\%$). After cyclic stretch for 72 hours, the rate of apoptosis increased to $25.7 \pm 2.5\%$, around 5.6 times greater than in control cells ($4.7 \pm 1.2\%$) (Fig. 2B).

Next we examined whether cyclic stretch could enhance ROS generation in LF cells. The results showed that cyclic stretch for 12 hours and 72 hours caused 190% and 542% increase in ROS level in LF cells, respectively, compared to the static (non-stretch) control (Fig. 2C).

To confirm that cyclic stretch enhances apoptosis in LF cells, we measured caspase-9 activity. Compared to control cells, caspase-9 activity was not increased in the cells after cyclic stretch for 12 hours. However, after cyclic stretch for 72 hours, caspase-9 activity increased to about 4.1 ± 0.3 fold higher than that of control cells ($P < 0.05$, Fig. 2D).

Discussion

Spinal creep is known to impair sensorimotor control mechanisms and increased the risk for low back disorder.^{15–20} LF of the spine demonstrates the development of creep during cyclic work. After the creep the biological responses follow, including the activation, differentiation, proliferation, and apoptosis in viable cells.²¹

Recently, mechanical forces have been shown to induce apoptosis in several cell types.^{22–25} Apoptosis is a cellular suicidal program crucial to the development and maintenance of multicellular organisms.⁸ To our knowledge, the investigation described in the current study is the first to report the apoptosis in LF cells after mechanical stretch *in vitro*. We found that cyclic stretch for 20% elongation induced apoptosis of human LF cells, and this is correlated with ROS overproduction and the activation of caspase-9 in human LF cells.

To quantify LF cell apoptosis induced by cyclic stretch, we performed flow cytometry analysis and found that cyclic stretch induced the apoptosis of LF cells. Compared to the control cells, the treatment group with stretch for 12 hours showed no significant increase in the rate of apoptosis, while the cells with stretch for 72 hours had a significantly higher rate of apoptosis. These observations are consistent with previous reports.^{22–25}

Cyclic stretch can induce ROS generation and ROS are well-known to enhance apoptosis.^{10,11} In this study, we found that cyclic stretch enhanced ROS production in LF cells in a time-dependent manner. Further studies are necessary to identify the sources of ROS that contribute to the effects of mechanical stretch on LF cells. The possible mechanism is that mechanical stretch promotes oxygen consumption and increases ROS production. Therefore, the application of antioxidants would reduce the accumulation of ROS and antagonize cyclic stretch induced apoptosis in LF cells.

Caspases are members of cysteine protease family involved in apoptosis.^{26–28} Caspase-9 is one of the key executors of apoptosis and induces apoptosis via the mitochondrial pathway.²⁹ In this study, we also assessed the apoptosis of LF cells based on the

Figure 2 The effects of cyclic stretch on human LF cells. **A** Cyclic stretch induces cell apoptosis in human lumbar LF cells. LF cells were subjected to 20% stretch at a frequency of 0.5 Hz for 12 and 72 hours, and those cells kept static were used as controls. Flow cytometric analysis of apoptosis in LF cells stained with annexin V and PI. The intensity of PI staining (y-axis) was plotted versus FITC intensity (x-axis): viable cells in the lower left quadrant (FITC⁻/PI⁻); early apoptotic cells in the lower right quadrant (FITC⁺/PI⁻); late apoptotic/necrotic cells in the upper right quadrant (FITC⁺/PI⁺); and necrotic cells in the upper left quadrant (FITC⁻/PI⁺). Shown were representative pictures of the following groups: a static control for 12 hours, b static control for 72 hours, c cyclic stretch group for 12 hours, and d cyclic stretch group for 72 hours. **B** Quantization of apoptotic incidences of human lumbar LF cells induced by cyclic stretch. Compared with static control cells (12 hours, $1.4 \pm 0.1\%$), the apoptotic rate of LF cells slightly increased to $1.7 \pm 0.2\%$. After cyclic stretch for 72 hours, the apoptotic rate of LF cells was increased to $25.7 \pm 2.5\%$, significantly greater than in the control group ($4.7 \pm 1.2\%$). * $P < 0.05$. **C** Intracellular ROS generation in human lumbar LF cells subjected to cyclic stretch. Cyclic stretch enhanced ROS level, compared to the static control. The data were expressed as the percentage of stretched control. Columns and bars represented the mean and SD, respectively. * $P < 0.05$. **D** The caspase-9 activity in human lumbar LF cells subjected to cyclic stretch. Compared to static control, cyclic stretch slightly increased the activity of caspase-9 after 12 hours, but after 72 hours, caspase-9 activity was significantly increased to 4.1 ± 0.3 fold higher. * $P < 0.05$.

detection of caspase-9 activity. We observed that cyclic stretch increased the activity of caspase-9 in LF cells in a time-dependant manner. Notably, stretch did not increase caspase-9 activation before 12 hours compared with the non-stretch group, but increased caspase-9 activation after continuously stretch for 72 hours. We believe that cyclic stretch induced caspase-9 activation to promote the apoptosis of LF cells. To confirm it, it is necessary to examine whether caspase-9 inhibitors could block cyclic stretch induced apoptosis of LF cells.

To our knowledge, this is the first study investigating LF cell apoptosis induced by cyclic stretch. There are several limitations of our present study. First, we only used 20% of external stress at 0.5 Hz and observed the changes at 12 hours and 72 hours. Further investigations using a range of stimulation response over a range of time are needed. Second, our study is descriptive and lacks mechanistic insight. In future studies we will employ antioxidants and ROS blockers to elucidate the role of ROS in mediating cyclic stretch induced apoptosis of LF cells.

In summary, we demonstrated that cyclic stretch with 20% elongation at 0.5 Hz promotes the apoptosis of LF cells. Our findings also demonstrate that increases in ROS production and caspase-9 activation are positively correlated with the effects of cyclic stretch on LF cells. We speculate that the mitochondrial apoptotic pathway may be crucial in our observations. Our findings would provide clues on novel strategies to prevent the development of lumbar LF degeneration.

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Disclaimer Statements

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Conflicts of interest None.

Ethics approval None.

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