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Research Article / Araştırma

Cytokine gene expression in ligamentum flavum hypertrophy

Ligamentum flavum hipertrofisinde sitokin gen ekspresyonu

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ABSTRACT

Aim: Lumbar spinal canal stenosis is a disease is an important reason of injury in the elderly and the most important reason of spinal surgery in patients above the age of 65. Ligamentum flavum hypertrophy plays a critical role in the pathogenesis of lumbar spinal canal stenosis. Inflammatory agents induce the ligamentum flavum hypertrophy and increased scar tissue formation. Even though its prevalence, lumbar spinal stenos does not have a universal definition in this day and age and its generally accepted radiologic diagnosis criteria is lacking. **Material and Methods:** In our study, 25 samples of hypertrophied LF tissue were obtained from the patients with the diagnosis of lumbar spinal canal stenosis while 25 samples were obtained from the patients who underwent surgery for lumbar disc herniation forming control group. Samples were investigated for histological study. Lumbar spinal canal stenosis and control groups were investigated for the expression of inflammatory cytokine genes by semiquantitative RT-PCR method. **Results**: Cytokines were found both in lumbar spinal canal stenosis and control groups. Any statistically significant difference was not found between lumbar spinal canal stenosis and control groups in terms of some cytokines. However, low levels of IL-1 beta were found in lumbar spinal canal stenosis group. **Conclusions**: Spinal canal stenosis is the most important reason of pain and decrease in functional capacity in elderly patients. Therefore, effective therapeutic strategies are needed. Drug resistance is common in fibrotic tissue. Antifibrotic drugs can be an important part of a targeted drug development strategy.

ÖZ

Amaç: Giriş: Lomber spinal kanal darlığı, yaşlılarda önemli bir yaralanma nedenidir ve 65 yaş üstü hastalarda spinal cerrahinin en önemli nedeni olan bir hastalıktır. Ligamentum flavum hipertrofisi lomber spinal kanal darlığının patogenezinde kritik rol oynar. İnflamatuar ajanlar, ligamentum flavum hipertrofisini indükler ve skar dokusu oluşumunu arttırır. Lomber spinal stenozların yaygınlığına rağmen günümüzde evrensel bir tanımı yoktur ve genel kabul görmüş radyolojik tanı kriterleri eksiktir. **Gereç-Yöntem**: Çalışmamızda çalışmamızda lomber spinal kanal stenozu tanısı alan hastalardan 25 hipertrofik LF dokusu örneği ile vaka grubu, lomber disk hernisi nedeniyle ameliyat edilen hastalardan alınan 25 örnek ile control grubu oluşturuldu. Numuneler histolojik çalışma için incelendi. Lomber spinal kanal darlığı ve kontrol gruplarında inflamatuar sitokin genlerinin ekspresyonu semikantitatif RT-PCR yöntemi ile araştırıldı. **Bulgular**: Hem lomber spinal kanal stenozunda hem de kontrol gruplarında sitokinler tespi edildi. Lomber spinal kanal darlığı ve kontrol grupları arasında incelenen sitokinler açısından istatistiksel olarak anlamlı fark bulunamadı. Ancak lomber spinal kanal darlığı grubunda düşük IL-1 beta seviyeleri bulundu. **Sonuç**: Spinal kanal darlığı, yaşlı hastalarda ağrının ve fonksiyonel kapasitenin azalmasının en önemli nedenidir. Bu nedenle, etkili terapötik stratejilere ihtiyaç vardır. İlaç direnci fibrotik dokuda yaygındır. Antifibrotik ilaçlar, hedefe yönelik bir ilaç geliştirme stratejisinin önemli bir parçası olabilir.

ARTICLE INFO/MAKALE BİLGİSİ

Key Words: Lumbar Spinal Canal Stenosis, Ligamentum Flavum Hypertrophy, Spinal Stenosis, Cytokine, Fibrosis.

Anahtar Kelimeler: Lomber Spinal Kanal Stenozu, Ligamentum Flavum Hipertrofisi, Spinal Stenoz, Sitokin, Fibroz.

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INTRODUCTION

Lumbar spinal canal stenosis (LSCS) is a disease caused due to bone hypertrophy, ligamentous hypertrophy, disc protrusion, spondylolisthesis or these issues existing alongside with spinal canal and foraminal stenosis. It is an important reason of injury in the elderly and the most important reason of spinal surgery in patients above the age of 65. It is a common reason for leg and back pain. Lumbar radiculopathy symptoms may occur when lateral indentation and neural foramina narrow. Even though its prevalence, lumbar spinal stenos does not have a universal definition in this day and age and its generally accepted radiologic diagnosis criteria is lacking (1, 2).

LSCS is a developmental or acquired condition where the neural arc diameter is smaller than normal and more triangle than round. The disc's protrusion on the front and the ligamentum flavum's (LF) hypertrophy on the back stretch over the nerve roots and in result, prevent the flow of cerebrospinal fluid around the

caudal by minimizing the thecal arc's premises. LSCS may be found in one segments or multiple segments. The most frequently seen segment is L 4-5. LSCS, can be classified according to congenital, idiopathic, degenerative and metabolic diseases (2, 3).

LF, covers an important part of the anterior and side walls of the spinal canal. LF hypertrophy is accepted as an important reason of the development of LSCS. Fibrosis is the main cause of ligamentum flavum hypertrophy and it mainly stems from mechanical stress. Histopathologically, there is around 60-70% elastic fibrils in a normal ligamentum flavum's extracellular matrix. However, decrease in elastin fibrils, increase in collagen fibrils and ossification is observed in ligamentum flavum that suffered hypertrophy. It was shown that upregulation of some cytokines has an important role in the development of hypertrophy and that inflammatory agents cause ligamentum flavum hypertrophy and increased scar tissue formation (4, 5).

Cytokines are peptide or glycoprotein molecules produced and secreted by nearly every cell to regulate immunity reactions. The secrete of proinflammatory cytokines causes the release of other cytokines into the activation and production of immunity cells (6, 7).

The aim of this study is to study the change in the expression of COX-2, IL-15, IL-8, TNF-alpha, IL-1 beta, IL-6, IL-1 alpha genes that affect the inflammatory process in hypertrophic ligamentum flavum tissues compared to normal ligamentum flavum tissues.

MATERIALS - METHODS

Selection of the Case and Control Groups

The study was conducted on patients that consulted a tertiary health center's neurosurgery clinic and was decided on a lumbar spinal surgery. 25 people with a diagnosis of LSCS constituted the case group and 25 people that was monitored with lumbar disc herniation (LDH) and was not diagnosed with LSCS constituted the control group.

Study Design

The participants' demographic information was recorded as descriptive statistics. Pre-surgery ligament thickness was measured based on the available lumbar Magnetic Resonance images. LF tissues that was taken out by cutting or tearing with rongeurs during LDH and LSCS surgeries of participants in the control and case groups which were not used again and thrown out after surgery were used for research during the study. No additional damage was done to participants' tissues. No procedure that would extend surgery time, cause complications or reduce the quality-of-life post-surgery was performed.

Exclusion Criteria for the Study

Participants that have a history of analgesic use in the last month (drugs that effect the inflammatory process),

Work in jobs that require prolonged standing times and heavy-duty jobs,

Suffered trauma on the lumbar area due to accidents like car crashes and falling from a height,

Have a history of epidural steroid injunction, spinal anesthesia and lumbar spinal surgery have been excluded from the study.

Neuroradiologic Evaluation

The available MR images of the case and control groups were analyzed. No new lumbar MR imagining was performed. The thickness of the middle part of the LF that will undergo surgical operation was measured in accordance with the line that passes through the T2-weighted axial sections' facet joint. Measurements were done automatically with the Picture Archiving and Communication System (PACS). After the measurements participants, under 2.5 mm were included in the control (LDH) group and participants above 2.5 mm were included in the case (LSCS) group. Measurements were taken three times and the average values were regarded as the LF thicknesses (Figure 1).

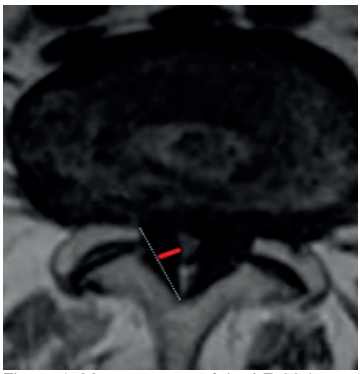

Figure 1. Measurement of the LF thickness

Histopathological Analysis

The LF samples of the case and control groups were taken during the surgical operations. Part of the LF tissues determined in a 10% formalin solution and embedded in paraffin blocks. After taking 4µm thick samples, the samples underwent hematoxylin and eosin staining, following that, they underwent masson trichrome staining to indicate fibrosis levels. The severity of the ligamentum flavum fibrosis was ranked according to the guide presented by Kosaka and friends (8).

According to this guide;

Normal tissues without fibrosis constituted grade 0,

Tissues with fibrosis less than 25% constituted grade 1,

Tissues with fibrosis between 25%-50% constituted grade 2,

Tissues with fibrosis between 50%-75% constituted grade 3,

Tissues with fibrosis over 75% constituted grade 4.

All histomorphological analysis were performed by two independent pathologists.

Real-Time PCR

Primarily, ovarian tissues were homogenized for analysis. For this purpose, a tissue homogenizer (Next Advance, USA) was used. According to the manufacturer's instructions, RNA isolation was performed using the PureLink RNA Mini Kit (Invitrogen, USA).

Accordingly:

The appropriate volume of lysis buffer containing 2-mercaptoethanol was added to the sample. At room temperature, the lysate below was placed in the collection tube and centrifuged for two minutes at 12,000 g.

1.5 volumes of 100% ethanol and tissue lysate were added to an appropriately sized RNase-free tube and vortexed.

700 µL of the sample was transferred to the cartridge (Spin Cartridge) and transferred to the collection tube, centrifuged at 12,000 g for 15 seconds at room temperature, and the liquid part was discarded. 350 µL wash buffer 1 was added to the cartridge (Spin Cartridge), centrifuged for 15 seconds at 12,000 g at room temperature.

80 µL of DNase mixture was added to the surface of the cartridge membrane and incubated for 15 minutes at room temperature.

Once more, wash buffer 1 was added and centrifuged. 500 µL of washing buffer 2 was added to the cartridge with ethanol, centrifuged at room temperature, and the liquid part was discarded.

The cartridge was placed in a recovery tube (Recovery Tube), added RNase-free water, and incubated for one minute at room temperature. Centrifuged for 2 minutes at 12,000 x g, the liquid portion was discarded.

The quantity and quality of purified total RNA were determined with the Quant-iT™ RiboGreen™ RNA Test Kit using a fluorescent microplate reader (UV absorbance 260 nm).

The obtained RNA was stored at -80oC.

cDNA synthesis was done in a palm cycler device.

While interpreting our results, the concentration value of our target genes was proportioned to the concentration value of the reference (housekeeping) gene, and the variation of the results obtained compared to the control group were examined. In our study, the beta-actin gene was used for this purpose.

Expression levels of COX-2, IL-15, IL-8, TNF-alpha, IL-1 beta, IL-6, IL-1 alpha genes were studied by using the synthesized cDNA. The housekeeping gen was Alpha actin. Semiquantitative RT PCR reactions was performed by using hydrolysis probs stained with a fluorescence paint called FAM (Roche Diagnostics GmbH, Mannheim, Germany). Gene expression analysis was done through a machine called The Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany) 480 II System. The data was corrected with the 2-ΔΔCt method.

Statistical Analysis

The data was transferred to the SSPS 22.0 statistical package program and studied through that. Age, LF thickness and body mass index (BMI) was presented as descriptive statistics. The Mann Whitney U test was used to compare age, ligament thickness, ligament fibrosis levels, BMI and cytokine levels between the case and control groups. Fibrosis grades and levels were compared with the Ki-2 test. Statistical analysis results were given as average ± standard deviation. Statistical significance was assumed as ($p<0.05$).

Ethics

This study was carried out in a tertiary health center by the Decision (2013.12.06 no:13/68) of a tertiary health center's Ethics Committee. All the participants signed an informed consent form.

RESULTS

28 participants were women and 22 were men. The descriptive statistics containing participants' age, LF thickness and BMI values and the comparison between the groups are shown in Table-1. As predicted, the LF thickness of the LSCS group was high enough to be considered statistically significant compared to the LDH group.

The ligament hypertrophy levels in the lumbar vertebrae of both of the groups was examined. It was observed that hypertrophy was most frequently seen in L4-L5 levels in the case group and L5-S1 levels in the control group (Figure 2)

Fibrosis in the ligament was studied histopathologically and graded. The results were compared, and no statistically significant differences was found between the fibrosis levels. Fibrosis grading of the groups can be found in Figure 3. Grading was done between 0-4 based on the severity of the fibrosis. An example for grade 0 (lowest) fibrosis and grade 4 (highest) fibrosis can be found in Figure 4.

Gene Expression

Results found on COX-2, IL-15, IL-8, TNF-alpha, IL-6, IL-1 alpha and IL-1 beta through RT PCR are shown in Table 2 and Figure 5. COX-2, IL-15, IL-8, TNF-alpha, IL-6, IL-1 alpha and IL-1 beta expressions increased in

Table 1. Descriptive statistics of the groups and their comparison

		Lowest	Highest	Average±SD	p
Age	Case	21	86	53.66±15.465	>0.05
	Control	21	58	43.04±9.90	
LF thickness	Case	0.28	0.64	0.42±0.098	**<0.01**
	Control	0.13	0.24	0.19±0.029	
BMI	Case	21.5	38.3	28.06±4.12	0.3
	Control	17.7	38.6	26.86±4.41	

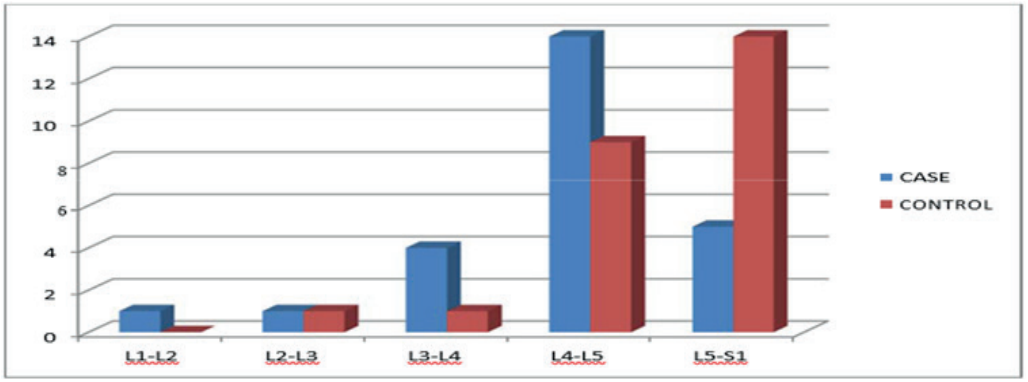

Figure 2. Ligament hypertrophy levels in lumbar vertebrae.

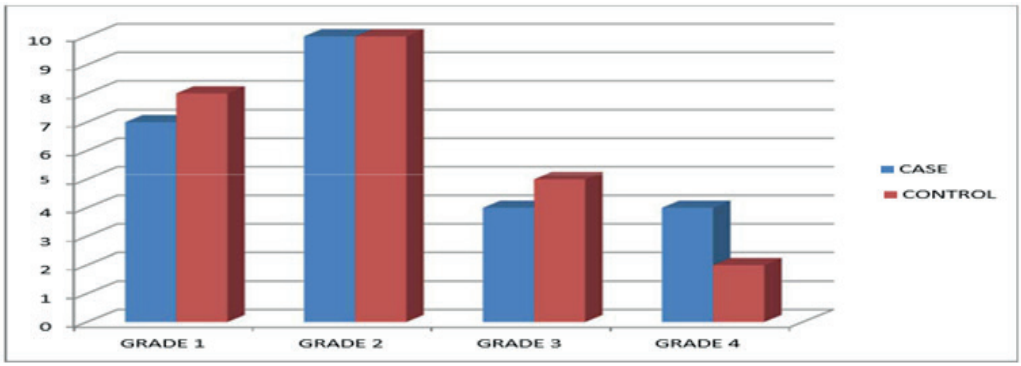

Figure 3. Grading of fibrosis in the case and control groups

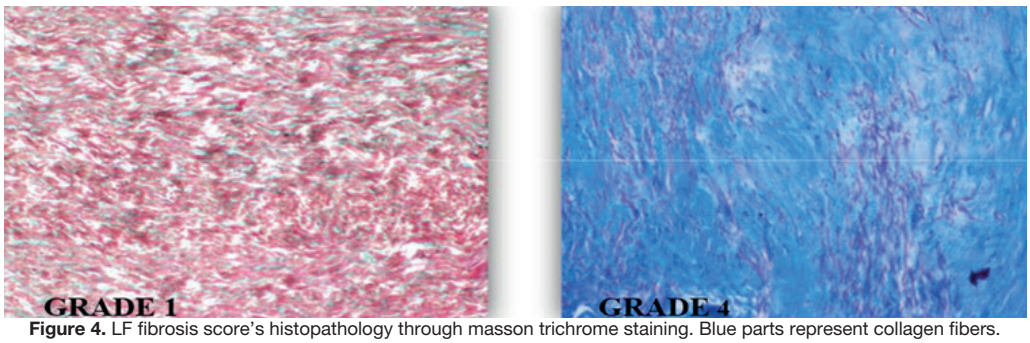

Figure 4. LF fibrosis score's histopathology through masson trichrome staining. Blue parts represent collagen fibers.

Table 2. Expression analysis results of cytokine in LSCS and LDH groups

	CASE		CONTROL		p*
	Number	Average±SD	Number	Average±SD	
IL-1 beta	(n=13)	0.005±0,00	(n=15)	6.18±13.69	<0.01
IL-6	(n=19)	0.032±0.03	(n=23)	0.06±0.10	0.60
IL-8	(n=21)	0.11±0.09	(n=24)	0.22±0.17	0.16
COX-2	(n=14)	0.007±0.00	(n=18)	0.01±0.20	0.28
TNF-alpha	(n=19)	0.13±.023	(n=23)	0.11±0.11	0.21
IL-15	(n=10)	0.00±0,00	(n=13)	0.00±0.00	0.62
IL-1 alpha	(n=3)	0.0006±0.00	(n=7)	0.0003±0.00	0.90

* Mann-Whitney U test

both groups, no significant difference was observed. However, IL-1 beta expression is very low in the case group but increases in the control group. The difference in IL-1 beta expression between the groups is also statistically significant (p<0.01).

DISCUSSION

When the descriptive statistics including age, LF thickness and BMI values of the participants are examined, it is observed that the case group is older than the control group, but this situation is not statistically significant. It is expectable that the case group is older. LSCS is a pathology especially seen in the elderly, and this feature is more prominent than LDH. LF thickness was found to be statistically significant in the LSCS group compared to the LDH group. This result is expected since the case and control groups of our study were chosen based upon the LF thickness.

It is accepted that chronic low-grade inflammation associated with obesity causes disc degeneration through different mechanisms. Degeneration due to obesity is associated with LF thickness, obesity indirectly increases LF thickness. LF thickness was found to be greatly increased in patients with a BMI of 25 kg/m2 and above. There are studies showing a positive relationship between disc degeneration and obesity. There are also studies that show the opposite. It is emphasized that there is no correlation between BMI and LF thickness in these studies. The results obtained from our study also support that there is no relationship between BMI and LF thickness (9-11).

LF levels in the lumbar vertebrae were examined and was determined that hypertrophy was most commonly seen at the L4-L5 level in the case group and at the L5-S1 level in the control group. This finding is supported by other studies on the subject. In one of these studies, it was reported that hypertrophy was most common in L4-L5 in women, and no information was given about men. In our study, the levels in the lumbar vertebrae were examined without differentiating between men and women (11, 12).

In terms of fibrosis levels, the groups were in similar levels to each other. Grade 4 fibrosis was observed more frequently in the case group than in the control group. The tissues at the highest level of fibrosis caused a blue color with the masson trichrome stain. The blue color is due to collagen fibers. Hypertrophy causes loss of LF elastic fibers and an increase in collagen fibers. The increase in collagen synthesis and deposition is a key cellular event in the fibrosis process. In an in vitro study, it was shown that mechanical stress causing LF hypertrophy increased collagen gene expression in LF cell culture. Results of another study showed that connective tissue growth factor treatment induced protein synthesis and increased gene expression of type I and III collagen. In our study, the presence of fibrosis

was examined, but analysis regarding the release and accumulation of collagen were not performed (13, 14).

COX-2, IL-6, IL-8, IL-15, TNF-alpha and IL-1 alpha expression increased in both groups, no significant difference was found. However, IL-1 beta expression was extremely low in the LSCS group. IL-1 beta expression was substantially higher in the LDH group compared to the LSCS group. A significant difference between the groups in terms of IL-1 beta expression was determined. While IL-6 expression is more apparent than the other expressions in the LSCS group; IL-1 beta expression is more apparent in the LDH group.

IL-1β is a potent proinflammatory cytokine that is crucial for host defense responses like infection and injury. It is the best characterized and most researched member of the IL-1 family. It is produced and secreted by various cell types. Monocytes and macrophages are prominent in production. It is produced as an inactive precursor called pro-IL-1β in response to molecules carried by pathogens. Pro-IL-1β is activated by the proinflammatory protease caspase-1. IL-1β is produced without a signal sequence. The mechanisms of IL-1β secretion are still not fully understood due to the diversity of secretory stimuli, cell types that secrete IL-1β, and their genetic diversity. In the secretion stage, the traditional protein secretion way is not followed, one or more non-traditional secretion ways are used. The ways of secretion are determined by the strength of the inflammatory stimulus. It is secreted in extremely small amounts when it is perceived that the extracellular requirement is low or only the extracellular IL needs to be supplemented. When extreme inflammatory stress conditions are sensed, large amounts of active IL-1βcan be rapidly released across the plasma membrane. The mechanisms of these two events mentioned as examples are completely different from each other. Therefore, the fact that LSCS and LDH are two different pathologies are thought to be the key to the difference in IL-1β secretion. The strength of the inflammatory stimulus is different in different pathologies. The secretion way and amount of IL-1β are determined by the strength of the inflammatory stimulus. An inflammatory response is created proportional to the power perceived by the body, and the extracellular IL-1β levels increase to this extent. According to the results obtained in our study, the inflammatory stimulus perceived in the case of LSCS is not at a level to trigger IL-1β secretion mechanisms (15, 16).

Among the reasons for backpain, intervertebral disc degeneration is the most common. Intervertebral disc degeneration is the basis of LDH. It is emphasized that, inflammatory processes increased in severity due to IL-1β have an important part in intervertebral disc degeneration and therefore in LDH development. It was found that IL-1β was expressed in high amounts in intervertebral disc cells during LDH. It is estimated that this is the reason why there was a high IL-1β expression in the LDH group in our study (17-19).

There are studies stating that one of the cytokines that trigger fibrosis, which is the key to the development of LSCS, is IL-1β. Some of these are cell culture studies or animal experiments. Since our study was conducted on human tissues, the results obtained in these studies were not compared with those obtained in our study. In a review study that examines the subject in depth, it was stated that inflammation is the most important event of the fibrosis phenomenon, progressive LF fibrosis is caused by chronic inflammatory response, and important cytokines that play a role in LF fibrosis are listed. Among the cytokines listed, IL-1β is absent. This is a finding parallel to our study (20–22).

The results of a study design similarly to ours and conducted by Yabe et al. support our results. According to this, immunoreactivities of type I and III collagen and gene expression related to chondrogenesis and proteoglycan synthesis were found to be higher in the LSCS group than in the LDH group. In contrast, no significant difference was found between the groups in gene expression related to inflammation. This result is parallel to those obtained in our study, except for IL-1β (3).

Our study has strengths and constraints. A total of 50 participants is one of our strengths; it allowed us to do highly reliable analysis. Another strength is that analysis done in RT PCR were supported with histochemical analysis. Examining a high number of cytokines also puts our study forward. The limitation of our study is that the immunoreactivities of different collagen types and the potential roles of the metalloproteinase family, which is currently predicted to have an important place in the pathogenesis of LF hypertrophy, were not studied.

CONCLUSIONS

As the elderly population grows, spine diseases' incidence increase. Spinal canal stenosis is the most important reason of pain and decrease in functional capacity in elderly patients.

To date, hypotheses regarding the mechanism of LF hypertrophy have been put forward. Based on the positive clinical response of the patients to anti-inflammatory therapy, it was accepted that scarring due to inflammation caused LF hypertrophy.

The process of fibrosis is very complex. There are many different molecular and cellular mechanisms

that contribute to the process. Cytokines and similar factors are released from cells in LF tissues, resulting in inflammation. Inflammation triggers tissue fibrosis through collagen molecules and eventually LF hypertrophy develops.

Fibrosis is a cause of high morbidity and mortality worldwide. Therefore, effective therapeutic strategies are needed. Drug resistance is common in fibrotic tissue. It is necessary to develop targeted drugs for effective treatment.

Antifibrotic drugs can be an important part of a targeted drug development strategy. In order to be successful in these strategic studies, many critical questions regarding the pathogenesis and mechanisms of LF fibrosis need to be answered. It is evaluated that these answers can be reached by further examination of the complex molecular mechanisms at the center of the fibrogenic process.

This article is adapted from Dr. Engin Fidanci's medical specialty thesis. Original title of thesis is "Investigation of COX-2, IL-15, IL-8, TNF-alpha, IL-1 beta, IL-6, IL-1 alpha gene Expressions in Patients with and without Ligamentum Flavum Hypertrophy".

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