The Role of Degenerated Intervertebral Discs in Hypertrophy and Ossification of the Ligamentum Flavum in Humans

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The Role of Degenerated Intervertebral Discs in Hypertrophy and Ossification of the Ligamentum Flavum in Humans

Directed by Professor Hak-Sun Kim

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벌써 2년이라는 시간이 흘러 석사 졸업 논문 마무리를 하고 있는 지금 이 순간이 너무나도 기쁘지만, 처음 임상연구센터에 발을 디뎠을 때의 결심을 떠올려 봅니다. 열심히 노력하였지만 섭섭함과 아쉬움을 애써 뒤로하고 이 작은 결실을 이루도록 도와주신 여러 고마운 분들에게 감사의 말씀을 전합니다.

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중한 친구 현진이에게 고마운 마음을 전하고 싶습니다.

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ABSTRACT

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(Directed by Professor Hak-Sun Kim)

Spinal stenosis is caused, in part, by hypertrophy and ossification of the ligamentum flavum and facet joints. Hypertrophy and ossification of the ligamentum flavum are induced by the degenerative processes that occur with aging and the increased collagen synthesis that results from mechanical stretching. The degree of hypertrophy and ossification of the ligamentum flavum is usually correlated with the amount of intervertebral disc (IVD) degeneration. This implies a possible relationship between disc degeneration or herniation and hypertrophy of the ligamentum flavum in lumbar spinal stenosis. A herniated disc results in the spontaneous production of inflammatory cytokines, such as IL-1, IL-6, NO, PGE_2, and TNF-α. Therefore, we hypothesized that cytokines from a herniated IVD affect cellular proliferation, matrix synthesis, and osteogenesis in the ligamentum flavum, causing hypertrophy and ossification of the ligament. Therefore, this study examined the effect of a secreted cocktail of inflammatory cytokines from herniated discs on ligamentum flavum cells, to identify the role of degenerated discs in the pathogenesis of ligamentum flavum hypertrophy and ossification in spinal stenosis.

Specimens from the interlaminar portion of the ligamentum flavum and herniated lumbar disc tissues were collected during surgery on 27 patients (age range: 49-78 years) with lumbar spine stenosis. Then, the supernatant was collected from herniated disc tissue and used as a cytokine cocktail that was administered to ligamentum flavum cultures. We refer to this as this conditioned medium. Ligamentum flavum cell cultures were treated with cytokines and conditioned medium, and incubated for 48 h. Saline treatment served as a control. Tests including the MTT assay, the determination of ^{3}H-thymidine incorporation and the expression of types I, III, V, and XI collagen and osteocalcin mRNA, and Von Kossa, Alizarin Red-S, and alkaline phosphatase (ALP) staining were performed.

No cytotoxicity was observed. DNA synthesis was increased significantly in the treatment with cytokines and conditioned medium. The expression of types I, III, V, and XI collagen and osteoclacin mRNA was highly upregulated in the treatments with cytokines and conditioned medium. The ligamentum flavum cells treated with cytokines and conditioned medium stained positively for Von Kossa, Alizarin Red-S, and ALP stains. Combined, disc degeneration or herniation has a pathogenetic significance in hypertrophy and ossification of the ligamentum flavum. Disc herniation can affect the ligamentum flavum via inflammatory cytokines and cause hypertrophy and ossification of the ligamentum flavum.

Key words: intervertebral disc, degeneration, ligamentum flavum, hypertrophy, ossification

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Ⅰ. Introduction

Lumbar spinal stenosis results from intervertebral disc herniation and degenerative changes in the posterior structures of the lumbar spine, including hypertrophy of the facet joints and ligamentum flavum (LF). It causes severe disability by compressing the cauda equina and nerve roots.[1-5] Since the LF covers most of the posterior and lateral parts of the lumbar spinal canal, morphological and histological changes in the LF might play a role in the pathogenesis of lumbar spinal canal encroachment.[6, 7]

Nevertheless, there have been few studies of the mechanism of LF hypertrophy. Most studies have addressed the morphologic or histologic

changes in the LF.[8-13]

Yoshida *et al*.[14] examined 45 cases of lumbar spinal stenosis using computed tomography and an immunohistochemical study. As controls, 10 cases of acute disc herniation were used. Statistically significant differences in thickness and transverse area were found in the lumbar stenosis specimens compared with the controls. The pathology of LF hypertrophy includes fibrocartilaginous change due to the proliferation of type II collagen, ossification, calcium crystal deposition, collagen degeneration, and elastic fiber and chondroid metaplasia of ligament fibroblasts.[15-17]

Chondroid metaplasia in LF hypertrophy plays an important role in ligament ossification, as cartilage differentiation, hypertrophy, and cell death are followed by bone formation in the bone morphogenetic pathway.[18]

Polgar first reported ossification of the LF.[19, 20] The ossification of spinal ligaments (OSL) is a common form of myeloradiculopathy that is characterized by heterotopic bone formation in the spinal ligaments, which are normally composed of fibrous tissue.[21, 22]

Ossification of the LF is often associated with ossification of the

posterior longitudinal ligament (OPLL), ankylosing spinal hyperostosis (ASH), and diffuse idiopathic skeletal hyperostosis (DISH).[23, 24] DISH is a common diathesis in middle-aged and elderly patients, and is characterized by bone proliferation along the anterior aspect of the spinal column, and at extraspinal sites of the ligament and tendon attachments.[25, 26]

Hence, LF ossification appears to be a part of ossification of the spinal ligaments. Many reports have examined the etiology of OSL and the systemic and local factors thought involved in the pathogenesis of OSL, but the exact mechanism remains unclear.

Sakou *et al*.[27] reported that slight LF ossification is observed more frequently in the dried vertebral bones of aged individuals; LF ossification might be induced by the degeneration of the LF with aging. Such ossification might be induced pathologically and cause radiculopathy or myelopathy in LF ossification patients.

Several investigators have postulated that mechanical stress to the spine contributes to accelerating ossification[28-34] and inducing collagen synthesis, which is mediated by TGF-β1 in mesangial[35, 36] and smooth muscle cells.[37]

In spite of the significance of the LF in the pathogenesis of spinal disease, there has been no thorough biochemical analysis of the degenerate LF.

In clinical imaging, the degrees of LF hypertrophy and LF ossification usually appear to coincide with those of intervertebral disc (IVD) degeneration. Ugarriza *et al.*[38] showed that its association with cervical disc herniation may produce acute or subacute clinical spinal cord compression. Tanaka *et al.*[39, 40] reported that when disc tissue was applied to an osteoblast culture, osteoblast growth was stimulated, as shown by increased alkaline phosphatase (ALP) activity, type I collagen production, and ^{3}H-thymidine incorporation. This suggests a relationship between IVD degeneration or herniation and LF hypertrophy in lumbar spinal stenosis.

Kang *et al.*[41] reported that a herniated IVD spontaneously produces inflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor alpha (TNF-α), and matrix metalloproteinase.

Therefore, we hypothesized that cytokines from a herniated IVD affect the cellular proliferation, matrix synthesis, and osteogenesis of LF

fibroblasts, ultimately causing LF hypertrophy and ossification.

Accordingly, the objectives of this study were to demonstrate a biological effect of inflammatory cytokines on the metabolism of LF cells, then to demonstrate the local effect of a cocktail derived from herniated discs on LF cells, and finally to propose a role of degenerated discs in the pathogenesis of LF hypertrophy and ossification in spinal stenosis.

Ⅱ. Materials and Methods

1. Materials

A. Patient data and tissue acquisition procedures

LF tissue was obtained from 27 patients (age range: 49 to 78 years) during surgery for lumbar spinal stenosis. IVD tissue was obtained from patients (age range: 58 to 68 years) during surgical disc procedures. The details of the 27 patients are listed in Table 1. All of the patients had

Table 1. Clinical features of the cases studied

Case	Age	Sex	Diagnosis	Operation level
1	63	F	spinal stenosis disc degeneration	L4-5-S1, L4-5, L5-S1
2	62	F	spinal stenosis disc degeneration	L4-5-S1 L4-5, G_{IV}
3	60	M	spinal stenosis , degenerative spondylolisthesis disc degeneration	L4-5 G_{V}
4	69	M	Spinal stenosis disc degeneration	L4-5 L4-5 G_{IV}
5	59	F	Spinal stenosis disc degeneration , disc herniation	L4-5 G_{V}
6	64	F	spinal stenosis , degenerative spondylolisthesis disc degeneration	L3-4-5 L4-5 G_{IV}
7	64	F	Spinal stenosis disc degeneration , disc herniation	L4-5 L4-5
8	52	F	spinal stenosis disc degeneration	L3-4-5 L3-4- 4-5 G_{IV}
9	73	F	spinal stenosis disc degeneration	L4-5 L4-5 G_{V}
10	68	F	spinal stenosis , degenrative spondylolisthesis disc degeneration	L4-5 L4-5 G_{IV}
11	49	F	spinal stenosis , degenrative spondylolisthesis disc degeneration	L4-5 L4-5 G_{IV}
12	63	M	spinal stenosis , disc hernation disc degeneration	L3-4 G_{IV}
13	59	F	spinal stenosis disc degenration	L3-4-5 L3-4 4-5 G_{V}

14	58	F	spinal stenosis , degenerative spondylolisthesis	L4-5
			disc degeneration	GIV
15	61	M	Spinal stensosis	L4-5-S1
			disc degeneration	GV
16	63	F	spinal stenosis degenrative spondylolisthesis	L4
			disc degenration	GIV
17	78	F	spinal stenosis	L4-5
			disc degeneration	GIV
18	62	F	spinal stenosis	L3-4-5
			disc deegenration	GIV
19	61	M	Spinal stenosis , disc hernation	L4-5
			disc degenration	GV
20	58	F	spinal stenosis	L4-5-S1
			disc degenration	GIV
21	73	M	spinal stenosis	L4-5
			disc degenration	GIII
22	65	F	spinal stenosis	L3-4-5
			disc degenration	GV
23	62	M	spinal stenosis	L3-4-5
			disc degenration	GIV
24	62	M	spinal stenosis	L3-4-5
			disc degenration	GIV
25	78	F	spinal stenosis	L4-5
			disc degenration	GV
26	77	M	spinal stenosis	L3-4-5
			disc degenration	GV
27	76	F	spinal stenosis	L4-5
			disc degenration	GV

severe stenosis of the lumbar spine and IVD herniation. The thickened

LF and herniated IVD were noted on magnetic resonance imaging preoperatively and visualized directly during surgery. The operating surgeon (SHM, HML) attempted to obtain tissue *en-bloc* from the central portion of the LF to minimize tissue damage and optimize the harvest of ligament only. The IVD specimen consisted of nucleus pulposus or annulus fibrosus, although in most cases these could not be distinguished with certainty and the disc fragment probably represented a mixture of nucleus and annulus. The LF and IVD tissue specimens were washed with Hank's balanced salt solution (HBSS, Gibco-BRL, Grand Island, NY, USA) to remove blood and bodily fluid contaminants, and were then transported in sterile HBSS to the laboratory, within 20 min of surgical removal.

B. Materials

Hank's balanced salt solution, Dulbecco's Modified Eagle medium and Ham's F-12 medium, fetal bovine serum, 1% v/v penicillin, streptomycin, nystatin, Trypsin-EDTA, and PBS were purchased from Gibco-BRL (Grand Island, NY, USA). 0.2% Pronase, 0.004% deoxyribonuclease II type IV, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dipfenyltetrazolium

bromide) reagent, dimethylsulfoxide (DMSO), 3% silver nitrate, 40 mM Alizarin Red-S, 0.1 mg/ml naphthol AS-MX phosphate, and 0.6 mg/ml fast blue BB salt were obtained from Sigma (St. Louis, MO, USA). Bacterial 0.02% collagenase type II was purchased from Worthington Biochemical Corp (Lakewood, NJ, USA). Sterile nylon mesh filter (pore size: 75 μm) and 24-well plates were purchased from Falcon (Franklin Lakes, NJ, USA). IL-1α (5 μg), IL-6 (10 μg), and TNF-α (10 μg) were obtained from R&D Systems (Minneapolis, MN, USA). Prostaglandin E_2 (PGE_2, 1 mg) and SNAP (NO donor, S-nitroso-N-acetylpenicillamine, 20 mg) were obtained from Calbiochem (Darmstadt, Germany). ^{3}H-thymidine was purchased from Amersham Pharmacia (Uppsala, Sweden). The scintillation spectrophotometer was from Packard (Downers Grove, IL, USA). RNeasy® Mini Kits were obtained from QIAGEN (Valencia, CA, USA). The DNA thermal cycler was from Perkin-Elmer (Norwalk, CT, USA).

2. Ligamentum flavum cell culture

LF cells were isolated from the ligament as described previously.[42] Briefly, the dissected specimens were minced with a scalpel into ca. 2 mm^3

pieces. Then, the LF tissues were digested for 60 min at 37°C under gentle agitation in medium composed of equal parts of Dulbecco's Modified Eagle medium and Ham's F-12 medium (DMEM/F12) containing 5% heat-inactivated fetal bovine serum (FBS) with 0.2% pronase and 0.004% deoxyribonuclease II type IV (DNase). The tissue was washed three times with DMEM/F12 and digested overnight under the same conditions, except that the pronase was replaced with bacterial 0.02% collagenase type II. The cells were filtered through a sterile nylon mesh filter (pore size: 75 μm), counted in a hemocytometer, and plated in 24-well plates at a density of approximately 5×10^5 cells/ml. Primary cultures were sustained for 2 to 3 weeks in DMEM/F12 containing 10% FBS, 1% v/v penicillin, streptomycin, and nystatin in a 5% CO_2 incubator with humidity. The culture medium was changed twice a week.

3. Study design

To analyze the biologic response of human LF (hLF) cells to inflammatory cytokines, hLF cell cultures were organized into three groups: the (1) cytokine, (2) conditioned medium, and (3) control groups. In the first group, 30 ng/ml IL-1α, 30 ng/ml IL-6, 100 ng/ml TNF-α, 10

μM PGE_2, and 100 μM SNAP were added to hLF cell cultures. In the second group, conditioned medium was added to the cultured cells. In the third group, hLF cells were cultured without inflammatory cytokines or conditioned medium.

Each culture was subjected to an MTT assay to estimate cell cytotoxicity, a ^{3}H-thymidine incorporation assay to analyze DNA synthesis, and RT-PCR to determine the expression of types I, II, III, V, and XI collagen and osteocalcin mRNA. Alkaline phosphatase (ALP), Von Kossa, and Alizarin Red-S staining were used to demonstrate the expression of osteogenic markers and the formation of bone nodules.

4. Preparation of conditioned medium

The IVD tissue was minced and incubated in 30 ml DMEM-F12 (200 mg tissue/ 1 mL DMEM-F12 medium) for 72 h in 5% CO_2 at 37°C with humidity. The culture medium was changed and collected daily. We refer to the collected medium as "conditioned medium".

5.Treatment with inflammatory cytokines and conditioned medium

At confluence, hLF cell cultures were rinsed with phosphate-buffered

saline (PBS) three times and exposed to the optimum dose of inflammatory cytokines and conditioned medium. Then, culture medium was added to each well of 24-well plates with cytokines, and the hLF cells were incubated for 48 h in 5% CO_2 at 37°C with humidity.

6. MTT assay

After incubation for 48 h, the viability of hLF cells in the monolayer at the bottom of each well was assessed using the MTT assay,[43] as follows, in triplicate: 200 µl MTT reagent (2 mg/mL culture medium) were added to each well and incubated for 3 h in 5% CO_2 at 37°C with humidity. The supernatant was discarded and replaced with DMSO to dissolve the formazan product, which was measured at 550 nm in a spectrophotometric plate reader.

7. DNA synthesis analysis

After incubation for 48 h, hLF cells were labeled with ^{3}H-thymidine (5 µCi/mL) for the last 4 h of the 48-h treatment, as described previously.[44] Then, the hLF cell layer was washed three times with PBS,

treated with Trypsin-EDTA for 15 min, and the labeled hLF cells were harvested. The radioactivity was measured in a scintillation spectrophotometer.

8. Reverse-transcription polymerase chain reaction analysis

Expression of the types I, II, III, V, and XI collagen and osteocalcin genes was examined using quantitative RT-PCR.[45, 46] Briefly, hLF cells were disrupted in lysis buffer containing guanidine isothiocyanate and homogenized. Total RNA was eluted by selective binding to a silica gel-based membrane using an RNeasy® mini kit. Reverse transcription of RNA into cDNA was performed by incubating 1 μl of RNA in a reaction mixture containing 0.5 mg/mL cDNA reaction product and was used as the template to co-amplify β-actin, types I, II, III, V, and XI collagen, and osteocalcin. PCR was performed using a DNA thermal cycler. The primer sequences and PCR conditions used are shown in Tables 2 and 3, respectively.[47-51]

Table 2. Sequences of the RT-PCR primers used

Primer	Sequence	Length	Size(bp)
ß-actin	5'-GGC GGA CTA TGA CTT AGT TG-3'	20	238
	5'-AAA CAA CAA TGT GCA ATC AA-3'	20	
collagen type I	5'-CCT GTC TGC TTC CTG TTA AC-3'	20	177
	5'-AGA GAT GAA TGC AAA GGA AA-3'	20	
collagen type II	5'-CTA CTG GAG TGA CTG GTC CTA A-3'	22	320
	5'-ACC ATC TTT TCC AGA AGG AA-3'	20	
collagen type III	5'-CTG CCA TCC TGA ACT CAA GAG TGG-3'	24	447
	5'-CCA TCC TCC AGA ACT GTG TAG G-3'	22	
collagen type V	5'-GGA TGA GGA GGT GTT TGA-3'	18	345
	5'-GCC CCT TCA CTG GTT TCA-3'	18	
collagen type XI	5'-GCT GAA AGT GTA ACA GAG GG-3'	20	452
	5'-GGT TCT CCT TTC TGT CCT TT-3'	20	
osteocalcin	5'-CAC TCC TCG CCC TAT TGG CC-3'	20	237
	5'-GCC AAC TCG TCA CAG TCC GG-3'	20	

Table 3. RT-PCR conditions

Primer	Conditions			Cycle
	Denatuation	Annealing	Polymerization	
ß-actin	94℃ 1min	94℃ 5sec→ 53℃ 5sec→ 72℃ 30sec	72℃ 2min	24
collagen type I	94℃ 1min	94℃ 5sec→ 48℃ 5sec→ 72℃ 30sec	72℃ 2min	21
collagen type II	94℃ 1min	94℃ 5sec→ 50℃ 5sec→ 72℃ 30sec	72℃ 2min	41
collagen type III	94℃ 9min	94℃ 30sec→ 54℃ 30sec→ 72℃ 1min	72℃ 7min	30
collagen type V	94℃ 9min	94℃ 30sec→ 60℃ 30sec→ 72℃ 1min	72℃ 7min	35
collagen type XI	94℃ 9min	94℃ 30sec→ 54℃ 30sec→ 72℃ 1min	72℃ 7min	40
osteocalcin	94℃ 1min	94℃ 5sec→ 60 ℃ 5sec→ 72℃ 30sec	72℃ 2min	30

9. Von Kossa, Alizarin Red-S, and alkaline phosphatase staining

For Von Kossa staining, hLF cells were fixed with 0.1 M sodium cacodylate and then washed. Silver nitrate (3%) was added to the cell cultures in a dark room and the cells were incubated for 30 min at room temperature in the dark and then exposed to light for 1 h. For Alizarin

Red-S staining, hLF cells were fixed for 2 h with ice-cold 70% ethanol. After washing with distilled water, the cells were stained with 40 mM Alizarin Red-S, pH 4.2, for 10 min at room temperature. The stained cell layers were then rinsed five times with distilled water, and washed with PBS for 15 min. For alkaline phosphatase (ALP) staining, hLF cells were fixed for 10 min with 3.7% formaldehyde at room temperature. After washing with PBS, the cells were incubated for 30 min with a mixture of 0.1 mg/ml naphthol AS-MX phosphate, 0.5% N,N-dimethylformamide, 2 mM $MgCl_2$, and 0.6 mg/ml fast blue BB salt in 0.1 M Tris-HCl, pH 8.5, at room temperature in the dark. Histochemical staining was compared qualitatively.

10. Statistical analysis

The values were assessed using analysis of variance (ANOVA) and the *t*-test to evaluate the differences and were considered significant at $p < 0.05$.

Ⅲ. Results

1.The hLF cells cultured with a given cytokine dose

A.Cytotoxicity

The hLF cells showed 200 to 250% cytotoxicity in the groups treated with cytokines and 175% in the control group. The cytotoxicity of hLF cells treated with cytokines did not differ from that of untreated hLF cells in any of the experiments (Fig. 1).

B.DNA synthesis

In the group treated with cytokines, DNA synthesis was enhanced in hLF cells compared with the controls, as shown by the increased ^{3}H-thymidine incorporation. Moreover, DNA synthesis was significantly increased in the hLF cells treated with IL-1α, IL-6, TNF-α, SNAP, and the supernatant from herniated IVD (Fig. 2).

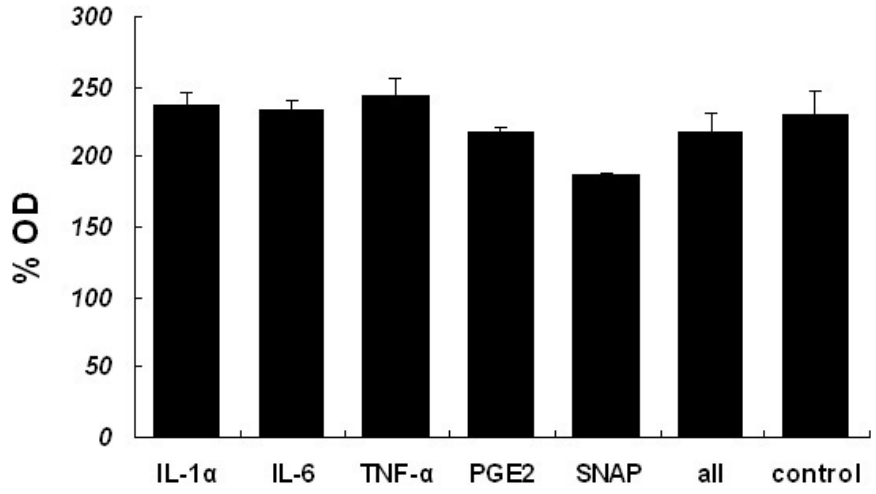


Figure 1. Cytotoxicity of hLF cells in response to IL-1α, IL-6, TNF-α, PGE_2, and SNAP (NO donor). Combined cytokines, herniated IVD medium, and control. The hLF cells were cultured in 24-well plates until confluence, and then treated with various cytokines for 48 h. Cell cytotoxicity was measured using the MTT assay. No cytotoxicity was detected.

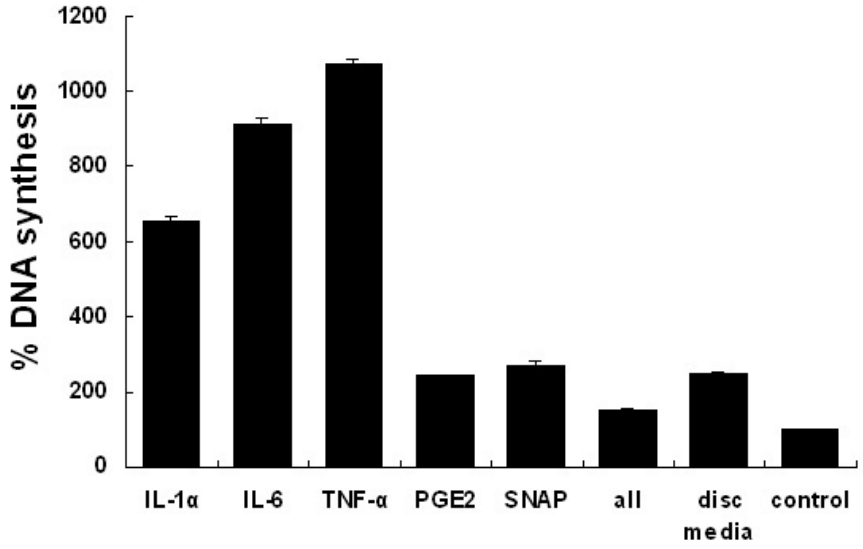


Figure 2. The effect of cytokines on DNA synthesis in hLF cells. The hLF cells were cultured in 24-well plates until confluence. After confluence, the cells were exposed to a given dose of various cytokines (IL-1α, IL-6, TNF-α, PGE_2, and SNAP (NO donor)) for 48 h and labeled with ^3H-thymidine for the final 4 h. Then, the incorporated radioactivity was determined. DNA synthesis was significantly increased with cytokine treatment.

C.Effect of cytokines on the expression of collagen and osteocalcin

mRNA

The hLF cells treated with cytokines expressed types I, III, V, and XI collagen and osteocalcin mRNA (Fig. 3A).

The hLF cells cultured with SNAP showed upregulation of types I, III, and V collagen mRNA, and a significant increase in osteocalcin mRNA expression. The expression of type III collagen was upregulated in hLF cells treated with the combined cytokines. Moreover, the expression of types I and XI collagen mRNA was upregulated in hLF cells treated with the supernatant from herniated IVD compared with the controls (Fig. 3B).

D.Effect of cytokines on the expression of the osteogenic phenotype

The hLF cells treated with IL-6, TNF-α, PGE_2, SNAP, and the upernatant from herniated IVD stained positively for Von Kossa stain (Fig. 4), but not for Alizarin Red-S or ALP stains (data not shown).

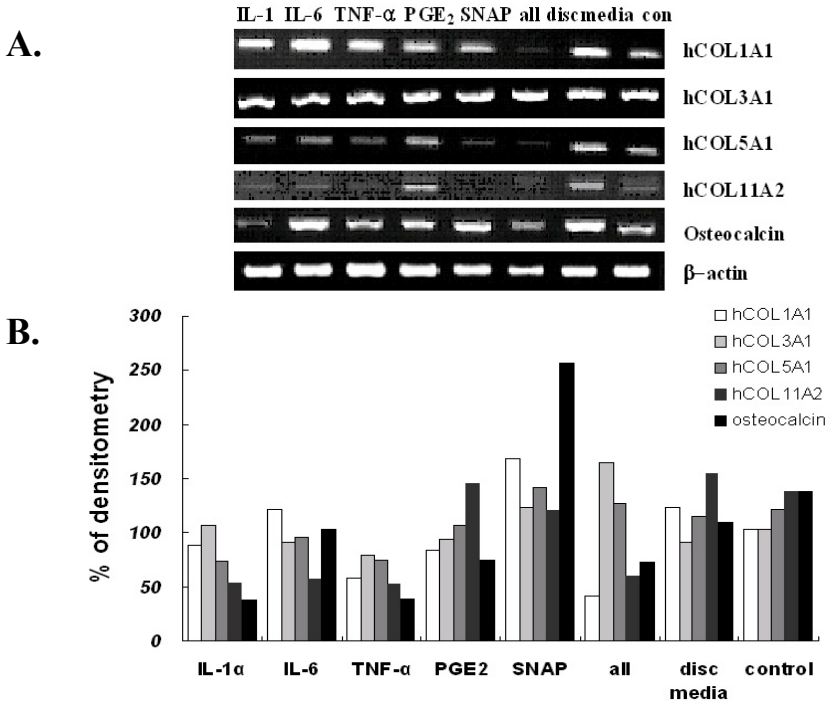


Figure 3. Effect of cytokines on the expression of hCOL1A1, hCOL3A1, hCOL5A1, hCOL11A1, and osteocalcin mRNA in hLF cells. A. The hLF cells were exposed to a given dose of cytokines: IL-1α, IL-6, TNF-α, PGE_2, and SNAP (NO donor). Total RNA was isolated from cells and subjected to RT-PCR. The PCR products were separated on 2% agarose gels containing ethidium bromide, and then observed on an ultraviolet transilluminator. B. The expression of each band seen in A was quantified using an image analyzer. The results are presented as the percentage of the mRNA level relative to β-actin for each band. The hLF cells cultured with SNAP showed upregulation of types I, III, V collagen and osteocalcin mRNA expression. The expression of types I and XI collagen mRNA was upregulated in hLF cells treated with the supernatant from herniated IVD compared with the controls (hCOL1A1: human collagen type I, hCOL3A1: human collagen type III, hCOL5A1: human collagen type V, hCOL11A2: human collagen type XI, con: control)

Von Kossa staining

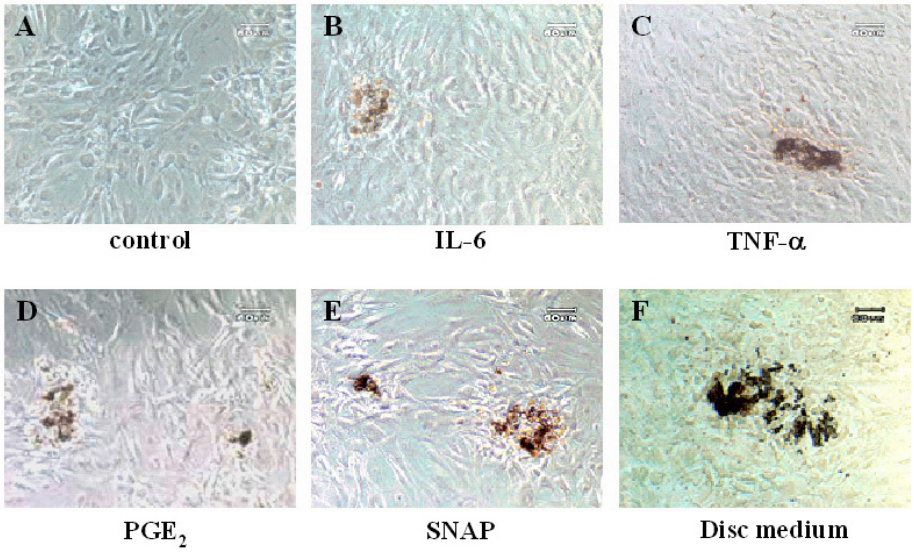


Figure 4. The hLF cells were cultured in a 24-well culture plate until confluence, and then treated with various cytokines for 48 h: IL-1α, IL-6, TNF-α, PGE_2, and SNAP (NO donor). The cells stained positively with Von Kossa stain (original magnification ×100).

2. The hLF cells cultured with conditioned medium

A. Morphology of hLF cells

The hLF cells were mainly polygonal to oval cells arranged in sheets. There were a few spindle-shaped cells (Fig. 5A). The proportions and phenotypic characteristics did not differ from specimen to specimen or with the number of cell passages. The hLF cells treated with conditioned medium included more polygonal to oval cells than spindle-shaped cells (Fig. 5B). These cells showed significant signs of cellular proliferation (Fig. 5B) compared with the untreated hLF cells (Fig. 5A).

B. Cytotoxicity

The control hLF cells showed 50 to 96% cytotoxicity, versus 80 to 113% for the conditioned medium group. There was considerable variation among the patients. Nevertheless, the toxicity of hLF cells treated with cytokines did not differ significantly from that of untreated hLF cells in any of the experiments (Fig. 6).

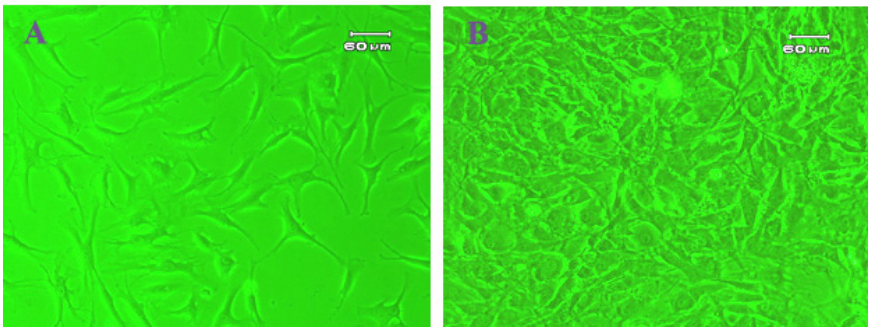


Figure 5. The morphology of hLF cells. A. The hLF cells cultured in a 24-well culture plate. B. The hLF cells treated with conditioned medium for 48 h (original magnification ×100). The hLF cells treated with conditioned medium included more polygonal to oval cells than spindle-shaped cells, reflecting significantly increased cellular proliferation compared with the untreated hLF cells.

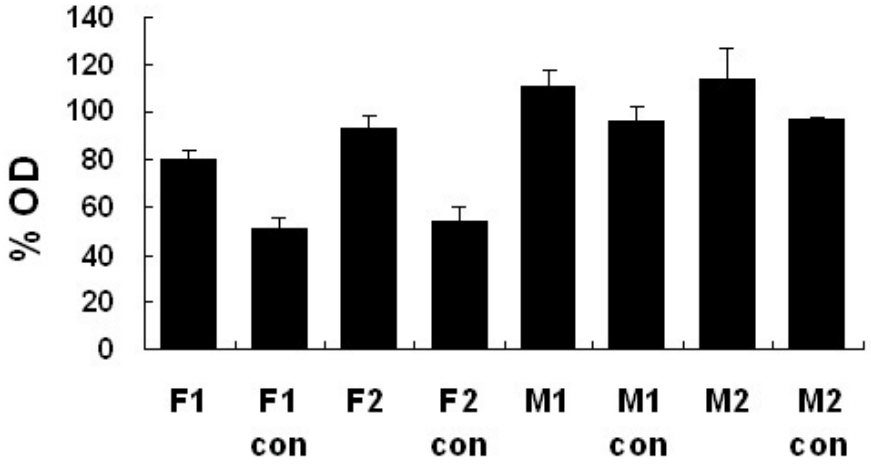


Figure 6. Cell cytotoxicity of hLF cells in response to conditioned medium. The hLF cells were cultured in a 24-well plate until confluence, and then treated with conditioned medium for 48 h. Cell cytotoxicity was measured using the MTT assay. No cytotoxicity was detected. (F1, F2, M1, M2: the numbers refer to specific female and male patients, con: control)

C. DNA synthesis

In the group treated with conditioned medium, the hLF cells showed a significant increase in DNA synthesis, compared with the control (Fig. 7).

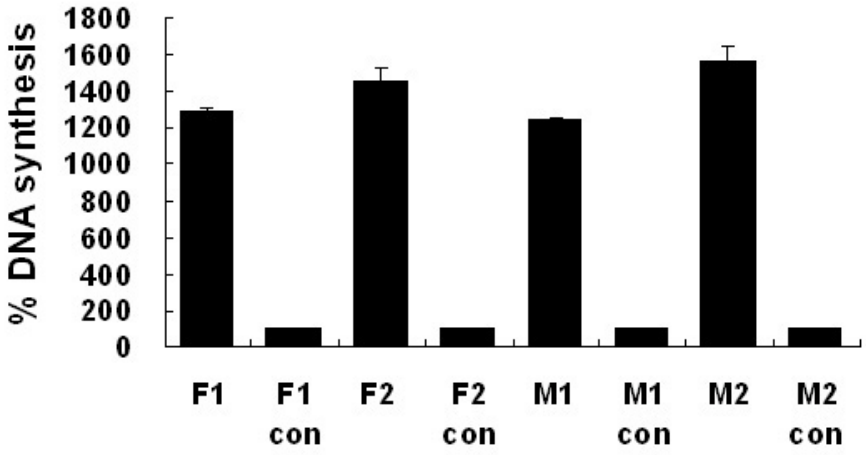


Figure 7. The effect of conditioned medium on DNA synthesis in hLF cells. the hLF cells were cultured in a 24-well plate until confluence. After confluence, the cells were exposed to conditioned medium for 48 h, labeled with ^{3}H-thymidine for the final 4 h, and the amount of incorporated radioactivity was determined. DNA synthesis was significantly increased in cells treated with conditioned medium. (F1, F2, M1, M2: the numbers refer to specific female and male patients, con: control.)

D. Effect of conditioned medium on the expression of collagen and osteocalcin mRNA

The hLF cells treated with conditioned medium expressed types I, III, V, and XI collagen and osteocalcin mRNA; collagen type II mRNA was absent (Fig. 8A). The OD of hLF cells treated with conditioned medium showed the upregulation of types I, III, and V collagen mRNA expression, and significant increases in the type XI collagen and osteocalcin mRNA expression, compared with controls. To minimize the effects of variation among patients, all the data were normalized with respect to the untreated controls (Fig. 8B).

E. Effect of conditioned medium on expression of the osteogenic phenotype

Although there was individual variation in the intensity of Von Kossa, Alizarin Red-S, and ALP staining, the conditioned medium had a uniformly positive effect (Fig. 9). As the effects of Von Kossa, Alizarin Red-S, and ALP stains can be delayed, hLF cells were treated with conditioned medium for 7 days. These LF

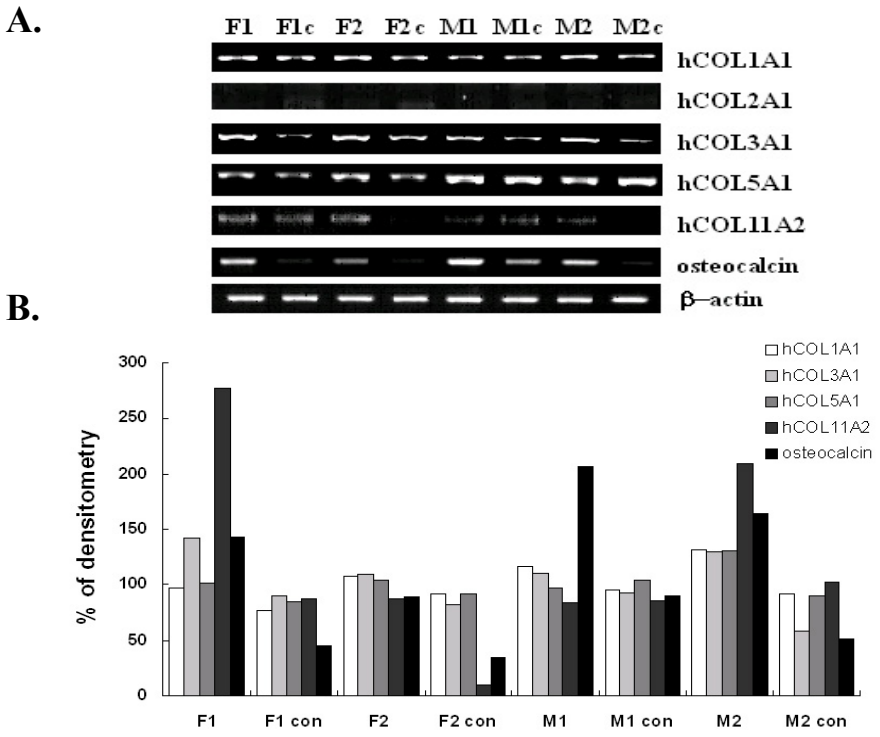
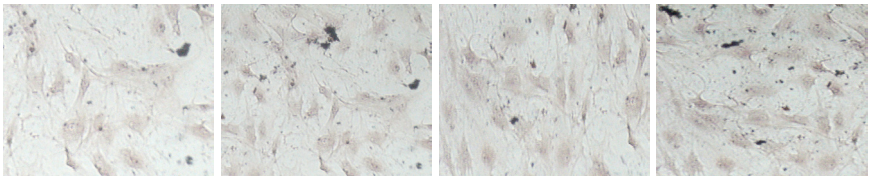


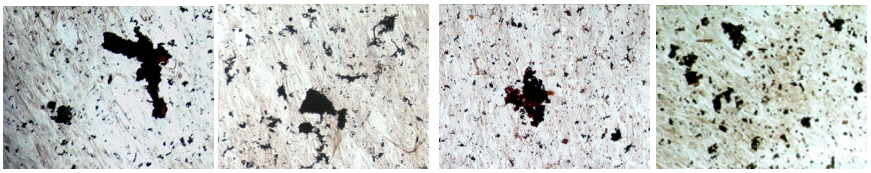
Figure 8. Effect of conditioned medium on the expression of hCOL1A1, hCOL2A1, hCOL3A1, hCOL5A1, hCOL11A1, and osteocalcin mRNA in hLF cells. A. The hLF cells were exposed to conditioned medium and total RNA isolated from cells, and then subjected to RT-PCR analysis. B. The expression of each band shown in A was quantified using an image analyzer. The results are presented as the percentage of the mRNA level in each band relative to β-actin. The expression of types I, III, V, and XI collagen and osteoclacin mRNA was strongly upregulated when treated with conditioned medium. (hCOL1A1: human collagen type I, hCOL2A1: human collagen type II, hCOL3A1: human collagen type III, hCOL5A1: human collagen type V, hCOL11A2: human collagen type XI, F1, F2, M1, M2: the numbers refer to specific female and male patients, F1c, F2c, M1c, M2c: the numbers of control female and male patients, con: control)

A. Von Kossa staining

(A) Control group



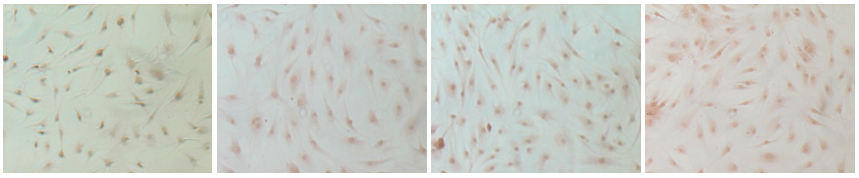
(B) Conditioned (disc) medium group



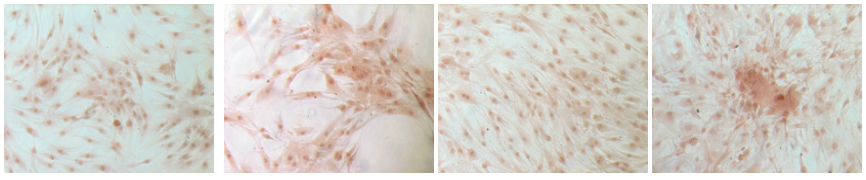
Patient 1 Patient 2 Patient 3 Patient 4

B. Alizarin red-S staining

(A) Control group



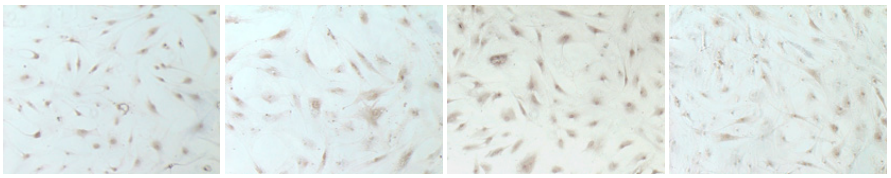
(B) Conditioned (disc) medium group



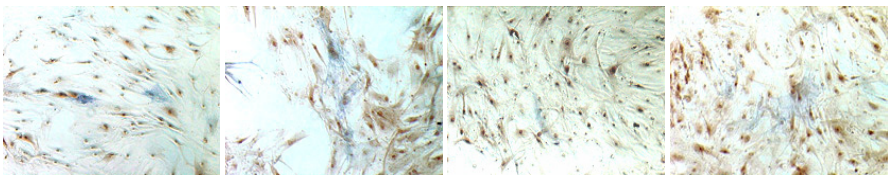
Patient 1 Patient 2 Patient 3 Patient 4

C. Alkaline Phosphatase staining

(A) Control group



(B) Conditioned (disc) medium group

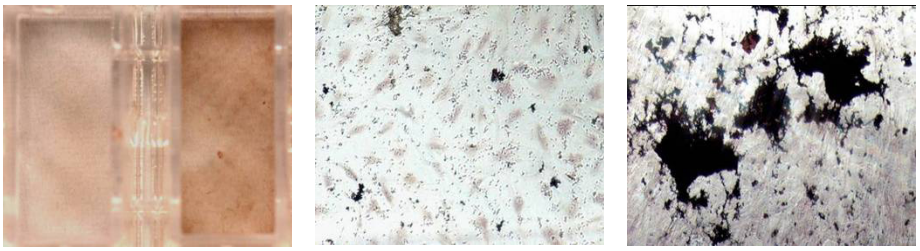


Patient 1 **Patient 2** **Patient 3** **Patient 4**

Figure 9. The hLF cells were cultured in 24-well culture plates until confluence, and then treated with conditioned medium for 48 h. They stained positively for A: Von Kossa, B: Alizarin Red-S, and C: ALP stains (original magnification ×100).

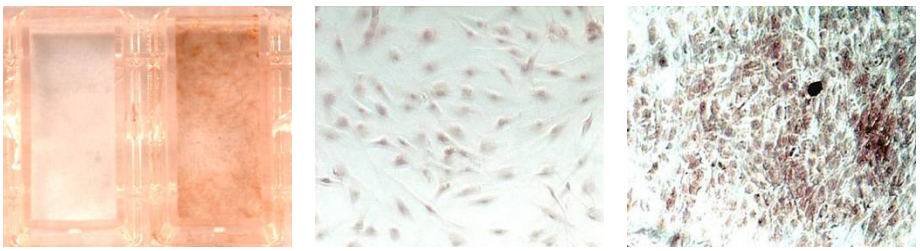
cells stained more strongly with Von Kossa and Alizarin Red-S, but less so for ALP relative to the other stains (Fig. 10).

A. Von Kossa staining



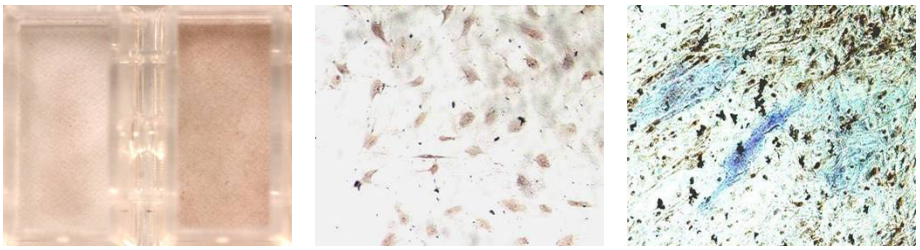
control conditioned medium

B. Alizarin red-S staining



control conditioned medium

C. Alkaline Phosphatase staining



control conditioned medium

Figure 10. The hLF cells were cultured in a 4-well chamber slide, and then treated with conditioned medium for 7 days. The cells stained more positively than in Fig. 9. A: Von Kossa, B: Alizarin Red-S, C: ALP stains (original magnification ×100).

Ⅳ. Discussion

Hypertrophy of the LF is a characteristic feature of lumbar spinal stenosis, causing compression of the cauda equina or nerve roots. Two mechanisms, not entirely exclusive, have been proposed for hypertrophy of the LF: one involves degenerative changes secondary to the aging process and the other is based on mechanical stress, such as instability.[16, 52]

A number of authors have reported that LF hypertrophy plays a major role in the pathogenesis of lumbar canal stenosis.[53] Histological and immunohistochemical studies have revealed that an increased number of collagen fibers are present in the degenerate LF.[16] A biochemical analysis also showed increased collagen in a degenerate LF specimen.[15] These reports indicate that hypertrophy of the LF is characterized by a decrease in elastic fibers and an increase in collagen content.

By contrast, some articles have reported that the LF contracts and folds in on itself as the interlaminar space narrows due to spinal disc collapse.[54]

Ossification of the LF is a well-known clinical entity. Ono *et al*.[55]

showed that bone morphogenic protein-2 (BMP-2) and transforming growth factor-β (TGF-β) were crucial in the process of ossification of the LF. They act on progenitor cells in the ligament, causing them to proliferate, form cartilage, and then ossify. The direction of calcification is from the dorsum of the ligament towards the spinal canal.

The question of whether LF hypertrophy is an early stage of LF ossification arises.[56] Motegi *et al.*[57] reported that LF hypertrophy is often considered an early stage of LF ossification because of the apparent clinicopathological similarities. Epstein[58, 59] proposed "Ossification of LF in evolution", which was defined as small areas of ossification in hypertrophy of the LF. LF hypertrophy might result from the process of LF degeneration, whereas LF ossification is an atypical form of diffuse idiopathic skeletal hyprostosis.[60] Therefore, LF hypertrophy should be considered an early stage or atypical pattern of LF ossification, making it less necessary to establish an independent clinical category for LF hypertrophy.

In spite of the significance of the LF in the pathogenesis of spinal disease, there has been no thorough biochemical analysis of the degenerate LF.

To investigate whether inflammatory cytokines affect LF fibroblasts, we examined the cellular proliferation, matrix synthesis, and osteogenesis in LF hypertrophy and ossification. Due to limitations in the availability of fresh human IVD tissue and cancellous bone chips, we made and used conditioned medium. DNA synthesis increased significantly with the hypertrophy of LF cells treated with cytokines and conditioned medium.

Moreover, the expression of types I, III, and V collagen mRNA was upregulated, while there was no apparent change in collagen type II, which is distributed mainly at the ligament insertion of the facet in the lateral recess, where chondrocytes exist. Only a few chondrocytes are present in the interlaminar portion of the LF.[16] Specchia *et al.*[42] reported that cultured LF cells from patients with spinal stenosis included chondroblastic cells, which possessed type II collagen immunoreactivity, while the cells from patients without spinal canal stenosis did not. This demonstrates that inflammatory cytokines and conditioned medium affect LF hypertrophy significantly. In addition, the expression of collagen type XI and osteocalcin mRNA were upregulated significantly. Koga *et al.*[61, 62] showed that the COL11A2 gene located in chromosome 6 may be responsible for genetic susceptibility to LF ossification, and may be a novel marker of ectopic ossification.

Human LF cells cultured with inflammatory cytokines and conditioned medium stained strongly with Von Kossa stain, and with Alizarin Red-S stain. Kazuhito *et al.*[63] found that bone-specific alkaline phosphatase was not correlated with LF ossification. Several studies have indicated that bone-specific alkaline phosphatase is an early marker in osteoblast differentiation and that osteocalcin is a late marker.[64, 65] Therefore, the stage of osteoblast differentiation may be associated with the activity of general ectopic bone formation in patients with LF ossification. These findings suggest that inflammatory cytokines and conditioned medium have significant effects on the pathogenesis of LF ossification.

Ⅴ. Conclusion

This study investigated whether inflammatory cytokines from herniated IVD affect hypertrophy and ossification of the LF.

1. **No recognizable cytotoxicity was seen.**
2. **DNA synthesis was significantly increased in treatments with cytokines and conditioned medium.**
3. **The expression of types I, III, V, and XI collagen and osteoclacin mRNA was highly upregulated in treatments with cytokines and conditioned medium.**
4. **The hLF cells treated with cytokines and conditioned medium stained positively with Von Kossa, Alizarin Red-S, and ALP stains.**

Taken together, IVD degeneration or herniation has a pathogenetic significance in hypertrophy and ossification of the LF. IVD herniation affects the LF via inflammatory cytokines and causes hypertrophy and ossification of the LF.

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Abstract in Korean

인간 황색 인대의 비후와 골화에 미치는 퇴행된 인간 추간판 조직의 역할

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척추관 협착증은 추간판 탈출증, 후관절 비후와 함께 황색인대의 비후 및 골화의 결과등에 의해 일어난다. 황색 인대의 비후와 골화는 가령에 따른 퇴행적 변화에 의해 유도되고, 물리적 확장(mechanical stretching)에 의해 교원질 합성이 증대된다. 황색 인대의 비후와 골화의 정도는 보통 추간판 퇴행의 정도와 일치한다. 이와 같은 점으로 보아 요추 척추관 협착증에서 추간판 퇴행 또는 탈출증과 황색 인대의 비후는 관련 깊을 것으로 보인다. 탈출된 추간판은 자발적으로 IL-1α, IL-6, TNF-α, PGE_2, NO등과 같은 inflammatory cytokines 을 생산한다. 본 연구는 이런 cytokines 이 황색 인대의 섬유아 세포를 자극시켜 세포 증식, 기질 생성, 골형성등을 유도하는 기전을 설명함으로써 척추관 협착증에서의 황색 인대 비후와 골화의 병리기전에 대한 퇴행된 추간판의 역할을 밝히고자 하였다.

세포 배양에 필요한 표본은 척추관 협착증으로 감압술을 시행한 27명의 환자의 척추 황색 인대 조직과 탈출된 추간판 조직을 모

아 이용하였다(연령 49-78세). 황색 인대 세포는 효소 처리법으로 분리하였으며 계대수가 3을 넘지 않도록 하였다. 추간판 조직은 기본 배지에 넣고 48시간 배양한 뒤 상층액만 모았다. 이것을 conditioned medium라 명명하였다. 황색 인대 세포에 대한 cytokine의 역할을 보기 위해 IL-1α (30 ng/ml), IL-6 (30 ng/ml), TNF-α (100 ng/ml), PGE_2 (10uM), NO (100uM)과 conditioned medium를 48시간 처리하였다. 대조군으로써 saline 을 처리하였다. MTT assay 와 ^{3}H-thymidine incorporation 을 통해 세포 독성과 세포 증식을 확인하였고, RT-PCR 을 통해 제 1, 2, 3, 5, 11형 교원질과 osteocalcin 의 mRNA 발현을 측정하였다. ß-actin 은 mRNA 량의 표준화를 위해 사용하였다. Alkaline phosphatase staining, Alizarin Red-S staining, Von Kossa staining 을 이용하여 황색 인대 세포의 골형성능을 확인하였다.

세포독성효과는 없었으며, cytokine 과 conditioned medium를 처리했을 때 황색 인대 세포의 DNA 합성이 의의있게 증가하였고, 제 1, 3, 5, 11형 교원질, osteocalcin mRNA 발현량이 크게 증가하였고, Von Kossa, Alizarin Red-S, alkaline phosphatase로 염색되었다. 종합해 보면, 추간판의 퇴행 또는 탈출증은 황색 인대의 비후 또는 골화와 pathogenic significance 가 있다는 것을 알 수 있다. 결론적으로, 추간판 탈출증은 inflammatory cytokine 을 통해 황색 인대에 영향을 끼치며, 황색 인대의 비후와 골화의 원인이 될 수 있다.

핵심되는 말 : 추간판, 변성, 황색 인대, 비후, 골화.