Comparative expression of matrix associated genes and inflammatory cytokines associated genes according to disc degeneration

: Analysis of living human nucleus pulposus

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: Analysis of living human nucleus pulposus

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Jeong Yoon Park

<TABLE OF CONTENTS>

ABSTRACT ····· 1
I. INTRODUCTION ····· 4
II. MATERIALS AND METHODS ····· 7
1. Clinical materials ····· 7
2. Isolation of disc cells and culture ····· 12
3. Real-time polymerase chain reaction assay ····· 14
4. Western blot analysis ····· 20
5. Statistical analysis ····· 21
III. RESULTS ····· 22
1. Patient Characteristics ····· 22
2. Quantitation of mRNA Levels ····· 22
3. Expression by Western blot assay ····· 25
IV. DISCUSSION ····· 26
V. CONCLUSION ····· 29
REFERENCES ····· 30
ABSTRACT (IN KOREAN) ····· 35

LIST OF FIGURES

Figure 1. Rectangular cutting around the annulus ·····8
Figure 2. Annulus and nucleus ····· 9
Figure 3. Melting curve of aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, osteocalcin, TNF-α, and IL-1β ····· 15
Figure 4. mRNA levels of aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, osteocalcin, TNF-α, and IL-1β ····· 24
Figure 5. Western blot result for aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase and osteocalcin ····· 25

LIST OF TABLES

Table 1. Classification of Disc Degeneration according to Pfirrmann's Grading System ····· 11
Table 2. Primer sequence ····· 13
Table 3. Quantitation of mRNA Levels ····· 23

ABSTRACT

Comparative expression of matrix associated genes and inflammatory cytokines associated genes according to disc degeneration
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This study was conducted to investigate the expressions of various genes associated with matrix synthesis, and to examine relations between the expressions of inflammatory cytokines and

degrees of disc degeneration in human discs. Degenerated discs were obtained from 18 patients who underwent discectomy for lumbar disc herniation. Disc degeneration was graded by T2-weighted MRI using Pfirrmann's grading system. Discs were allocated to two groups; Group I (9 patients) – mildly degenerated discs (grades 2 and 3), and Group II (9 patients) – severely degenerated discs (grades 4 and 5). Cells from the nucleus pulposus were isolated and then cultured as monolayers. The mRNA expressions of aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, osteocalcin, TNF-α, and IL-1β in the two groups were compared by real-time PCR, and those of matrix associate proteins (aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, osteocalcin) were compared by western blotting. mRNA expressions in Group I were upregulated versus Group II to the following extents; 1.83 times for aggrecan, 1.82 times for Type II collagen, 1.80 times for Sox9, 1.41 times for Type I collagen, 1.38 times for alkaline phosphatase, 1.80 times for osteocalcin, and. Furthermore, western blotting showed that aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, and osteocalcin were higher in Group I. However, the mRNA levels of TNF-α and IL-1β

were 1.26 and 1.11-fold upregulated in Group II. Mildly degenerated discs showed greater matrix, chondrogenic, and osteoblastic gene expressions than severely degenerated discs, indicating that the ability to produce matrix-associated proteins is greater for cells in mildly degenerated than in severely degenerated discs. However, inflammatory cytokines genes associated with disc degeneration were expressed at higher levels in the severely degenerated group. This study shows that a reduction in matrix synthesis and an increase of inflammatory cytokine levels occurs during disc degeneration.

Key Words**:** Degenerative disc, Aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, osteocalcin, TNF-α, IL-1β

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1. INTRODUCTION

The pathogenesis of intervertebral disc degeneration is a complex process that disrupts the well-defined organizational and biochemical balance due to the replacement of normal nucleus pulposus disc matrix by more

fibrotic, less cartilaginous material.[1-5]

Disc degenerative changes can be associated with pain, and treatment options for disc degeneration are limited. Many trials of potential molecular and biologic therapies have been conducted in this context. Previous studies have evaluated three main biochemical changes; 1) decreased matrix synthesis, 2) increased catabolism, and 3) change in growth factors and cytokines associated with altered disc cell phenotypes during disc aging and degeneration.[1, 2, 6-9] Of the various genes associated with matrix synthesis, Type I and Type II collagens (fibrillar molecules), aggrecan (consists of a core protein to which sulfated-glycosaminoglycans), Sox-9 (which upregulates both aggrecan and Type II collagen), osteocalcin and alkaline phosphatase (markers of osteogenic genes), are important.[2, 7, 8, 10] And, of the cytokines associated with disc degeneration, the pro-inflammatory cytokines; tumor necrosis factor-α (TNF-α) and interleukin (IL) also play important roles.[6, 11-13] However, no study has investigated the expressions of genes associated with disc matrix synthesis and of cytokines associated with disc degradation with respect to disc degeneration in human discs at the same time.

This study was conducted to investigate the expressions of matrix associate genes and cytokine expressions in discs showing various degrees of

degradation. The purpose of this study was to identify important factors of disc degeneration among genes involved in matrix synthesis and among pro-inflammatory cytokines, and to suggest a strategy for a molecular therapy.

II. MATERIALS AND METHODS

1. Clinical materials

Disc specimens obtained from 18 patients that underwent discectomy for lumbar disc herniation unresponsive to conservative therapy were utilized in the present study. Only nucleus pulposus samples were taken during surgery. After dissecting the disc space, the author made a rectangular cut around the annulus fibrosus with a sharp knife (Figure 1), and using a pituitary forceps, removed the disc with annulus and nucleus *en block* (Figure 2A). After confirming the margin of the annulus and nucleus, only the nucleus was excised for this study (Figure 2B). MRI (Magneton Vision 1.5T, Siemens, Erlangen, Germany) was performed on all patients. PACS software and a PACS workstation (Centricity 2.0, General Electrics Medical Systems, Milwaukee, WI) were used by an independent neurosurgeon and neuroradiologist not involved in treatment to disc degeneration. Disc degeneration was graded using routine T2-weighted MR images using Pfirrmann's grading system (Table 1).[14] Discs were classified into two groups. Group I (9 patients) contained mildly degenerated discs (grades 2 and 3), and Group II (9 patients) contained severely degenerated discs (Grades 4 and 5).

This study was approved by the Institutional Review Board (IRB) of Gangnam Severance Hospital, Yonsei University College of Medicine (No. 3-2008-0173).

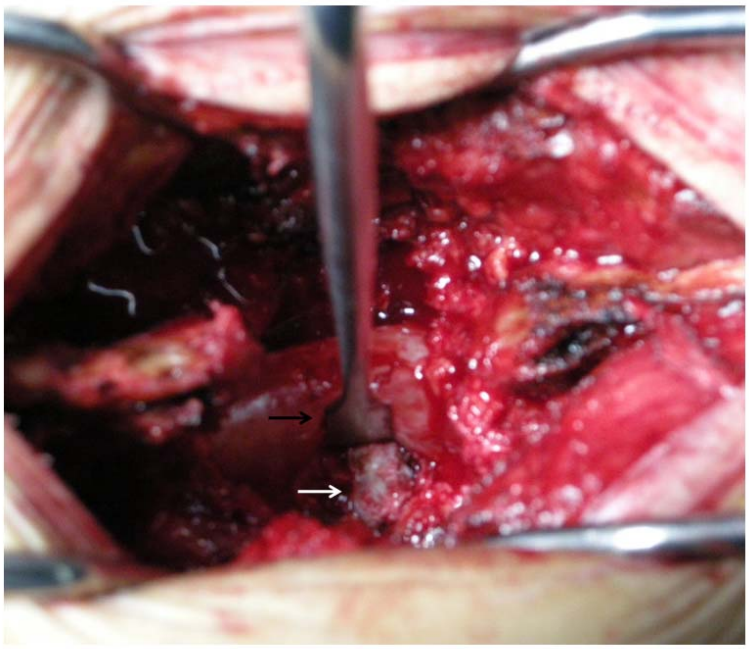

Figure 1 After dissecting the disc space, a rectangular cut was made around the annulus fibrosus with a sharp knife. The black arrow indicates dura and

white arrow the disc.

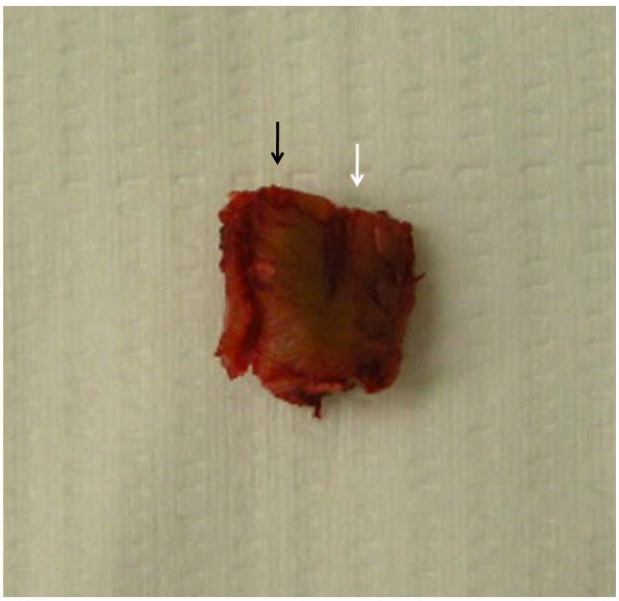

Figure 2A Using a pituitary forceps, the disc with annulus and nucleus were removed en bloc. The black arrow indicates the annulus and the white arrow the nucleus.

Figure 2B After confirming the margin between the annulus and nucleus, the nucleus was excised. The black arrow indicates the annulus and the white arrow the nucleus.

Table 1. Classification of Disc Degeneration according to Pfirrmann's Grading System

Grade	Structure	Distinction of Nucleus and Anulus	Signal intensity	Height of Intervertebral Disc
I	Homogeneous, bright white	Clear	Hyperintense, isointense to cerebrospinal fluid	Normal
II	Inhomogeneous with or without horizontal bands	Clear	Hyperintense, isointense to cerebrospinal fluid	Normal
III	Inhomogeneous, gray	Unclear	Intermediate	Normal to slightly decreased
IV	Inhomogeneous, gray to black	Lost	Intermediate to hypointense	Normal to moderately decreased
V	Inhomogeneous, black	Lost	Hypointense	Collapsed disc space

2. Isolation of disc cells and culture

All reagents were purchased from GibcoBRL (Grand Island, NY). During lumbar discectomy, intervertebral disc materials were excised, and as mentioned above to ensure sample homogeneity, disc tissues were acquired from the nucleus pulposus not from the annulus. Tissues were dissected into small pieces and incubated in 5% CO_2/95% room air at 37 ^{o}C in Dulbecco's Modified Eagle Medium and in Ham's F-12 (DMEM/F-12) media. To isolate cells, disc tissues were digested in DMEM/F-12 media containing 0.2% protease (Sigma Chemical, St. Louis, MO) for 1 hour, followed by 0.025% collagenase (Sigma) for 12 hours. Cell passage less than twice were used in subsequent experiments.

Disc cells ($2x10^5$ cells/well) were grown as monolayer cultures for 6 days in DMEM/F-12 media containing 10% fetal bovine serum (FBS), 10 U/ml penicillin, 10 g/ml streptomycin, and 0.2 mmol/L L-glutamine. The mRNA expressions of aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, osteocalcin, TNF-α, and IL-1β were then constructed using complete mRNA sequences obtained from the National Center for Biotechnology Information. The forward and reverse primer sequences of all genes examined in this study are summarized in Table 2.

Table 2. Primer sequence

	Primer	Sequence
1. Aggrecan	Forward	CTGCTTCCGAGGCATTTCAG
	Reverse	CTTGGGTCACGATCCACTCC
2. Type II collagen	Forward	GGTCTTGGTGGAAACTTTGCT
	Reverse	GGTCCTTGCATTACTCCCAAC
3. Sox 9	Forward	AGCGAACGCACATCAAGAC
	Reverse	GCTGTAGTGTGGGAGGTTGAA
4. Type I collagen	Forward	GTCGAGGGCCAAGACGAAG
	Reverse	CAGATCACGTCATCGCACAAC
5. Alkaline phosphatase	Forward	ATGGGATGGGTGTCTCCACA
	Reverse	CCACGAAGGGGAACTTGTC
6. Osteocalcin	Forward	CACTCCTCGCCCTATTGGC
	Reverse	CCCTCCTGCTTGGACACAAAG
7. TNF-α	Forward	CGAACATCCAACCTTCCCAAC
	Reverse	TGGTGGTCTTGTTGCTTAAAGTTC
8. IL-1β	Forward	CGGCCACATTTGGTTCTAAGA
	Reverse	AGGGAAGCGGTTGCTCATC
9. GAPDH*	Forward	ATGGGGAAGGTGAAGGTCG
	Reverse	GGGGTCATTGATGGCAACAATA

*GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a house keeping gene.

3. Real-time polymerase chain reaction assay

We used an ABI Prism 7300 (Applied Biosystems, USA) that detects SYBR Green fluorescent dye incorporated in double stand DNA. The 20 μL reaction volumes contained 25ng of cDNA (obtained by RT-PCR) and 5 pmole of each primer (aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, osteocalcin, TNF-α, and IL-1β). Forty real-time PCR cycles were performed for denaturation (95 ^{o}C for 30 seconds), annealing, and elongation (60 ^{o}C for 60 seconds). To confirm amplification specificity, PCR products were subjected to melting curve analysis (Figure 3). Threshold cycles (Ct) of aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, osteocalcin, TNF-α, and IL-1β were standardized versus glyceraldehyde phosphate dehydrogenase (GAPDH). The mRNA expressions of Group I and Group II tissues are reported as ratios (Group I/Group II).

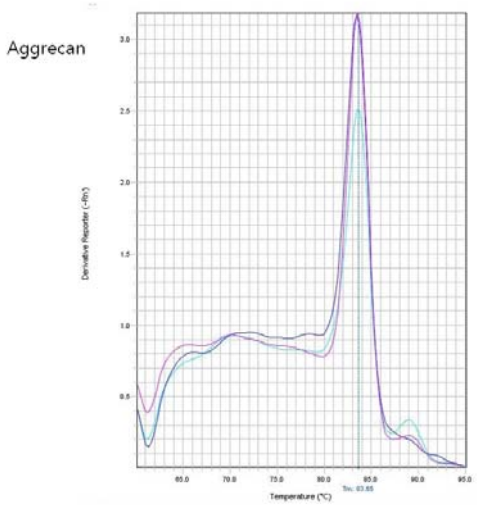

Figure 3A Melting curve for aggrecan.

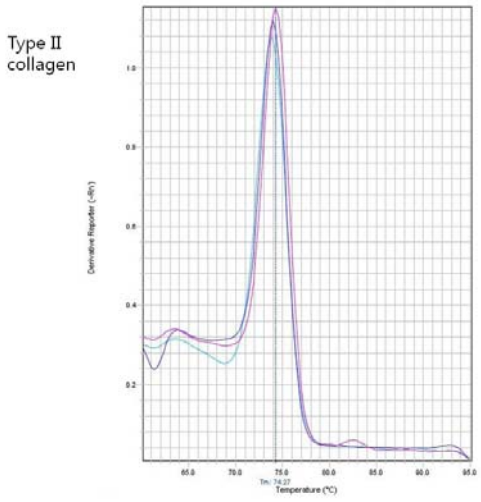

Figure 3B Melting curve for Type II collagen.

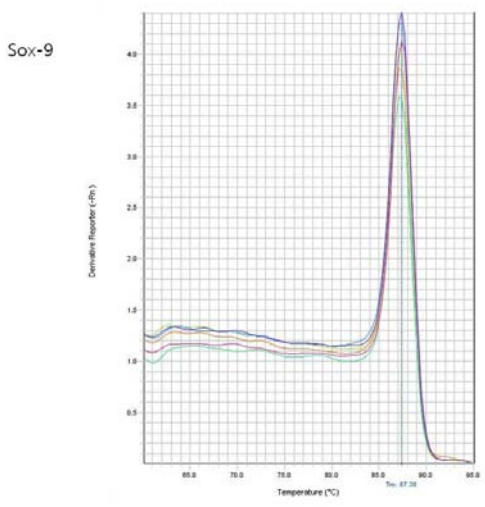

Figure 3C Melting curve for Sox9.

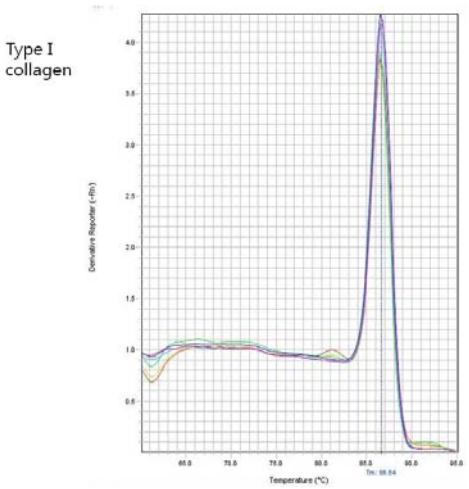

Figure 3D Melting curve for Type I collagen.

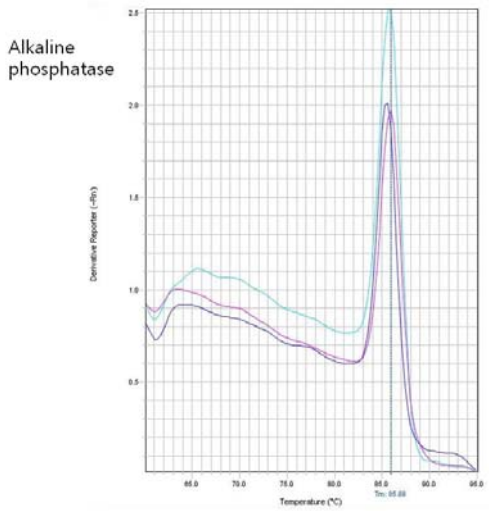

Figure 3E Melting curve for alkaline phosphatase.

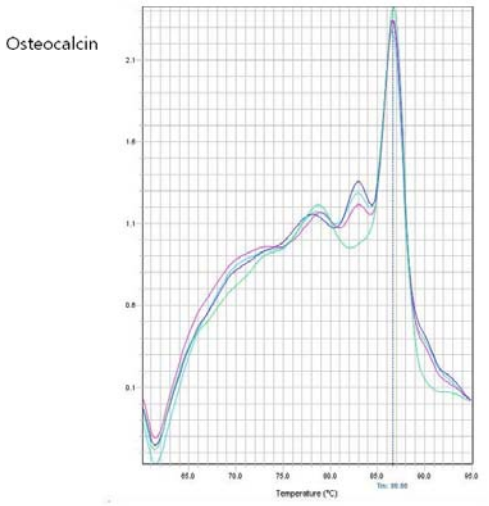

Figure 3F Melting curve for osteocalcin.

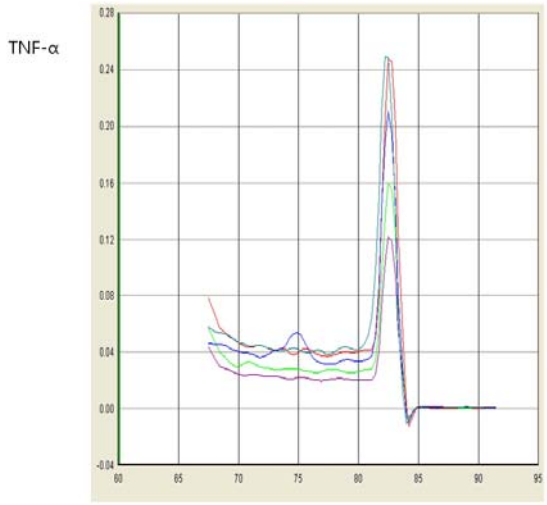

Figure 3G Melting curve for TNF-α.

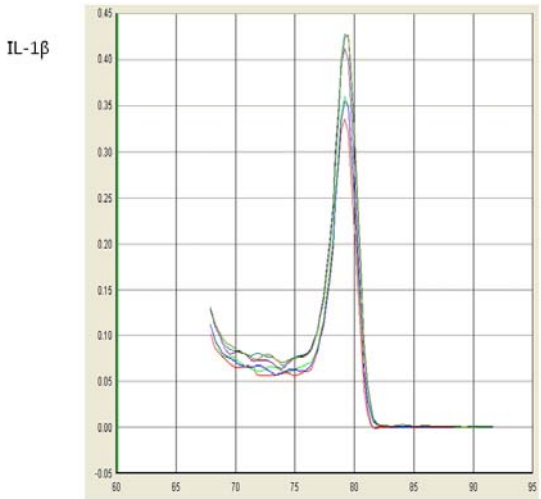

Figure 3H Melting curve for IL-1β.

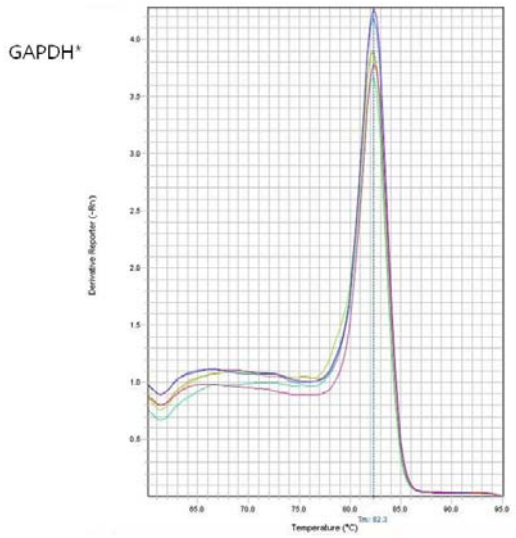

Figure 3I Melting curve for glyceraldehydes phosphate dehydrogenase (GAPDH).

4. Western blot analysis

We also compared matrix associated protein synthesis (aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase and osteocalcin) by western blotting in the two groups. Protein concentrations of total lysates were determined using Bradford protein assays (Bio-Rad Laboratory, Richmond, CA). Equal amounts of protein per lane were separated on 10% SDS-polyacrylamide gels and subsequently transferred to nitrocellulose membranes (Bio-Rad, USA) at 330 mA for 2 h using a transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3). The membranes were then blocked with blocking buffer, washed 3 times, and incubated. Immune complexes were detected using the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). To accomplish this, primary antibodies against aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase and osteocalcin were applied at optimized concentrations. Membranes were then treated with secondary antibodies conjugated to goat anti mouse (1:1000; Jackson Immunoresearch, USA) followed by enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ, USA) to allow proteins to be detected.

5. Statistical analysis

Intergroup comparisons were analyzed using Wilcoxon's Signed Rank test. *P* values of less than 0.05 were considered statistically significant. All analyses were carried out using SPSS Ver. 12.00K (SPSS, Inc., Chicago, IL).

III. RESULTS

1. Patient Characteristics

The discs of 18 patients were classified into two Groups. Group I (9 patients) contained mildly degenerated discs (Grade 2, 5 patients; Grade 3, 4 patients), and Group II (9 patients) contained severely degenerated discs (Grade 4, 7 patients; Grade 5, 2 patients). Mean patient age in Group II (56.9±13.9 years) was significantly higher than in Group 1 (43.1±13.3years) ($P<0.05$).

2. Quantitation of mRNA Levels

We assayed the mRNA levels of genes specific for aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, osteocalcin, TNF-α, and IL-1β. mRNA gene expression in Group I (mildly degenerated discs) was significantly greater for aggrecan (1.83-fold*), Type II collagen (1.82-fold*), Sox9 (1.80-fold*), Type I collagen (1.41-fold*), alkaline phosphatase (1.38-fold*), and osteocalcin (1.80-fold**) (Table 3) (Fig 4). However, mRNA levels of TNF-α (1.26-fold*) and IL-1β (1.11-fold*) were significantly greater in Group II (Table 3) (Fig. 4) (* indicates $P<0.01$ and ** indicates $P<0.05$).

Table 3. Quantitation of mRNA Levels

	Group I (Mean ± SD)		Group II (Mean ± SD)	
	ΔC_T Mean	Standard deviation	ΔC_T Mean	Standard deviation
aggrecan*	7.3827	2.5399	4.0349	2.0052
Type II collagen*	7.5175	2.5581	4.1343	2.2314
Sox9*	5.3146	1.781	2.9539	1.906
Type I collagen*	4.8026	1.8008	3.4069	0.9467
alkaline phosphatase*	6.8565	2.9718	4.9791	2.4063
osteocalcin**	2.9765	1.3423	1.6542	3.0829
TNF-α*	7.7311	1.6683	9.7616	2.1651
IL-1β*	6.1267	0.714	6.8105	0.9926

* indicates $P<0.01$ and ** indicates $P<0.05$.

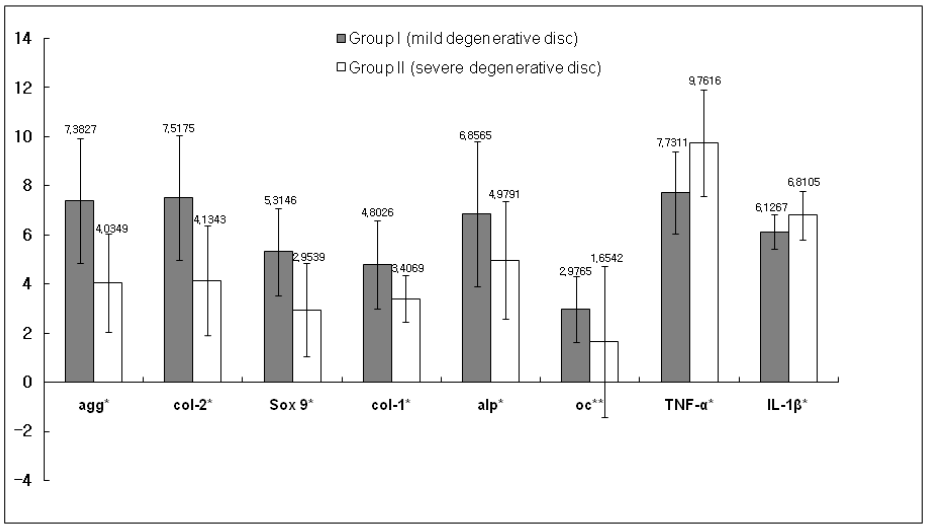

Figure 4 mRNA levels of genes specific for aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, osteocalcin, TNF-α, and IL-1β in Group I (mildly degenerated discs) and Group II (severely degenerated discs). * indicates $P<0.01$ and ** indicates $P<0.05$.

3. Expression by Western blot assay

When we investigated the matrix associate proteins (aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase and osteocalcin) levels by Western blotting, Group I was found to express higher protein levels than Group II (Figure 5).

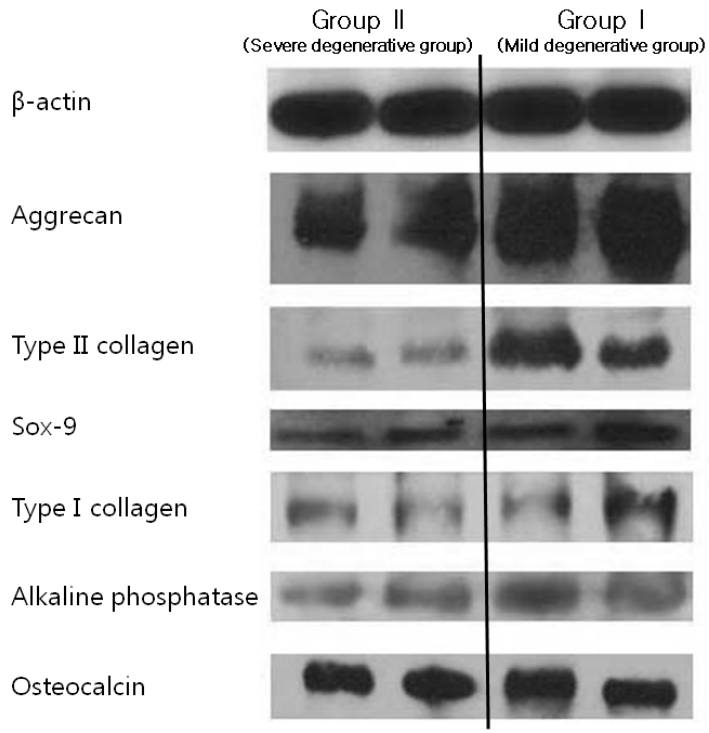

Figure 5 Western blot results for aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase and osteocalcin in Groups I and II.

IV. DISCUSSION

The integrity of intervertebral discs is dependent on the balance between matrix synthesis and degradation. During aging or when affected by disease, disc cells undergo substantial degenerative changes, which including cell type and cell phenotype alterations, the latter of which is characterized by a compromised ability to synthesize normal matrix components and by enhanced catabolism.[4, 6, 15] Previous studies evaluated three types of biochemical change caused by an altered disc cell phenotype during disc aging and degeneration [1, 4, 6, 11, 15], namely, a decrease in matrix synthesis, increased catabolism, and changes in growth factors and cytokines.[2, 6-8] In the present study, we sought to determine which of these types of biochemical change is initially responsible for disc degeneration. Previous studies have shown that various genes (aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, osteocalcin, and others) are downregulated in degenerated discs.[4, 6, 10, 15] Of the various genes associated with matrix synthesis, Type I and Type II collagens act as fibrillar molecules, aggrecan consists of a core protein to which sulfated-glycosaminoglycans, Sox-9 upregulates both aggrecan and Type II collagen, osteocalcin and alkaline phosphatase are markers of osteogenic genes.[2, 7, 8, 10] Here, we investigated above genes that have been most strongly associated with matrix synthesis.

Furthermore, it is also known that various pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-1α, nitro oxide, prostaglandin E_2, and others) are upregulated during disc degeneration.[1, 6, 9, 16, 17] Of these cytokines, TNF-α and IL-1β are well known to be associated with disc degradation and discogenic back pain.[18-20] TNF-α is an important initiator of matrix degeneration, whereas IL-1β plays a greater role in pathological degradation.[12] Accordingly, we focused on these cytokines.

Molecular therapies for disc degeneration have been devised based on phenotype changes in degenerated discs.[1] Four different classes of molecules are currently being investigated in this context, namely, anti-catabolics, mitogens, morphogens, and intracellular regulators.[1, 3, 8, 13, 16, 20-22] However, molecular therapy trials conducted to date have been conducted on individual candidates.[1, 3, 8, 13, 16, 20-22] This is the first study to evaluate both disc matrix synthesizing factors and cytokines associated with disc degeneration in human discs with respect to the extent of disc degeneration. The present study shows that the genes of matrix factors are downregulated but that those of pro-inflammatory cytokines are simultaneously upregulated during disc degeneration. These results concur with those of a previous study. [1, 4, 6, 10, 11, 15, 16] Our western blotting results also show that matrix associated protein levels reduced in accord with degree of disc degeneration.

From this study, we had some clue for disc degeneration. Initially we were interested in answering the question "Which of the three types of biologic changes is primarily responsible for the initiation of disc degeneration?" The answer appears to be that these changes co-occur. This indicates that current strategies for molecular therapies should be modified. A number of molecules have been studied *in vitro* and *in vivo* in this context with encouraging results.[1, 3, 8, 9, 16, 20, 22, 23] However, previous trials have focused on single candidates, whereas our results indicate that a molecular therapy should address all factors associated with disc degeneration simultaneously.

This study has several limitations that require consideration. First, the sample size was too small to examine in detail relations between matrix and cytokine components and disc degeneration at different levels. In addition, to determine the mechanism of disc degeneration, longitudinal studies are required to identify the major factors responsible for disc degeneration, such as, reduced matrix synthesis, increased catabolism, and expressional changes of growth factors and inflammatory cytokines. Furthermore, these studies should include a range of disc degenerations from normal to most severe. In addition, the findings of the present suggest that future molecular therapies should be composite therapies that address all factors associated disc

degeneration.

V. CONCLUSION

Our results show that mildly degenerated disc cells more potently express matrix, chondrogenic, and osteoblastic genes than severely degenerated disc cells. In addition, it shows that catabolic cytokines associated with disc degeneration were more highly expressed in severely degenerated discs. Furthermore, decreases in matrix synthesis, increase of inflammatory cytokine levels were found to occur simultaneously during disc degeneration. Our findings indicate that developmental strategies regarding molecular therapy should simultaneously address all factors associated with disc degeneration.

VI. REFERENCES

1. Yoon ST, Patel NM. Molecular therapy of the intervertebral disc. Eur Spine J 2006;15(3):379-88.

2. Kuh SU, Zhu Y, Li J, Tsai K, Fei Q, Hutton WC, et al. A comparison of three cell types as potential candidates for intervertebral disc therapy: annulus fibrosus cells, chondrocytes, and bone marrow derived cells. Joint Bone Spine 2009;76(1):70-4.

3. Evans C. Potential biologic therapies for the intervertebral disc. J Bone Joint Surg Am 2006;88(2):95-8.

4. Antoniou J, Steffen T, Nelson F, Winterbottom N, Hollander AP, Poole RA, et al. The human lumbar intervertebral disc: evidence for changes in the biosynthesis and denaturation of the extracellular matrix with growth, maturation, ageing, and degeneration. J Clin Invest 1996;98(4):996-1003.

5. Peng B, Hao J, Hou S, Wu W, Jiang D, Fu X, et al. Possible pathogenesis of painful intervertebral disc degeneration. Spine 2006;31(5):560-6.

6. Zhao C, Wang L, Jiang L, Dai L. The cell biology of intervertebral

disc aging and degeneration. Ageing Res Rev 2007;6(3):247-61.

7. Diefenderfer DL, Brighton CT. Microvascular pericytes express aggrecan message which is regulated by BMP-2. Biochem Biophys Res Commun 2000;269(1):172-8.

8. Yoon ST, Kim KS, Li J, Park JS, Akamaru T, Elmer WA, et al. The effect of bone morphogenetic protein-2 on rat intervertebral disc cells in vitro. Spine 2003;28(16):1773-80.

9. Fassett DR, Kurd MF, Vaccaro AR. Biologic solutions for degenerative disk disease. J Spinal Disord Tech 2009;22(4):297-308.

10. Adam M, Deyl Z. Degenerated annulus fibrosus of the intervertebral disc contains collagen type II. Ann Rheum Dis 1984;43(2):258-63.

11. Lee S, Moon CS, Sul D, Lee J, Bae M, Hong Y, et al. Comparison of growth factor and cytokine expression in patients with degenerated disc disease and herniated nucleus pulposus. Clin Biochem 2009;42(15):1504-11.

12. Millward-Sadler SJ, Costello PW, Freemont AJ, Hoyland JA. Regulation of catabolic gene expression in normal and degenerate human intervertebral disc cells: implications for the pathogenesis of intervertebral

disc degeneration. Arthritis Res Ther 2009;11(3):R65-R.

13. Kuh SU, Zhu Y, Li J, Tsai K, Fei Q, Hutton WC, et al. Can TGF-beta1 and rhBMP-2 act in synergy to transform bone marrow stem cells to discogenic-type cells? Acta Neurochir 2008;150(10):1073-9.

14. Pfirrmann CW, Metzdorf A, Zanetti M, Hodler J, Boos N. Magnetic resonance classification of lumbar intervertebral disc degeneration. Spine 2001;26(17):1873-8.

15. Antoniou J, Goudsouzian NM, Heathfield TF, Winterbottom N, Steffen T, Poole AR, et al. The human lumbar endplate. Evidence of changes in biosynthesis and denaturation of the extracellular matrix with growth, maturation, aging, and degeneration. Spine 1996;21(10):1153-61.

16. Sguin CA, Pilliar RM, Roughley PJ, Kandel RA. Tumor necrosis factor-alpha modulates matrix production and catabolism in nucleus pulposus tissue. Spine 2005;30(17):1940-8.

17. Rutges JP, Kummer JA, Oner FC, Verbout AJ, Castelein RJM, Roestenburg HJA, et al. Increased MMP-2 activity during intervertebral disc degeneration is correlated to MMP-14 levels. J Pathol 2008;214(4):523-30.

18. Hoyland JA, Le Maitre C, Freemont AJ. Investigation of the role of IL-1 and TNF in matrix degradation in the intervertebral disc. Rheumatology 2008;47(6):809-14.

19. Yoshida T, Park JS, Yokosuka K, Jimbo K, Yamada K, Sato K, et al. Up-regulation in receptor for advanced glycation end-products in inflammatory circumstances in bovine coccygeal intervertebral disc specimens in vitro. Spine 2009;34(15):1544-8.

20. Weiler C, Nerlich AG, Bachmeier BE, Boos N. Expression and distribution of tumor necrosis factor alpha in human lumbar intervertebral discs: a study in surgical specimen and autopsy controls. Spine 2005;30(1):44-53.

21. Yoon ST, Park JS, Kim KS, Li J, Attallah-Wasif ES, Hutton WC, et al. ISSLS prize winner: LMP-1 upregulates intervertebral disc cell production of proteoglycans and BMPs in vitro and in vivo. Spine 2004;29(23):2603-11.

22. Fei Q, Jiang X, Chen T, Li J, Murakami H, Tsai K, et al. Changes with age and the effect of recombinant human BMP-2 on proteoglycan and collagen gene expression in rabbit anulus fibrosus cells. Acta Biochim Biophys Sin 2006;38(11):773-9.

23. Sheikh H, Zakharian K, De La Torre RP, Facek C, Vasquez A, Chaudhry GR, et al. In vivo intervertebral disc regeneration using stem cell-derived chondroprogenitors. J Neurosurg Spine 2009;10(3):265-72.

ABSTRACT (IN KOREAN)

추간판의 퇴행정도에 따른 기질관련 유전자와 염증매개 cytokine관련 유전자의 발현 정도의 비교

: 생체 추간판 수핵을 이용한 분석

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박 정 윤

본 연구는 생체의 추간판의 노화에 따른 기질생성과 관련된 다양한 유전자와 염증매개 cytokine의 발현 정도를 비교하기 위한 연구이다. 위 실험을 위하여 추간판 탈출증등의 질환으로 인하여 추간판 제거수술이 필요하였던 18명의 환자로부터 퇴행성 추간판을 획득하였다. 얻어진 추간판은 수술 전 실시한 MRI T2 영상을 이용한 Pfirrmann의 퇴행성 변화척도를 이용하여 퇴행 정도를 분류하였다. 퇴행 정도에 따라 그룹 I (Grade 2 와 Grade 3의 미약한 퇴행성 변화)과 그룹 II

(Grade 4 와 Grade 5의 심한 퇴행성 변화)로 분류하였다. 각각의 그룹은 9명씩 포함되었으며, 얻어진 추간판중 수핵만을 분리하여 monolayer cultures를 실시하였다. 얻어진 세포로부터 Aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, osteocalcin, TNF-α 와 IL-1β 의 mRNA의 발현 정도를 real-time PCR를 이용하여 그룹간에 비교하였다. 또한 aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase 와 osteocalcin 의 직접적인 단백질 생성을 western blot을 이용하여 그룹간에 비교하였다. Aggrecan는 1.83배, Type II collagen은 1.82배, Sox9은 1.80배, Type I collagen은 1.41배, alkaline phosphatase는 1.38배, osteocalcin은 1.80배로 기질과 관련된 mRNA는 그룹 I (미약한 퇴행성변화)에서 그룹 II (심한 퇴행성변화)보다 높게 나타났다. Western blot에서의 단백질 분석도 aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase 와 osteocalcin 은 그룹 I 에서 그룹 II보다 높게 나타났다. 그러나 반대로, TNF-α는 1.26배, IL-1β는 1.11배로 염증매개

cytokine들은 그룹 II에서 그룹 I보다 높게 나타났다. 미약한 퇴행성 추간판은 심한 퇴행성 추간판보다 기질관련 유전자의 발현이 높게 발현되었으며, 또한 이와 관련된 단백질의 생성도 보다 강력하게 생산되었다. 그러나 염증매개 cytokines들은 심한 퇴행성 변화를 보이는 추간판에서 높게 발현되었다. 결론적으로 기질 생산의 감소와 염증매개 cytokine의 증가는 추간판의 퇴행성 변화에 동시에 나타나는 변화라는 것을 증명하였다.

핵심되는 말: 퇴행성 추간판, Aggrecan, Type II collagen, Sox9, Type I collagen, alkaline phosphatase, osteocalcin, TNF-α, IL-1β