Molecular biologic response in the degenerative living human nucleus pulposus cells treated with cytokines

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Molecular biologic response in the degenerative living human nucleus pulposus cells treated with cytokines

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When I began this dissertation, I thought it would be easy because there were many professors and researchers who could give me their hands. But, it was not easy to do and to continue the experiment once I started to work at Ajou university hospital. I was stuck in operations and could not even try to do the experiment. Unfortunately, I needed to change my topic and plan for initial experiment of my doctoral degree because the result from the preliminary study and the hypotheses seldom correspond. I felt that I was standing in front of a huge mountain I never go over.

In those days, the Basic Experimental Association was organized by some young and active professors, and professor Kuh who is one of foundation member, suggested me to join the experiment which he had been doing. So I restarted working on my doctoral degree, and eventually I could go over this huge mountain. I really appreciate to professor Kuh again. I am heartily thankful to my supervisor, professor Yoon, professor Shin, and professor Kim whose encouragement, guidance, and support from the initial to the

final level enabled me to develop an understanding of the subject and to complete this work. I really thank to researcher Park who always helped my experiment and taught me how to do in every step.

Lastly, I would like to express my special thank to my family soo-kyung, min, and tark whose love and support enabled me to complete this work.

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There are at least four different classes of molecules that are currently being investigated for disc therapy: anti-catabolics, mitogens, morphogens, and intracellular regulators. The result of biological therapy can be also different according to therapeutic modalities and to the degree of degeneration which has a relation to chemical composition and histologic changes of intervertebral disc (IVD). The objective of this study was to investigate the molecular biologic responses of various genes and proteins relating disc degeneration to cytokines that influence disc-cell metabolism and phenotype. The responsiveness according to the degree of disc degeneration in living human IVD to these cytokines was also evaluated.

Living human disc specimens were obtained from 12 patients who underwent discectomy. Disc degeneration was graded on routine T2-weighted MRI using the Pfirrmann's grading system. The disc specimens were classified into two Groups. Group 1 (6 patients) was mild degeneration of IVD and Group 2 (6 patients) was severe degeneration of IVD. Intervertebral disc materials were taken from the patients during the discectomy. Each disc cells (2×10^5 cells/well) were grown as monolayer cultures for 6 days. After 6 days, mRNA expression of aggrecan, type I collagen, type II collagen, Sox9, alkaline phosphatase, osteocalcin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were constructed using the complete mRNA. The gene expression was analyzed after treatment using four cytokines. Recombinant human bone morphogenic protein-2 (rhBMP-2) and transforming growth factor-β (TGF-β) were used as morphogens for the disc cells. Interleukin-1β (IL-1β) and Tumor necrosis factor-α (TNF-α) were treated as inflammatory mediators implicated in disc degeneration. The mRNA expression from disc cell culture without cytokines was used as a baseline control value. The mRNA expression of Group I was compared to Group II and reported as a ratio. The immunoreactivity of IVD for rhBMP-2 and TGF- β were analyzed to check the chondrogenic activity.

Four cytokines, including rhBMP-2, TGF-β, TNF-α, and IL-1β were used as morphogenic cytokines and inflammatory implicators in this study. The responsiveness to these four cytokines between Group 1 (mild degenerative IVD) and Group 2 (severe degenerative IVD) were checked into gene and protein expression and showed statistical difference. The mRNA gene expression in Group 1 was significantly greater for aggrecan, type I collagen, type II collagen, alkaline phosphatase, osteocalcin, and Sox9 than the mRNA gene expression in Group 2 when they were not treated with cytokine. The mRNA levels of gene for these molecules

after treatment of morphogens also revealed significant increment in both Groups which were much higher in Group 1 than in Group 2. There was no statistical significance in both Groups after treatment of inflammatory implicators. Micrographic findings of rhBMP-2 and TGF-β immunoreactive IVD cells for aggrecan, alkakine phosphatase, type I collagen, type II collagen, osteocalcin, and Sox9 revealed similar results in both Groups. The average numbers of immunofluorescence positive stained IVD cells for alkaline phosphatase were increased after treatment of rhBMP-2 and TGF-β in Group 1.

The treatment of rhBMP-2 and TGF-β increased the expression of the various genes associated with matrix synthesis, including aggrecan, alkaline phosphatase, type I collagen, type II collagens, osteocalcin, and Sox9. The treatment of TNF-α and IL-1β decreased the expression of these genes. The molecular biologic responsiveness to the treatment of rhBMP-2, TGF-β, TNF-α and IL-1β in the degenerative living human IVD can be different according to the degree of degeneration of IVD.

Key words: intervertebral disc, degeneration, cytokine, rhBMP-2, TGF-β, TNF-α, IL-1β

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I. INTRODUCTION

Degeneration of the intervertebral disc (IVD) is one of the natural aging processes. The healthy IVD has high water content in the nucleus pulposus (NP) as its matrix is rich in large aggregating proteoglycans.[1-2] With degeneration, there is a progressive loss of the proteoglycan matrix, which naturally imbibes water, causing dehydration and desiccation within the NP, and the IVD becomes a more fibrotic and less cartilaginous structure.[3-6]

Degeneration of the IVD is clinically associated with low back pain and other important disease conditions of the spine.[1] Treatment options range from pain

management to invasive procedures such as discectomy, intradiscal electrothermal therapy, fusion, and spinal arthroplasty. However, these options address the clinical symptoms of degeneration of IVD rather than target the pathophysiological pathways involved in the degenerative process.[3,7-11]

Genetic, mechanical, and biologic factors are widely regarded as important contributors to the degenerative process.[12-19] Although the exact pathophysiology of disc degeneration is not completely understood, the structural changes within the IVD have been well described.[20-21] To better characterize the degenerative process and to prevent or reverse the degenerative changes in the disc matrix by altering the disc matrix metabolism, many investigators have attempted biological experiments and treatment of disc degeneration including the use of cellular components, matrix-derivatives, and molecules influencing disc cell metabolism and phenotype.[22-26] There are at least four different classes of molecules that are currently being investigated for disc therapy: anti-catabolics, mitogens, morphogens, and intracellular regulators. All of these molecules have some in vitro data, but few have been tested in vivo in an animal model with disc degeneration to verify the biological mechanisms of each molecule. There is no clinically proven biological therapy for degeneration of human IVD.[12,14-16,22-23,26-33] The results of biological therapy may also be different according to therapeutic modalities and to the degree of degeneration, which has a relation to the chemical composition and histologic changes of IVD.[20-21,32,34-35]

The aging process changes the expression level and spatial distribution of transforming growth factor (TGF) and bone morphogenic protein (BMP) molecules and receptors.[17,36] Okuda et al. supported this notion by demonstrating that the responsiveness of intervertebral cells to insulin like growth factor-1 (IGF-1) and TGF-β decreases with advancing age in rabbit disc cells.[24] Thompson *et al.* were the first to

demonstrate that exogenous administration of a growth factor, TGF-β, can significantly increase proteoglycan synthesis by NP cells *in vitro*.[37] This prompted further investigation into other growth factors such as IGF-1, BMP-2, and BMP-7, all of which have been shown to enhance the anabolic functions of IVD cells by up-regulating proteoglycan synthesis.[38-39] Degeneration of IVD may be a reflection of molecular biologic change of the IVD with age and the degree of IVD degeneration may be an influencing factor for responsiveness to treatment of cytokines.

The objective of this study was to investigate the molecular biologic response of various genes and proteins relating disc degeneration to cytokines that influence disc-cell metabolism and phenotype. The responsiveness according to the degree of disc degeneration in living human IVD to these cytokines was also evaluated.

II. MATERIALS AND METHODS

1. Study design

Living human disc specimens were obtained from 12 patients who underwent discectomy for degenerative lumbar disc herniation and who were unresponsive to conservative therapy. Exclusion criteria included infection, metabolic bone disease and neoplastic disease. MRI (Magneton Vision 1.5T, Siemens, Erlangen, Germany) was taken performed in all patients. We used the PACS software and PACS workstation (Centricity 2.0, General Electrics Medical Systems, Milwaukee, WI, USA) for the review by an independent neurosurgeon and neuroradiologist. Disc degeneration was graded on routine T2-weighted MRI using the Pfirrmann's grading system (Table 1). The disc specimens were classified into two Groups. Group 1 (6 patients) was mild degeneration of IVD (Grade II and III), and Group 2 (6 patients) was severe degeneration of IVD (Grade IV and V). This study was approved by the Institutional Review Board (IRB) of Gangnam Severance Hospital, Yonsei University College of Medicine (No. 6-2008-0290).

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

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

2. Isolation of disc cells and culture

Unless otherwise stated, all reagents were purchased from GibcoBRL (Grand Island, NY, USA). Intervertebral disc materials were taken from patients during the discectomy. To make the samples homologous, disc materials were acquired from the nucleus pulposus and not the annulus. Tissues from each disc were dissected into small pieces and incubated (5% CO_2, 95% room air at 37 ^{o}C) in Dulbecco's Modified Eagle Medium and Ham's F-12 (DMEM/F-12) media. To isolate the cells, disc tissue were digested in DMEM/F-12 media with 0.2% protease (Sigma Chemical, St. Louis, MO, USA) for 1 hour, followed by 0.025% collagenase (Sigma Chemical, St Louis, MO, USA) for 12 hours. Cells from less than 2 passages were used for each experiment.

Each disc cells ($2x10^5$ cells/well) were grown as monolayer cultures for 6 days in DMEM/F-12 media with 10% fetal bovine serum (FBS) + 10 U/ml penicillin + 10 g/ml streptomycin + 0.2 mmol/L L-glutamine. After 6 days, mRNA expression of aggrecan, type I collagen, type II collagen, Sox9, alkaline phosphatase, osteocalcin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were constructed using the complete mRNA sequence from the National Center for Biotechnology Information. Forward and reverse primer sequences of aggrecan, type I collagen, type II collagen, Sox9, osteocalcin, and alkaline phosphatase are summarized in Table 2.

Table 2. Primer sequence for aggrecan, type I collagen, type II collagen, Sox9, osteocalcin, and alkaline phosphatase used in quantitative real time PCR

		Primer nucleotide Sequence (5′-3′)
1. Aggrecan	Forward	CTGCTTCCGAGGCATTTCAG
	Reverse	CTTGGGTCACGATCCACTCC
2. Type I collagen	Forward	GTCGAGGGCCAAGACGAAG
	Reverse	CAGATCACGTCATCGCACAAC
3. Type II collagen	Forward	GGTCTTGGTGGAAACTTTGCT
	Reverse	GGTCCTTGCATTACTCCCAAC
4. Sox9	Forward	AGCGAACGCACATCAAGAC
	Reverse	GCTGTAGTGTGGGAGGTTGAA
5. Alkaline phosphatase	Forward	ATGGGATGGGTGTCTCCACA
	Reverse	CCACGAAGGGGAACTTGTC
6. Osteocalcin	Forward	CACTCCTCGCCCTATTGGC
	Reverse	CCCTCCTGCTTGGACACAAAG
7. GAPDH*	Forward	ATGGGGAAGGTGAAGGTCG
	Reverse	GGGGTCATTGATGGCAACAATA

*GAPDH was used as a house keeping gene.

3. Treatment of cytokines

Each experimental Group was also cultured in a chamber slide in an incubator (5% CO_2, 95 % room air at 37˚C) at 3×10^4 cells/chamber. When the cell culture became confluent, the media were replaced with DMEM/F-12 media containing 1% FBS+10 U/ml penicillin+10 g/ml streptomycin+0.2 mmol/l L-glutamine+5 μg/ml vitamin C. The gene expressions after treatment with four cytokines were analyzed. TGF-β and rhBMP-2 were used as morphogens for the disc cell. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were treated as inflammatory mediators implicated in disc degeneration. The mRNA expression from disc cell culture without cytokines was used as a baseline control value. Disc cells were cultured in monolayer and treated with rhBMP-2 100 ng/ml (R&D System, Minneapolis, MN, USA), TGF-β 10 ng/ml (Invitrogen, Carlsbad, CA, USA), IL-1β (Invitrogen, Carlsbad, CA, USA) and TNF- α (invitrogen, Carlsbad, CA, USA) for 6 days. On days 3, the culture media were changed with the same concentration of rhBMP-2, TGF-β, IL-1β and TNF-α in each well.

4. Real-time polymerase chain reaction (PCR) assay

ABI Prism 7300 (Applied biosystem, Foster city, CA, USA) was used to detect SYBR Green fluorescent dye incorporated in double stand DNA. A 20 μL reaction volume included 25ng of cDNA of RT-PCR and 5 pmole of each primer (aggrecan, alkaline phosphatase, type I collagen, type II collagen, osteocalcin, and Sox9). Forty real-time PCR cycles were performed for denaturation (95˚C for 30 seconds),

annealing, and elongation (60°C for 60 seconds). To confirm amplification specificity, PCR products were subjected to a melting curve analysis. Threshold cycles (Ct) of aggrecan, alkaline phosphatase, type I collagen, type II collagen, osteocalcin, and Sox9 were standardized according to glyceraldehydes phosphate dehydrogenase (GAPDH). The mRNA expression of Group 1 were compared to Group 2 and reported as a ratio.

5. Immunofluorescence staining with antibodies

Each cell ($3x10^4$ cells/well) was grown as monolayer cultures in the DMEM/F-12 culture media containing 1% FBS+10 U/ml penicillin+10 g/ml streptomycin+0.2 mmol/l L-glutamine+5 μg/ml vitamin C for 3 days in the incubator (5% CO_2, 95% room air at 37°C). The cultured cells were fixed with 100% EtOH and then washed with 10mM PBS solution (Sigma, St. Louis, MO, USA). Monoclonal anti-aggrecan, anti-alkaline phosphatase, anti-type I collagen, anti-type II collagen, anti-osteocalcin, and anti-Sox9 were applied respectively at 4^oC overnight. After washing, secondary antibody conjugated with fluorescein isothiocyanate (FITC) was applied to the wells (room temperate, 2 hours). The immunoreactivity of IVD for rhBMP-2 and TGF-β were analyzed to check the chondrogenic activity.

The wells were then rinsed, mounted and photographed with fluorescence photomicroscope (NIKON microphot-SA, Japan). For immunofluorescence, fluorochromes on the sections were exited using a 510nm emission filter for green fluorescent protein (GFP), and 580 nm emission filter for secondary antibody with FITC. The average number of immunofluorescence positive stained IVD cells for anti-aggrecan, anti-alkaline phosphatase, anti-type I collagen, anti-type II collagen,

anti-osteocalcin, and anti-Sox9 were counted respectively in both Group 1 and Group 2. Five out of nine wells were scanned using light microscopy. The number of each immunofluorescence positive stained IVD cells in five wells was calculated in the number of nine wells. The values of the average number of each immunofluorescence positive stained IVD cells were compared as a ratio with their average number without treatment of cytokine.

6. Statistical analysis

The student t-test and Wilcoxon's Signed Rank test were used for interGroup comparisons. Values are reported as mean SD. A value of $P < 0.05$ was considered statistically significant. All analyses were carried out using SPSS Version 12.0 (SPSS Inc., Chicago, IL, USA).

III. RESULTS

1. Degeneration of IVD

Using Pfirrmann's grading system, the discs of 12 patients were classified in to 2 Groups. Group 1 (6 patients) contained mildly degenerated discs (grade II; 3 patients and grade III; 3 patients) and Group 2 (6 patients) contained severely degenerated discs (grade IV; 2 patients and grade V; 4 patients). Mean patient age in Group 2 (59.9 ± 13.9 yr) was significantly higher than Group 1 (43.1 ± 13.3 yr) ($P < 0.05$).

2. Quantitation of mRNA Levels

The mRNA levels of genes specific for aggrecan, type I collagen, type II collagen, alkaline phosphatase, osteocalcin, and Sox9 which were produced from forty real-time PCR cycles were analyzed, and their melting curves were checked to confirm the amplification specificity. Melting curves of those mRNA showed uniform patterns with one peak (Figure 1).

The mRNA gene expression in Group 1 (mild degenerative IVD) was significantly greater for aggrecan (3.28-fold*), type I collagen (2.56-fold*), type II collagen (3.36-fold*), alkaline phosphatase (5.47-fold*), osteocalcin (1.46-fold), and sox9 (1.45-fold**) than the mRNA gene expression in Group 2 (severe degenerative IVD). The differences of mRNA gene expression for aggrecan, type I collagen, type II collagen, alkaline phosphatase, and Sox9 were statistically significant. (* indicates $p < 0.01$ and ** indicates $p < 0.05$) (Table3, Figure 2).

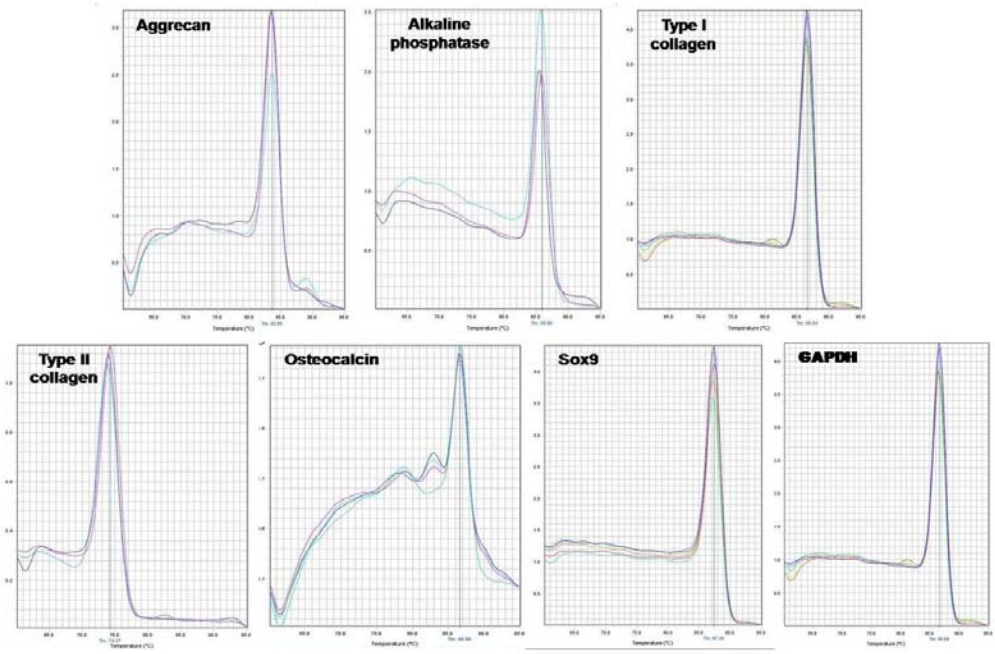


Figure 1. Melting curves of genes specific for aggrecan, type I collagen, type II collagen, alkaline phosphatase, osteocalcin, and Sox9. Melting curves of those mRNA showed uniform patterns with one peak.

Table 3. The mRNA gene expression in Group 1 (mild degenerative IVD) was significantly greater for aggrecan (3.28-fold*), type I collagen (2.56-fold*), type II collagen (3.36-fold*), alkaline phosphatase (5.47-fold*), osteocalcin (1.46-fold), and Sox9 (1.45-fold**) than the mRNA gene expression in Group 2 (severe degenerative IVD).

	Group I (Mean ± SD)		Group II (Mean ± SD)	
	ΔC_T Mean	Standard deviation	ΔC_T Mean	Standard deviation
Aggrecan*	7.4655	3.6230	2.2734	1.4268
Type I collagen*	6.9288	2.4729	2.7064	1.4765
Type II collagen*	9.4161	2.7463	2.8008	1.7049
Alkaline phosphatase*	7.1195	3.4158	1.2949	1.0856
Osteocalcin	6.9099	2.5996	4.7282	0.9566
Sox9**	4.7773	1.0143	3.2852	0.9069

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

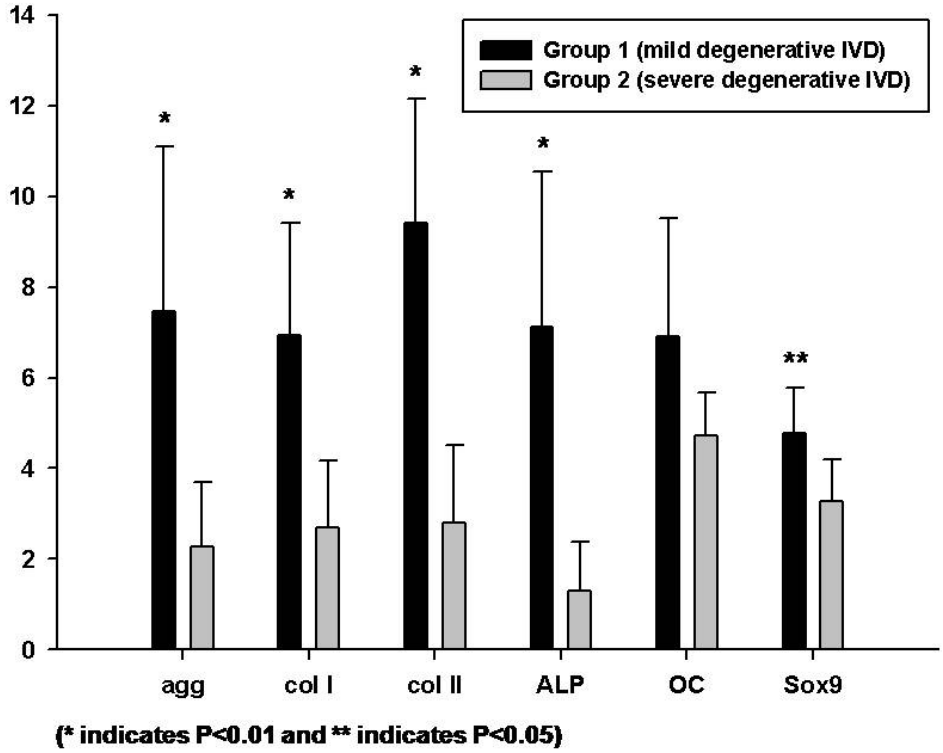


Figure 2. The mRNA gene expression in Group 1 (mild degenerative IVD) was significantly greater for aggrecan, type I collagen, type II collagen, alkaline phosphatase, osteocalcin, and Sox9 than the mRNA gene expression in Group 2 (severe degenerative IVD).

3. mRNA levels of aggrecan, type I collagen, type II collagen, alkaline phosphatase, osteocalcin, and Sox9 after treatment of cytokines

In order to confirm the response to cytokines, the mRNA levels of these genes without cytokine treatment were used as a control. The values of each mRNA level of these genes were compared as a ratio with their mRNA levels without cytokine treatment.

The mRNA levels of gene for aggrecan after treatment of morphogens were increased by 5.46* fold with rhBMP-2 and 3.55* fold with TGF-β in Group 1 and increased by 2.67* fold with rhBMP-2 and 2.30* fold with TGF-β in Group 2. The mRNA expressions of gene for aggrecan after treatment of inflammatory mediators were decreased by 0.48* fold with TNF-α and 0.61* fold with IL-1β in Group 1 and decreased by 0.65* fold with TNF-α and 0.51* fold with IL-1β in Group 2. Response to rhBMP-2 in Group 1 was greater 2.03† fold than that of Group 2 and showed statistical difference between the two groups. But, response to others showed no statistical difference between the two groups († indicates $p < 0.05$) (Figure 3).

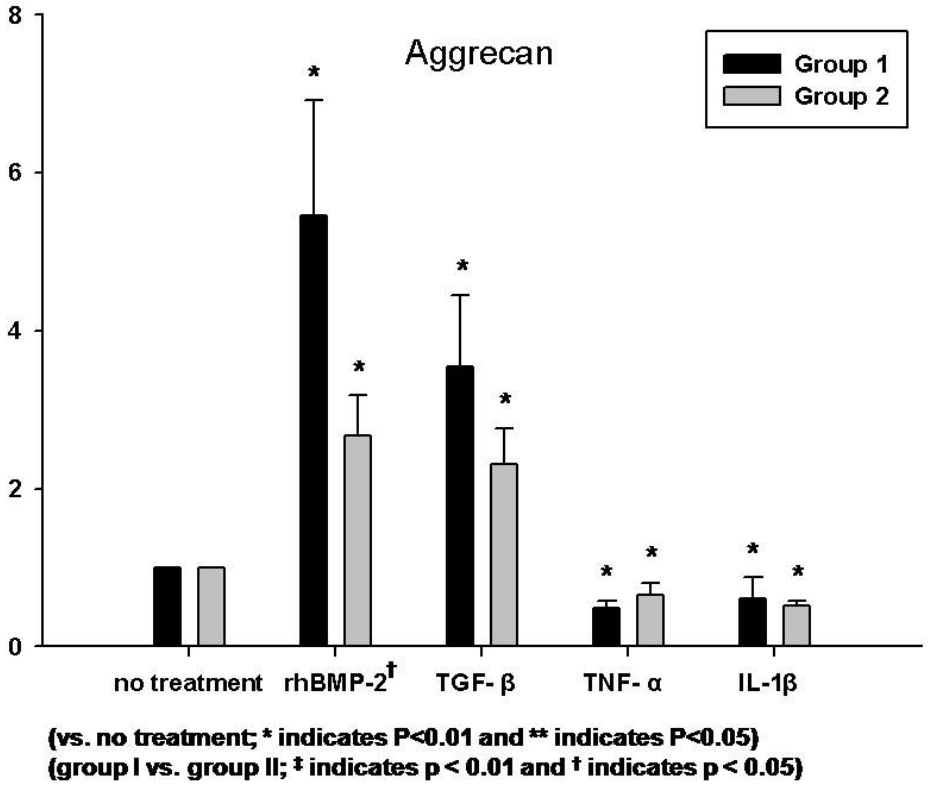


Figure 3. The mRNA levels of gene for aggrecan after treatment of morphogens were increased with rhBMP-2 and TGF-β in both Group 1 and 2. The mRNA expressions of gene for aggrecan after treatment of inflammatory mediators were decreased with TNF-α and IL-1β in both Group 1 and 2. Response to rhBMP-2 in Group 1 was greater than that of Group 2 and showed statistical difference between the two groups. Response to others showed no statistical difference between the two groups.

The mRNA levels of gene for alkaline phosphatase after treatment of morphogens were increased by 4.82* fold with rhBMP-2 and 4.63* fold with TGF-β in Group 1 and increased by 2.62* fold with rhBMP-2 and 2.31* fold with TGF-β in Group 2. The mRNA expressions of gene for alkaline phosphatase after treatment of inflammatory mediators were decreased by 0.59 fold with TNF-α and 0.54* fold with IL-1β in Group 1, and decreased by 0.66 fold with TNF-α and 0.62* fold with IL-1β in Group 2. Response to rhBMP-2 and TGF-β in Group 1 was greater by 1.84† times and 2.00† times each than those in Group 2, and showed statistical difference between the two groups. But response to others demonstrated no statistical difference between the two groups (Figure 4).

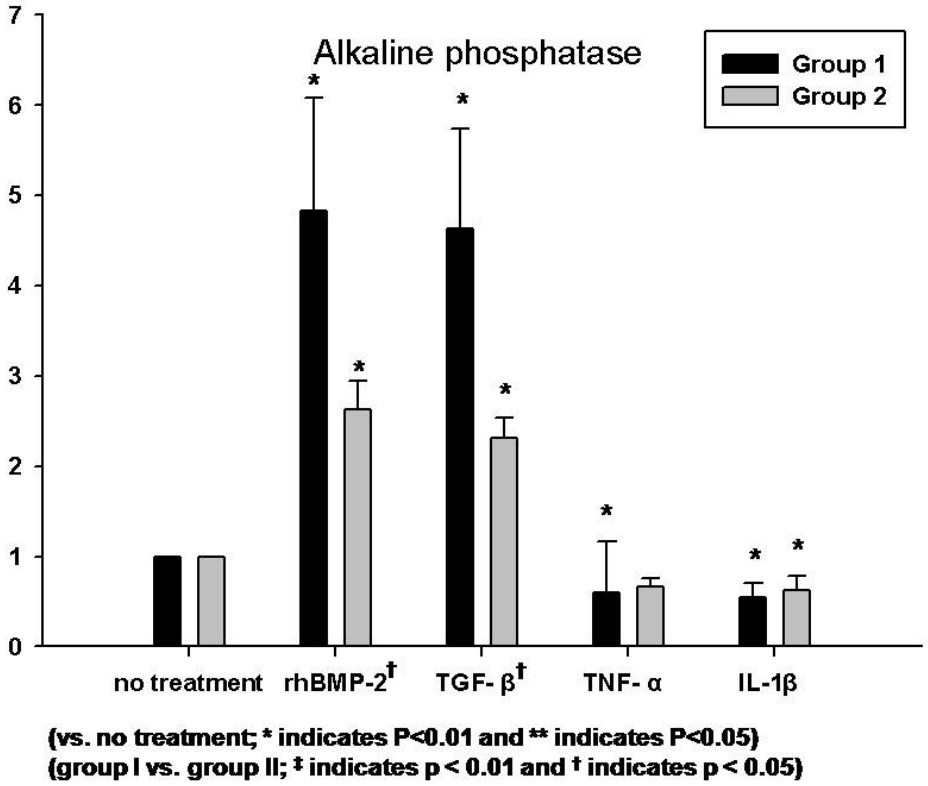


Figure 4. The mRNA levels of gene for alkaline phosphatase after treatment of morphogens were increased with rhBMP-2 and TGF-β in both Group 1 and 2. The mRNA expressions of gene for alkaline phosphatase after treatment of inflammatory mediators were decreased with TNF-α and IL-1β in both Group 1 and 2. Response to rhBMP-2 and TGF-β in Group 1 was greater than that of Group 2 and had statistical difference between the two groups. Response to others demonstrated no statistical difference between the two groups.

The mRNA levels of gene for type I collagen after treatment of morphogens were increased by 4.35* fold with rhBMP-2 and 3.87* fold with TGF-β in Group 1 and increased by 1.84* fold with rhBMP-2 and 2.54* fold with TGF-β in Group 2. The mRNA gene expressions for type I collagen after treatment of inflammatory mediators were decreased by 0.84* fold with TNF-α and 0.75* fold with IL-1β in Group 1 and decreased by 0.70 fold with TNF-α and 0.77 fold with IL-1β in Group 2. Response to all cytokines showed no statistical difference between the two groups (Figure 5).

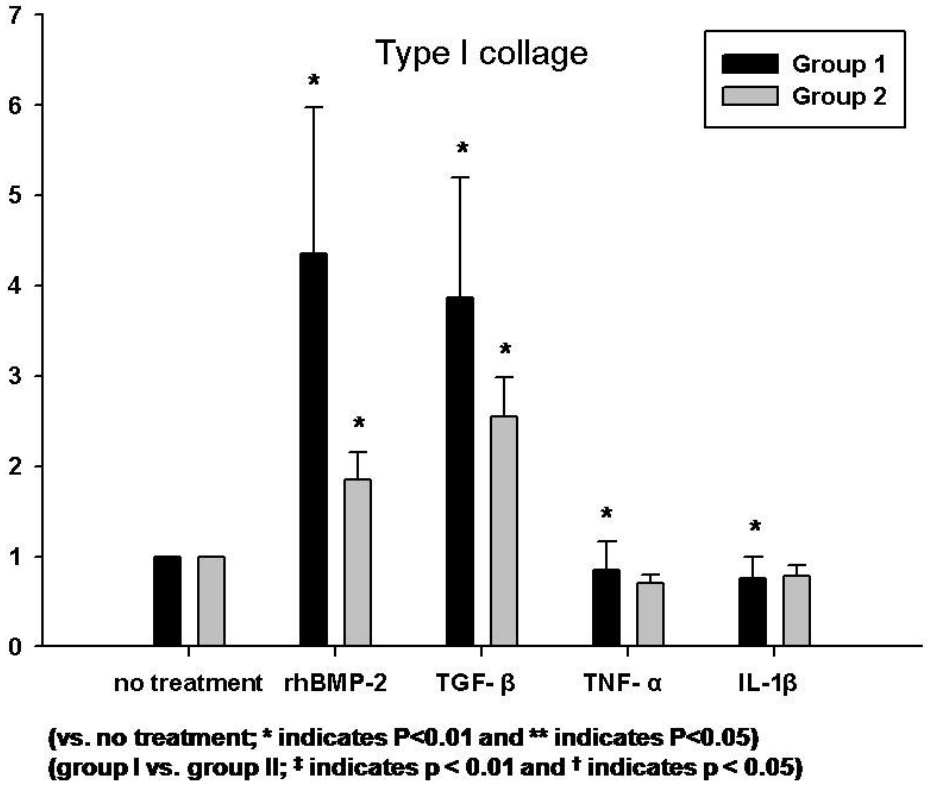


Figure 5. The mRNA levels of gene for type I collagen after treatment of morphogens were increased with rhBMP-2 and TGF-β in both Group 1 and 2. The mRNA gene expressions for type I collagen after treatment of inflammatory mediators were decreased with TNF-α and IL-1β in both Group 1 and 2. Response to all cytokines showed no statistical difference between the two groups.

The mRNA levels of gene for type II collagen after treatment of morphogens were increased by 3.92* fold with rhBMP-2 and 3.59* fold with TGF-β in Group 1 and increased by 2.51* fold with rhBMP-2 and 2.93* fold with TGF-β in Group 2. The mRNA gene expressions for type II collagen after treatment of inflammatory mediators were decreased by 0.56* fold with TNF- α and 0.49* fold with IL-1β in Group 1 and decreased by 0.67 fold with TNF-α and 0.67* fold with IL-1β in Group 2. Response to rhBMP-2 in Group 1 was greater 1.55‡ times than that of Group 2 and showed statistical difference between the two groups. But response to others showed no statistical difference between the two groups (‡ indicates $p < 0.01$) (Figure 6).

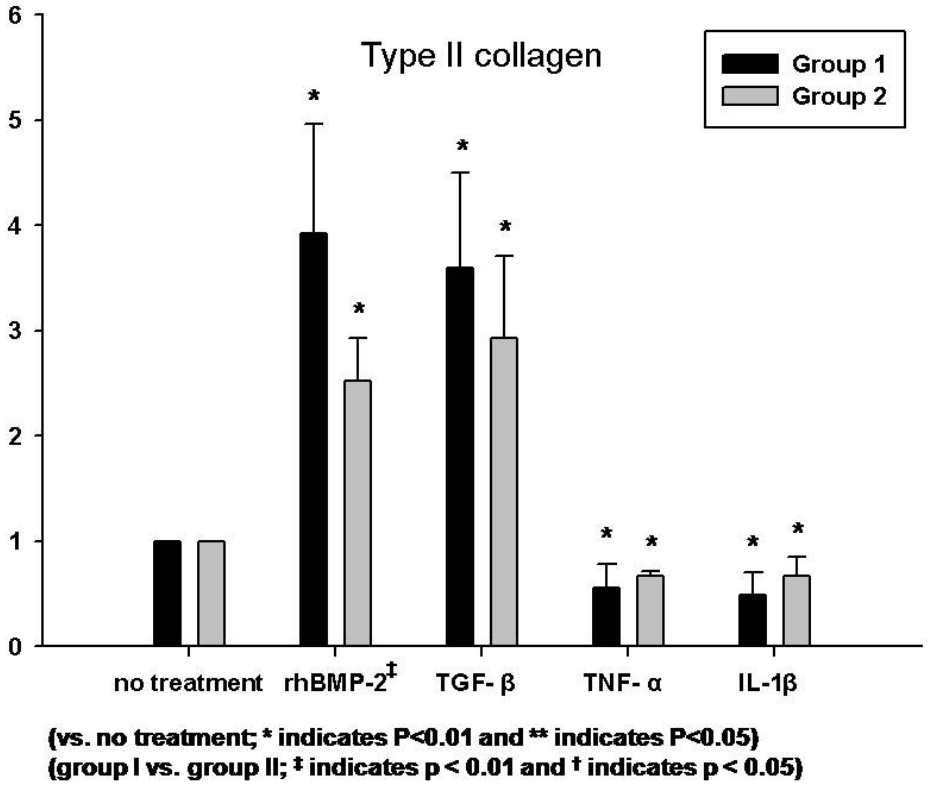


Figure 6. The mRNA levels of gene for type II collagen after treatment of morphogens were increased with rhBMP-2 and TGF-β in both Group 1 and 2. The mRNA expressions of gene for type II collagen after treatment of inflammatory mediators were decreased with TNF-α and IL-1β in both Group 1 and 2. Responsiveness to rhBMP-2 in Group 1 was greater than that of Group 2 and showed statistical difference between the two Groups. Response to others showed no statistical difference between the two groups.

The mRNA levels of gene for osteocalcin after treatment of morphogens were increased by 4.83* fold with rhBMP-2 and 3.04* fold with TGF-β in Group 1 and increased by 2.46* fold with rhBMP-2 and 1.77* fold with TGF-β in Group 2. The mRNA gene expressions for osteocalcin after treatment of inflammatory mediators were decreased by 0.52* fold with TNF-α and 0.65* fold with IL-1β in Group 1 and decreased by 0.76* fold with TNF-α and 0.67* fold with IL-1β in Group 2. Response to TGF-β in Group 1 was greater 1.72† times than that of Group 2 and showed statistical difference between the two groups. But response to others showed no statistical difference between the two groups (Figure 7).

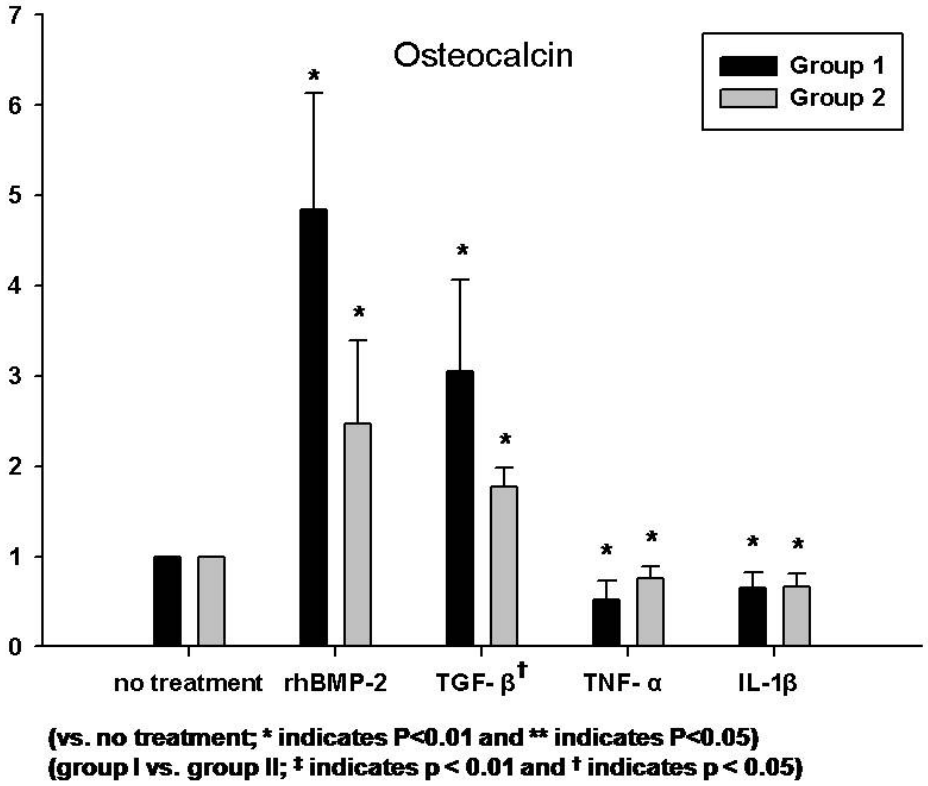


Figure 7. The mRNA levels of gene for osteocalcin after treatment of morphogens were increased with rhBMP-2 and TGF-β in both Group 1 and 2. The mRNA expressions of gene for osteocalcin after treatment of inflammatory mediators were decreased with TNF-α and IL-1β in both Groups 1 and 2. Response to TGF-β in Group 1 was greater than that of Group 2 and showed statistical difference between the two groups. Response to others showed no statistical difference between the two groups.

The mRNA levels of gene for Sox9 after treatment of morphogens were increased by 5.17* fold with rhBMP-2 and 3.69* fold with TGF-β in Group 1 and increased by 2.44* fold with rhBMP-2 and 2.41** fold with TGF-β in Group 2. The mRNA gene expressions for Sox9 after treatment of inflammatory mediators were decreased by 0.53* fold with TNF-α and 0.68** fold with IL-1β in Group 1 and decreased by 0.64* fold with TNF-α and 0.68** fold with IL-1β in Group 2. Response to TGF-β in Group 1 was greater $1.72^{\dagger}$ times than that in Group 2 and showed statistical difference between the two groups. But response to others showed no statistical difference between the two groups (Figure 8).

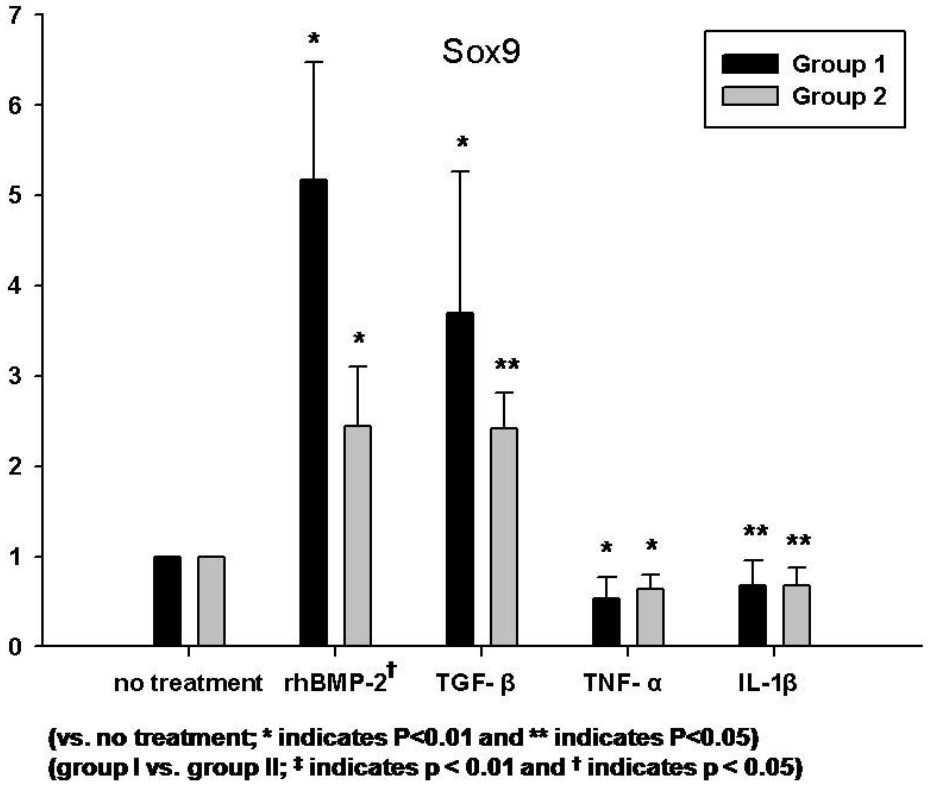


Figure 8. The mRNA levels of gene for Sox9 after treatment of morphogens were increased with rhBMP-2 and TGF-β in both Group 1 and 2. The mRNA expressions of gene for Sox9 after treatment of inflammatory mediators were decreased with TNF-α and IL-1β in both Group 1 and 2. Response to rhBMP-2 in Group 1 was greater than that of Group 2 and showed statistical difference between the two groups. Response to others showed no statistical difference between the two groups

4. Immunoreactivity of IVD for rhBMP-2 and TGF-β

The average numbers of immunofluorescence positive stained IVD cells for aggrecan, according to the control Group (no treatment), rhBMP-2 100 ng/ml Group and TGF-β 10 ng/ml Group, were 6.20 x 10^3, 2.61 x 10^4*, 2.31 x 10^4*, respectively, in Group 1, and these were 5.04 x 10^3, 1.08 x 10^4*, 9.76 x 10^3* respectively, in Group 2. The average numbers of immunofluorescence positive stained IVD cells for aggrecan were increased 4.21* times with rhBMP-2 and 3.72* times with TGF-β in Group I and increased 2.14* times with rhBMP-2 and 1.94* times with TGF-β in Group II. Response to rhBMP-2 and TGF-β in Group I was greater 1.96† times and 1.92† times than those of Group II and showed statistical difference between the two groups (Table 4).

Table 4. The average numbers of immunofluorescence positive stained IVD cells for aggrecan, alkaline phosphatase, type I collagen, type II collagen, osteocalcin, and Sox9 in both Group 1 and Group 2.

	Group I (ratio)		Group II (ratio)	
	rhBMP-2	TGF-β	rhBMP-2	TGF-β
Aggrecan	4.21*	3.72*	2.14*	1.94*
Alkaline phosphatase	3.73*	3.66*	2.52*	2.29*
Type I collagen	4.11*	3.94*	1.833*	2.010*
Type II collagen	3.62*	3.47*	2.38*	2.56*
Osteocalcin	3.54*	3.30*	2.38*	1.97*
Sox9	4.19*	3.85*	2.25*	2.06*

(vs. no treatment : * indicates $P<0.01$, Group I vs. Group II : † indicates $p < 0.05$)

The average numbers of immunofluorescence positive stained IVD cells for alkaline phosphatase were 6.57 x 10^3, 2.45 x 10^4*, 2.41 x 10^4* respectively in Group 1 and were 4.86 x 10^3, 1.22 x 10^4*, 1.12 x 10^4* respectively in Group 2. The average numbers of immunofluorescence positive stained IVD cells for alkaline phosphatase were increased 3.73* times with rhBMP-2 and 3.66* times with TGF-β in Group 1 and increased 2.52* times with rhBMP-2 and 2.29* times with TGF-β in Group 2. Response to rhBMP-2 and TGF-β in Group 1 was greater 1.48† times and 1.59† times than those of Group 2 and showed statistical difference between the two groups.

The average numbers of immunofluorescence positive stained IVD cells for type I collagen were 6.50 x 10^3, 2.67 x 10^4*, 2.56 x 10^4* respectively, in Group 1, and were 4.32 x 10^3, 7.92 x 10^3*, 8.69 x 10^3* respectively, in Group 2. The average numbers of immunofluorescence positive stained IVD cells for type I collagen were increased 4.11* times with rhBMP-2 and 2.01* times with TGF-β in Group 1 and increased 1.83* times with rhBMP-2 and 1.95* times with TGF-β in Group 2. Response to rhBMP-2 and TGF-β in Group 1 was greater 2.24† times and 1.96† times than those of Group 2 and showed statistical difference between the two groups.

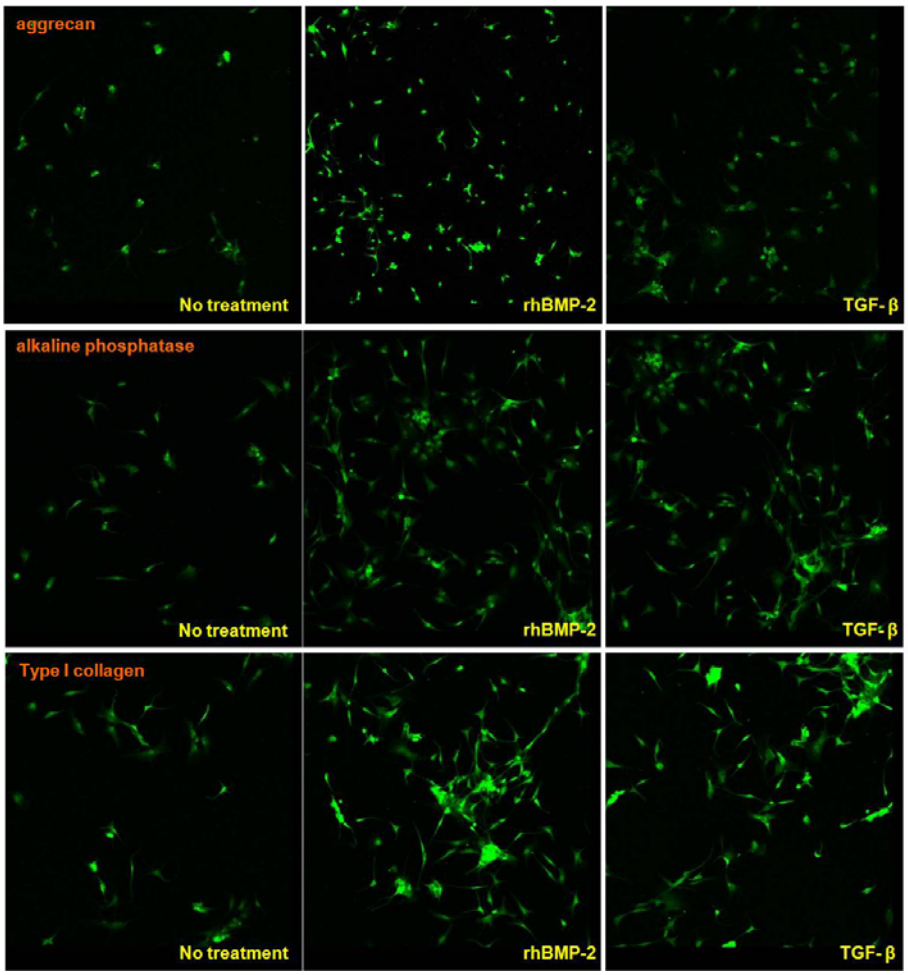
The average numbers of immunofluorescence positive stained IVD cells for type II collagen were 6.98 x 10^3, 2.53 x 10^4*, 2.42 x 10^4* respectively in Group 1 and were 6.21 x 10^3, 1.47 x 10^4*, 1.59 x 10^4* respectively in Group 2. The average numbers of immunofluorescence positive stained IVD cells for type II collagen were increased 3.62* times with rhBMP-2 and 3.47* times with TGF-β in Group 1 and increased 2.38* times with rhBMP-2 and 2.56* times with TGF-β in Group 2. Response to rhBMP-2 and TGF-β in Group 1 was greater 1.53† times and 1.35 times than those of Group 2 and showed statistical difference between the two groups.

The average numbers of immunofluorescence positive stained IVD cells for

osteocalcin were 6.30 x 10^3, 2.23 x 10^4*, 2.08 x 10^4* respectively, in Group 1, and were 5.58 x 10^3, 1.37 x 10^4*, 1.10 x 10^4* respectively, in Group 2. The average numbers of immunofluorescence positive stained IVD cells for osteocalcin were increased 3.54* times with rhBMP-2 and 3.30* times with TGF-β in Group 1 and increased 2.38* times with rhBMP-2 and 1.97* times with TGF-β in Group 2. Response to rhBMP-2 and TGF-β in Group 1 were greater 1.49† times and 1.68† times than those of Group 2 and showed statistical difference between the two groups.

The average numbers of immunofluorescence positive stained IVD cells for Sox9 were 6.60 x 10^3, 2.77 x 10^4*, 2.54 x 10^4* respectively, in Group 1 and were 5.76 x 10^3, 1.29 x 10^4*, 1.19 x 10^4* respectively, in Group 2. The average numbers of immunofluorescence positive stained IVD cells for Sox9 were increased 4.19* times with rhBMP-2 and 3.85* times with TGF-β in Group 1 and increased 2.25* times with rhBMP-2 and 2.06* times with TGF-β in Group 2. Response to rhBMP-2 and TGF-β in Group 1 was greater 1.86† times and 1.87† times than those of Group 2 and showed statistical difference between the two groups.

(A)


aggrecan
No treatment
rhBMP-2
TGF-β
alkaline phosphatase
No treatment
rhBMP-2
TGF-β
Type I collagen
No treatment
rhBMP-2
TGF-β

(B)

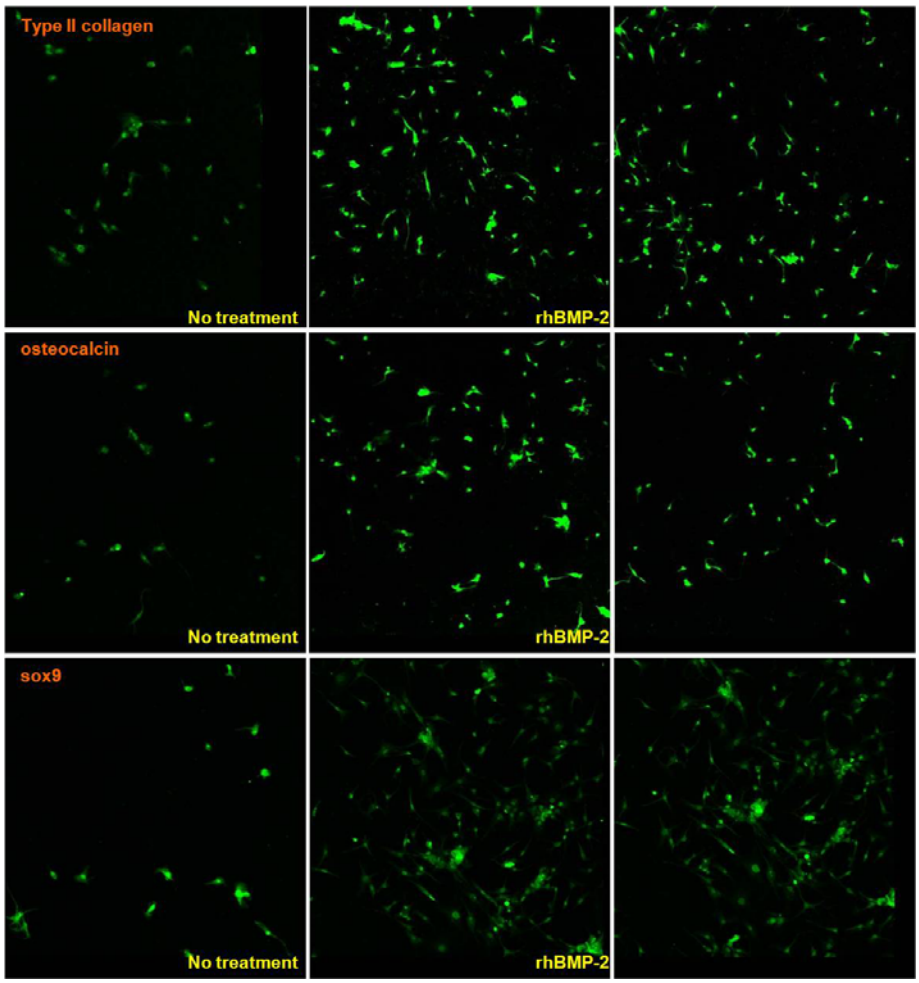
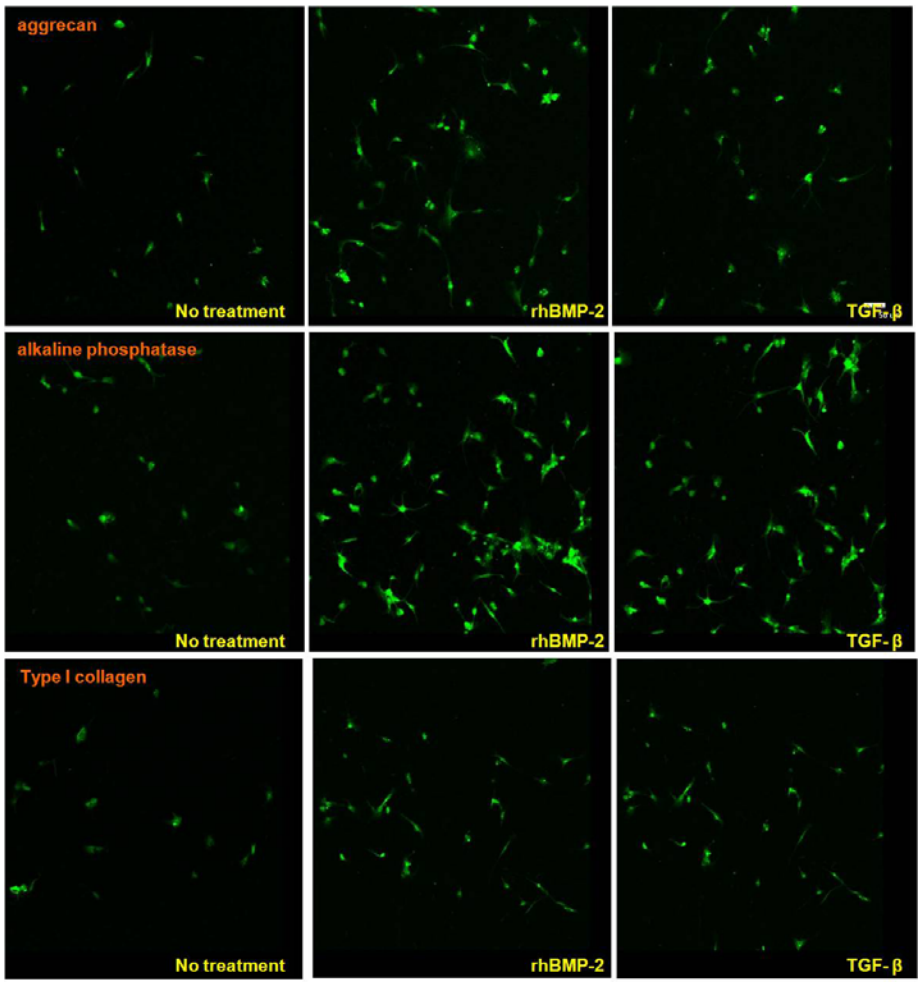


Figure 9. Immunostaining of human IVD cells for aggrecan, alkakine phosphatase and type I collagen (A) and for type II collagen, osteocalcin, and Sox9 (B) in Group 1 (mild degenerative IVD) after rhBMP-2 and TGF-β treatment.

(A)


aggrecan
No treatment
rhBMP-2
TGF-β
alkaline phosphatase
No treatment
rhBMP-2
TGF-β
Type I collagen
No treatment
rhBMP-2
TGF-β

(B)

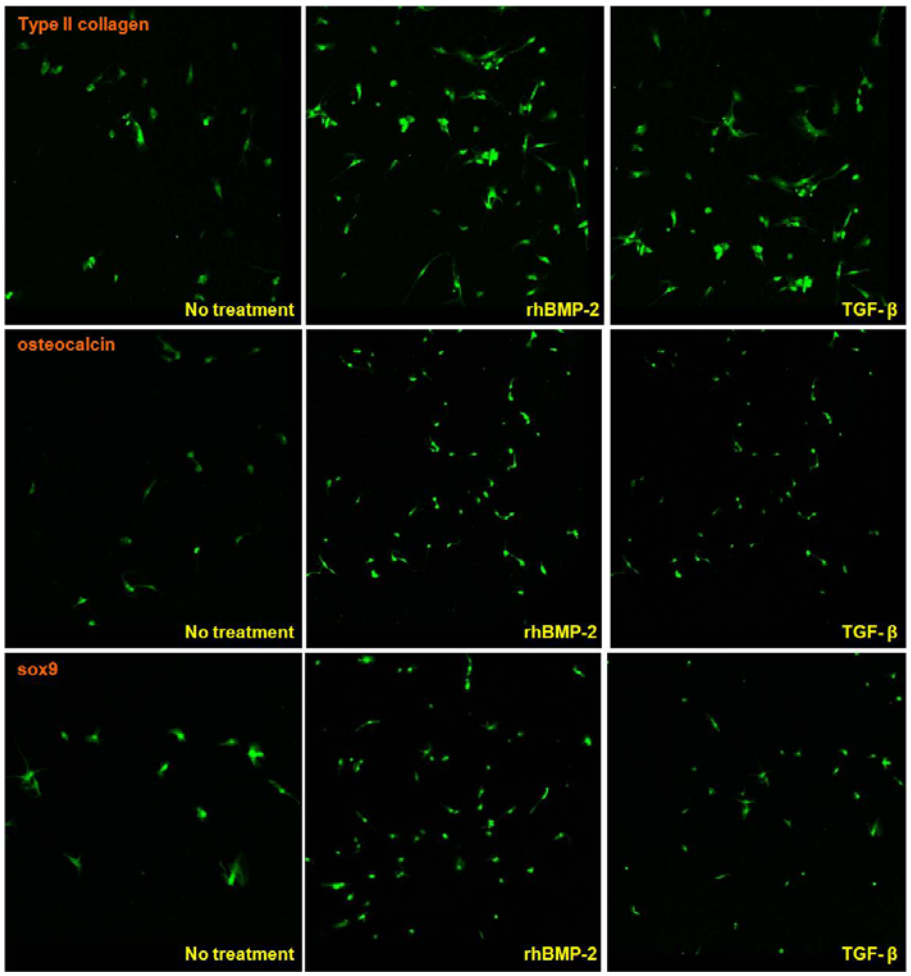


Figure 10. Immunostaining of human IVD cells for aggrecan, alkakine phosphatase and type I collagen (A) and for type II collagen, osteocalcin, and Sox9 (B) in Group 2 (mild degenerative IVD) after rhBMP-2 and TGF-β treatment.

IV. DISCUSSION

Disc degenerative changes may be associated with pain, and treatment options for disc degeneration are limited. Among the various genes associated with matrix synthesis, types I and II collagens act as fibrillar molecules, aggrecan consists of a core protein to sulfated glycosaminoglycans, Sox9 upregulates both aggrecan and type II collagen, osteocalcin, and alkaline phosphatase are markers of osteogenic genes.[40-44] Proinflammatory cytokines, such as TNF-α and interleukin (IL), are well known to be associated with disc degradation.[5,45-47]

With degeneration, IVD shows downregulation of various genes for aggrecan, type II collagen, sox9, type I collagen, alkaline phosphatase, osteocalcin and others.[5,43,45] Various inflammatory mediators have been also implicated in the degeneration of the IVD and discogenic pain, including nitric oxide (NO), interleukins, matrix metalloproteinases (MMPs), prostaglandin E2 (PGE2) and a group of cytokines.[32] TNF-α and IL-1β are well known to be associated with disc degradation and discogenic back pain.[20,33,48] TNF-α is an important initiator of matrix degeneration, whereas IL-1β plays a greater role in pathological degradation.

Many growth factors, including insulin-like growth factor-1 (IGF-1), TGF-1, BMP-2, and BMP-7 have been extensively investigated by the cartilage research community, and have been shown to positively influence the metabolism and healing potential of cartilage.[25,49-51] With this success and the similar chondrocytic composition of the IVD, investigations into the effects of growth factors on IVD cells were performed by researchers. All of these molecules have some in vitro data, but few have been tested in vivo in an animal model with disc degeneration to verify the biological mechanisms of each molecule. There is no clinically proven biological

therapy for degeneration of human IVD.

Four cytokines, including rhBMP-2, TGF-β, TNF-α, and IL-1β were used as morphogenic cytokines and inflammatory implicators in this study. The response to these four cytokines between Group 1 (mild degenerative IVD) and Group 2 (severe generative IVD) were checked into gene and protein expression and demonstrated statistical difference. The mRNA gene expression in Group 1 was significantly greater for aggrecan, type I collagen, type II collagen, alkaline phosphatase, osteocalcin, and Sox9 than the mRNA gene expression in Group 2 before cytokine treatment. The mRNA gene levels for these molecules also revealed significant increment in both groups after treatment of morphogens, which were much higher in Group 1 than in Group 2. There was no statistical significance in both groups after treatment of inflammatory implicators. Micrographic findings of rhBMP-2 and TGF-β immunoreactive IVD cells for aggrecan, alkakine phosphatase, type I collagen, type II collagen, osteocalcin, and Sox9 revealed similar result in both groups. The average numbers of immunofluorescence positive stained IVD cells for alkaline phosphatase were increased after treatment of rhBMP-2 and TGF-β in Group I. Microscopically, IVD cells also showed strong immunoreactivity and well demarcated configuration. This result suggests several features. The treatment of rhBMP-2 and TGF-β increases the expression of the various genes associated with matrix synthesis, including aggrecan, alkaline phosphatase, types I collagen, type II collagens, osteocalcin, and Sox9. The treatment of TNF-α and IL-1β decreases the expression of these genes. The molecular biologic response to treatment of rhBMP-2, TGF-β, TNF-α and IL-1β in the degenerative living human IVD may be different according to the degree of IVD degeneration. Even though IVD cells in Group 1 (mild degenerative IVD) had greater molecular biologic response to treatment of cytokines than Group 2 (severe generative

IVD) in this *in vitro* study, several factors should be considered in classifying IVD cells by degree of degeneration in living humans, such as IVD cell count, the degree of hydration, elasticity, existence of vacuum disc, intradiscal pressure, and etc.. Therefore, the molecular biologic response to treatment of rhBMP-2, TGF-β, TNF-α and IL-1β in degenerative living human IVD can be different in an *in vivo* study.

V. CONCLUSION

Degeneration of IVD is a complex process that disrupts this well-defined organization and biochemical balance. Many different biological treatment modalities have been studied to treat degenerative disc disease. Some of the bioactive molecules have been investigated for clinical application. In this study, treatment of rhBMP-2 and TGF-β modulate molecular biologic response in degenerative living human IVD cells, and the molecular biologic response is different according to the degree of IVD degeneration. Cytokine treatment may be an effective therapy in severe degenerative IVD as well as in mild degenerative IVD.

Despite the positive results obtained *in vitro*, the potential clinical use of rhBMP-2 and TGF-β for treatment of degenerative IVD is limited due to their short biologic half-life. Chronic conditions like IDD may require more prolonged and sustained cytokine levels to have a therapeutic effect. This has led investigators to contemplate the potential use of gene therapy as a treatment modality.

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Abstract (in korean)

퇴행성 추간반과 비퇴행성 추간반에서 Cytokine을 이용한 세포치료에 따른 추간반 재생능의 차이

(지도교수 김 긍 년)

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퇴행성 추간반의 세포치료요법으로 최근에 연구되고 있는 방법들로는 항이화작용 물질, 세포의 유사분열을 촉진하는 물질, 세포의 형태형성을 제어하는 물질, 세포내 조절인자로 크게 4가지 다른 군들이 있다. 본 연구는 추간반 세포의 대사와 형질에 영향을 주는 사이토카인에 대한 추간반의 퇴행성 변화와 관련된 다양한 유전자와 단백질들의 분자생물학적인 반응을 연구하고, 인간의 추간반 퇴행성변화의 진행단계에 따른 반응성을 연구하였다.

본 연구는 퇴행성 요추 추간반 탈출증으로 추간반 절제술을 시행 받은

12명의 환자에서 얻어진 살아있는 추간반 표본을 이용하였다. 추간반의 퇴행성 변화 정도는 자기공명영상을 이용한 Pfirrmann's grading system을 적용하였다. 제 1집단은 6개의 시료로 추간반 퇴행성 정도가 2단계와 3단계에 해당하는 경도의 퇴행성 추간반을 가지는 군이고, 제 2집단은 6개의 시료로 4단계와 5단계에 해당하는 심한 퇴행성 추간반을 가지는 군이다. 각 시료는 섬유륜을 제거하고, 잘게 쪼개서 단층으로 6일간 배양되었고, 이후 아그레칸, 제 I형 콜라겐, 제 II형 콜라겐, Sox9, 알칼리성 인산 가수분해 효소, 오스테오칼신과 글리세르 알데히드-3-인산탈수소 효소에 대한 mRNA 발현을 확인하였다. 제 1집단과 제 2집단에 사람 재조합 골형성 단백질-2, 전환 성장인자-β, 인터루킨-1β, 종양괴사인자-α를 각각 처치한 후, 각각의 유전자에 대한 mRNA 발현을 확인하였다. 두 집단간의 mRNA 발현 정도는 비율로 분석하였다. 또한 네 가지 사이토카인을 처치한 후 면역형광염색을 통해 추간반 세포 내 사이토카인에 대한 추간반 세포 내 아그레칸, 제 I형 콜라겐, 제 II형 콜라겐, Sox9, 알칼리성 인산 가수분해 효소, 오스테오칼신과 글리세르 알데히드-3-인산탈수소 효소관련 단백질의 발현을 확인하였다. 두 집단간에 유전자와 단백의 발현은 통계적으로 의미 있는 차이를 보였다. 아그레칸, 제 I형 콜라겐, 제 II형 콜라겐, Sox9에 대한 mRNA 발현은 경도의 퇴행성 변화를 보인 집단에서 심한 퇴행성 변화를 보인 집단에 비해 의미 있는 증가를 보였다. 사이토카인 처리 후에는 제 1, 2집단 모두에서 각각의 유전자에 대한 mRNA 발현 및 면역형광염색에서도 사람 재조합 골형성 단백질-2과 전환 성장인자-β 처치 후 제 1, 2집단

모두에서 추간반 세포의 발현 및 면역반응성이 의미 있게 증가하였고, 증가의 정도는 경도의 퇴행성 변화를 보인 집단에서 심한 퇴행성 변화를 보인 집단에 비하여 통계적으로 유의하게 증가하였다.

결론적으로 퇴행성 추간반 치료를 위해 연구중인 물질 중 사이토카인에 대한 추간반의 반응성은 퇴행성 변화와 상관없이 통계적으로 의미 있는 증가를 보였고, 경도의 퇴행성 변화를 보이는 추간반 세포가 심한 퇴행성 변화를 보이는 추간반 세포에 비하여 현저히 많은 증가를 나타내었다. 또한 심한 퇴행성 변화를 보이는 추간반 세포도 통계적으로 의미 있는 발현 양상을 보였으므로, 다른 치료방법의 복합요법 없이 사이토카인을 이용한 단독 요법에도 그 효과를 기대할 수 있을 것으로 추정된다.

핵심되는 말 : 추간반, 퇴행, 사이토카인, 사람재조합골형성단백질-2, 전환성장인자-β, 인터루킨-1β, 종양괴사인자-α