Zonal response of human intervertebral disc to bone morphogenetic protein-2

Hyung Chan Kim

Department of Medicine

The Graduate School, Yonsei University

Zonal response of human intervertebral disc to bone morphogenetic protein-2

Directed by Professor Hwan-Mo Lee

The Master's Thesis
Submitted to the Department of Medicine,
The Graduate School of Yonsei University
In partial fulfillment of the requirements for the degree of Master of Medicine

Hyung Chan Kim

June 2005

This certifies that the Master's Thesis of Hyung Chan Kim is approved

---

[Thesis supervisor : Hwan-Mo Lee]

[Thesis Committee Member#1: Seong-Hwan Moon]

[Thesis Committee Member#2:   Hwal Suh]

The Graduate School

Yonsei University

August 2005

Acknowledgements

It took so long for me to receive this master's degree. It's been eight years since I failed in an entrance examination for graduate school in 1997 when I was in second year of resident. When I did not have anywhere to go after finishing military service since I did not have enough knowledge or technique in orthopedic surgery, Professor Lee Hwan-mo took me as a spine surgery research instructor and gave me a chance to challenge for master's degree once more. I can't thank you enough for Professor Lee for his constant encouragement and teaching as well as leading me to learn spine surgery and Professor Moon Sung-hwan for his easy and graceful attitude and comfortable teaching like older brother. I thank Professor Seo Hwal in department of medical engineering for his elaborate proofreading of my thesis. Also, I thank you for Kim Hyang for her careful teaching when I was lack of laboratory basics. I also want to thank Kwon Un-Hye for her kind and active attitude that made me to work at the laboratory comfortably. I want to thank my parents who raised me as who I am right now and also I want to say that I love them. Finally, I want to thank my wife for marrying me who is very busy with raising our two daughters Joo-won and Do-won. I think finishing my master's degree thesis is not the end of my learning. I look forward to get more and more advice from everyone and I also want to thank everyone that I could not put all of their names on this thank you note.

CONTENTS

ABSTRACT 1

Ⅰ. INTRODUCTION 3

Ⅱ. MATERIALS AND METHODS 6
1. Materials 6
2. Intervertebral disc cell culture 7
3. Incorporation of isolated cells into alginate beads 9
4. Depolymerization of alginate bead 9
5. Preparation of recombinant human BMP-2 10
6. Cellular proliferation 10
7. Newly synthesized proteoglycan 11
8. Reverse transcription-polymerase chain reaction for Osteocalcin 12
9. Statistical analysis 14

Ⅲ. RESULTS 15
1. Cellular morphology 15
2. DNA synthesis of human NP, TZ, and AF cells with rhBMP-2 15
3. Proteoglycan synthesis of human NP, TZ, and AF cells with rhBMP-2 17
4. Expression of osteocalcin mRNA 18

Ⅳ. DISCUSSION 19
Ⅴ. CONCLUSION 22
VI . REFERENCES 23

ABSTRACT (In Korean) 26

LIST OF FIGURES

Figure 1. Topography of human intervertebral disc 7

Figure 2. Photos of human intervertebral disc cell from each zone 15

Figure 3. Proliferation assay of intervertebral disc from each zone 16

Figure 4. Proteoglycan synthesis of intervertebral disc cell from each zone 17

Figure 5. Densitometric assay of RT-PCR 18

LIST OF TABLES

Table 1. Sequences of the RT-PCR primers used 13

Table 2. RT-PCR conditions 13

ABSTRACT

Zonal response of human intervertebral disc to bone morphogenetic protein-2

Hyung Chan Kim

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Hwan-Mo Lee)

Study design. In vitro experiment using bone morphogenetic protein-2 (BMP-2) and human intervertebral disc (IVD) cells from nucleus pulposus, annulus fibrosus, and transitional zone.

Objectives. To demonstrate the differential effect of BMP-2 on mRNAs expression (collagen type I, collagen type II, aggrecan, and osteocalcin), proteoglycan synthesis, expression of alkaline phosphatase, bone nodule formation in human IVD cells from nucleus, annulus, and transitional zone.

Summary of Background Data. BMP-2 was widely known as a powerful agent for osteoinduction and a crucial growth factor for early chondrogenesis and maintenance of cartilaginous phenotype. BMP-2 has been proven to be effective in stimulating proteoglycan synthesis in articular chondrocytes and IVD cells from nucleus pulposus. Nevertheless, the effect of BMP-2 on IVD cells from different region of disc i.e. nucleus pulposus, annulus fibrosus, and transitional zone was not thoroughly elucidated.

Materials and Methods. Human IVDs were harvested from nucleus pulposus, annulus fibrosus, and transitional zone from surgical disc specimen. Disc tissue was enzymatically digested. Then IVD cells were cultured three-dimensionally in alginate beads. Recombinant human BMP-2 (rhBMP-2) was produced by Chinese hamster ovary cells after transduction of BMP-2 cDNA, then concentrated and purified. Then IVD cell cultures from nucleus pulposus(NP), transitional zone (TZ), and annulus fibrosus(AF) were exposed to rhBMP-2. Reverse transcription-polymerase chain reaction for mRNA expression of osteocalcin was performed. Newly synthesized proteoglycan was measured by ^{35}S-sulfate incorporation on Sephadex G-25M in PD 10 columns.

Results. Cells from AF exhibited mitogenic effect with rhBMP-2. However there was no significant increase in DNA synthesis in cultures from NP and TZ with rhBMP-2. Cells from NP showed increase in newly synthesized proteoglycan while cells from TZ and AF demonstrated no significant increase in response to rhBMP-2. In RT-PCR, IVD cells from all zones demonstrated no siginificant expression of osteocalcin mRNA expression.

Conclusions. BMP-2 clearly showed mitogenic effect in the cells from AF, while stimulate proteoglycan synthesis in the cells from NP without evidence of the expression of osteogenic phenotype. Taken together, this study raised the possibility of rhBMP-2 to be used an anabolic agent for mitogenesis and regenerating matrix of IVD depending on different zone.

Key Words: Bone morphogenetic protein-2 (BMP-2), intervertebral disc (IVD) cell, proteoglycan synthesis, zonal effect

Zonal response of human intervertebral disc to bone morphogenetic protein-2

Hyung Chan Kim

Department of Medicine

The Graduate School, Yonsei University

(Directed by Professor Hwan-Mo Lee)

Ⅰ. INTRODUCTION

Bone morphogenetic protein (BMP)s are pleiotropic morphogens and also regulate hematopoiesis, stimulate extracellular matrix synthesis, and influence cell survival maintenance and death.[1] In bone morphogenetic pathway, cartilage differentiation, hypertrophy, and cell death are followed by bone formation. In this regard, BMPs are cartilage morphogenetic protein since cartilage is formed first.[1] Also BMPs have a profound role in the maintenance of articular cartilage phenotype.[2, 3] Those observations have prompted the research toward applicability of BMPs on cartilage repair [4,5] and regeneration of intervertebral disc (IVD). [6] In the research of

articular cartilage, adenovirus-mediated BMP gene transfer to articular chondrocytes resulted in increased synthesis of proteoglycan and collagens.[7] Meanwhile, for the purpose of inducing intervertebral fusion, direct injection of BMP-2 resulted in ossification of anulus fibrosus while no apparent changes in nucleus pulposus of IVD. [8] In contrast, in order to stimulate matrix regeneration of IVD which has mainly chondrogenic components i.e. proteoglycan and collagens, adenovirus-mediated transfer of BMP-2 encoding gene has been reported to up regulate proteoglycan synthesis in human IVD cells. [6, 9, 10]

Since BMP-2 has diverse effects on mesenchymal tissue, the effect of BMP-2 on IVD should be validated before attempting clinical application of BMP-2 in intradiscal therapy. In previous study, BMP-2 has been proven to stimulate proteoglycan synthesis and up-regulate chondrogenic phenotypes i.e. collagen type II and aggrecan mRNAs expression while it rendered no recognizable effect on osteocalcin mRNA expression in human disc cells from nucleus pulposus[11]. Thus the effect of BMP-2 on nucleus pulposus of IVD appears to be chondrogenic not osteogenic. There are three different zones in human intervertebral disc, which are nucleus pulposus(NP), transitional zone(TZ), and annulus fibrosus(AF) respectively.[12] It is quite mandated to demonstrate the effect of BMP-2 on cells from different region of the disc i.e. NP, TZ, and AF, since each region of the IVD exhibited different cellular phenotype, matrix composition, oxygen tension, and nutrient concentration.

If different zone of IVD might react to BMP-2 differentially, BMP-2 therapy should be performed in zone specific manner. On the other hand, if three different regions of the disc i.e. NP, TZ, and AF reacted in uniform fashion i.e. matrix synthesis, upregulation of chondrogenic phenotype, intradiscal BMP-2 therapy could be performed with wide range of safety.

Accordingly, the purposes of this study were to demonstrate zonal effect of BMP-2 on three different zones in human disc ie. NP, AF, and TZ, in terms of cellular proliferation, proteoglycan synthesis, and the expression of osteogenic phenotype.

Ⅱ. MATERIALS AND METHODS

All of the experimental protocols were approved by the human subjects Institutional Review Board of the institution.

1. MATERIALS

Lumbar IVD tissues were obtained from five patients (age range: 35 to 52 years) during surgical disc procedures which included posterior lumbar interbody fusion and anterior interbody fusion. Classification of the IVD of each patient as grade of degeneration was performed based on magnetic resonance images of each disc as described in the literature. Grade III and IV degenerations were included in this study to minimize the effect of degeneration grades on the expression of phenotype and matrix synthesis. An attempt was made by the operating surgeon (SHM, HML) to carefully differentiate tissue into NP, TZ, AF. In detail, tissues within 1cm from outer annulus and anterior or posterior longitudinal ligament was designated as AF, tissue from central part of disc approximately 2cm diameter size was designated NP, then intervening tissue from annulus and nucleus was regarded as TZ (Figure 1). Herniated disc material was strictly excluded from current study. The disc 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, less than 20 minutes following surgical removal.

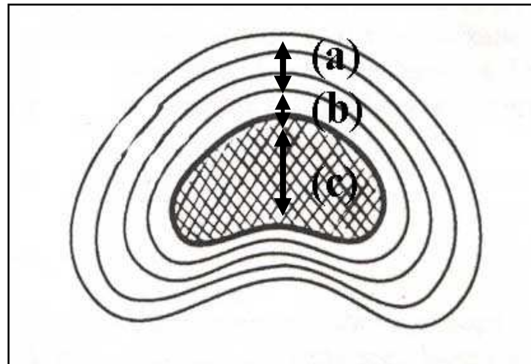


Figure 1. Topography of human (a) anulus fibrosus(AF), (b) transitional zone(TZ), (c) nucleus pulposus(NP).

2. Intervertebral disc cell culture

Harvested specimens were divided into three parts on NP, TZ and AF (Figure1.) Any obvious granulation tissue, dense outer anulus, and cartilaginous endplates were removed carefully from the disc tissue specimens. Disc cells were isolated as described .[13] Briefly, the dissected specimens were minced with a scalpel into pieces of approximately two cubic millimeters in volume. Disc tissues were digested for 60 minutes at 37° C under gentle

agitation in a medium composed of equal parts of Dulbecco's Modified Eagle Medium and Ham's F-12 medium (DMEM/F12, Gibco-BRL, Grand Island, NY, USA) containing 5% heat-inactivated fetal bovine serum (FBS, Gibco-BRL, Grand Island, NY, USA) with 0.4% protease (Sigma, St. Louis, MO, USA) and 0.004% deoxyribonuclease II type IV (DNase, Sigma, St. Louis, MO, USA). The tissue was then washed 2 times with DMEM/F12 and digested overnight under the same conditions, except that the pronase was replaced with bacterial 0.025% collagenase type II (Worthington Biochemical Corp., Lakewood, NJ, USA). Cells were filtered through a sterile nylon mesh filter (pore size: 70um) and then were counted in a haemocytometer and plated in 24 well plates (Falcon, Franklin Lakes, NJ, USA) at a density of approximately 6 x 10^4 cells/ml. Primary cultures were sustained for 2 to 3 weeks in DMEM/F12 containing 10% FBS, 1% v/v penicillin, streptomycin and nystatin (all antibiotics from Gibco-BRL, Grand Island, NY, USA) in a 5% CO_2 incubator with humid condition (Figure2). Culture medium was changed twice a week. Cell viability was determined by trypan blue exclusion test. Secondary cultures after trypsinization of primary cultures were exclusively utilized to minimize the effect of subculture on the expression of phenotype.

3. Incorporation of isolated cells into alginate beads

The preparation of IVD cells from each zone in alginate beads was performed as described elsewhere. [6, 14, 15] Briefly, isolated cells from primary culture with trypsinization were resuspended in sterile 0.15M NaCl containing 1.2% low-viscosity alginate (Sigma, St. Louis, MO, USA) at a density of two million cells per milliliter, then slowly expressed through a 22 gauge needle in a drop-wise fashion into 102mM $CaCl_2$ solutions. After gelation, the beads were allowed to polymerize further for a period of 10 minutes in the $CaCl_2$ solution. And then the polymerized beads were once washed in 10 volume of 0.15M NaCl and 3 times washed in 10 volumes of DMEM/F12 medium. The beads were finally placed in complete culture medium. Ten beads were cultured in each well of a 24-well plate.

4. Depolymerization of alginate bead

To remove cells from the alginate bead, the wells were rinsed twice with 0.15M NaCl with gently pipetting. The beads were incubated for 1 minute with the rinse solution and aspirated off. The three times volume of dissolving buffer (55mM sodium citrate and 0.15M NaCl) to alginate bead was added to the wells and incubated at 37 ˚C for 10 minutes with shaking.

5. Preparation of recombinant human BMP-2

Recombinant human BMP-2 (rhBMP 2) was prepared as described elsewhere. [16] Briefly cDNA for BMP-2A was inserted into a mammalian expression vector pcDNA3.1/hygro (6.7kb). Chinese hamster ovary cells were transduced by pcDNA3.1/hygro/BMP-2A by Lipofectamine PLUS(Gibco-BRL, Grand Island, NY, USA). Transformants were selected with Hygromycin-containing medium(Gibco-BRL, Grand Island, NY, USA). Then supernatant was harvested every 24 hours for 4 days and applied to Heparin-Sepharose (Amersham Pharmacia, Uppsala, Sweden) column. Finally, recombinant protein was concentrated by ultrafiltration with YM10 (Amicon, Bedford, MA, USA) membrane. Purity assay was performed on silver stained gel.

6. Cellular proliferation

DNA synthesis was measured by the [^{3}H]-thymidine incorporation. 5 uCi/ml of [^{3}H]-thymidine (Amersham Biosciences, Buckinghamshire, UK; 25 Ci/mmol specific activity) was added to control and the cultures for 24h. The medium was then discarded and the beads were depolymerized with dissolving solution. The cells were filtered onto glass fiber filters (Whatman GF/C, Maidstone, England), and transferred

to scintillation vial. The filters were completely dried and transferred into scintillation vial. Each vial was added 3ml of scintillation cocktail solution (Fisher, Fair Lawn, New Jersey, USA) and counted in a Packard liquid scintillation counter. The results of each experiment, expressed as cpm/well, are the means of three parallel cultures.

7. Newly synthesized proteoglycan

5 uCi/ml of [^{35}S]-sulfate (Amersham Biosciences, Buckinghamshire, UK; 25 Ci/mmol specific activity) was added to control and treated cultures for 24h. At the end of culture the medium was collected and the beads were dissolved with 28mM EDTA/0.15M NaCl. The cells were then placed in an extraction media (8M guanidine HCl solution, 5mM sodium acetate (pH5.8), proteinase inhibitor) at 4℃ for 48hours. Aliquots (200ul) of the cell extracts were eluted on Sephadex G-25M in PD-10 columns (Amersham Pharmacia, Uppsala, Sweden). Fractions (1ml) were colleted in scintillation vial and mixed with 6ml scintillation cocktail solution (Fisher, Fair Lawn, New Jersey, USA). Five fractions were collected per sample, and three meddle fractions were counted in a Packard liquid scintillation counter

8. Reverse transcription-polymerase chain reaction for osteoclacin

Total cellular RNA was isolated using RNeasy Mini Kit (QIAGEN, GmbH, Germany) according to the manufacturer's protocol. cDNA was synthesized from 1μg total RNA using RT-premix system (Bioneer, Dae-jeon, South Korea) with the oligo(dT) priming method in a 50μl reaction mixture. 1μl aliquots were amplified in a 20μl reaction mixture that contained 1U Taq DNA polymerase (Bioneer, Dae-jeon, South Korea), 250Mm of each dNTP, 10mM Tris-HCl(pH9.0), 40mM KCl, and 1.5mM $MgCl_2$. The primers used for human β-actin, and osteocalcin. Primer sequence of each gene was listed on Table 1. The same reaction profile was used for all primer sets: an initial denaturation at 94℃ for 1 min, followed by the appropriate cycles of: 94℃ for 5 sec; 47-62℃ for 5 sec; and 72℃ for 30 sec; and an additional 2 min extension step at 72℃ after the last cycle (Table2). PCR products (3ul) were analyzed by electrophoresis in 2 % agarose gels, and detected by staining with ethidium bromide. The intensity of the products was quantified using the BioImage Visage 110 system (BioRad, Hercules, CA, USA).

Table 1. Sequences of primers for reverse transcription polymerase chain reaction for β-actin, aggrecan, collagen type I, type II, and osteocalcin.

Primer	Sequence	Length	Size(bp)
Human β-actin	5'-GGCGGACTATGACTTAGTTG-3' 5'-AAACAACAATGTGCAATCAA-3'	20 20	238
Human aggrecan	5'-GAA TCT AGC AGT GAG ACG TC-3' 5'-CTG CAG CAG TTG ATT CTG AT-3'	20 20	541
Human collagen type Ⅰ	5'-CCT GTC TGC TTC CTG TTA AC-3' 5'-AGA GAT GAA TGC AAA GGA AA-3'	20 20	182
Human collagen type Ⅱ	5'-CAG GAC CAA AGG GAC AGA AA-3' 5'-TTG GTC CTT GCA TTA CTC CC-3'	20 20	328
Human osteocalcin	5'-CAC TCC TCG CCC TAT TGG CC-3' 5'-GCC AAC TCG TCA CAG TCC GG-3'	20 20	299

Table 2. RT-PCR conditions of β-actin, aggrecan, collagen type I, type II, and osteocalcin.

Primer	Conditions			Cycle
	Denaturation	Annealing	Polymerization	
Human β-actin	94℃ 5 sec	53℃ 5 sec	72℃ 30 sec	24
Human aggrecan	94℃ 5 sec	47℃ 5 sec	72℃ 30 sec	26
Human collagen type Ⅰ	94℃ 5 sec	48℃ 5 sec	72℃ 30 sec	21
Human collagen type Ⅱ	94℃ 5 sec	48℃ 5 sec	72℃ 30 sec	40
Human osteocalcin	94℃ 1 min.	62℃ 1min	72℃ 1 min.	35

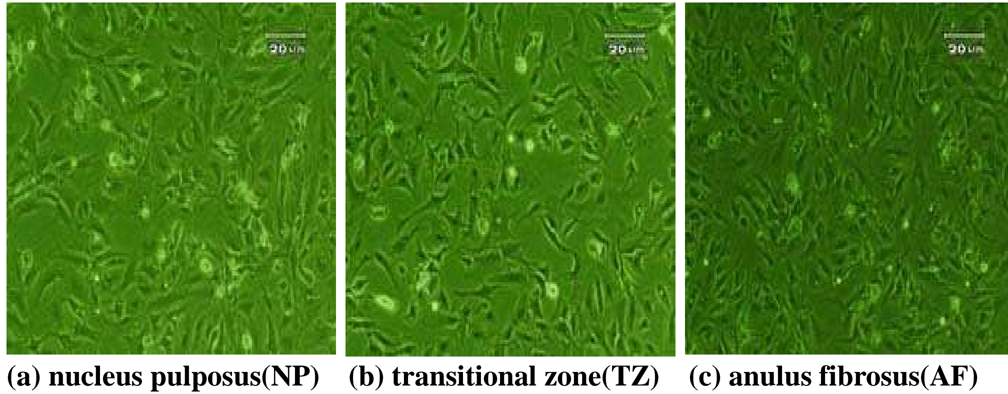
9. Statistical analysis.

One-way analysis of variance with Fisher's protected LSD post-hoc test was performed to test difference in densitometric data and [^{35}S]-sulfate labeled proteoglycan. Significance level was set as $p<0.05$.

Ⅲ. RESULTS

1. Cellular morphology

Cells from NP showed more polygonal and round shape comparing those of AF. Cells from AF demonstrated more slender and elongated shape than those of NP. However there was no marked distinction among cells from NP, TZ, and AF in terms of morphology in monolayer culture. (Figure 2)



(a) nucleus pulposus(NP) **(b) transitional zone(TZ)** **(c) anulus fibrosus(AF)**

Figure 2. Photography of human NP, TZ, and AF cells at 7 days after primary monolayer culture (original magnification x 100)

2. DNA synthesis of human NP, TZ, and AF cells with rhBMP-2

Human NP and TZ cell culture in three dimensional alginate beads with various

concentrations of rhBMP-2 (50, 100, 1000 ng/ml) showed no significant increase in DNA synthesis compared to control culture without rhBMP-2. In contrast, human AF cell culture showed seven-fold increase in DNA synthesis compared to control ($p<0.05$) (Figure3)

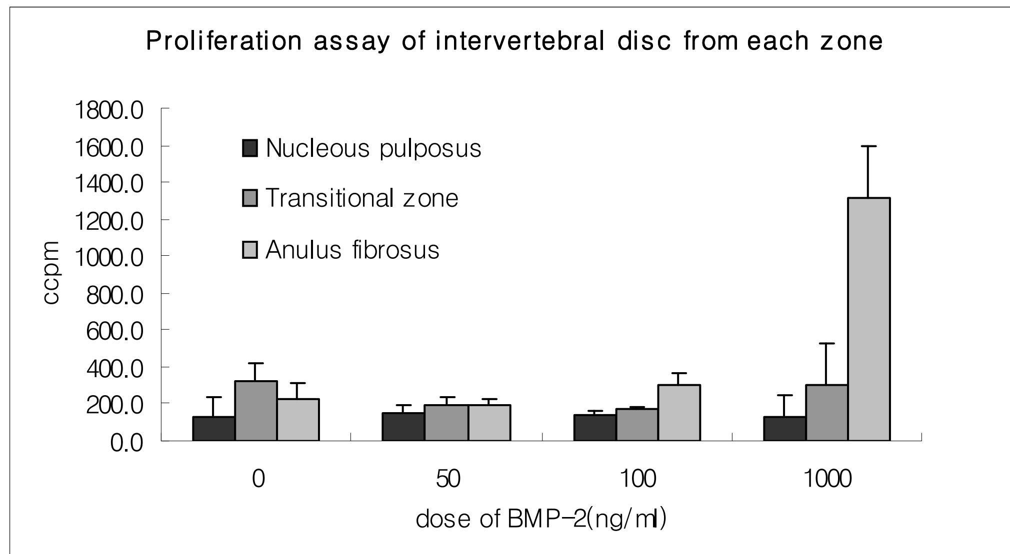


Figure 3. Human NP, TZ, and AF cells were cultured in three dimensional alginate beads with various concentration of rhBMP-2 (0, 50, 100, 1000ng/ml). DNA synthesis was analyzed with [^{3}H]-Thymidine incorporation. Human NP and TZ cell culture showed no significant increase in DNA synthesis compared to control culture without rhBMP-2. In contrast, human AF cell culture showed 7-fold increase in DNA synthesis compared to control cultures ($P>0.05$).

3. Proteoglycan synthesis of human NP, TZ, and AF cells with rhBMP-2

Human NP cell culture in three dimensional alginate beads with rhBMP-2 (1000ng/ml) showed 55% increase in proteoglycan synthesis ($p<0.05$), compared to control culture without rhBMP-2. In contrast, human TZ and AF cell cultures with various dose of rhBMP-2 demonstrated no significant increase in proteoglycan synthesis compared to control culture (Figure 4).

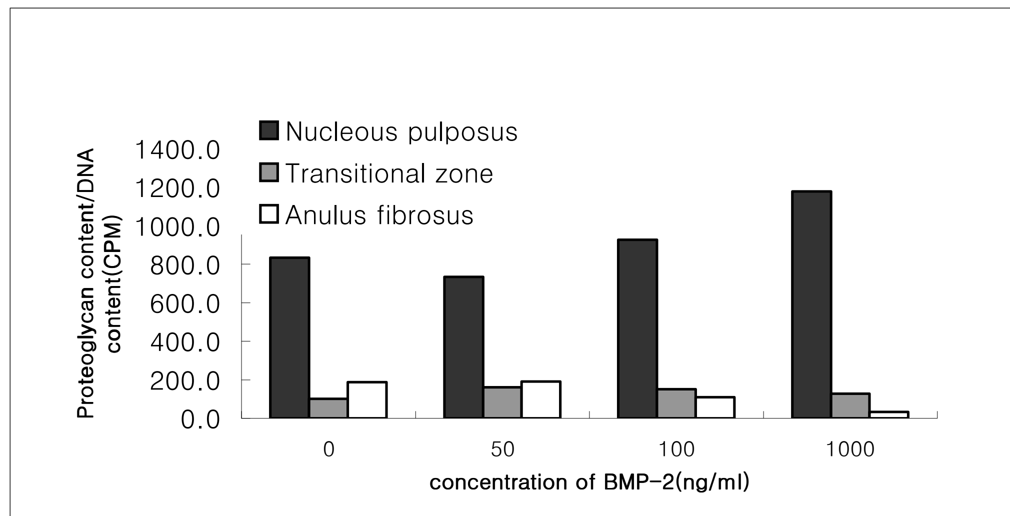


Figure 4. Human NP, TZ, and AF cells were cultured in three dimensional alginate beads with various concentration of rhBMP-2 (0, 50, 100, 1000ng/ml). Proteoglycan synthesis was analyzed with [^{35}S]-Sulfate incorporation. Human NP cell culture with high dose of rhBMP-2 (1000ng/ml) showed 55% increase in proteoglycan synthesis, compared to control culture without rhBMP-2. In contrast, human TZ and AF cell cultures with various dose of rhBMP-2 demonstrated no significant increase in proteoglycan synthesis compared to control culture ($p<0.05$)

4. Expression of osteocalcin mRNA,

In densitometric assay of reverse transcription-polymerase chain reaction, human NP, TZ, and AF cell cultures in three dimensional alginate beads with 1000ng/ml rhBMP-2 showed no statistically significant changes in mRNA expression of osteocalcin compared to each cell control without rhBMP-2 (Figure 5).

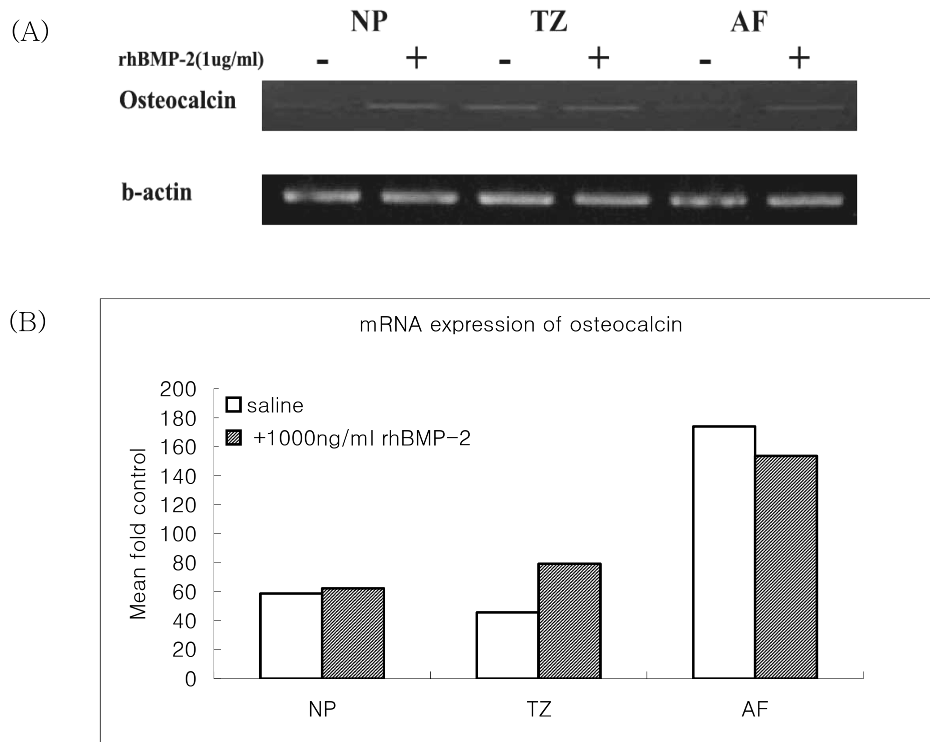


Figure 5. In densitometric assay of reverse transcription-polymerase chain reaction, human NP, TZ, and AF cells were cultured in three dimensional alginate beads with 1000ng/ml rhBMP-2 showed no statistically significant changes in mRNA expression of osteocalcin compared to control. mRNA expression was normalized by ß-actin mRNA expression.

Ⅳ. DISCUSSION

The spectrum of disc disease includes simple disc degeneration, internal disc derangement, disc herniation, radiculopathy, myelopathy, and spinal stenosis. Theoretically, the loss of proteoglycan may directly affect the biomechanical functions of IVDs, altering loading of the facet joint and other structures, causing degenerative changes of the spine. [17] Indeed, there has been no effective treatment in degenerative disc disease since treatment were largely symptomatic measures or palliative procedures i.e. discectomy and spinal fusion. Recently therapy based on biologic approaches to stimulate matrix synthesis or prevent matrix degradation of the IVD has gained popularity in spinal research field. [14,15,18,19] One promising approach to achieve this objective relies upon the ability the certain growth factors to up-regulate synthesis of matrix components.

Efficient and reliable methods to deliver exogenous gene(s) to the IVD have become available with recent developments in molecular biology and viral vector technology. The optimal combination of exogenous genes for the management of degenerative disc disease is largely unknown. While the cDNAs of anabolic growth factors as well as catabolic cytokines and enzymes are available, most research efforts to date have focused on enhancing the anabolic aspects of disc metabolism—hence the emphasis has been on anabolic growth factors. Transforming growth factor-β1(TGF-

β1), insulin like growth factor-1(IGF-1), osteogenic protein-1, platelet derived growth factor, BMP-2, fibroblast growth factor, and epithelial growth factor were known to be a candidate for regenerating matrix of degenerated disc. [6, 14, 18, 19] Previous study demonstrated that rhBMP-2 up-regulated expressions of aggrecan, collagen type I, and collagen type II mRNA without expression of osteocalcin mRNA in human IVD cells 20). Furthermore, human IVD cells cultured in monolayer demonstrated neither visible expression of osteogenic marker nor bone nodule formation in given dose of rhBMP-2 and culture period as evidenced by negative alkaline phosphatase and Alizarin red-S stain. [11] However, it still remains unanswered whether the effect of rhBMP-2 to cells from different regions of IVD. In other words, different responses of cells from NP, TZ, and AF of IVD to rhBMP-2 were major concern before initiating BMP-2 gene therapy or protein therapy to regenerate IVD. Hence, in this study, we elucidated responsiveness of cells from different zone of IVD i.e., NP, TZ, and AF to rhBMP-2 in vitro in terms of DNA synthesis, proteoglycan synthesis, and finally expression of osteogenic phenotype. The expression of osteogenic phenotype or pure chondrogenic phenotype to rhBMP-2 has prime importance in IVD regeneration, since IVD is made of fibrocartilageous tissue and provide mechanism of amphiarthrosis.

The findings of the current study demonstrated that rhBMP-2 has differential effects on different zone of IVD i.e., NP, TZ, and AF, while has no recognizable expression of osteogenic phenotype in all three region of IVD. Interestingly, rhBMP-2

exhibited more cellular proliferation i.e., seven fold increase compared to control culture, in AF, while it showed no significant increase in DNA synthesis in TZ and NP. In addition rhBMP-2 demonstrated increased proteoglycan synthesis in the culture from NP than TZ and AF. In RT-PCR, the expression of osteocalcin mRNA, maker for osteogenic phenotype, was not documented in cell cultures from all three region of IVD. Taken together, rhBMP-2 demonstrated cellular proliferation in cells from AF, proteoglycan synthesis in cells from NP, and no evident expression of osteocalcin mRNA expression in cells from NP, TZ, and AF.

BMP-2 and BMP-2 encoding gene appear to be a therapeutic and/or prophylactic agent for IVD degeneration with least possibility of bone formation. In previous study, adenovirus-mediated transfer of BMP-2 encoding gene alone and with combination with TGF-β1 and IGF-1 encoding gene to human IVD cells was proven to be effective in up regulating proteoglycan synthesis.[6] Moreover in cell cultures from NP rhBMP-2 proved to stimulate proteoglycan synthesis and up regulate chondrogenic phenotypes i.e. collagen type II and aggrecan mRNAs expression while it rendered no recognizable effect on osteocalcin mRNA expression in human disc cells from nucleus pulposus.[11] Results of the current study, together with previous ones, clearly demonstrated that rhBMP-2 protein and gene transfer of BMP-2 encoding gene provide a mechanism for upregulating matrix synthesis in human IVD cells.

V. CONCLUSION

BMP-2 clearly showed mitogenic effect in the cells from AF, while stimulate proteoglycan synthesis in the cells from NP without evidence of the expression of osteogenic phenotype. Taken together, this study raised the possibility of rhBMP-2 to be used an anabolic agent for mitogenesis and regenerating matrix depending on different zones of human IVD .

VI. REFERENCES

1. Reddi AH. Bone morphogenetic proteins: From basic science to clinical applications. J Bone Joint Surg [Am] 2001;83s:S1-6.
2. Asahina I, Sampath TK, Nishimura I, Hauschka PV. Human osteogenic protein-1 induces both chondroblastic and osteoblastic differentiation of osteoprogenitor cells derived from new born rat calvaria. J Cell Biol 1993;123:921-933.
3. Baur ST, Mai JJ, Dymecki SM. Combinational signaling through BMP receptor IB and GDF5: Shaping of the distal mouth limb and the genetics of distal limb diversity. Development 2000;127:605-619.
4. Flechtenmacher J, Huch K, Thonar EJ, Mollenhauer JA, Davies SR, Schmid TM, et al. Recombinant human osteogenic protein 1 is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. Arthritis Rheum 1996;39:1896-1904.
5. Sellers RS, Peluso D, Morris EA. The effect of recombinant human bone morphogenetic protein-2 on healing of full thickness defects on articular cartilage. J Bone Joint Surg [Am] 1997;79:1452-1463.
6. Moon SH, Nishida K, Gilbertson LG. Biologic response of human intervertebral disc cells to gene therapy cocktail. Proc Orthop Res Soc, 2000, Orlando, FL.
7. Smith P, Shuler FD, Georgescu HI, Ghivizzani SC, Johnstone B, Nixibizi C, et al.

Genetic enhancement of matrix synthesis by articular chondrocytes. Arthritis Rheuma 2000;43:1156-1164.

8. Muschik M, Schlenzka D, Ritsila V, Tennstedt C, Lewandrowski KU. Experimental anterior spine fusion using bovine bone morphogenetic protein: a study in rabbits. J Orthop Sci 2000;5:165-170.

9. Lipson SJ, Muir H. Proteoglycans in experimental intervertebral disc degeneration. Spine 1981;6:194-210.

10. Melrose J, Ghosh P, Taylor TK, Hall A, Osti OL, Vernon-Roberts B, et al. A longitudinal study of the matrix changes induced in the intervertebral disc by surgical damage to the annulus fibosus. J Orthop Res 1992;10:665-676.

11. Kim DJ, Moon SH, Kim H, Kwon UH, Park MS, Han KJ, Hahn SB, Lee HM: Bone Morphogenetic Protein-2 Facilitates Expression of Chondrogenic not Osteogenic Phenotype of Human Intervertebral Disc Cells. Spine, 2003;25: 2573-2579.

12. Eyre D, Benya P, Buckwalter J. Intervertebral disc: Part B. Basic science perspective. In New Perspectives in Low Back Pain. Park Ridge, IL: American Academy of Orthopaedic Surgeons, 1989; 147-207.

13. Chelberg MK, Banks GM, Geiger DF, Oegema TR Jr. Identification of heterogenous cell populations in normal human intervertebral disc. J Anat 1995;186:43-53.

14. Nishida K, Kang JD, Gilbertson LG, Moon SH, Suh JK, Vogt MT, et al. Modulation of the biologic activity of the rabbit intervertebral disc by gene therapy: An in-vivo

study of adenovirus-mediated transfer of the human TGF- ß1 encoding gene. Spine 1999;24:2419-2425.

15. Nishida K, Kang JD, Suh JK, Robbins PD, Evans CH, Gilbertson LG.. Adenovirus-mediated gene transfer to nucleus pulposus cells: Implication for the treatment of intervertebral disc degeneration. Spine 1998;23:2437-2443.

16. Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, et al. Recombinant human bone morphogenic protein induces bone formation. Proc Natl Acad Sci 1990;87:2220-2224.

17. Butler D, Trafimow JH, Andersson GB. Discs degenerate before facets. Spine 1990;15:111-113.

18. Osada R, Ohshima H, Ishihara H, Yudoh K, Sakai K, Matsui H, et al. Autocrine/paracrine mechanism of insulin-like growth factor-1 secretion, and the effect of insuline-like growth factors-1 on proteoglycan synthesis in bovine intervertebral discs. J Orthop Res 1996;14:690-699.

19. Thompson JP, Oegema TR Jr, Bradford DS. Stimulation of mature cannine intervertebral disc by growth factors. Spine 1991;16:253-260.

ABSTRACT (In Korean)

인간 척추 추간판의 수핵 중심부, 이행부, 외측 섬유륜으로 부터 분리한 세포의 BMP-2에 대한 반응

< 지도교수 이 환 모 >

연세대학교 대학원 의학과

김 형 찬

인간의 척추 추간판은 수분을 함유하고있는 당단백이 다량 함유되어 있는 수핵과 이것을 감싸고 있는 섬유륜과 중간사이의 전이층으로 구분된다. 퇴행된 추간판은 당단백 과 수분의 함유가 감소 되어 있으며, 이로 인해 추간판의 높이가 줄어들며 외부 충격에 효과적으로 대처할 수 없어 추가적인 손상이 발생하게 된다. 척추질환의 가장 많은 원인이 되는 추간판의 퇴행성 병변은 일반적인 병리현상으로 재생은 불가능한 것으로 알려져 있다. BMP-2 는 골유도 및 증식, 연골합성, 및 연골의 표현에 강력한 유발인자로 알려져 있으며 이미 골유합 촉진제로써 임상적으로도 사용되고 있다. 또한 인간 추간판 세포의 당단백 합성을 증식시키는 효과도 있어 퇴행성 인간 추간판 질환의 치료제로써 가능성이 매우 높은 것으로 알려져 있다. BMP-2의 인간추간판에 대한 효과가 당단백 재생을 유도할 것인지,

아니면 골 형성을 유도할 것인지에 대한 다양한 연구들이 이미 진행되고 발표되었으나 추간판내의 특정부위에 대한 비교연구는 그 동안 이뤄지지 않았다. 따라서 본 연구의 목적은, 인간 추간판 세포를 수핵, 전이부, 섬유륜 각각 3부위에서 따로 채취 분리하여 시험관내에서 각각의 BMP-2에 대한 반응을 알아보고 부위에 따른 차이점, 소위 zonal effect 존재를 확인하고자 했다. 추간판 조직은 5명의 환자로부터 획득하였으며 단계적 효소 소화법으로 각 부위별 세포를 분리하여 배양하였다. 재조합 인간 BMP-2는 유전자 재조합 방법으로 생산하였다. 다양한 용량의 BMP-2를 세포배양에 적용하였고, alkaline phsphatase 활성, von Kossa 염색 , 등을 수행하였다.

BMP-2 투여 후 섬유륜 세포군에서 cDNA 합성이 증가되어 mitogenic effect를 확인 할 수 있었으나 수핵 세포군 및 전이부 세포군에서는 이러한 현상이 관찰되지 않았다. 당단백 합성은 수핵 세포군에서 의미는 있는 증가 양상을 관찰할 수 있었으나 섬유륜 및 전이부 에서는 관찰되지 않았다. 그러나 모든 부위에서 골 형성에 관련된 mRNA 발현은 관찰되지 않았다. 본 실험 결과, 재조합 인간 BMP-2는 인간 추간판내 각 부위별 특이적인 zonal effect 를 확인할 수 있었으며 분열증식 및 기질의 합성을 증가시키는 효과적인 동화인자의 가능성을 다시 확인하였다.

핵심되는 말 ; BMP-2, 추간판세포, 당단백합성 , 기질생성, zonal effect