Human intervertebral disc engineering with atelocollagen scaffold and by adenovirus-mediated gene transfer

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Human intervertebral disc engineering with atelocollagen scaffold and by adenovirus-mediated gene transfer

Directed by Professor Seong-Hwan Moon

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ABSTRACT

Human intervertebral disc engineering with atelocollagen scaffold and by adenovirus-mediated gene transfer

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Tissue engineering is regarded as a new experimental technique for the biological treatment about degenerative intervertebral disc (IVD) diseases. Among artificial scaffolds, atelocollagen has an advantage in safety over others. And growth factors were known to stimulate matrix synthesis of IVD cells, among of them, transforming growth factor-β1 and bone morphogenetic protein-2 are the best candidates for IVD regeneration. Gene transfer to IVD

cells provided sustained mechanism for matrix synthesis. Hence, the object of this study was to examine the cellular proliferation, proteoglycan (PG) synthesis, and phenotypical expression of IVD cells transduced by adenovirus transforming growth factor beta-1(Ad/TGF-β1) construct and adenovirus bone morphogenetic protein-2(Ad/BMP-2) construct and seeded on type I atelocollagen scaffolds.

The IVD cells were isolated by sequential enzymatic digestion. Atelocollagen scaffold were prepared by lypophilization and cross-linking method. Ad/TGF-β1 or Ad/BMP-2 treated IVD cells were cultured for 7days. At 3 and 7 days, cellular proliferation, newly synthesized proteoglycan, mRNA expressions and the inner morphology of cell seeded scaffolds was determined by scanning electron microscopy (SEM).

As result, transduced IVD cultures in atelocollagen type I with Ad/TGF-β1 and mixture group demonstrated increase in proteoglycan synthesis, but did not show significant change in mRNA expressions, compared to control. On the other hand, the IVD of transduced with Ad/ TGF-β1 in types I atelocollagen scaffolds decresed in cellular proliferation compared with others. And there were not showed osteocalcin mRNA expression in any groups.

Human IVD cells were viable in type I atelocollagen scaffolds. Type I atelocollagen scaffold was suitable for cell proliferation, extracellular matrix

synthesis. And Ad/TGF-β1 and Ad/BMP-2 combination was potential for IVD regeneration. Taken together, IVD cells in atelocollagen scaffolds, with therapeutic gene, provide a mechanism for tissue engineering of IVD.

Key words: gene therapy, tissue engineering, intervertebral disc, atelocollagen scaffold, Ad/TGF-β1, Ad/BMP-2

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Ⅰ. INTRODUCTION

Intervertebral disc (IVD) degeneration is a chronic process that the nucleus pulposus(NP) has been changed on account of multiple factors(malnutrition, mechanical stress and apoptosis, among others) [1]. Healthy NP is rich in proteoglycans(PG) and type II collagen, with a water content of over 85% [2]. But in adults, PG content decreases, and type I collagen becomes more prevalent. High concentration of PG in the NP helps to maintain disc height and contribute to the load-bearing ability of the disc [3, 4]. Therefore, decreased PG directly affects the biomechanical function of IVD.

Current treatment for IVD degeneration, includes conservative managements such as bed rest, NSAIDs, analgesia, and physical therapy and surgical operations. Rather than traditional treatment, by the technique of tissue engineering, composite IVD implants are fabricated as novel materials for disc replacement. Collagen is the most frequently used material for mammal cell culture carriers [5, 6]. Atelocollagen removed the antigenic telopeptide region, so it's immunogenic and safety for transplantation [7].

And gene therapy is accounted to be a potential application in treatment of IVD disorders. The delivery of exogenous gene to target cells results in gene expression and subsequent production of proteins which makes up for the weak of protein injection. Including the growth factors, transforming growth factor-β1(TGF-β1) [8, 9] and bone morphogenetic protein-2(BMP-2) [10] are the best applicant for IVD regeneration. IVD engineering by BMP-2 and TGF-β1 using atelocollagen and rabbit IVD cells were attempted [11], nevertheless, human IVD cells and gene transfer approach was not tried before.

Hence, in this study, we elucidated the effect of adenoviral TGF-β1 and BMP-2 gene transfer to human IVD cells cultured in atelocollagen scaffolds.

Ⅱ. MATERIALS AND METHODS

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

1. Materials

Lumbar IVD tissue was obtained from 3 patients (Age range 50-65 years) during surgical discectomy performed for herniated lumbar disc. Classification of the IVD of each patient was performed based in the literature [12]. IVDs with grade III and IV degeneration were exclusively harvested to minimize the effect of IVD degeneration on transgene expression and PG synthesis. An attempt was made by the surgeons (HML, SHM) to carefully obtain tissue from the central aspect of the disc to optimize harvest of only the nucleus pulposus and the inner annulus fibrosus.

The IVD tissue specimens were transported in sterile GBSS (GBSS, Gibco-BRL, Grand Island, NY, USA) to the laboratory, less than 20 minutes after surgical removal and then washed with GBSS to remove blood and bodily fluid contaminants.

2. Intervertebral disc cell culture

IVD was shredded with scissors and digested in Ham's F-12 medium (F-12, Gibco-BRL, Grand Island, NY, USA) containing 1% (v/v) penicillin, streptomycin, nystatin (all antibiotics from Gibco-BRL, Grand Island, NY, USA), 0.2% (w/v) Protease, 0.004% (w/v) DNase (Sigma, ST. Louis, MO, USA) for an hour at 37℃ under gentle agitation. The tissue was then washed 2 times with DMEM/F-12 and digested in Ham's F-12 containing 1% (v/v) antibiotics, 0.02% (w/v) collagenase type II, 0.004% (w/v) DNase (Sigma, St. Louis, MO, USA) for 2 to 3 hours under the same conditions. The digested tissue was passed through a sterile cell strainer (Falcon, Franklin Lakes, NJ, USA) with a pore size of 70um. The filtrated was centrifuged at 1,500 rpm for 5 minutes to separate the cells. The resulting cell suspensioins were placed in T-25 at 1×10^6 cells per well and grown in 7ml Dulbecco's modified eagle medium and Hams F-12 medium (DMEM/F-12, Gibco-BRL, Grand Island, NY, USA) supplemented with 10% heat activated fetal bovine serum (FBS, Gibco-BRL, Grand Island, NY, USA), 1% (v/v) antibiotic-antimycotic, and 25ug/ml ascorbic acid at 37℃ in an atmosphere of 5% CO_2 and 95% air. Culture medium was changed every other day for 3 weeks and fresh ascorbic acid was added at each feeding. 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. Production of the adenoviral vector

Three different adenoviral constructs were prepared for this study: Adenovirus-lacZ construct(Ad/lacZ), adenovirus TGF-β1 construct(Ad/TGF-β1) and adenovirus BMP-2 (Ad/BMP-2). Each recombinant adenoviral vectro originated from replication-deficient Type 5 adenovirus, which lacked the E1 and E3 regions of genome [13]. All of the genes were cloned into the E1 region under the control of the human cytomegalovirus early promoter. Recombinant virus was grown in transformed human embryonic kidney 293 cells and purified by CsCL density gradient purification. Titers were determined by optical density at 260 nm (OD 260) and standard plaque assay [14, 15].

4. Production of type I atelocollagen scaffold

Fifty six ul of 1% type I atelocollagen (Dalim Tissen, Seoul, Korea) dispersion was poured into a 96-well plate (diameter 5mm), frozen at -70℃,

and then lyophilized at -50℃. The fabricated porous collagen matrixes were crosslinked in 50mM of 1-ethyl-(3-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Sigma Chemical Co., St. Louis, MO, USA) solution (H_2O-ethanol=5:95) for 24 hours. The matrixes obtained were washed in distilled water using a sonicator and then relyophilized at -50℃ [16, 17].

5. Gene transfer to intervertebral disc cell

At confluence, NP cells were organized into five groups: 1) control group, 2)Ad/lacZ group, 3) Ad/BMP-2 group, 4) Ad/TGF-β1 group, 5) Ad/BMP and Ad/ TGF-β1 1:1 mixture group. In group 1, 2ml of HBSS without adenovirus was added to the culture plate. In group 2 to 5, 2ml of HBSS with different adenovirus (100MOI) were added. Group 1 was used as the saline control. All cultures were incubated at 37℃, 5% CO_2 humidity under gentle agitation for 1 hour. Culture medium (DMEM-F12 containing 5% FBS 25ug/ml ascorbic acid) 10ml was then added and the transduced cells were further incubated.

6. Cell transplantation to atelocollagen scaffolds

Atelocollagen scaffolds were γ-irradiation for 10 minutes for sterilization.

After sterilization, they were soaked in culture media. Cell suspensions were imbibed by surface tension into each scaffold. 1×10^5 cells per 96-well size scaffold in 30ul of DMEM/F-12 containing 5% FBS, 25ug/ml ascorbic acid were seeded and incubated for 4 hours at 37℃, 5% CO_2. After 4 hours incubation, DMEM-F12 containing 5% FBS, 25ug/ml ascorbic acid were added and cultured for 7 days.

7. Cellular proliferation

Cellular proliferation was measured by Alamar Blue assay (AbD Serotec, Oxford, UK). Initial experiments were carried out to follow % AB reduction over time. AB was directly into culture media at a final concentration of 10% and the plate was returned to the incubator. Optical density of the plate was measured by ELISA at 570nm and 600nm with a standard spectrophotometer at 7day. The calculated % AB reduction is a cumulative value. As a negative control, AB was added to medium without cells.

8. Newly synthesized proteoglycan

5u Ci/ml of [^{35}S]-sulfate (Amersham Biosciences, Uppsala, Sweden; 25Ci/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 Biosciences, Uppsala, Sweden) under dissociative condition. Fractions (1ml) were colleted in scintillation vial and mixed with 6ml scintillation cocktail solution (Beckman Coulter Inc., Fullerton, CA, USA). Five fractions were collected per sample, and three meddle fractions were counted in a Packard liquid scintillation counter (Beckman Coulter Inc., Fullerton, CA, USA)

9. Reverse transcription-polymerase chain reaction analysis

Total cellular RNA was eluted by selective binding to a silica gel-based membrane using an RNeasy mini kit(QIAGEN, GmbH, Hiden, German). Reverse transcription of RNA into cDNA was performed incubating 1μl of

RNA in a reaction mixture containing 0.5mg/ml cDNA reaction product and was used as the template to co-amplify β-actin, aggrecan, collagen type Ⅰ, Ⅱ, and osteocalcin. PCR was performed using a DNA thermal cycler. Amplification reactions specific for the following cDNAs were performed: β-actin, aggrecan, collagen type I, type II, and osteocalcin. Primer sequence of each cDNA was listed on Table 2. PCR products (5ul) were analyzed by electrophoresis in 1.5 % 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).

10. Scanning electron microscopy (SEM)

Cellular and cellular scaffolds were observed at the 3 and 7 -day time point using SEM. Specimens for SEM were washed twice with sterile PBS and then fixed in 4% paraformaldehyde (w/v) for 2 days. After fixation, the samples were dehydrated in a graded series of ethanol (10-95%). The dehydrated samples were transferred to a vacuum desiccator until completely dry. The specimens were then gold sputter coated with a DESK II gold sputter coater (Denton) and examined using a Hitachi 3500 scanning electron microscope (Hitachi, Tokyo, Japan) in secondary electron mode at 15.0 kV.

11. Statistical analysis.

The numerical data from each experiment were the average from at least triplicate samples. The same experiments were repeated three times to ensure the repeatability of the methods used. One-way analysis of variance and Fisher's protected LSD post-hoc test, power analysis were performed to test difference in densitometric data, Alamar Blue assy, and [^{35}S]-sulfate labeled proteoglycan. Significance level was set as $p<0.05$.

TABLE 1. Sequences of the RT-PCR primers used

Human Primer	Sequence	Length	Size(bp)
β-actin	5'-GCC GGA CTA TGA CTT AGT TG-3'	20	238
	5'-AAA CAA CAA TGT GCA ATC AA-3'	20	
Aggrecan	5'-GAA TCT AGC AGT GAG ACG TC-3'	20	541
	5'-CTG CAG CAG TTG ATT CTG AT-3'	20	
Collagen type Ⅰ	5'-CCT GTC TGC TTC CTG TTA AC-3'	20	182
	5'-AGA GAT GAA TGC AAA GGA AA-3'	20	
Collagen type Ⅱ	5'-AAG ATG GTC CCA AAG GTG CTC G-3'	22	500
	5'-AGC TTC TCC TCT GTC TCC TTG C-3'	22	
Osteocalcin	5'-CAC TCC TCG CCC TAT TGG CC-3'	20	866
	5'-GCC AAA TCG TCA CAG TCC GG-3'	20	

TABLE 2. RT-PCR conditions

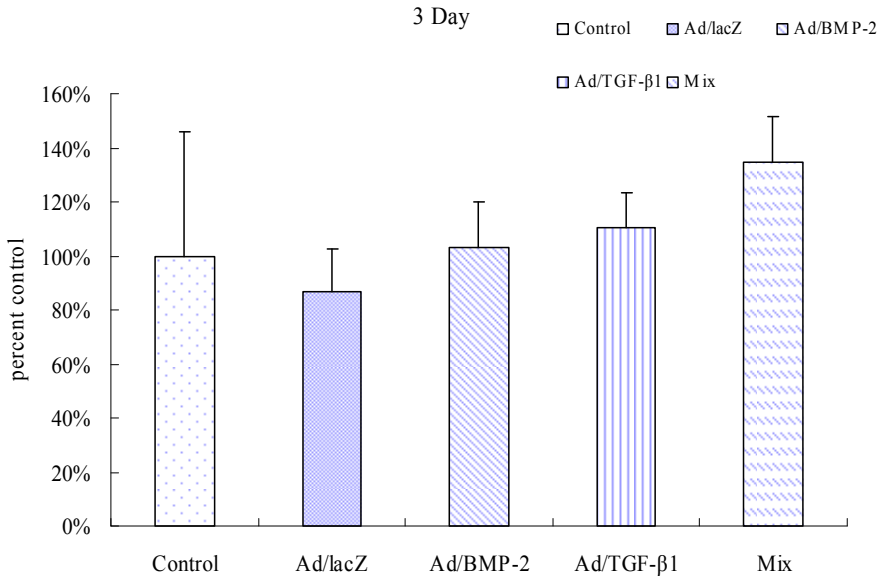
Human Primer	Conditions			Cycle
	Denaturation	Annealing	Polymerization	
β-actin	94℃ 5 sec	53℃ 5 sec	72℃ 30 sec	28
Aggrecan	94℃ 30 sec	47℃ 30 sec	72℃ 30 sec	30
Collagen type Ⅰ	94℃ 5 sec	52℃ 5 sec	72℃ 30 sec	25
Collagen type Ⅱ	94℃ 45 sec	54℃ 1 min	72℃ 1 min	35
Osteocalcin	94℃ 5 sec	46℃ 5 sec	72℃ 30 sec	30

Ⅲ. RESULTS

1. DNA synthesis

There were no statistical differences between control and experimental groups in DNA synthesis at 3 days, but Ad/TGF-β1 group was decreased at 7 days compared with control (Figure 1-A, -B).

A



B

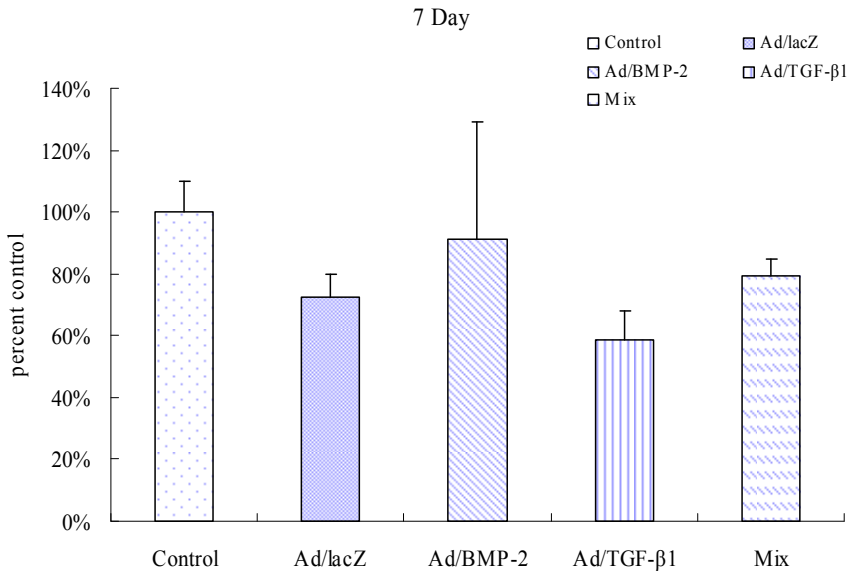
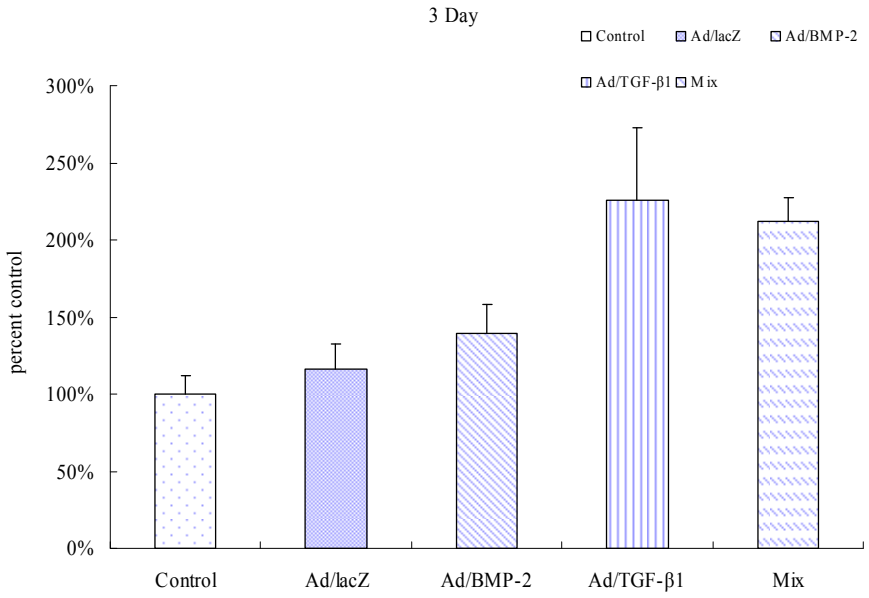


Fig.1A-B. DNA synthesis of transduced IVD cells seeded on atelocollagen scaffolds (*p<0.05). Percent control of DNA synthesis was measured by Alamar Blue assay. Control; human nucleus puplosus cells only, Ad/lacZ; transduced with Ad/lacZ (100MOI), Ad/TGF-β1; transduced with Ad/TGF-β1(100MOI), Ad/BMP-2; transduced with Ad/BMP-2 (100MOI), Mix; transduced with mixture of Ad/TGF-β1 and Ad/BMP-2)at the ratio of 1(50MOI):1(50MOI). The culture period was three and seven days each.

2. Newly synthesized proteoglycan normalized by DNA synthesis

Human NP cell cultures in atelocollagen type I scaffold with each Ad/TGF-β1 (5.4 fold increases) and mixture (4.3 fold increase) increased in proteoglycan synthesis at 3 and 7 days compared with control. But there's no significant change on Ad/BMP-2 group. (Figure 2)

A



B

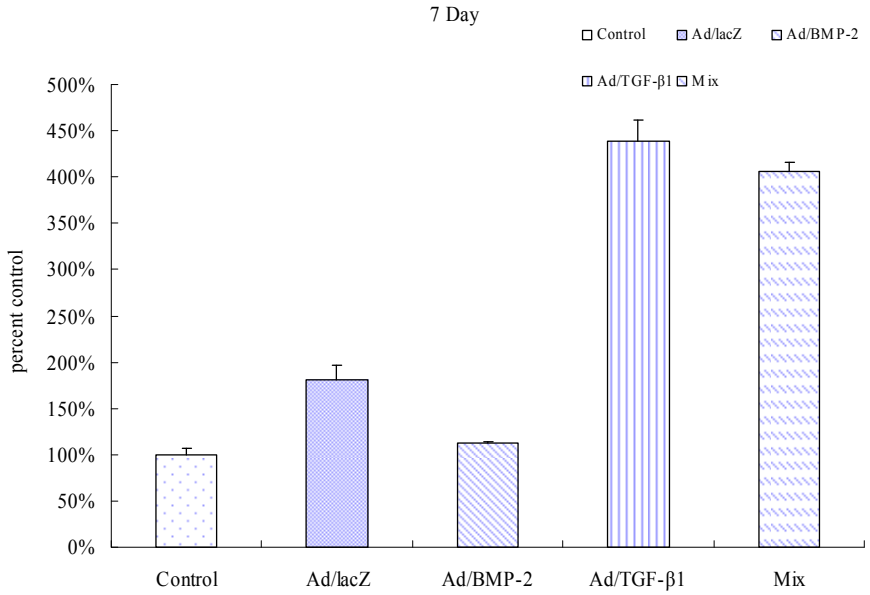


Fig. 2A-B. Newly synthesized proteoglycan of human nucleus pulposus cells seeded on typeI atelocollagen scaffolds (*p<0.05). Percent control of proteoglycan synthesis was measured by [^{35}S]-sulfate incorporation (CPM). Control; human nucleus puplosus cells only, Ad/lacZ; transduced with Ad/lacZ (100MOI), Ad/TGF-β1; transduced with Ad/TGF-β1(100MOI), Ad/BMP-2; transduced with Ad/BMP-2 (100MOI), Mix; transduced with mixture of Ad/TGF-β1 and Ad/BMP-2)at the ratio of 1(50MOI):1(50MOI). The culture period was three and seven days each.

3. mRNA expression of aggrecan, collagen type I, II, and osteocalcin

In densitometry assay of reverse transcription-polymerase chain reaction, Ad/BMP-2 transduced human NP cell cultures in atelocollagen type I scaffold showed no statistically significant increase of mRNA expression of collagen type I, II and aggrecan compared with control. However Ad/TGF-β1 group showed up-regulation of collagen type I, aggrecan mRNA expression, on the other hand, there were not shown osteocalcin mRNA expression in any groups.(Fig3, 4)

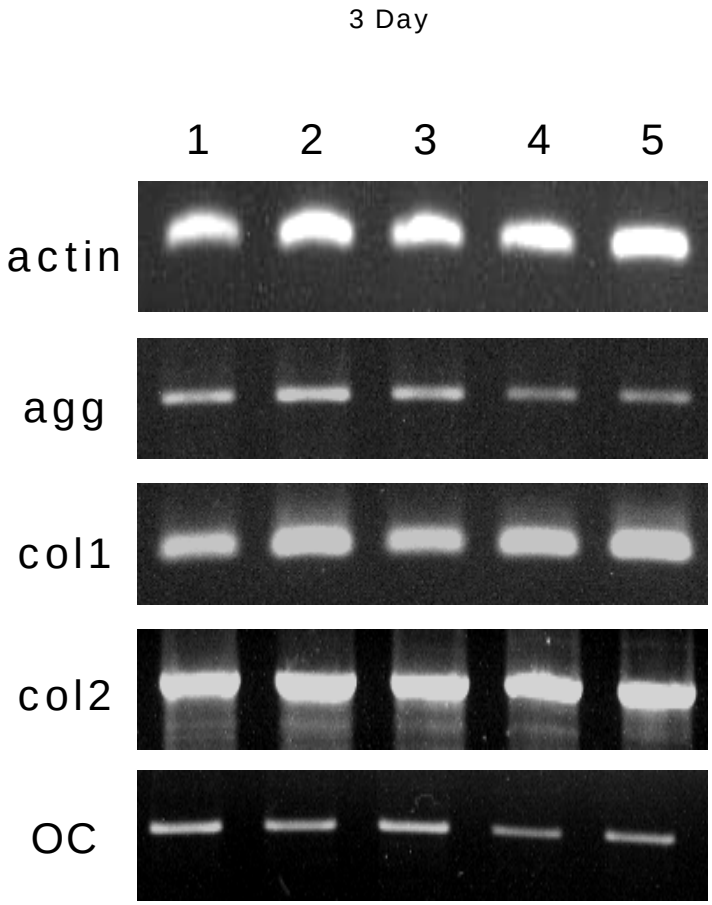
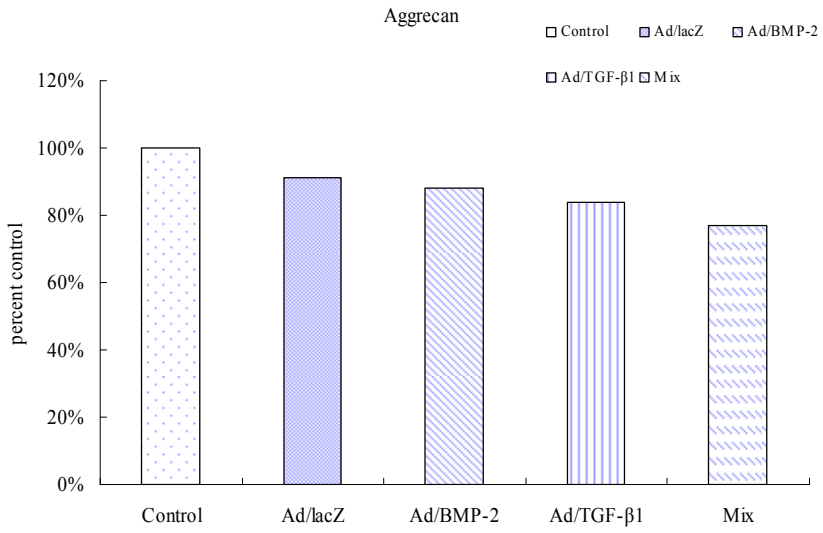
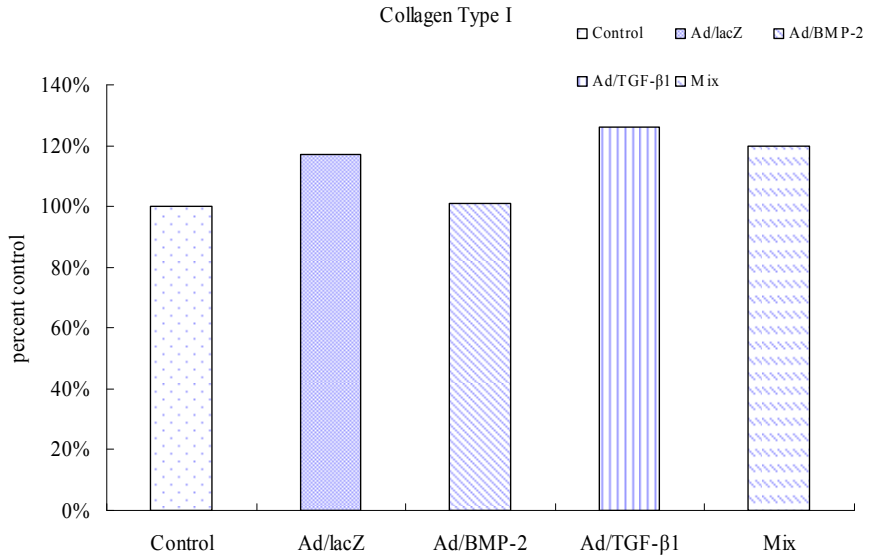


Fig.3. RT-PCR of beta-actin, aggrecan, collagentactin, aggrecan, collagen type I, II and osteocalcin. Total RNA was isolated from cells and subjected to RT-PCR. The PCR products were separated on 1.5% agarose gels containing ethidium bromide, and then observed on an ultraviolet transilluminater. 1: Control, 2,:Ad/lacZ 3:Ad/TGF-β 1,4:Ad/BMP-2,5:Mix

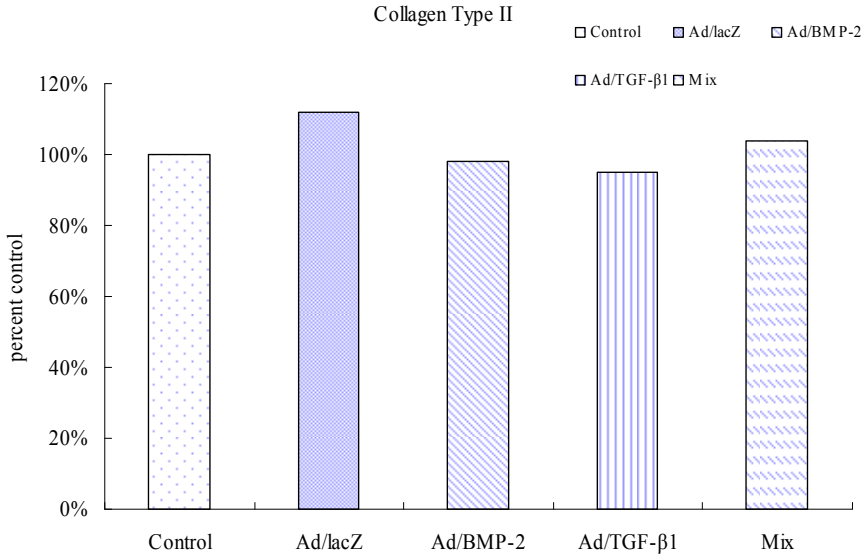
A


Aggrecan
Control
Ad/lacZ
Ad/BMP-2
Ad/TGF-β1
Mix
120%
100%
80%
60%
40%
20%
0%
percent control
Control
Ad/lacZ
Ad/BMP-2
Ad/TGF-β1
Mix

B


Collagen Type I
Control
Ad/lacZ
Ad/BMP-2
Ad/TGF-β1
Mix
140%
120%
100%
80%
60%
40%
20%
0%
percent control
Control
Ad/lacZ
Ad/BMP-2
Ad/TGF-β1
Mix

C


Collagen Type II
Control
Ad/lacZ
Ad/BMP-2
Ad/TGF-β1
Mix
120%
100%
80%
60%
40%
20%
0%
percent control
Control
Ad/lacZ
Ad/BMP-2
Ad/TGF-β1
Mix

D

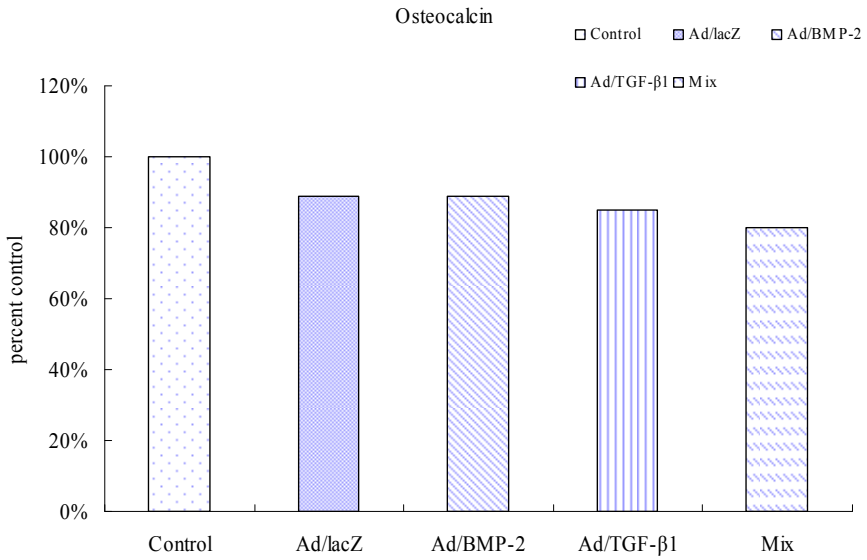
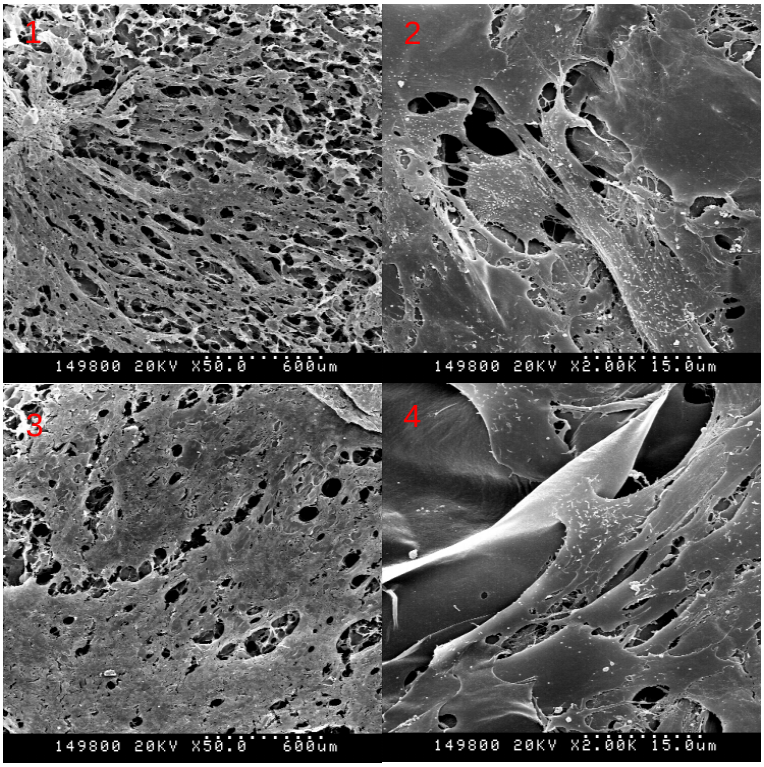

Osteocalcin
Control
Ad/lacZ
Ad/BMP-2
Ad/TGF-β1
Mix
120%
100%
80%
60%
40%
20%
0%
percent control
Control
Ad/lacZ
Ad/BMP-2
Ad/TGF-β1
Mix

Fig.4A-D. Densitometry of A; aggrecan, B; collagen type I, C; collagen type II, and D; osteocalcin mRNA expression in atelocollagen scaffolds. The expression of each PCR band was quantified using an image analyzer. The results are presented as the percentage of the mRNA level relative to beta-actin for each band.

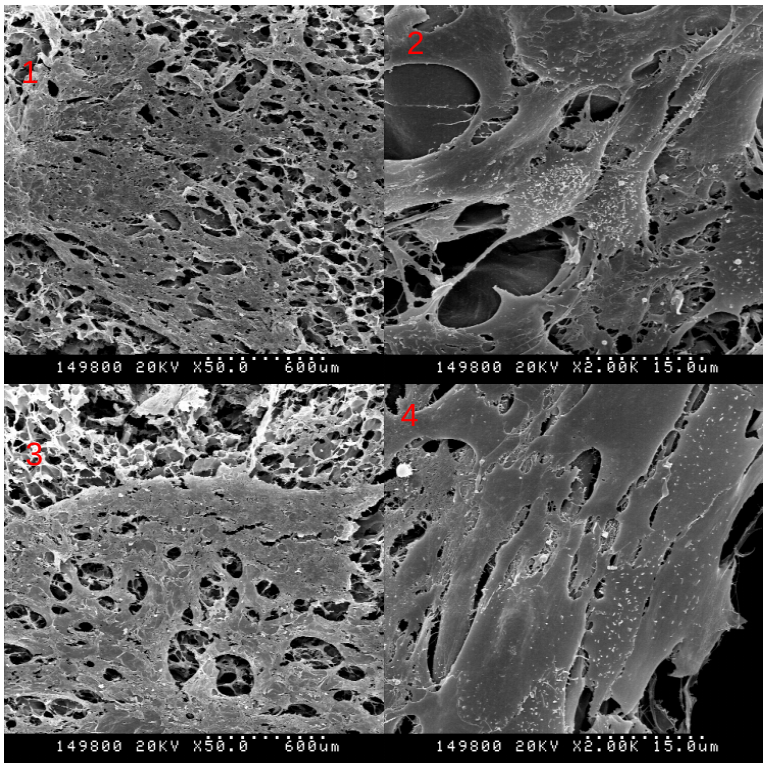
4. Scanning electron microscopy (SEM)

The SEM images of human IVD cells of porous atelocollagen type I were shown in figure5. When seeded on atelocollagen type I scaffold, the cells showed stable adhesion on each matrix and releasing of extra cellular matrixes on the surfaces. On the other hand, ttransduced human IVD cells growing on atelocollagen scaffold had the best cell to cell contact with neighboring cells, compared with control.

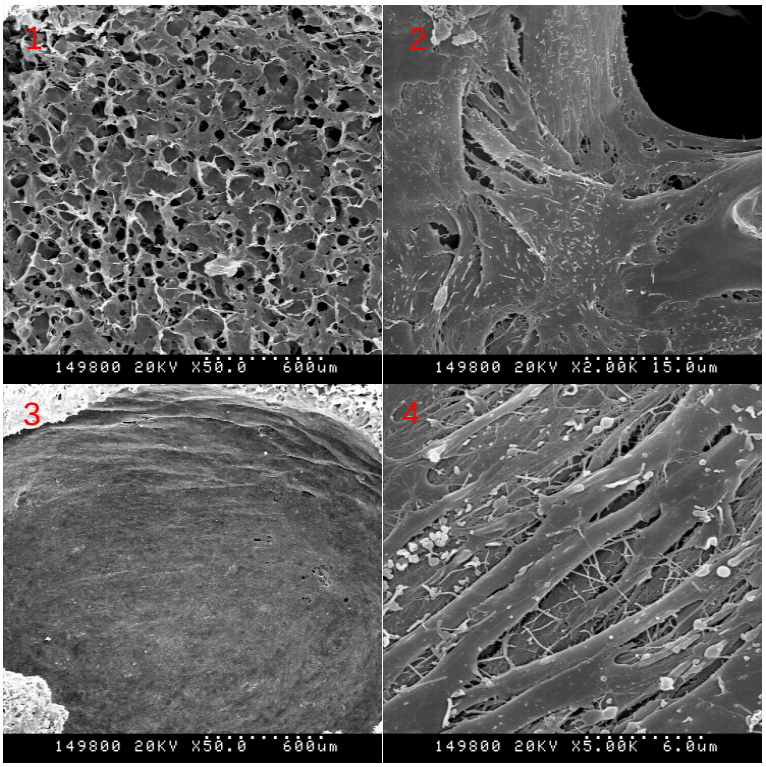
A


1
2
3
4
149800 20KV X50.0 600um
149800 20KV X2.00K 15.0um
149800 20KV X50.0 600um
149800 20KV X2.00K 15.0um

B


1
2
149800 20KV X50.0 600um
149800 20KV X2.00K 15.0um
3
4
149800 20KV X50.0 600um
149800 20KV X2.00K 15.0um

C


1
149800 20KV X50.0 600um
2
149800 20KV X2.00K 15.0um
3
149800 20KV X50.0 600um
4
149800 20KV X5.00K 6.0um

D

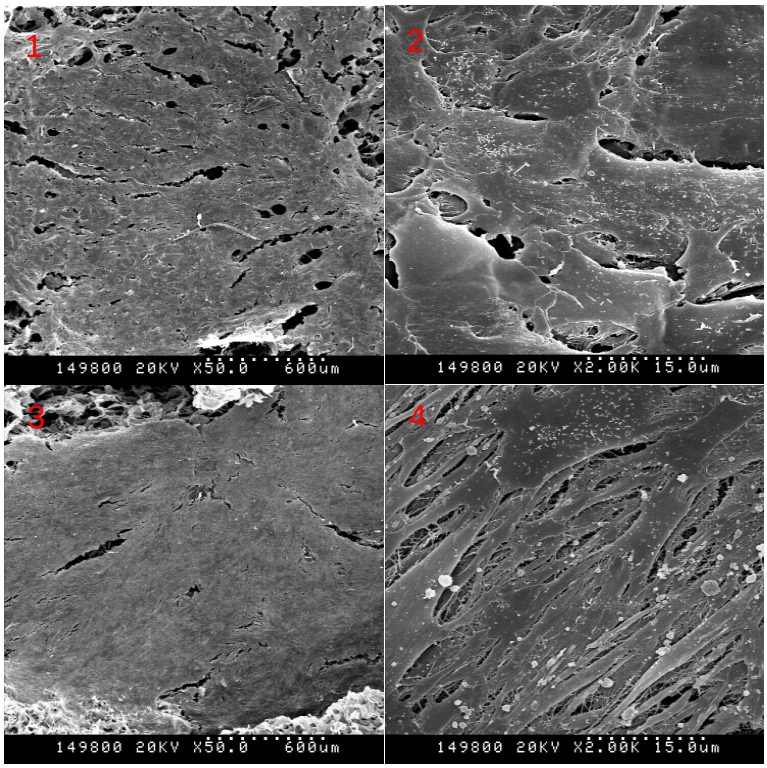


Fig.5A-D. Morphology of porous atelocollagen type I scaffold on scanning electron microscopy (SEM). (A) control, (B) Ad/BMP-2 (C) Ad/TGF-β1 (D) Mix; 1(X50), 2(X2000) were on 3 days and 3(X50), 4(X2000) were on 7 days.

Ⅳ. DISCUSSION

IVD is a well-encapsulated, avascular organ consisting of nucleus pulposus(NP) scattered within an extracellular matrix and collagen fiber annulus fibrosus(AF) surrounding NP. The healthy NP is abundant in PG and type II collagen. But when got on in years, PG contents decrease, and type I collagen becomes more prevalent. The PG and collagens are produced by cells within the AF, NP, or chondrocytes from the endplates remains. However, chondrocytes when cultured in monolayer are known to express a fibroblastic differentiated morphology and lose their ability to synthesize matrix of collagen distribution [18, 19]. Human IVD cells are known to possess chondrocyte phenotype [20, 21], so IVDs also express fibroblastic morphology in monolayer but regain in a three-dimensional environment [22, 23].

Recently, several groups have attempted to produce IVD-like materials for implantation [24, 25, 26].Among of them, PG synthesis proportion in atelocollagen is better [4]. Atelocollagen is a collagen gel that removed the antigenetic telopeptide region by pepsin digestion and differential salt precipitation during purification. So atelocollagen was chosen as a cell carrier because of the advantages of immunogenic and safety to transplant. Moreover

atelocollagen is suitable for gene therapy cause of slow releasing of DNA [27, 28, 29].

On the other hand, TGF-β1 and BMP-2 is well known for accelerating the synthesis of PG in IVD. TGF-β1 and BMP-2 both function in the TGF-family pathway, nevertheless, after receptor activation, Smad2 and Smad4 for TGF-β1, Smad1 and Smad4 for BMP-2 were activated and formed Smad4 complexion [30]. Therefore, the same signal molecular pathway will enhance the biological response such as PG synthesis. This demonstrates the ability of the gene therapy for disc regeneration.

In this experimental study, transduced human IVD cells were seeded on atelocollagen scaffolds. In the final result, cellular proliferation was not active in mixture group, on the other hand newly synthesized proteoglycan was rich in mixture group. In the same way Ad/TGF-β1 group stimulated proteoglycan synthesis. All of transducer culture groups did not show significant change in ECM component mRNA expression. Moreover in any combination of growth factor, they did not show up-regulation of osteocalcin mRNA expression. Therefore It can be concluded that Ad/TGF-β1 and Ad/BMP-2 gene therapy render only increased synthesis of chondrogenic matrix without an osteogeneic effect on IVD cells in atelocollagen scaffold.

These experimental results demonstrated that transduced cells seeding on the atelocollagen type I scaffolds are suitable for IVD cell transplantation. Therefore the following study in the future will be necessary to experiment of IVD cell based tissue engineering in vivo.

Ⅴ. CONCLUSION

Atelocollagen type I scaffold was suitable for cell proliferation and extracellular matrix synthesis. And Ad/TGF-β1 and Ad/BMP-2 combination was potential for IVD regeneration. Taken together, IVD cells in atelocollagen scaffolds, with therapeutic gene, provide a mechanism for tissue engineering of IVD.

REFERENCES

1. Sakai D, Mochida J, Iwashina T, Hiyama A, Omi H, Imai M et al. Regenerative effects of transplanting mesenchymal stem cells embedded in atelocollagen to the degenerated intervertebral disc. Biomaterials 2006;27:346-53
2. Sobajima S, Kim JS, Gilvertson LG, Kang JD. Gene therapy for degenerative disc disease. Gene Ther 2004;11:390-401
3. Urban JP, McMullin JF. Swelling pressure of the lumbar intevertebral discs: influence of age, spinal level, composition and degeneration. Spine 1988;13:179-87
4. Urban JP, McMullin JF. Swelling pressure of the lumbar intevertebral discs: influence of proteoglycan and collagen contents. Biorheology 1985;22:145-57
5. Cleator GM, Beswich TS. The probable role of collagen when used as a growth surface for cell culture. Cytobios 1972;20;231-9
6. Stephenson EM. A cell culture substrate obtained from heat-fused collagen. Experimentia 1975;31:1473-4
7. Sakai D, Mochida J, Yamamoto Y, Nomura T, Okuma M, Nishimura K et al. Transplantation of mesenchymal stem cells embedded in atelocollagen gel to the intervertebral disc: a potential therapeutic model for disc degeneration. Biomaterials 2003;24:3531-41

8. Thompson JP, Oegema Jr TR, Bradford DS. Stimulation of mature canine intervertebral disc by growth factors. Spine 1991;16:253-60

9. Gruber HE, Fisher EJ, Desai B, Stasky AA, Hoelscher G, Hanley E. Human intervertebral disc cells form the annulus: three-dimensional culture in agarose or alginate and responsiveness to TGF-beta. Exp Cell Res 1997;235:13-21

10. Osada R, Ohshima H, Ishihara H, Yudoh K, Sakai K, Matsui H, et al. Atutocrine/paracrine mechanism of insulin-like growth factor-1 secretion, and the effect of insulin-like growth factors-1 on proteoglycan sythesis in bovine intervertebral discs. J Orthop Res 1996;14:690-9

11. Takegami K, Thonar EJ, An Hs, Kamada H, Masuda K. Osteogenic protein-1 enhances matrix replenishment by intervetebral disc cells previously exposed to interleukin-1. Spine 2002;27:1318-25

12. Eyre D, Benya P, Buckwalter J. Intervertebral disc: part B. Basic science perspective. In: Frymoyer JW, Gordon SL, eds. New perspectives in Lowe Back Pain. Park Ridge, Ill American Academy of Orthopaedic Surgeons; 1989:147-07

13. Robbins PD, Ghivizzani SC. Viral vectors for gene therapy. Pharmacol Ther 1998;80:35-7

14. Graham FL,van der Eb Aj. A new techinque for assay of infectivity of human adenovirus 5 DNA. Virology 1973;52:456-7

15. Mittereder N, March KL, Trapnell BC. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J Virol 1996;70:7498-509

16. Park SN, Lee HJ, Lee KH, Suh H. Biological characterization of EDC-crosslinked collagen hyaluronic acid matrix in dermal tissue restoration. Biomaterials 2003;24:1631-41

17. Park SN, Kim JK, Suh H. Evaluation of antibiotic-loaded collagen-hyaluronic acid matrix as a skin substitute, Biomaterials 2004;25:3689-98

18. Uchio Y, Ochi M, Matsusaki M, Kurioka H, Katsube K. Human chondrocyte proliferation and matrix synthesis cultured in Atelocollagen gel. J Biomed Mater Res 2000;50:138-43

19. Kimurar T, Yasui N, Ohsawa S, Ono K. Chondrocytes embedded in collagen gels maintain cartilage phenotype during long-term cultures. Clin Orthop 1984;186:231-40

20. Horner HA, Roberts S, Bielby RC, Menage J, Evans H, Urban JPG. Cell from different regions of the intervetebral disc. Spine 2001;26:2543-9

21. Gruber HE, Stasky AA, Hanley Jr EN. Characterization and phenotypic stability of human disc cells in vitro. Matrix Biol 1999;14:359-68

22. Chiba K, Andersson GB, Masuda K, Momohara S, Williams JM, Thonar EJ. A new culture system to study the metabolism of the intervertebral disc in vitro. Spine 1998;23:1821-7

23. Chiba K, Andersson GB, Masuda K, Thonar EJ. Metabolism of the extracellular matrix formed by intervetebral disc cells cultured in alginate. Spine 1997;15:2885-93

24. Alini M, Li W, Markovic P, Aebi M, Spiro RC, Roughley PJ. The potential and limitations of a cell-seeded collagen /hyaluronan scaffold to engineer an intervertebral disc-like matrix. Spine 2003;28:446-54

25. Mizuno H, Roy AK, Vacanti CA, Kojima K, Ueda M, Bonassar LJ. Tissue-engineered composites of annulus fibrosus and nucleus pulposus for intervertebral disc replacement. Spine 2004;29:1290-7

26. Seguin CA, Grynpas MD, Pilliar RM, Waldman SD, Kandel RA. Tissue engineered nucleus pulposus tissue formed on a porous calcium polyphosphate substrate. Spine 2004;29:1299-06

27. 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 transforming growth factor β1 encoding gene. Spine 1999;24:2419-25

28. Grunder T, Gaissmaier C, Fritz J, Stoop R, Hortschansky P, Mollenhauer J, et al. Bone morphogenetic protein (BMP-2) enhances the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads. Osteoarthritis cartilage 2004;12:559-67

29. Moon SH, Gibertson LG, Nishida K, Knaub M, Muzzonigro T, Robbins PD, et al. Human intervertebral disc cells are genetically modifiable by adenovirus-mediated gene transfer. Spine 2000;25:2573-9

ABSTRACT (In Korean)

아데노바이러스를 이용한 유전자치료법과 아텔로콜라겐 지지체를 매개로 한 인간 추간판의 조직공학적 재생

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남 미 란

연 구 계 획: in vitro 연구

연 구 목 적: 아텔로콜라겐 지지체에 유전자가 전달된 인간 추간판 세포를 이식하여 세포의 세포 분열, 표현형 발현, 기질 생성 효과를 알아보기 위함.

연 구 배 경: 퇴행성 추간판 질환의 생물학적 치료를 위하여 새로운 기술로 조직공학이 각광을 받고 있으며, 이는 신체의 조직과 기관의 재생을 가능케 하는 전도유망한 기술로 평가되고 있다. 뿐만 아니라 유전자 전달을 이용한 유전자 치료 기술도

지속적인 생물학적 자극을 줄 수 있어 추간판 조직의 재생에 적합한 기술로 인정을 받고 있다.

대상 및 방법: 인간 추간판 조직에서 추간판 부위를 분리하고 순차적 효소처리에 의해 추간판 세포를 분리, 배양한다. 1%의 제 1 형 아텔로콜라겐을 96-well plate 에 넣고, -70℃에서 냉동시킨 후 다시 -50℃에서 냉동건조 처리를 한다. 이렇게 제작된 다공성의 교원질 물질은 교차 결합 방식에 의해 지지체로 완성된다. 재조합된 아데노바이러스로 인간 추간판 세포를 감염시키고, 지지체의 표면 장력을 이용하여 감염된 추간판 세포들이 아텔로콜라겐 지지체로 이식하여 배양한다. 세포가 이식된 배양한 지 각각 3 일, 7 일 째 되는 날, Alamar Blue Assay 을 이용하여 세포의 분열을 측정하고, [^{35}S]-sulfate incorporation 을 통해서 기질 생성량을 측정한다. 또한 RT-PCR 을 통해 기질성분인 aggrecan, 제 1 형, 2 형 교원질 그리고 골성인자인 osteocalcin 의 mRNA 발현 정도를 알아보고, 주사전자 현미경 관찰을 통해 세포가 이식된 아텔로콜라겐 지지체의 내부 형태를 관찰한다.

결 과: Ad/TGF-β1 로 감염시킨 제 1 형 아텔로콜라겐 지지체 배양군은 세포의 기질 생성이 증가하였지만 기질 성분인 aggrecan, 2 형 교원질의 mRNA 발현은 대조군에 비하여

선명하게 증가하지 않았다. 한편, 골성장인자인 osteocalcin 의 mRNA 의 발현은 모두 유의적인 변화를 나타내지 않았다. 형태학적으로는 Ad/ TGF-β1 군과 Ad/ TGF-β1 와 Ad/BMP-2 를 혼합하여 감염시킨 군에서 세포 외 기질의 발현이 활발히 진행이 됨이 관찰되었다.

결 론: 제 1 형 아텔로콜라겐 지지체에 이식 배양된 인간 추간판 세포는 생존 가능하였고, 세포의 증식과 기질의 생성이 활발하였다. 또한 두 지지체의 배양군들은 Ad/TGF-β1 및 Ad/BMP-2 에 생물학적으로 반응하였다. 따라서 재조합된 아데노바이러스로 감염시킨 인간 추간판 세포를 아텔로콜라겐 지지체에서 배양하여 조직공학적으로 추간판을 재생시킬 수 있다.

핵심되는 말: 유전자 치료, 조직공학, 척추 추간판 세포, 아텔로콜라겐 지지체, Ad/TGF-β1, Ad/BMP-2