Tissue engineering of the intervertebral disc with cultured nucleus pulposus cells using atelocollagen scaffold and growth factors

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Directed by Professor Hak-Sun Kim

The Master's Thesis

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This certifies that the Master's Thesis of Kwang Il Lee is approved.

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The Graduate School

Yonsei University

December 2006

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ABSTRACT

Tissue engineering of the intervertebral disc with cultured nucleus pulposus cells using atelocollagen scaffold and growth factors

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(Directed by Professor Hak-Sun Kim)

Study design: In vitro experimental study.

Objectives: To examine the cellular proliferation, synthetic activity, and phenotypical expression of intervertebral disc (IVD) cells seeded on types I and II atelocollagen scaffolds with the stimulation of TGF-β1 and BMP-2.

Summary of literature review: Recently, tissue engineering is regarded as a new experimental technique for the biological treatment about degenerative IVD diseases and has been highlighted as a promising

technique for the regeneration of tissues and organs in human body. Research on cell transplantation in artificial scaffolds should be validated in terms of cell viability and proliferation, maintenance of characteristic phenotype, and biologically active growth factor.

Materials and Methods: Lumbar IVD were harvested from 10 New Zealand white rabbits, with the nucleus pulposus (NP) cells were isolated by sequential enzymatic digestion. Each of 1% types I and II atelocollagen dispersions were poured into a 96-well plate (diameter 5mm), frozen at -70℃, and then lyophilized at -50℃. Fabricated porous collagen matrices were made using the cross-linking method. Cell suspensions were then treated with TGF-β1 (10ng/ml) or BMP-2 (100ng/ml) or both. After 1, 2, and 4 week culture periods, the DNA synthesis was measured by [^{3}H]-thymidine incorporation and newly synthesized proteoglycan was measured by incorporation of [^{35}S]-sulfate. Reverse transcription-polymerase chain reactions for the mRNA expressions of aggrecan, types I, II collagens, and osteocalcin were performed. The inner morphology of cell seeded scaffolds was determined by scanning electron microscopy (SEM).

Results: The NP cell cultures in atelocollagen type II with TGF-β1 demonstrated increase in proteoglycan synthesis and upregulation of aggrecan, types I and II collagen mRNA expressions, compared to control. IVD cultures in type I atelocollagen scaffold with growth factors exhibited

an increase in DNA synthesis and up regulation of types I, II collagen mRNA expressions. With all combinations of growth factor, the IVD cultures in types I and II atelocollagen scaffolds showed no upregulation of the osteocalcin mRNA expression. Furthermore there was no synergistic effect of TGF-β1 and BMP-2 in matrix synthesis and mRNA expression of matrix components. The SEM images showed stable cell adhesion on each matrix and releasing of extracellular matrices on cell surfaces. **Conclusion:** NP cells from rabbit were viable in types I and type II atelocollagen scaffolds. Type I atelocollagen scaffold was suitable for cell proliferation, but type II atelocollagen scaffold was suitable for extracellular matrix synthesis. The NP cells in both scaffolds were biologically responsive to growth factors. Taken together, NP cells in atelocollagen scaffolds, with anabolic growth factors provide a mechanism for tissue engineering of IVD.

Key words: intervertebral disc, atelocollagen scaffold, TGF-β1, BMP-2, tissue engineering

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Ⅰ. INTRODUCTION

Intervertebral disc (IVD) degeneration is caused by loss of water content in nucleus pulposus (NP), resulting from decreased proteoglycan and type II collagen in IVD.[1-7] Degeneration of IVD results in several spinal diseases which are internal disc derangement, hernia of IVD, lumboscral radiculopathy, and spinal canal stenosis moreover increases health care costs. Nevertheless there is only a halfway cure which is disc excision or spinal fusion without regenerating disc itself.

Recently, tissue engineering emerges as new alternatives for biological

treatment of degenerative IVD diseases and is regarded as a promising technique to regenerate tissues and organs in human body.[8-9] Researches on cell transplantation in artificial scaffolds has provided valuable information in optimal condition for tissue engineering.[10-13] In this way, IVD cell transplantation in artificial scaffold have to be validated in terms of cell viability, proliferation, maintenance of characteristic phenotype, and stimulatory effect of biologically active growth factor.

To regenerate articular cartilage and IVD, growth factors i.e., transforming growth factor- β1 (TGF- β1) and Bone morphogenetic protein-2 (BMP-2) proved to be effective in proteoglycan synthesis, collagen synthesis, and cell proliferation.[14-17] Moreover annulus fibrosus cell seeded on atelocollagen scaffold demonstrated increased proteoglycan synthesis compared to cells in monolayered culture.[18-21]

The objective of the current experimental study was to elucidate biologic effect of atelocollagen and growth factors in matrix synthesis and phenotypical expression of IVD cells. Therefore, rabbit NP cells were transplanted in each of types I and II atelocollagen scaffolds which were made by removal of telopeptide in insoluble collagen and stimulated by TGF- β1 and BMP-2.

In this study, rabbit NP cells were transplanted in each of types I and II atelocollagen scaffolds which were made by removal of telopeptide in

insoluble collagen and conditionally stimulated by TGF-β1 and BMP-2. With this experimental design, we investigated NP cell proliferation, newly synthesized proteoglycan, and expression of chondrogenic phenotype.

Ⅱ. MATERIALS AND METHODS

All experimental protocols were approved by Institutional Review Board and Animal Experimentation Committee of the institution.

1. Materials

IVD form lumbar spines were obtained from 30 four-week-old female New Zealand white rabbits weighing about 3.5kg. Euthanasia was induced using ketamine HCl 50mg/ml. The rabbits were placed supine and the abdominal region of each animal was shaved, prepared, and draped in a sterile fashion. The discs in the lumbar region were exposed via a transperitoneal approach. The spinal column was dissected from the surrounding muscles under sterile conditions. The spine was sectioned between each of the lumbar discs form L1 to L7. The muscles and tendons were removed, and the column was sectioned transversally in the middle of each disc. The NP was removed from both halves of each disc with blunt forceps and pooled.

2. Intervertebral disc cell culture

NP was shredded with scissors and digested in Ham's F-12 medium (F-12, Gibco-BRL, Grand Island, NY) containing 1% (v/v) penicillin, streptomycin, nystatin (all antibiotics from Gibco-BRL, Grand Island, NY), 0.4% (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, 0.025% (w/v) collagenase type II, 0.004% (w/v) DNase (Sigma, St. Louis, MO, USA) for 3 hours under the same conditions. The digested tissue was passed through a sterile cell strainer (Falcon, Franklin Lakes, NJ) with a pore size of 100um. The filtrated was centrifuged at 1,500 rpm for 5 minutes to separate the cells.

The resulting cell suspensioins were placed in 6-well plates at 1×10^6 cells per well and grown in 3ml Dulbecco's modified eagle medium and Hams F-12 medium (DMEM/F-12, Gibco-BRL, Grand Island, NY) supplemented with 10% heat activated fetal bovine serum (FBS, Gibco-BRL, Grand Island, NY), 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 atelocollagen scaffolds

Each 56ul of 1% type I and type II atelocollagen (RBC I, Regenmed, 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℃.[22-23]

4. Preparation of BMP-2

After gene recombination of human BMP-2 by the pcDNA3.1/hygro expression vector, recombinant pcDNA3.1/hygro/BMP-2 gene was transfected to CHO (Chinese hamster ovary) cells by lipofectamine (Gibco-BRL, Grand Island, NY).[24] The transfected CHO cells were grown in DMEM/F-12, supplemented with 10% FBS. When the cells were 80-100%

confluent, the medium was replaced with serum-free DMEM/F-12 and medium was harvested every 24 hour for 4 days. Thirty-seven liters of conditioned medium was directly applied to an 80ml heparin sepharose (Pharmacia Biotech, NJ, USA) column. The resin was washed with 0.15M NaCl / 6M urea / 20mM Tris, pH 7.4, and then developed with a linear gradient to 1M NaCl / 6M urea / 50mM Tris, pH 7.4. The fractions with highest specific activity were pooled and concentrated by ultrafiltration with a YM10 membrane (Millipore Corp, MA, USA). Protein concentration was determined by amino acid analysis and aliquots were stored at -70℃.

5. Cell transplantation to atelocollagen scaffolds

Atelocollagen scaffolds were soaked in 70% EtOH for overnight. After washing two times with DPBS, they were soaked in culture media. Before transplanting the cell, the culture medium was aspirated perfectly. Cell suspensions were imbibed by surface tension into each scaffold consisting of atelocollagen type I and type II. 5×10^5 cells per 96-well in 30ul of DMEM/F-12 containing 10% FBS, 25ug/ml ascorbic acid and 1% antibiotics were seeded in each matrix, followed by gentle centrifugation of the entire plate at 1,000 rpm for 3 minutes, which had revealed optimal penetration of the cells into the pores at the air-side of the matrix. The cultures were

incubated at 37℃, 5% CO_2.

6. Stimulation of growth factors

After incubation in 37℃, 5% CO_2 atmosphere for 4 hours, the each culture medium was added with 5% FBS including TGF-β1 of 10ng/ml, BMP-2 of 100ng/ml and the mixture of both factors in the ratio of 1:1. Mixed medium was changed every other day for two weeks. The control group was only cell seeded type I and type II atelocollagen scaffolds without any growth factors.

7. Cell morphology

The morphology of the rabbit NP cells seeded in atelocollagen scaffolds was examined using light microscopy (Microscope digital camera, Olympus DP-12, Seoul, Korea) at each evaluation time point.

8. Cellular proliferation

DNA synthesis was measured by the [^{3}H]-thymidine incorporation. 5u Ci/ml of [^{3}H]-thymidine (Amersham Biosciences, Uppsala, Sweden;

25Ci/mmol specific activity) was added to control and treated cultures for 24h. The medium was then discarded and the cells were trypsinized with trypsin/EDTA. The trypsinized cells were filtered onto glass fiber filters (Whatman GF/C; Maidstone, England), and transferred to scintillation vial. Filters were dried and counted in 3ml of scintillation cocktail solution (Beckman Coulter Inc. USA) in a Packard scintillation counter (Packard #1900 TR, Mariden, CT). The results of each experiment, expressed as cpm/well, are the means of three parallel cultures.

9. 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. USA). Five fractions were collected per sample, and three meddle fractions were counted in a Packard liquid scintillation counter (Packard #1900 TR, Mariden, CT)

10. 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. 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. The same reaction profile was used for all primer sets: an initial denaturation at 94℃ for 1 minute, followed by 25~40 cycles of: 94℃ for 5 seconds; 47~50℃ for 5 seconds; and 72℃ for 30 seconds; and an additional 2 min extension step at 72℃ after the last cycle. 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 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).

11. Scanning electron microscopy (SEM)

Acellular and cellular scaffolds were observed at the 2-week 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.

12. 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, [^{3}H]-thymidine labeled DNA, and [^{35}S]-sulfate labeled proteoglycan. Significance level was set as $p<0.05$.

TABLE 1. Sequences of the RT-PCR primers used

Rabbit Primer	Sequence	Length	Size(bp)
β-actin	5'-GCC ATC CTG CGT CTG GAC CT-3'	20	227
	5'-GTG ATG ACC TGG CCG TCG GG-3'	20	
Aggrecan	5'-AGG TGT TGT GTT CCA CTA TC-3'	20	605
	5'-CTT CGC CTG TGT AGC AGA TG-3'	20	
Collagen type Ⅰ	5'-AGA AGG AGT AAC CTC CAA GG-3'	20	321
	5'-ATG ACC AAA GGT GCA ATA TC-3'	20	
Collagen type Ⅱ	5'-GCA CCC ATG GAC ATT GGA GG-3'	20	367
	5'-GAC ACG GAG TAG CAC CAT CG-3'	20	
Osteocalcin	5'-AAG AGA TCA TGA GGA GCC TG-3'	20	420
	5'-AGG AAA CAA GCA CTG TGC AT-3'	20	

TABLE 2. RT-PCR conditions

Rabbit Primer	Conditions			Cycle
	Denaturation	Annealing	Polymerization	
β-actin	94℃ 5 sec	58℃ 5 sec	72℃ 30 sec	30
Aggrecan	94℃ 30 sec	50℃ 30 sec	72℃ 90 sec	35
Collagen type Ⅰ	94℃ 5 sec	50℃ 5 sec	72℃ 30 sec	30
Collagen type Ⅱ	94℃ 5 sec	46℃ 5 sec	72℃ 30 sec	30
Osteocalcin	94℃ 5 sec	46℃ 5 sec	72℃ 30 sec	30

Ⅲ. RESULTS

1. Morphology of nucleus pulposus cells in atelocollagen scaffolds

In the cell-seeded constructs, the nucleus pulposus cells showed a spherical appearance, making small colonies, as are often seen in three-dimensional culture of human NP cells. However, there were differences between constructs in the number of colonies and amount of extracellular matrix (ECM). Cells cultured in atelocollagen type II scaffold showed abundant ECM-producing colonies. On the other hand, in the atelocollagen type I scaffold, most of the cells were spherical but with reduced colony formation. (Figure. 1)

A **B**

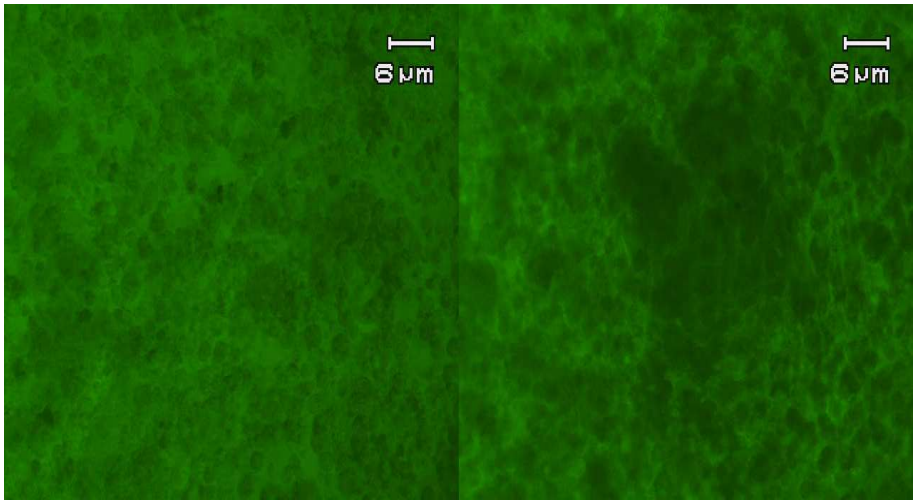
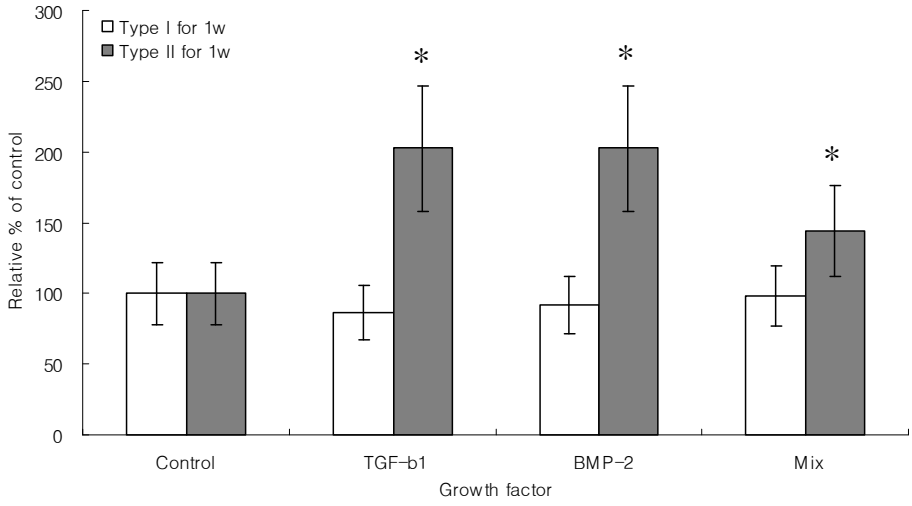


Fig. 1A-B. Rabbit nucleus pulposus cells cultured for 7 days in atelocollagen scaffolds. (A) The cells in atelocollagen type I scaffold, (B) those in atelocollagen type II scaffold. The cells express spherical appearance with colonization most frequently seen in atelocollagen type II scaffold. Bar = 6um.

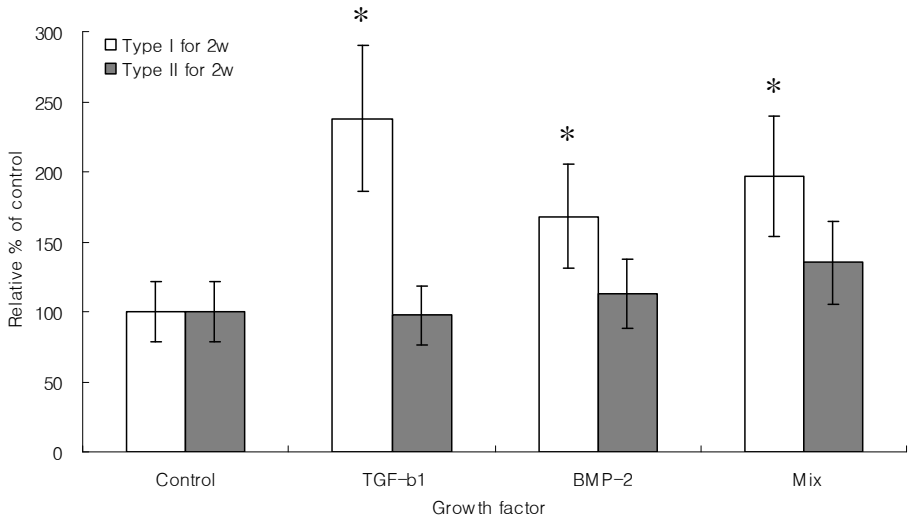
2. DNA synthesis

Rabbit NP cell cultures in atelocollagen type II scaffold with each TGF-β1, BMP-2, and both combination increased in DNA synthesis on 1 week culture compared to control and type I scaffold group however DNA synthesis of same condition in atelocollagen type I scaffold increased on 2, 4 weeks culture compared with control and type II scaffold group. (Figure. 2)

A



B


Type I for 2w
Type II for 2w
Relative % of control
300
250
200
150
100
50
0
Control
TGF-b1
BMP-2
Mix
Growth factor
*
*
*

C

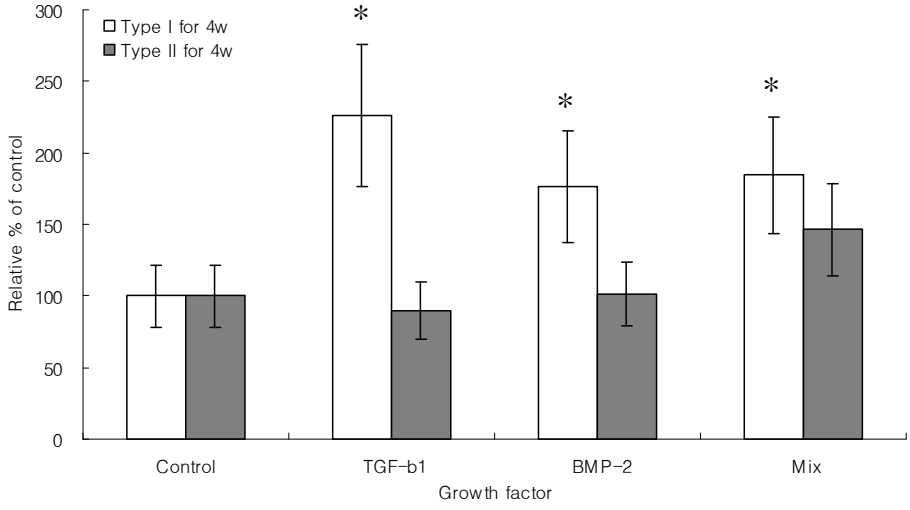
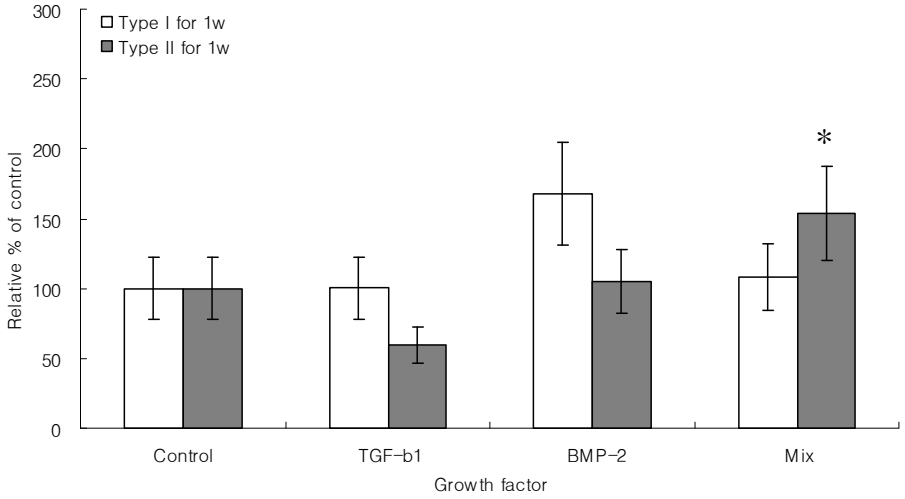

Type I for 4w
Type II for 4w
Relative % of control
300
250
200
150
100
50
0
Control
TGF-b1
BMP-2
Mix
Growth factor
*
*
*

Fig. 2A-C. DNA synthesis of rabbit nucleus pulposus cells seeded on atelocollagen scaffolds (*p<0.05). The rabbit NP cells were seeded on types I and II atelocollagen scaffolds. Percent control of DNA synthesis was measured by [^{3}H]-thymidine incorporation (CPM). Control; the cultures without growth factor stimulation, TGF-β1; with TGF-β1 of 10ng/ml, BMP-2; with BMP-2 of 100ng/ml, Mix; with mixture of TGF-β1 and BMP-2 in the ratio of 1:1. The culture period was each 1, 2, 4 week.

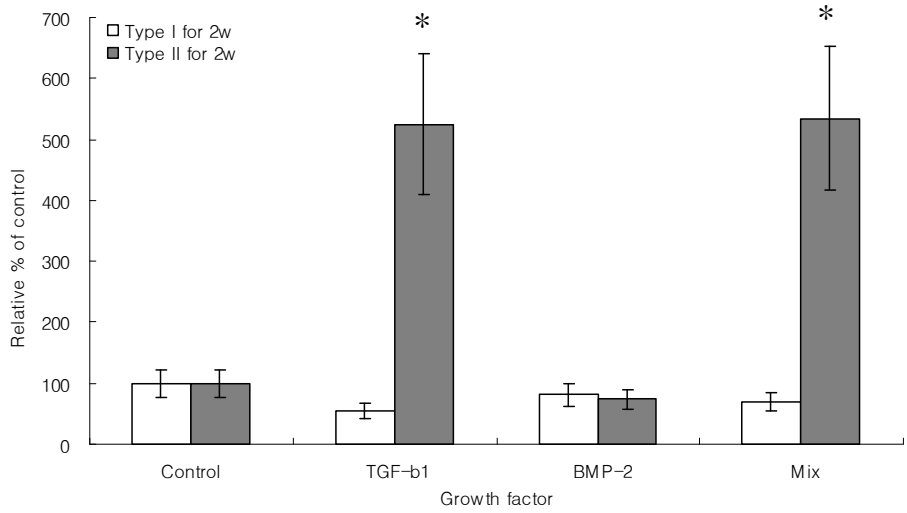
3. Newly synthesized proteoglycan normalized by DNA synthesis

Rabbit NP cell cultures in atelocollagen type II scaffold with each TGF-β1 and mixture increased in proteoglycan synthesis on 2, and 4 week culture compared with control and type I scaffold group. However rabbit NP cell cultures in atelocollagen type II scaffold with each TGF- β1 and mixture demonstrated no difference in proteoglycan synthesis at 1 week. NP cell culture in atelocollagen type I scaffold with each growth factor stimulation showed decrease in proteoglycan synthesis at 4 week comparing with control group. (Figure 3)

A


Type I for 1w
Type II for 1w
Relative % of control
300
250
200
150
100
50
0
*
Control
TGF-b1
BMP-2
Mix
Growth factor

B


Type I for 2w
Type II for 2w
Relative % of control
700
600
500
400
300
200
100
0
*
*
Control
TGF-b1
BMP-2
Mix
Growth factor

C

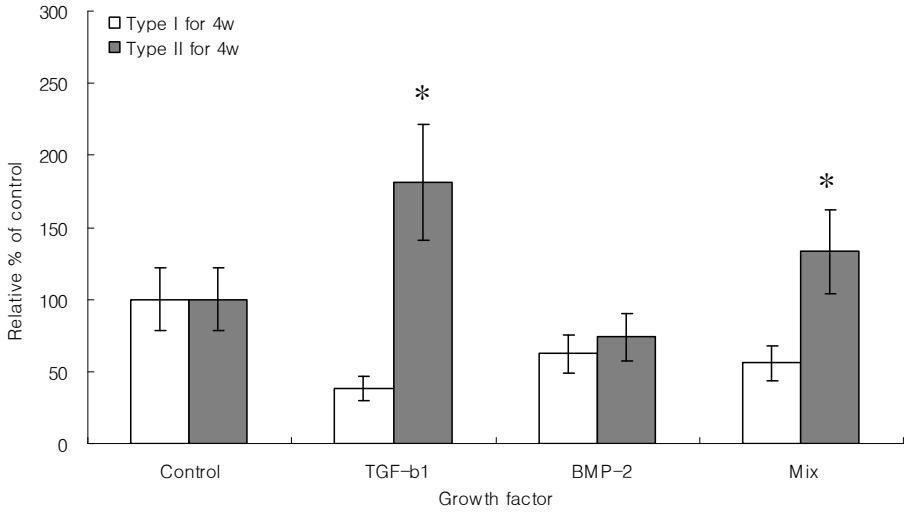


Fig. 3A-C. Newly synthesized proteoglycan of rabbit nucleus pulposus cells seeded on atelocollagen scaffolds (*p<0.05). The rabbit NP cells were seeded on types I and II atelocollagen scaffolds. Percent control of proteoglycan synthesis was measured by [^{35}S]-sulfate incorporation (CPM). Control; the cultures without growth factor stimulation, TGF-β1; with TGF-β1 of 10ng/ml, BMP-2; with BMP-2 of 100ng/ml, Mix; with mixture of TGF-b1 and BMP-2 in the ratio of 1:1. The culture period was each 1, 2, 4 week.

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

In densitometry assay of reverse transcription-polymerase chain reaction, Rabbit NP cell cultures in atelocollagen type I scaffold with TGF-β1 and the mixture of TGF-β1 and BMP-2 showed statistically significant upregulation of collagen type I, aggrecan and collagen type II mRNA expression, compared with control. The cultures in atelocollagen type II scaffold with TGF- β1 and the mixture showed significant upregulation of aggrecan, collagen type I, and II mRNA expression, compared with control and culture groups of atelocollagen type I scaffold. In any combination of growth factor, NP cultures in atelocollagen type I and type II did not show upregulation of osteocalcin mRNA expression. Furthermore there was no synergistic effect of TGF-β1 and BMP-2 in mRNA expression of matrix components. (Figure 4,5)

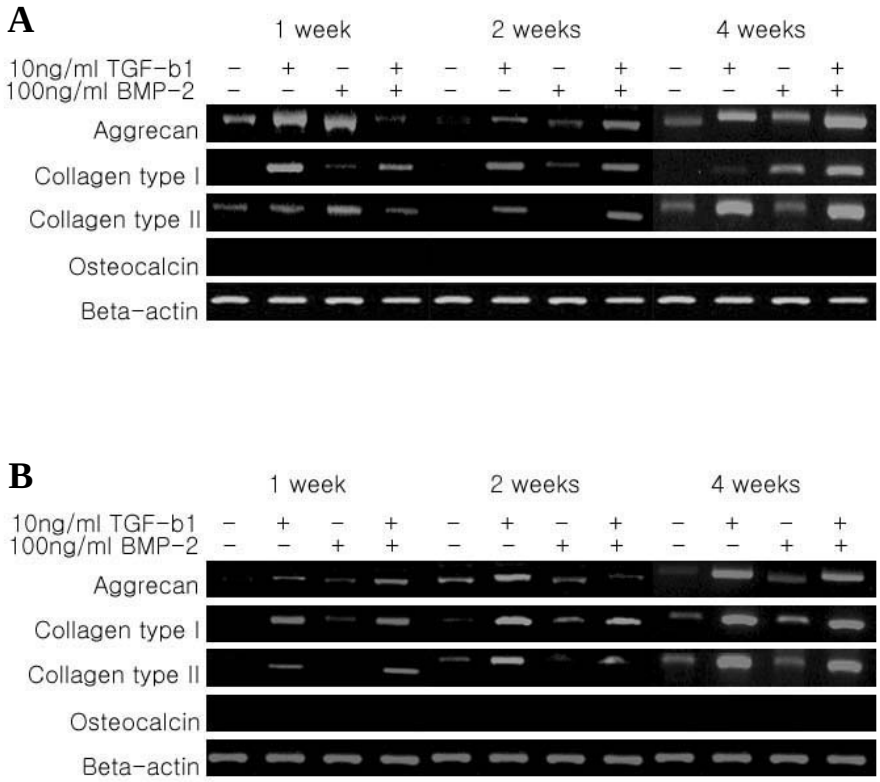
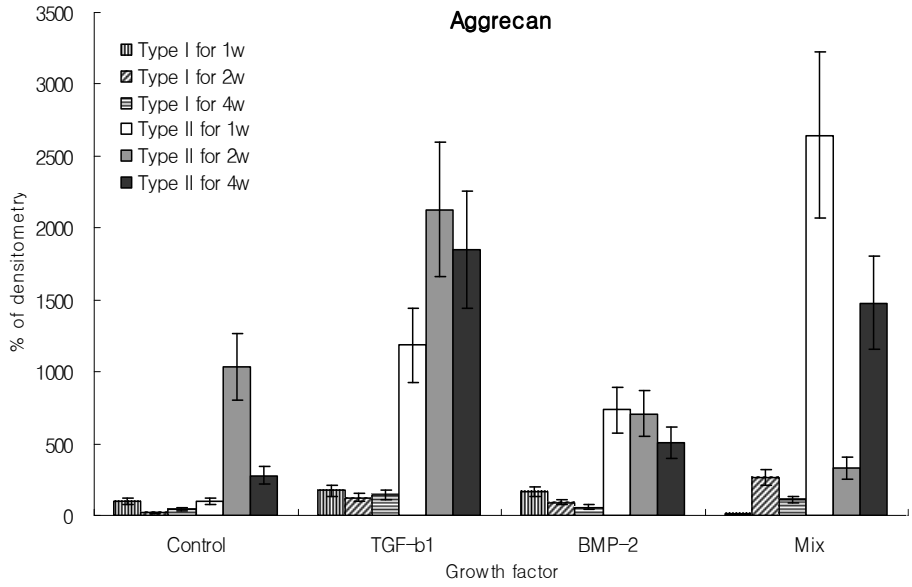
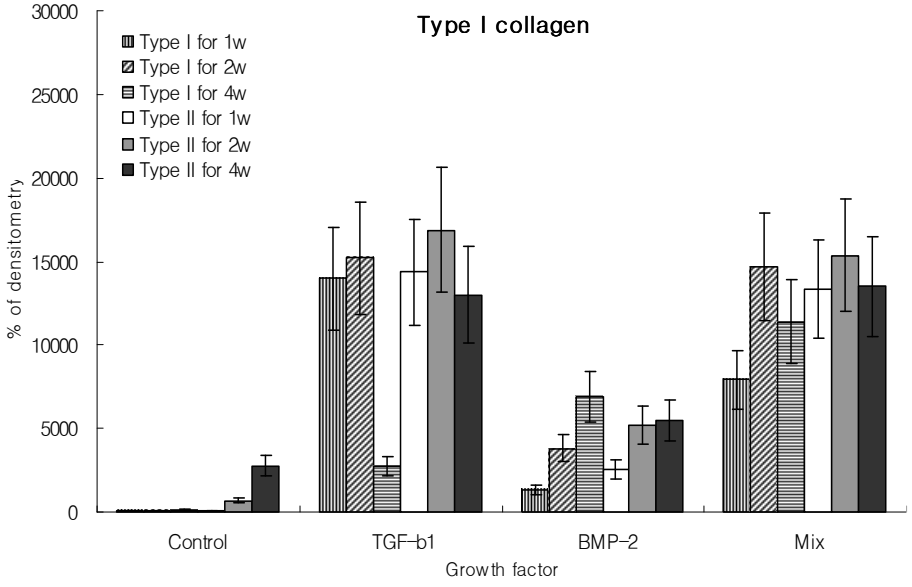


Fig. 4A-B. RT-PCR of beta-actin, aggrecan, collagen type I, II, and osteocalcin. Total RNA was isolated from cells and subjected to RT-PCR. The PCR products were separated on 2% agarose gels containing ethidium bromide, and then observed on an ultraviolet transilluminator. A; The PCR products of NP cultures on atelocollagen type I scaffold with each stimulation of growth factors for 1, 2, 4 weeks. B; Those of atelocollagen type II scaffold.

A


Aggrecan
Type I for 1w
Type I for 2w
Type I for 4w
Type II for 1w
Type II for 2w
Type II for 4w
% of densitometry
3500
3000
2500
2000
1500
1000
500
0
Control
TGF-b1
BMP-2
Mix
Growth factor

B


Type I collagen
Type I for 1w
Type I for 2w
Type I for 4w
Type II for 1w
Type II for 2w
Type II for 4w
% of densitometry
30000
25000
20000
15000
10000
5000
0
Control
TGF-b1
BMP-2
Mix
Growth factor

C

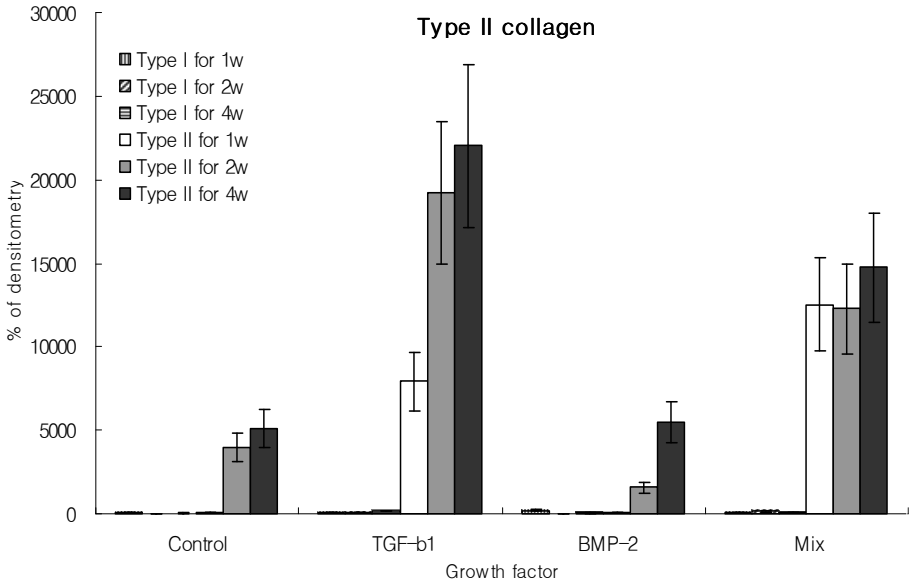


Fig. 5A-C. Densitometry of A; aggrecan, B; collagen type I, and C; collagen type II mRNA expression in atelocollagen scaffolds. The expression of each PCR band was quantified using an image analyzer. The results are presented as the percentage of the mRNA level relative to beta-actin for each band.

5. Scanning electron microscopy (SEM)

The SEM images of rabbit NP cells of porous atelocollagen type I and II scaffolds are shown in figure 6, 7. When seeded on both types of atelocollagen scaffold, the cells showed stable adhesion on each matrix and releasing of extra cellular matrices on cell surfaces. Moreover rabbit NP cells growing on atelocollagen scaffolds had proper cell-cell contact with neighboring NP cells. From the SEM pictures, it is concluded that atelocollagen type II scaffolds had a higher proteoglycan synthesis when compared to the atelocollagen type I scaffolds

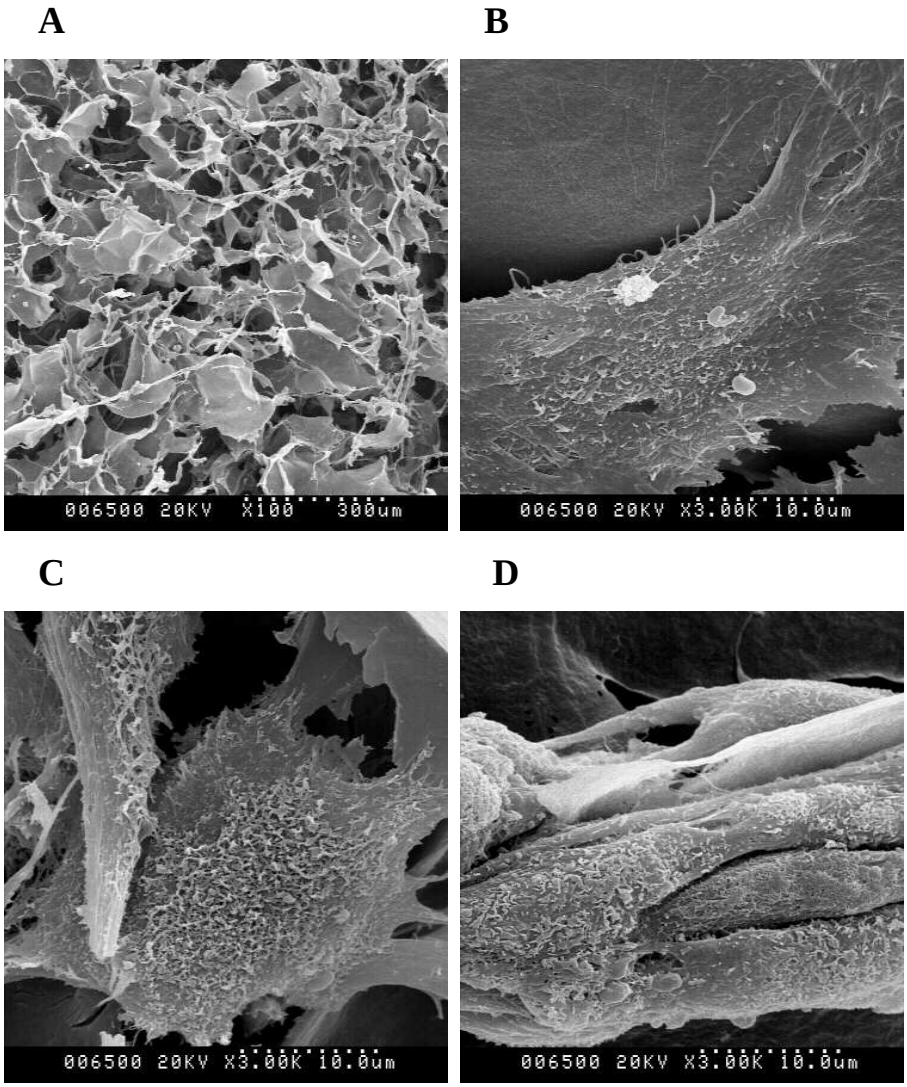


Fig. 6A-D. Morphology of porous atelocollagen type I scaffold on scanning electron microscopy (SEM). (A) The inside of type I scaffold (×100), (B) Rabbit NP seeded type I scaffold on 1 week-culture day (×3,000), (C) on 2 week-culture day (×3,000), (D) on 4 week-culture day (×3,000). All data were the culture groups with stimulation of mixed growth factors.

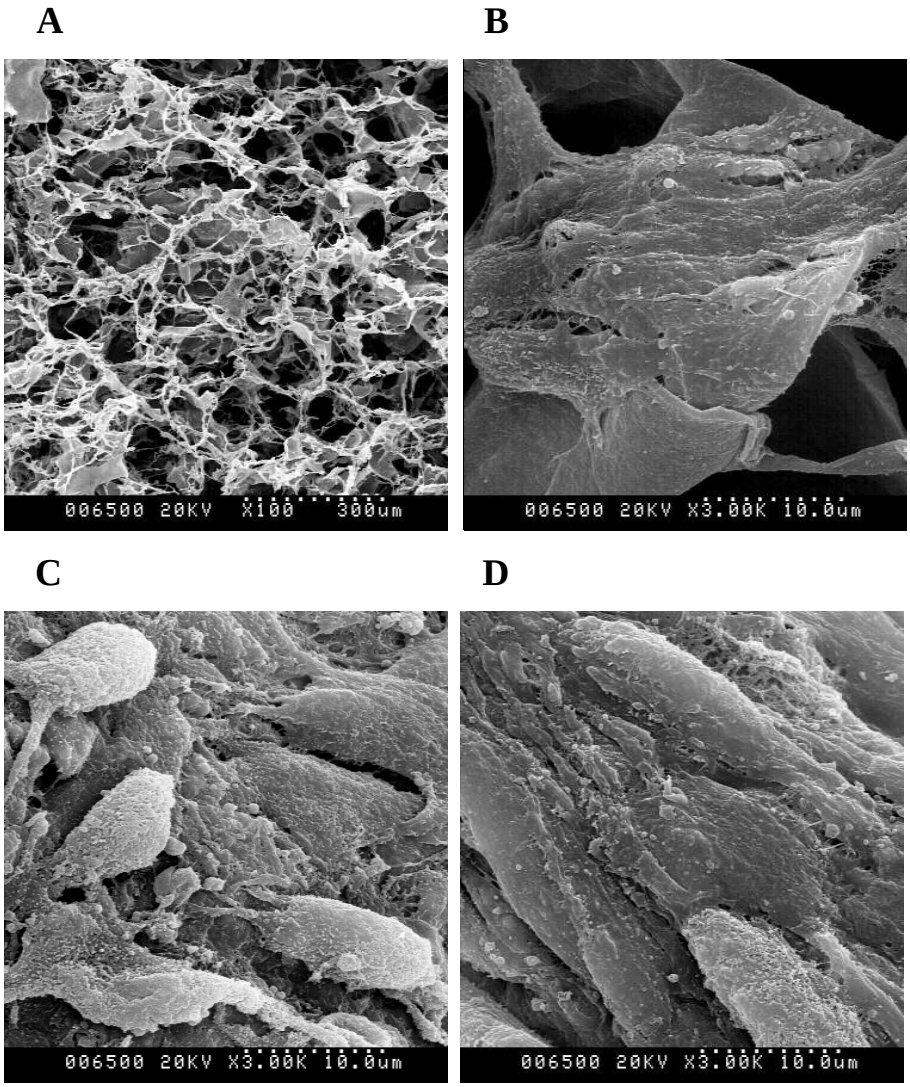


Fig. 7A-D. Morphology of porous atelocollagen type II scaffold on scanning electron microscopy (SEM). (A) The inside of type II scaffold (×100), (B) Rabbit NP seeded type II scaffold on 1 week-culture day (×3,000), (C) on 2 week-culture day (×3,000), (D) on 4 week-culture day (×3,000). All data were the culture groups with stimulation of mixed growth factors.

Ⅳ. DISCUSSION

Atelocollagen known as fibrous protein is lack of telopeptide, antigenic component so it prevents autoimmune response with cell transplantation.[25-26] Moreover atelocollagen is suitable for gene therapy cause of slow releasing of DNA.[27] On the other hand, TGF-β1 and BMP-2 is well known growth factors in accelerating the synthesis of proteoglycan in IVD. These scaffold and growth factors can provide a mechanism for IVD regeneration in terms of tissue engineering.[28-30]

In this experimental study, rabbit NP cells were seeded on atelocollagen scaffolds with growth factors. NP cellular proliferation was active in atelocollagen type I scaffold and more stimulated with TGF-β1. On the other hand, NP cells in atelocollagen type II scaffold demonstrated increased proteolgycan synthesis compared to those of atelocollagen type I. TGF-β1 also stimulated proteoglycan synthesis in NP cells seeded atelocollagen type II scaffold. NP cell cultures in atelocollagen type I and II demonstrated the upregulation of matrix component mRNA expression i.e., aggrecan, types I and II collagen mRNA. However cultures with each growth factor and combination of two growth factors did not demonstrate

osteocalcin mRNA expression.

These experimental results support the fact that that the atelocollagen type I and II scaffolds with growth factors are suitable for IVD regeneration. Therefore the following study in the future will be necessary to study about the mixture of atelocollagen type I and II matrices and in vivo tests with rabbit animals for stability and excellent effect on human.

Ⅴ. CONCLUSION

Atelocollagen type I scaffold was suitable for cell proliferation and type II scaffold was more suitable for extracellular matrix synthesis than type I atelocollagen. IVD cells in both scaffolds were biologically responsive to growth factors. Taken together, nucleus pulposus cells in atelocollagen scaffolds with anabolic growth factors provide a mechanism for tissue engineering of IVD.

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ABSTRACT (In Korean)

아텔로 콜라겐 지지체와 성장 인자로 배양된 수핵 세포를 이용한 조직 공학적 추간판의 재생

< 지도교수 ; 김 학 선 >

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이 광 일

연구계획 : 실험자 연구

연구목적 : 제 1 형, 2 형 아텔로콜라겐 지지체에 TGF-β1, BMP-2 를 투여하여 이식된 추간판 세포의 세포 증식, 기질 생성, 표현형 발현을 알아보기 위함.

연구배경 : 최근, 조직공학은 퇴행성 추간판 질환의 생물학적 치료를 위한 미래의 새로운 연구 기술로 언급되고 있으며, 이는 신체의 조직과 기관의 재생을 가능케 하는 전도유망한 기술로 각광받고 있다. 인공 지지체에 세포를 이식하는 연구는 세포의 생존 및 증식, 재생되려는 조직의 고유한 표현형 유지, 그리고 성장인자와 같은 생물학적인 자극 등의 조건들이 모두 조화를 이루어야만 한다.

대상 및 방법 : 뉴질랜드 흰 토끼 30 마리로부터 추간판 조직내의 수핵 부위를 분리하고 순차적 효소처리에 의해 수핵 세포를 배양한다. 각각 1%의 제 1 형, 2 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. 표면 장력을 이용하여 추간판 세포들이 아텔로콜라겐 지지체로 이식되고, 세포가 이식된 지지체에는 각각 10ng/ml 의 TGF-β1, 100ng/ml 의 BMP-2, 그리고 두 성장인자들이 1:1 로 혼합된 용액을 첨가한다. 배양한 지 각각 1, 2, 4 주 째 되는 날, [^{3}H]-thymidine incorporation 을 통해 DNA 의 양을 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, 주사 전자 현미경 관찰을 통해 세포가 이식된 아텔로콜라겐 지지체의 내부 형태를 관찰한다.

결과 : TGF-β1 이 투여된 제 2 형 아텔로콜라겐 지지체 배양군은 세포의 기질 생성이 증가하였고, 기질 성분인 aggrecan, 제 1 형, 2 형 교원질의 mRNA 발현도 대조군에 비해 유의하게 증가하였다. 한편, 제 1 형 아텔로콜라겐 지지체 배양군은 세포의 증식이 매우 활발하였으며 제 1 형, 2 형 교원질의 mRNA 발현도 증가하는 양상을 보였다. 그렇지만 어떤 지지체의 배양군에서도 추간판

연골 세포의 골성인자인 osteocalcin 의 mRNA 발현은 나타나지 않았으며, 기질 생성과 기질 성분의 mRNA 발현에 있어서 TGF-β1 과 BMP-2 간의 상호 상승작용은 나타나지 않았다. 한편 주사전자현미경 상으로 이식된 세포가 각각의 지지체에 안정적으로 부착된 상태이면서 세포 표면에 기질이 방출된 것을 확인하였다.

결론 : 제 1 형, 2 형 아텔로콜라겐 지지체에 이식 배양된 토끼의 추간판 수핵 세포는 생존 가능하였고, 제 1 형 아텔로콜라겐 지지체는 추간판 세포의 증식이 활발하였으며, 제 2 형 아텔로콜라겐 지지체는 세포외 기질 생성이 제 1 형 아텔로콜라겐 지지체에 비해 탁월하였다. 또한 두 지지체의 배양군들은 TGF-β1 및 BMP-2 성장인자에 생물학적으로 반응하였다. 따라서 성장인자가 처리된 아텔로콜라겐 지지체에서 자란 추간판 세포는 조직공학적 추간판의 재생에 적합하였다.

핵심되는 말 : 척추 추간판 세포, 아텔로콜라겐 지지체, TGF-β1, BMP-2, 조직공학