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관절연골 재생 치료에서 줄기세포의 생착 및 분화능 향상을 위한 트랜스글루타미나제-4 의 이용 검증

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김 종 근

A thesis of the Degree of Doctor of Philosophy

Enhancement of the stem cell engraftment and differentiation for cartilage regeneration using transglutaminase−4

August, 2021

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김종근의 의학박사 학위논문을 인준함.

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Enhancement of the stem cell engraftment and differentiation for cartilage regeneration using transglutaminase-4

by

Jong-Keun Kim, M.D.

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medicine (Orthopedic Surgery) at Seoul National University College of Medicine

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Abstract

Enhancement of the stem cell engraftment and differentiation for cartilage regeneration using transglutaminase−4

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Medicine (Orthopedic Surgery)

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Purpose: Although mesenchymal stem cells (MSCs) transplantation with a hydrogel type scaffold is a promising strategy for repairing damaged articular cartilage, MSC-based cartilage tissue engineering has numerous limitations, including poor implanted cell engraftment, phenotypic alteration, soft mechanical properties, in vivo degradation and loss of strength. Transglutaminase (TG) has been used to crosslink proteins to generate biocompatible tissue scaffolds in cell engineering. However, there are no published reports regarding the use of TG in improving the engraftment and differentiation of stem cell. This study aimed to investigate the efficacy of transplantation of synovium-derived mesenchymal stem cells (SDSCs) encapsulated in a hyaluronic acid/ collagen/ fibrinogen (HA/COL/FG) composite gel by supplementing recombinant human transglutaminase-4 (rhTG-4) in treating osteochondral defects.

Materials and Methods: Human SDSCs were prepared from the synovial tissues of knee from human donors who underwent total knee arthroplasty. HA/COL/FG composite gel was generated with a mixture ratio of HA: COL: FG = 0.5: 0.5: 2.0. RhTG−4 was prepared using baculovirus expression system. The adhesion assay was carried out by directly counting the number of attached cells to the fibronectin coating plate at different concentrations of rhTG−4. The distribution of adherent cells was analyzed by crystal violet staining. To visualize cell adhesion and cytoskeletal proteins, immunofluorescence assay was performed and cystamine was supplemented to inhibit the rhTG-4 activity. SDSCs cultured in the HA/COL/FG composite gel were assayed for cell proliferation and viability at different rhTG−4 concentrations. The proliferation assay was carried out by direct counting the number of cells. The Live/Dead Viability kit was used for the assessment of the viability. To evaluate the extra cellular matrix (ECM) hardness, the cells were incubated to generate micro mass, according to the concentrations of rhTG−4 and the hardness was evaluated using a nanoindentation instrument. RhTG−4 activity in the hydrogel was estimated by measuring incorporation of biotinylated pentylamine to N, N'−dimethylcasein.

In vitro chondrogenesis of SDSCs encapsulated HA/COL/FG composite gel supplementing rhTG-4 was verified. The expression of chondrogenesis-related genes (type I COL, type II COL type X COL, aggrecan, and SOX-9) was analyzed using reverse transcription- polymerase chain reaction (RT-PCR). To investigate the effect of integrin β1 on the TG-4-induced actin remodeling, the intracellular signal transduction pathway and the control of

chondrogensis-related genes expression, integrin β1 siRNA was transfected to inhibit integrin β1 expression. To evaluate the in vivo cartilage regeneration, fifty-six knee joints of rabbit osteochondral defect models were made and divided into 3 groups: control group, in which the defect was left untreated; Gel/SDSC group, in which the defect was filled with the HA/COL/FG composite gel loaded with human SDSCs; Gel/SDSC+TG−4 composite gels group, in which the defect was filled with the supplement of rhTG−4 in HA/COL/FG composite gels loaded with human SDSCs. After 12 weeks, tissue specimens were assessed by macroscopic and histological evaluation, and DiI−labeled SDSCs distribution in the center of defect was evaluated.

Results: RhTG-4 treatment improved the attachment of SDSCs with increased adhesion to fibronectin and in a concentration-dependent manner. The immunofluorescence assay revealed that pretreatment with cystamine markedly downregulated rhTG-4-induced integrin β1 activation and dynamic actin remodeling, demonstrating the possibility of modulating the phenotype. SDSCs proliferation was significantly increased in rhTG-4 group. Nanoindentation analysis revealed that rhTG-4 stimulation increased the hardness of 3D micro masses. Activity of rhTG-4 in hydrogel was maintained for 2 h.

In the group supplemented with rhTG-4, the relative expression levels of type II COL and aggrecan mRNA were significantly increased as determined by RT-PCR. Immunofluorescence analysis revealed that pretreatment with integrin β1 siRNA markedly decreased rhTG4-induced actin remodeling.

Intracellular signal transduction pathway and chondrogenesis-related genes including aggrecan and type II collagen were activated in an integrin β1 siRNA-dependent manner. In vivo study, Gel/SDSC+TG-4 group showed more repaired defect lesion compared to the other groups and yielded reconstructed tissue resembling native hyaline cartilage. The total macroscopic and histological scores were significantly higher and DiI-labeled SDSCs distribution was markedly increased at the center of defect in Gel/SDSC+TG-4 group.

Conclusion: RhTG−4 supplementation in the HA/COL/FG composite gel mediates cartilage regeneration by enhancing the engraftment and differentiation of SDSCs.

Keywords: transglutaminase−4, synovium−derived mesenchymal stem cells, engraftment, differentiation, cartilage regeneration, hydrogel

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List of Abbreviations and Symbols

MSCs	mesenchymal stem cells
TG	transglutaminase
Rh	recombinant human
SDSC	synovium−derived mesenchymal stem cell
HA	hyaluronic acid
COL	collagen
FG	fibrinogen
ECM	extra cellular matrix
BP	biotinylated pentylamine
RT	reverse transcription
ERK	extracellular signal-regulated kinase
JNK	c-Jun N-terminal Kinase
H&E	hematoxylin and eosin

Introduction

Tissue-engineered cartilage using mesenchymal stem cells (MSCs) has shown multiple differentiation potentials without dedifferentiation, easy availability, and rapid proliferation, rendering them a promising strategy for cartilage regeneration (1-3). Recently, injectable hydrogel-type scaffolds are effective for chondrogenesis of MSCs because these scaffolds have mechanical properties similar to those of articular cartilage components with the nutrition support (4-6). Although transplantation of MSCs encapsulated in hydrogel-type scaffolds is a promising strategy for the repair of damaged articular cartilage, hydrogel-type scaffold-based cartilage tissue engineering has numerous limitations including poor implanted cell engraftment, phenotypic alteration, overly soft mechanical properties, in vivo degradation and loss of strength in a high temperature or moisture environment (5, 7-9).

Transglutaminases (TGs) are calcium-dependent enzymes catalyzing bond formation between glutamyl and lysyl residues of proteins (10-13).TGs can be used to enhance tensility and enzymatic resistance of collagen-based material. Purified TGs have been used to crosslink proteins to generate biocompatible and injectable tissue scaffolds (8, 14-16). TGs are considered as natural substitutes for chemical agents widely used to produce scaffold materials, including formaldehyde or glutaraldehyde, which generate toxic byproducts (8). Among the TGs identified in humans, TG-2 has been well studied and implicated in various physiological phenomena including cell adhesion, differentiation, cell growth, and apoptosis in both intracellular and extracellular forms. However, intracellular TG-2 in the chondrocytes presents in the nucleus and cytoplasm and induces degeneration mediated by reactive

oxygen species and the cell hypertrophy (17, 18). Therefore, the risk of the occurrence of negative side effects on the MSCs induced by exogenous TG-2 should be considered. It was reported that transfection of TG-2 into chondrocytes promoted type X COL expression and matrix calcification consistent with cell hypertrophy (19). Compared to the TG-2, TG-4 is located in the extracellular area and plays a role as secretory protein (12, 13). Recent studies have indicated that overexpression of TG-4 increases the adhesion and migration showing the effective crosslinking ability (20, 21). Moreover, recent reports suggested TG-4 could induce intracellular signal transduction pathway such as the phosphorylation of FAK and paxillin, enhancing the cell-matrix adhesion (21) TG-4 interacted with integrin and initiates phenotype alteration. Considering these potential superiority of TG-4 over other TGs in terms of the cell adhesion and safety to the chondrocyte, the applications of TG-4 to stem cell treatment for cartilage injury or degeneration could be promising. However, there are no published reports regarding the efficacy of TG-4 in improving stem cell chondrogenesis.

We previously developed an injectable hyaluronic acid/ collagen/ fibrinogen (HA/COL/FG) composite gel that encapsulated SDSCs (22). Therefore, to achieve improved cartilage regeneration, we evaluated whether recombinant human TG-4 (rhTG-4) can affect the damaged cartilage by enhancing the engraftment and differentiation of synovium-derived mesenchymal stem cells (SDSCs). The hypothesis of this study was that rhTG-4 could improve SDSCs cartilage regeneration.

Chapter I.

Enhancement of the stem cell engraftment

I−1. Materials and methods

Isolation of SDSCs from human synovium

After obtaining the ethical approval from the institutional review board (protocol number: H-1104-110-359), isolation of human SDSCs was prepared using a previously described method (23) . Briefly, synovial tissues were harvested from the knees of human donors during total knee arthroplasty for degenerative arthritis. The synovial tissues were digested with 0.02% collagenase (Sigma-Aldrich, St. Louis, MO, USA) in a humidified 5% CO_2 atmosphere. Thereafter, cells were washed and re-suspended in a culture medium (high glucose DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco).

Preparation of composite hydrogel

HA/COL/FG composite gel was generated using a previously described method (17). Briefly, FG was dissolved in an aprotinin solution (Green Cross). The FG solution was mixed with COL and HA. The mixture ratio of composite gel was HA: COL: FG = 0.5: 0.5: 2.0.

Preparation of rhTG-4 using a baculovirus expression system

After cloning of human TG-4 into a baculovirus transfer vector pVL1392, expression of human TG-4 in Baculovirus Expression System (BD, Heidelberg, Germany) was performed in accordance with the manufacturer's instructions. Briefly, co-transfection of 5×10^5 SF21 insect cells was carried out using the baculovirus DNA (1 μg) and the recombinant transfer vector pVL1392/hTG-4 (2.5 μg) to generate a recombinant baculovirus. After

individual plaques were sonicated for 1 min, the supernatant was loaded on a NTA agarose column. Elution of His-tagged human TG-4 was achieved using an elution buffer.

Adhesion assay of SDSCs

The adhesion assay was carried out by directly counting the number of cells using the trypan blue staining. In total, 1×10^5 SDSCs were seeded into a fibronectin (2.5 μg/mL)-coating 12-well plate. RhTG-4 was then supplemented at different concentrations (0, 100, and 400 μg/mL). The well plates were incubated for 5, 15, 30, and 60 min at 37 °C in 5% CO_2. Unattached cells were washed out and the attached cells were enumerated using the trypan blue staining. To visualize the adherent cells, 50 μL of 0.5% crystal violet solution was added to each well in 12-well culture plate. The well plates were incubated for 20 min at room temperature. After washing with PBS, stained cells were visualized and photographed.

Immunofluorescence assay of adherent SDSCs

To visualize cell adhesion and cytoskeletal proteins, SDSCs were treated with 400 μg/mL rhTG-4 and plated on fibronectin-coated glass coverslips for 60 min. Cystamine (Sigma C8707) at 100 μM was supplemented in the culture media to inhibit the TG-4 activity (24, 25). Cells were fixed with 3% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100. After several washes with PBS cells were double-stained with 10 mg/mL polyclonal anti-beta actin antibody (Sigma) and 10 mg/mL mouse antibody against human integrin β1. After incubation with primary antibodies, cells were

stained with fluorescein-labeled goat anti–rabbit IgG and rhodamine-conjugated goat anti–mouse IgG (Chemicon). Stained cells were analyzed and photographed using a microscope (Nikon, Tokyo, Japan).

Proliferation assay of SDSCs in the hydrogel

SDSCs encapsulated in the supplement of TG−4 (0，100，400 μg/mL) on the HA/COL/FG composite gel were transferred to six-well culture plates and cultured in a defined chondrogenic medium (low glucose DMEM supplemented with 100 mM ascorbate-2-phosphate, 100 mg/mL pyruvate, 40 mg/mL proline, 100 nM dexamethasone, 100 ng/mL of BMP-2, and 10 ng/mL TGF-β1). The proliferation assay was carried out by counting the number of cells using trypan blue staining. A total of 6×10^6 SDSCs were encapsulated in composite gels. On days 1, 3, 7, 14, and 28 of cultivation, the SDSCs encapsulated in composite gels were digested with 100 mL of 0.1% collagenase (Sigma-Aldrich) for 3 h at 37°C in a 5% CO_2 atmosphere in the low-glucose DMEM containing 1% antimycotic-antibiotic solution and the number of cells was counted using trypan blue staining. The Live/Dead Viability kit (Molecular Probes) was used for the assessment of the viability of encapsulated SDSCs. On day 7 after cultivation of samples, they were treated in a solution containing 2mM ethidium homodimer-1, and 4mM Calcein AM in PBS for 40min. The samples were washed in PBS and thin slices of the stained samples were prepared on glass slides and observed using a fluorescence microscope. Live cells were indicated by a green color while dead cells by a red.

Evaluation of extra cellular matrix (ECM) hardness

SDSCs were harvested and resuspended into 96-well V-bottom culture plates at 1×10^6 cells/mL. The cells were incubated at 37 °C with 5% CO_2 for 24 h to generate micro mass cultures. Thereafter, chondrogenic differentiation was carried out using in a defined chondrogenic medium. Varying concentrations of rhTG-4 (0, 25, 50, 100 and 400 μg/mL) were then added. The medium was changed every 2 d, and micro masses were harvested on day 14. To evaluate the mechanical properties of the micro masses, the hardness (GPa) of 3D micro masses was evaluated using a nanoindentation instrument.

Activity of rhTG-4 in hydrogel

The solution mixed with biotinylated pentylamine (BP) and HA/COL/FG composite gels were prepared and rhTG−4 (400 μg/mL) was treated. RhTG−4 activity was estimated by measuring incorporation of BP to N, N'−dimethylcasein mediated by crosslinking of rhTG−4 (26).

Statistical analysis

All data are presented as mean ± standard deviation values. The continuous variables were analyzed with the student's t-test or Mann–Whitney U test to assess the differences in the mean values among different groups. All analyses were performed using SPSS 12.0 for Windows (version 23.0 IBM Corp., Armonk, NY, USA). P-values of < 0.05 were considered statistically significant.

I-1. Results

Effect of rhTG-4 on adhesion of SDSCs

Upon treating SDSCs with different doses (0, 100, and 400 μg/mL) of rhTG-4 and incubation time (5, 15, 30, and 60 min), the adhesion to fibronectin increased in a time- and concentration-dependent manner (Fig. 1A). At 60 min, the number of attached SDSCs to fibronectin increased approximately 4-fold in the 400 μg/mL TG-4-treated group, compared to the untreated group. The crystal violet assay at 5, 15, 30, and 60 min after culturing further confirmed the adhesion of SDSCs. The crystal violet assay revealed that rhTG-4 treatment increased the adhesion of SDSCs to fibronectin (Fig. 1B).

Change in SDSC phenotype by rhTG-4

In the immunofluorescence assay, changes in actin were observed. The actin morphology in the rhTG-4-treated group was more condensed and stratified, indicating the development of dynamic actin remodeling. In other words, the cell phenotype changed to one more favorable to adhesion. Pretreatment with the TG-inhibitor cystamine markedly downregulated integrin β1 expression and dynamic actin remodeling. These results indicate that rhTG-4 directly affected activation of integrin β1 and actin dynamics, leading to a change of cell phenotype and improved SDSC adhesion.

Effect of rhTG-4 on proliferation of SDSCs in hydrogel

SDSCs proliferation was significantly increased in the HA/COL/FG gel including rhTG−4 composite gel group (Fig. 3A); however, based on the number of SDSCs, this difference was not significant. The number of SDSCs

in the HA/COL/FG gel including rhTG−4 was relatively higher up to day 7 and thereafter this difference was not significantly increased to day 28. The Live/Dead stained assay on day 7 of culturing confirmed the viability of SDSCs encapsulated in HA/COL/FG gel including rhTG−4 (Fig. 3B). Fluorescence staining revealed high cell viability in HA/COL/FG gel including rhTG−4, indicating that TG−4 potentially increases the proliferation and vitality of the encapsulated SDSCs. The relative ratio of live cells/dead cells on day 7 was significantly higher in the HA/COL/FG gel, including rhTG−4 group, than in the HA/COL/FG gel (Fig. 3C).

Hardness of ECM in hydrogel

Nanoindentation analysis revealed that rhTG-4 stimulation increased the hardness of 3D micro masses. After 2 weeks of incubation, ECM hardness was improved in accordance with the dose of rhTG-4 (Fig. 4). Doses of 0, 25, 50, 100, and 400 μg/mL of rhTG-4 yielded a hardness of 0.13, 0.22, 0.57, 0.68, and 0.94 GPa, respectively.

Activity of rhTG-4 in hydrogel

Activity of rhTG-4 in hydrogel was high between 10 and 20 minutes. The effect was maintained for 2 hours without undesired side reactions (Fig. 5).

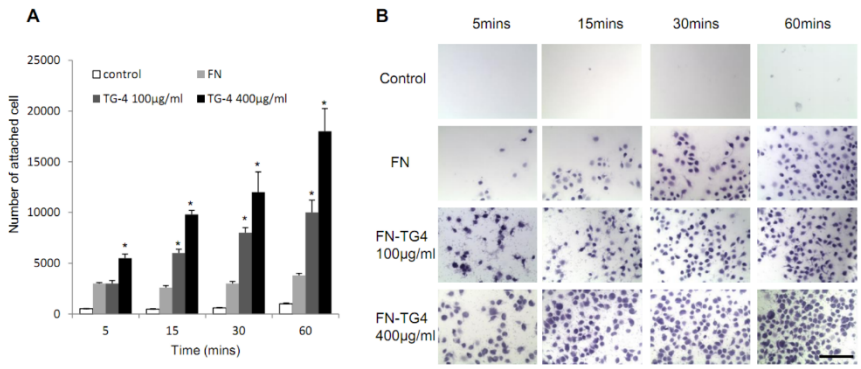

Figure 1. Adhesion assay of SDSCs treated with TG-4. TG-4 treatment improves the adhesion of SDSCs. In total, 3×10^4 SDSCs were seeded in fibronectin-coating 96-well plates. TG-4 was supplemented at different concentrations (0, 100, and 400 μg/mL). The well plates were incubated for 5, 15, 30, and 60 min at 37 °C in 5% CO_2. (A) Unattached cells were washed out, and attached cells were quantified by directly counting the number of cells using the trypan blue staining. (B) Representative photographs of the crystal violet assay. Scale bar: 100 mm. SDSCs, synovium-derived mesenchymal stem cells; TG, transglutaminase. *$p < 0.05$ represents a significant difference compared to the control group.

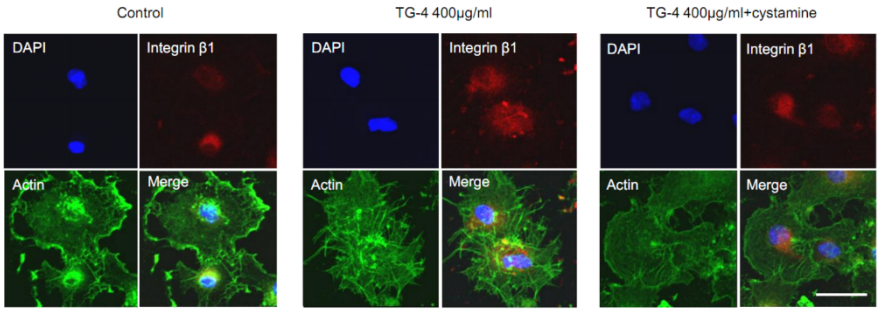

Figure 2. Immunofluorescence assay of SDSCs treated with TG-4. Treatment of TG4 induces integrin β1 and actin remodeling in SDSCs. One hour after 400 μg/mL TG-4 treatment of the SDSCs with or without the TG inhibitor; cystamine and immunofluorescence staining for integrin β1 and actin fiber expression levels was performed. Scale bar: 5 μm. SDSCs, synovium-derived mesenchymal stem cells; TG, transglutaminase. .

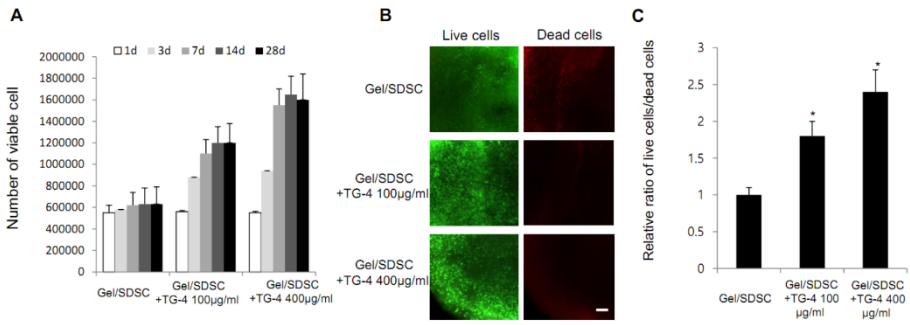

Figure 3. Proliferation assay of SDSCs treated with TG-4. (A) Proliferation rate of SDSCs encapsulated in the supplement of TG-4 (0,100, 400, μg/mL) on the HA/COL/FG composite gel at day 1, 3, 7, 14, and 28, after culturing and counting the number of cells using trypan blue staining. (B) Fluorescent microscopic images of SDSCs encapsulated in supplements of 400 μg/mL of rhTG-4 on the HA/COL/FG composite gel on day 7 after culturing via the Live/Dead assay. Scale bar: 100 μm (C) The relative ratio of live cells/dead cells at 7 d. The Live/Dead (green/red positive) cells were enumerated with the use of microscopy at the 40× objective. Green: live cells. Red: dead cells. Scale bar: 100 mm. SDSCs, synovium-derived mesenchymal stem cells; TG, transglutaminase; HA, hyaluronic acid; COL, collagen; FG, fibrinogen. *$p < 0.05$ represents a significant difference.

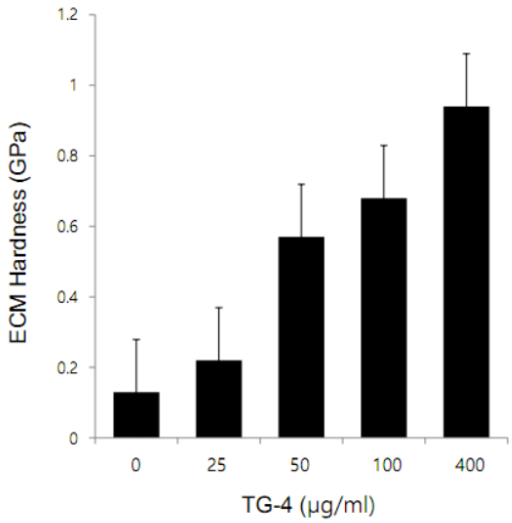

Figure 4. Hardness of ECM. Nanoindentation revealed that TG-4 stimulation increased the hardness of 3D micro masses. ECM, extracellular matrix; TG, transglutaminase.

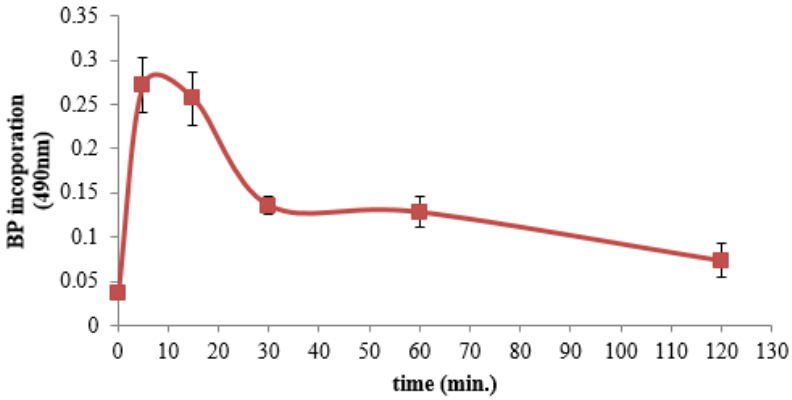

Figure 5. TG-4 activity in hydrogel. TG-4 activity was estimated by measuring incorporation of BP to N, N'-dimethylcasein. Activity of TG-4 in hydrogel was high between 10 and 20 minutes and maintained for 2 hours without side reactions. TG, transglutaminase; BP, biotinylated pentylamine

Chapter II.

Enhancement of the stem cell differentiation

II−1. Materials and Methods

Reverse transcription- polymerase chain reaction (RT-PCR)

After extraction of total RNA using RNeasy mini kit (Qiagen), reverse transcription was performed using the cDNA synthesis kit (Fermentas Life Sciences, Waltham, MA, USA). PCR amplification of type I COL, type II COL, type X COL, aggrecan, Sox9, or GAPDH was performed using AccuPower PCR PreMix (Bioneer) for 30 cycles. The following primers were used (15):

5’-CGTGGTGACAAGGGTGAGAC-3’ and

5’-TAGGTGATGTTCTGGGAGGC-3’ for type I COL,

5’-TTCAGCTATGGAGATGACAATC-3’ and

5’-AGAGTCCTAGAGTGACTGAG-3’ for type II COL,

5’-CCCTTTTTGCTGCTAGTATCC-3’ and

5’-CTGTTGTCCAGGTTTTCCTGGCAC-3’ for type X COL,

5’-TGAGGAGGGCTGGAACAAGTACC-3’ and

5’-GGAGGTGGTAATTGCAGGGAACA-3’ for aggrecan, and

5’-CCCGATCTGAAGAAGGAGAGC-3’ and

5’-GTTCTTCACCGACTTCCTCCG-3’ for Sox9. PCR fragments were normalized with the levels of GAPDH (5’-ATTGTTGCCATCAATGACCC-3’ and 5’-AGTAGAGGCAGGGATGATGTT-3’). PCR products were separated through agarose gel electrophoresis (1% agarose), and DNA bands were visualized with ethidium bromide.

Evaluation of chondrogenesis related gene expression in SDSCs

SDSCs encapsulated in the supplement of TG−4 (0,100, 400, μg/mL) on the HA/COL/FG composite were transferred to six-well culture plates and cultured in a defined chondrogenic medium. RT-PCR analysis was performed to evaluate the mRNA expression of type I, II, and X COL; aggrecan; and SOX9.

Evaluation of intracellular signal transduction pathway

SDSCs and HA/COL/FG composite gels were treated with 400 μg/mL rhTG-4. Integrin β1 siRNA was transfected to inhibit integrin β1 expression. An immunofluorescence assay was performed to visualize cell adhesion and cytoskeletal proteins. Western blotting was performed to evaluate the activation of mitogen-activated protein kinase (MAPK) by assessing the activation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal Kinase (JNK), and p38. RT-PCR analysis was performed to evaluate the mRNA expression of type I, II, and X COL; aggrecan; and SOX9.

Surgical procedure

New Zealand white rabbits (3.5–4.0 kg weighting, 8-month-old males) were anesthetized through intramuscular administration of xylazine hydrochloride (5 mg/kg; Bayer) and ketamine hydrochloride (35 mg/kg; Yuhan)). The anesthesia was maintained with isoflurane. The knee joints were incised by medial parapatellar approach, and the patella was eversed laterally to expose the femoral trochlea articular surface in rabbits. A full-thickness osteochondral defect model was established using a previously described method (17). Briefly, a full-thickness osteochondral defect (3 mm in depth and 4 mm in

diameter) was made in the trochlea groove of the distal femur of New Zealand white rabbits. Fifty-six knee joints were divided into three groups. The osteochondral defects of the control group (n = 16) were not treated with the HA/COL/FG composite gel; they served as controls. The osteochondral defects in knees of the Gel/SDSC group (n = 20) were filled with the HA/COL/FG composite gel (150 μL/defect) loaded with human SDSCs (2×10^7 cells/mL, 150 μL/defect). The osteochondral defects in the right knees of Gel/SDSC+TG-4 composite gels group (n = 20) were filled with the supplement of rhTG-4 in HA/COL/FG composite gels loaded with human SDSCs (2×10^7 cells/mL, 150 μL/defect). The experimental in vivo animal groups are summarized in Table 1. All experimental procedures related to the animal model were approved by the Institutional Animal Care and Use Committee (13-0333).

Macroscopic and histological assessment

For macroscopic scoring, the gross appearance of the defects was assessed through the international cartilage repair society (ICRS) cartilage repair assessment (degree of defect repair, macroscopic appearance, integration to border zone, and overall repair assessment) (27). Sagittal sections (5-mm thick) were stained with hematoxylin and eosin (H&E), and safranin-O/Fast Green. Histomorphologic findings (n = 8 at each time point) for each section were evaluated and scored by three investigators in accordance with the O'Driscoll histological grading parameters (cell morphology, matrix staining, structural integrity, thickness/defect filling, osteochondral junction, adjacent bonding, basal integration, cellularity, clustering/distribution, and adjacent

cartilage) (28). DiI-labeled SDSCs distribution in the center of defect was performed. DiI (1, 1'-Dioctadecyl-3, 3, 3', 3'-Tetramethylindocarbocyanine Perchlorate) staining was used for SDSCs distribution in cartilage. Fifty microliter of DiI (Molecular Probes) cell-labeling solution (2mg/ml) was added directly to 10ml culture media for labeling of 2×10^7 SDSCs. After 30 min of incubation at 37°C, the cells were washed in PBS twice. Then, the DiI-labeled SDSCs were encapsulated in the composite gels. After transplantation, DiI-labeled SDSCs distribution in cartilage was observed using a fluorescence microscope at 12 weeks.

Statistical analysis

All data are presented as mean ± standard deviation values. The continuous variables were analyzed with the student's t-test or Mann–Whitney U test to assess the differences in the mean values among different groups. All analyses were performed using SPSS 12.0 for Windows (version 23.0 IBM Corp., Armonk, NY, USA). P-values of < 0.05 were considered statistically significant.

Table 1. Summary of the experimental in vivo animal groups

Group		Number	Description
Defects only	1week (H&E,)	n = 8	control
	12week (H&E, safranin-O)	n = 8	
Gel/SDSC	1week (H&E)	n = 8	COL/HA/FG gel SDSCs 2 x 10^7 cells
	12week (H&E, safranin-O, DiI-labeling)	n = 12	
Gel/SDSC+TG-4	1week (H&E)	n = 8	COL/HA/FG gel SDSCs 2 x 10^7 cells 400 μg/ml rhTG-4
	12week (H&E, safranin-O, DiI-labeling)	n = 12	

II-2. Results

The chondrogenic effects of rhTG4-hydrogel

At 28 d after culturing in the supplement of rhTG-4 in HA/COL/FG gel group, the relative expression levels of type II COL (rhTG-4 100 μg: 4.00-fold, rhTG-4 400 μg: 4.5-fold) and aggrecan (rhTG-4 100 μg: 4.1-fold, rhTG-4 400 μg: 4.7-fold) mRNA were significantly increased, compared to the HA/COL/FG composite gel group; however, expression levels of type X COL, Sox9 and type I COL were not significantly altered compared to those in the HA/COL/FG composite gel group (Figs. 6 and 7).

Expression of genes associated with the intracellular signal transduction pathway

Immunofluorescence analysis revealed that pretreatment with integrin β1 siRNA markedly decreased rhTG4-induced actin remodeling (Fig. 8). MAPK was activated in an integrin β1 siRNA-dependent manner. ERK was not phosphorylated, and JNK and p38 did not depend on integrin β1 siRNA treatment (Fig. 8). Furthermore, chondrogenesis-related genes including aggrecan and type II collagen were downregulated (Fig. 9). These data indicate that rhTG-4 potentially regulates SDSCs adhesion and expression of chondrogenesis-related genes via the activation of intracellular signal transduction pathways.

The in vivo potential of HA/COL/FG gel including rhTG-4 to regenerate and repair osteochondral defects

The defect lesion in the control group was partially covered with dark brown tissue at 1 week after transplantation. The defect lesion in the Gel/SDSC and the Gel/SDSC+TG-4 groups were partially filled with white tissue similar to intact cartilage. At 12 weeks, the surface of the control group displayed irregular white tissue, with a depression in the center of the defects. In both the Gel/SDSC and Gel/SDSC+TG-4 gel groups, the transplanted area was mostly repaired by a cartilage-like tissue (Fig. 10). In the H & E staining, the Gel/SDSC group showed that the defect lesion was almost repaired with newly formed cartilage tissues except for a slight depression at the center. In the Gel/SDSC+TG-4 group, the defect lesion was more filled with white tissue and displayed substantial integration between the newly formed cartilage and the surrounding original cartilage. Moreover, the newly formed cartilage tissue on the defects had more hyaline cartilage, which was densely stained with safranin-O in the Gel/SDSC+TG-4 group (Fig. 11). At higher magnification, cells showed a columnar alignment and a similar morphology to normal chondrocytes in the Gel/SDSC+TG-4 group (Fig. 12). The total macroscopic and histological scores at 12 weeks were significantly higher in Gel/SDSC+TG-4, than in the control group and Gel/SDSC group. The macroscopic scores at 12 weeks for the control group, Gel/SDSC, and Gel/SDSC+TG-4 group were 3.40 ± 1.20, 5.20 ± 1.44, and 6.40 ± 1.36, respectively. The O'Driscoll histological scores at 12 weeks for the control group, Gel/SDSC group, and Gel/SDSC+TG-4 group were 11.00 ± 1.72, 15.00 ± 2.84, and 18.00 ± 2.24, respectively (Fig. 13). DiI-labeled SDSCs

distribution was markedly increased at the center of defect in the Gel/SDSC+TG-4 group showing that implanted stem cells survived (Fig. 14). No inflammatory or immunological reactions were encountered after transplantation of SDSCs throughout the experiment. These results indicate that the reconstructed tissue derived from the SDSCs encapsulated in the supplement of rhTG-4 on HA/COL/FG composite gel resembles hyaline cartilage.

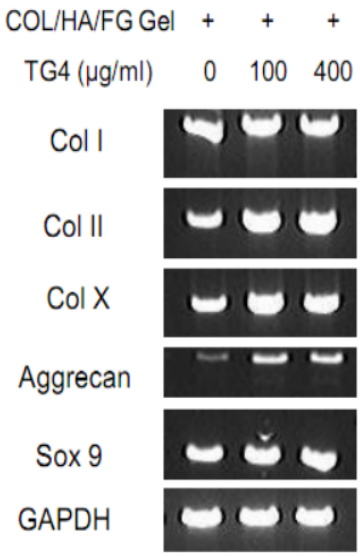

Figure 6. **RT−PCR analysis in SDSCs treated with TG−4**. Expression of type I, II, and X collagen, sox 9, and aggrecan genes in the SDSC−encapsulated HA/COL/FG composite gel upon supplementation of TG-4 (0,100, and 400 μg/mL) via RT-PCR analysis on day 28 after culturing. RT, reverse transcription; SDSCs, synovium-derived mesenchymal stem cells; TG, transglutaminase.

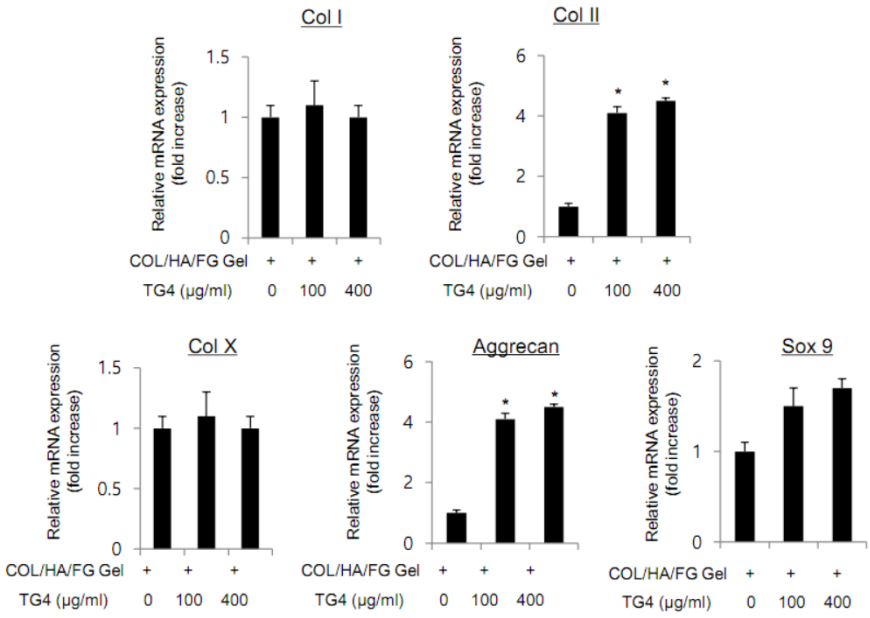

Figure 7. **Relative mRNA expression levels**. Type II and aggrecan mRNA were significantly increased upon TG−4 were supplementation (0,100, and 400 μg/mL). TG, transglutaminase. *$p < 0.05$ represents a significant difference compared to the control group.

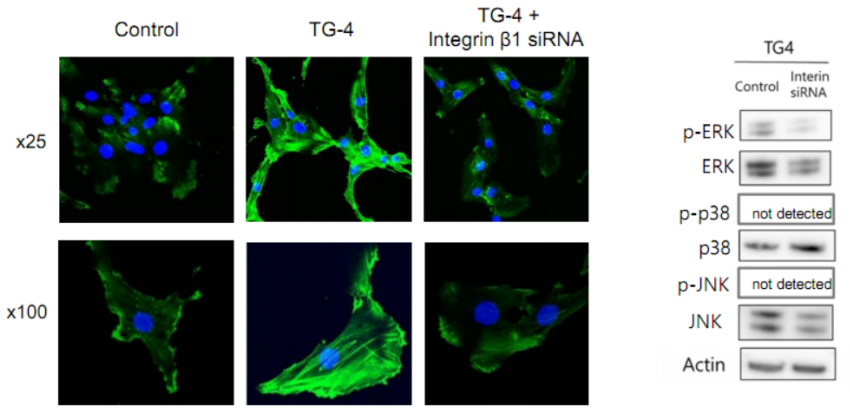

Figure 8. Expression of intracellular signal transduction pathway.

Immunofluorescence analysis revealed that pretreatment with integrin β1 siRNA markedly decreased TG4-induced the actin remodeling. ERK was not phosphorylated and JNK and p38 did not depend on integrin β1 siRNA treatment. TG, transglutaminase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal Kinase.

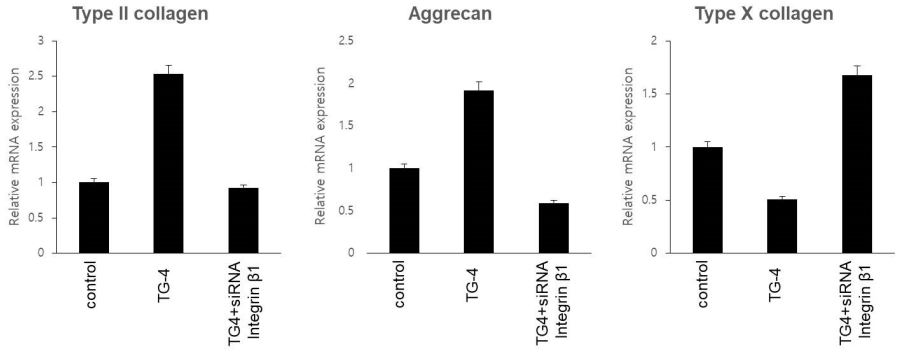

Figure 9. Relative expression levels of mRNA in the treatment integrin β1 siRNA. The expression of chondrogenesis-related genes including aggrecan and type II collagen was decreased. TG, transglutaminase.

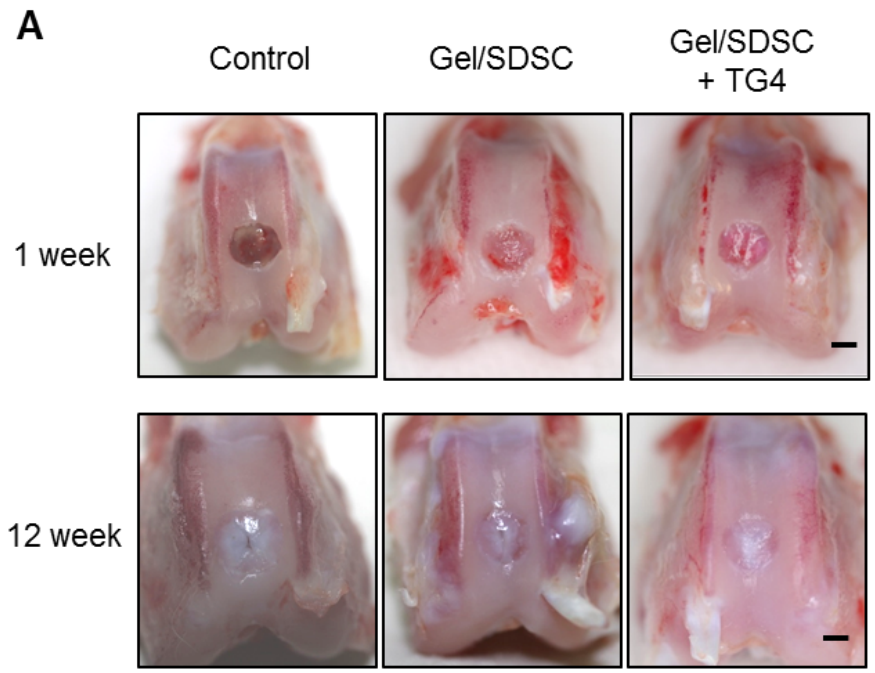

Figure 10. **Macroscopic appearance**. After transplantation of the SDSCs−encapsulated HA/COL/FG composite gel with TG−4, the defects on the trochlear groove was filled more compared to the other groups. Scale bar: 10 mm. SDSCs, synovium−derived mesenchymal stem cells; HA, hyaluronic acid; COL, collagen; FG, fibrinogen; TG, transglutaminase.

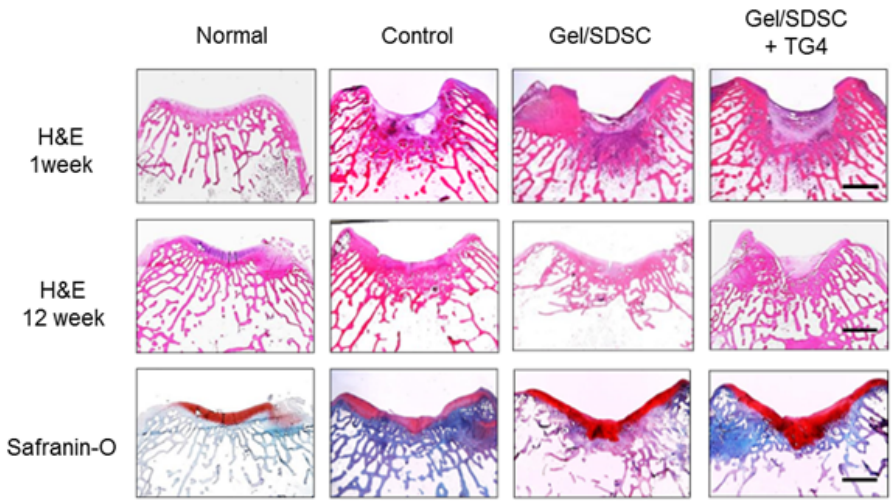

Figure 11. **Histologic appearance** (**1**). H&E staining (upper panel) and safranin−O staining (lower panel) of the defects lesion in the trochlear groove at 1 or 12 weeks after transplantation. In the Gel/SDSC+TG−4 group, the defect lesion was more filled with white tissue and displayed substantial integration. Moreover, the newly formed cartilage tissue on the defects had more hyaline cartilage, which was densely stained with safranin−O in the Gel/SDSC+TG−4 group Scale bar: 2 mm. H&E, hematoxylin and eosin; SDSC, synovium−derived mesenchymal stem cells; TG, transglutaminase.

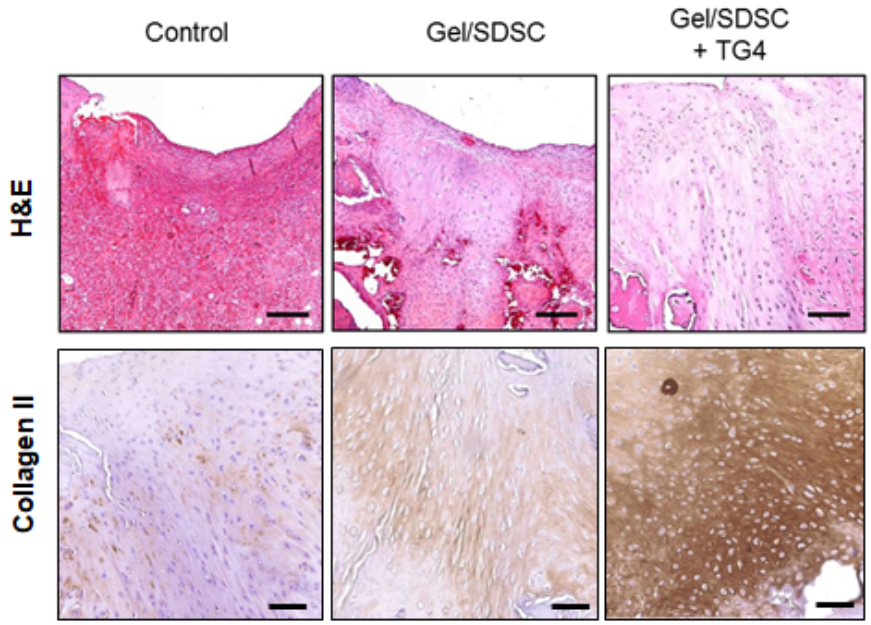

Figure 12. Histologic appearance (2). High-magnification images of the defect lesions (200×). The cells showed a columnar alignment and had a similar morphology to normal chondrocytes in the Gel/SDSC+TG-4 group. Scale bar: 100 μm. SDSC, synovium-derived mesenchymal stem cells; TG, transglutaminase.

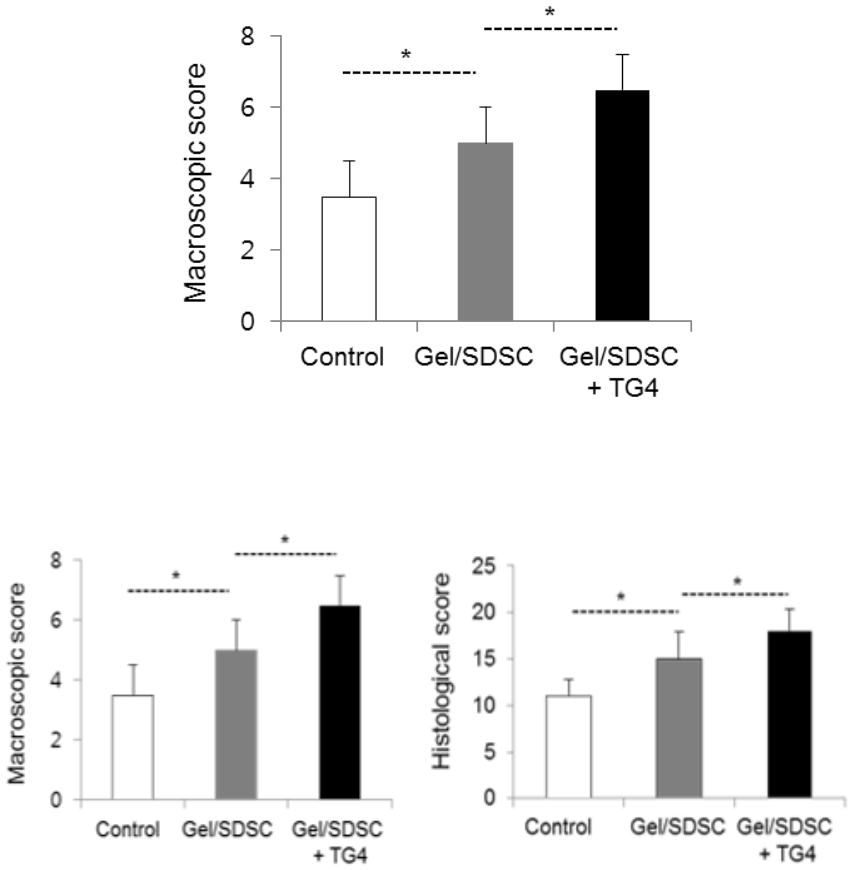

Figure 13. **Macroscopic scores and O'Driscoll histological scores**. Total macroscopic and histological scores at 12 weeks were significantly higher in Gel/SDSC+TG−4, than in the control group and Gel/SDSC group. SDSC, synovium−derived mesenchymal stem cells; TG, transglutaminase. *$p < 0.05$ represents a significant difference compared to the control group.

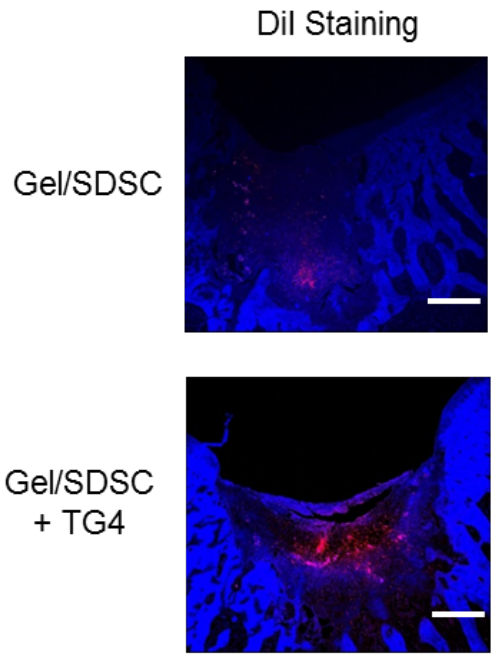

Figure 14. Confocal images of DiI. DiI-labeled SDSCs distribution was markedly increased at the center of defect in the Gel/SDSC+TG-4 group at 12 weeks after transplantation. Blue: DAPI, Red: DiI. Scale bar: 200 μm. SDSCs, synovium-derived mesenchymal stem cells; TG, transglutaminase.

Discussion

This study investigated the effect of rhTG-4 supplement in a HA/COL/FG composite gel encapsulated with SDSCs in vitro and in vivo for the repair of osteochondral defect. An important finding of the present study is that rhTG-4 improved the engraftment of SDSCs by the changing the integrin β1-mediated cell phenotype to one of abundant proliferation. In addition, rhTG-4 activated the intracellular signal transduction pathway mediated by integrin β1 and promoted expression of chondrogenesis-related genes, eventually leading to improved differentiation in vivo.

For cartilage regeneration, identifying appropriate stem cell sources that are easily accessible and have chondrogenic potential is important. SDSCs have been used and explored as an alternative stem cell source for cartilage repair and regeneration due to their chondrogenic potential and their ease of isolation from donors site without damage to the native cartilage tissue (29-33). MSCs are harvested from usually discarded portion during arthroscopy and arthroplasty surgery, such as infrapatellar fat pad and synovial membrane. The synovium itself can serve as a source of stem cells that are initiated following injury, while MSCs migrate to the wound site to participate in the regeneration response. Continuous layers of MSCs extended from the synovium contribute to cartilage regeneration when the chondral defects were created in the cartilage of rabbit (34). Previously studies conducted in our institution have also shown that the SDSCs could be differentiated into the desired lineage and expanded in the culture following the treatment of growth

factors (23, 35). Although the regeneration potential of bone marrow-derived MSCs (BM-MSCs) and SDSCs may be similar, the chondrogenic evaluation in an in vivo studies demonstrated that SDSCs produced more hyaline cartilage matrix than BM-MSCs (29, 30). Moreover, SDSCs have been shown to proliferate much faster than BM-MSCs when cultured with autologous human serum (36).

Hydrogel-type scaffolds are an effective system for chondrogenesis of MSCs including SDSCs, because these scaffolds have mechanical properties similar to those of articular cartilage (4-6). Collagen, hyaluronic acid, and fibrin are a major component of the cartilage ECM, and provide a favorable environment for chondrogenesis of MSCs (37-39). Furthermore, we previously developed an injectable hyaluronic acid/ collagen/ fibrinogen (HA/COL/FG) composite gel that encapsulated SDSCs (22). The combination of HA/COL/FG may mimic the cartilage extracellular matrix, providing a chondrogenic environment for the encapsulated SDSCs. Moreover, SDSCs encapsulated in HA/COL/FG composite gel led to the formation of hyaline cartilage in damaged articular cartilage in vivo. However, scaffold-based cartilage tissue engineering is associated with potential issues including poor implanted cell engraftment, phenotypic alteration, overly soft mechanical properties, in vivo degradation, and loss of strength at high temperatures or moisture (5, 7-9). To achieve effective transplantation of SDSCs, TG was assessed as potential stabilizer due to its ability to crosslinking protein or inducing cellular response.

TG is present as several forms in the human body. Major roles of all proteins

in the TG family are transamidase activity and catalyze the formation of N-ε-(γ-glutamyl) lysine protein crosslinks depending on calcium (10-13). Members of the TG family are expressed in numerous tissues and have different functions in processes including post-translational modification of proteins, cell adhesion and migration, ECM remodeling, and blood clotting (10, 11, 40-42). Among the TGs identified in humans, TG-2, also called tissue TG, is most widely distributed in the body and most extensively studied in the literature. TG-2, which is expressed in various tissues and cell types, occurs in intracellular and extracellular forms. In specific, intracellular TG-2 in chondrocytes is present in the nucleus and cytoplasm, inducing degeneration mediated by reactive oxygen species and cell hypertrophy (17, 18). Therefore, the risk of these negative effects occurring to SDSCs induced by exogenous TG-2 was a serious consideration in the present study. In addition, according to previous reports, transfection of TG-2 into chondrocytes promoted expression of type X COL and matrix calcification consistent with cell hypertrophy (19). Compared to TG-2, TG-4 has a relatively restricted distribution and activation pattern (12, 13). TG-4 is located in extracellular area and plays role of secretary protein. In rats, TG-4 participates in the formation of the vaginal plug in the genital tract making plug materials more adherent to the tissue by using its crosslinking ability (13). TG-4 levels in prostate tumor cells may be associated with their aggressiveness. For example, TG-4 overexpression in prostate tumor cells increases their adhesion and migration (20). Moreover, recent reports suggested TG-4 could induce intracellular signal transduction pathway such as the phosphorylation of FAK and paxillin, enhancing the cell-matrix adhesion (21). TG-4 interacted with integrin and initiates phenotype alteration. A neutralizing antibody to integrin-

β1 could abolish TG-4 induced cell-matrix adhesion. Matrix adhesion activities of TG-4 were observed in the TG-4 core domain because all constructs encoding core domains promote matrix adhesion, whereas the deletion of the core domain from TG-4 eliminates these activities (21). The interaction between cells and the ECM not only mechanistically supports cell adhesion to the matrix but also mediates cellular signaling. In fact, as a preliminary study in our institution, the adhesion and proliferation ability of TG-2 and TG -4 were compared and, in the Live/Dead assay, the TG-4-treated group showed more attached, viable, and active chondrocytes than the TG-2-treated group (Fig 16). Therefore, considering its potential superiority over other TGs for cell adhesion and chondrocyte safety, we hypothesized that recombinant TG-4 can enhance the cartilage regenerative potential of SDSCs by enhancing adhesion to matrix.

As shown in Figure 1-2, rhTG-4 increased the adhesion to fibronectin in a time- and concentration-dependent manner. Furthermore, to verify the role of TG-4 transamidation activity, a competitive substrate of the enzyme, cystamine was used (24, 25). RhTG-4 can induce the expression of integrin β1, dynamic actin fibers and phenotype modification of cell thus enhancing SDSCs adhesion (Fig. 3). In the additional adhesion assay conducted as supplementary study in our institution, rhTG-4 treatment also enhanced the SDSCs adhesion to HA and COL (Fig. 15). These results showed the improved adhesion could be correlated with phenotype modification apart from adhesion complex between integrin and fibronectin. Adhesion to the matrix is a key step in promoting proliferation, cell differentiation, and migration. Cellular adhesion is primarily mediated by adhesion-related

receptors including integrin. Specific receptor-ligand binding is important in cartilage tissue engineering, since these interactions result in an intracellular signaling, thus altering the status of the cells. Adhesion prevents apoptosis, induces proliferation, and regulates protein production by chondrocytes, as in the case of ECM proteins and growth factors (43-46).

ECM becomes harder as the dose of rhTG-4 increases, thus providing a stable environment for SDSCs survival and differentiation (Fig. 4). Although the stiffness optimum for differentiation and other behaviors varies significantly from cell to cell, it is generally accepted that increasing the ECM substrate stiffness correlates with increasing cell proliferation and differentiation (47-49). The mechanical properties of the ECM influence protein expression and posttranslational modification at the most basic level. After all, enhanced adhesion and stabilization of ECM in hydrogel by rhTG-4 led to improve the proliferation of SDSCs in the present study.

Activity of rhTG-4 in hydrogel was maintained for 2 hours without immunological or inflammatory reaction (Fig. 5). To date, no study has reported the proper duration and dose of maintenance in either in vitro or vivo environment. In the present study, rhTG-4 had positive effect on ECM in the composite hydrogel environment by showing an increased hardness of ECM where the crosslinking was modulated, and well proliferation of stem cell in rhTG-4 concentration-dependent manner. In addition, the proper activation time for rhTG-4 was demonstrated, avoiding undesired side reactions from prolonged residue exposure (26). Therefore, potential rhTG-4 safety issues and negative reaction between rhTG-4 and hydrogel can be mitigated to some

degree.

The rhTG4 increased the expression of aggrecans and type II collagen mRNA (Figs. 6 and 7). The present results suggest that rhTG-4 supplementation in the HA/COL/FG composite gel further increases the chondrogenic effects in vitro, compared to the HA/COL/FG composite gel group. Furthermore, the underlying mechanisms modulated by rhTG-4 are revealed herein, indicating that activation of the intracellular signal transduction pathway is mediated by integrin β1 while locking it with siRNA interrupted chondrogenesis. Binding with rhTG4 and integrin β1 in the cell wall induced actin remodeling and MAPK activation, eventually inducing chondrogenic mRNA expression (Figs. 8 and 9) It was already revealed that TG-2 affects integrin-mediated intracellular signaling independent from its enzymatic activity (50, 51). By interacting directly with integrin β, FAK tyrosine phosphorylation was activated. However, the regulation of the signal transduction pathway has not been entirely clear with TG-4. A recent study conducted with prostate cells suggested that TG-4 could induce intracellular signal transduction, such as phosphorylation of FAK and paxillin, enhancing cell-matrix adhesion (21). This is first study which revealed that binding with rhTG4 and integrin β1 in the cell wall induced actin remodeling and MAPK activation, eventually inducing chondrogenic mRNA expression (Figs. 8 and 9).

In vivo, rhTG-4 can enhance cartilage regeneration of SDSC encapsulated in hydrogels. Macroscopic and histologic appearance of defect lesion was repaired successfully in the Gel/SDSC+TG-4 group (Figs. 10, 11 and 12).

Furthermore, as shown in Figure 12, the macroscopic scores and O'Driscoll histological scores at 12 weeks were significantly higher in the Gel/SDSC+TG-4 group than in the control group and Gel/SDSC group. The effectiveness of stem cell based treatment will depend on the retention of cell viability after transplantation (52). Therefore, the fate of transplanted SDSCs on regenerated cartilage should be investigated without remaining unknown. By conducting the confocal microscopy with detecting DiI-labeled SDSCs distribution in the center of defect, this study confirmed whether that the transplanted cells were alive and localized. The DiI-labeled SDSCs distribution was markedly increased at the center of defect in the Gel/SDSC+TG-4 gel group (Fig. 14). Furthermore, host cell (DiI-unlabeled cells/DAPI positive cells) recruitment was further increased at the center of the defect in the Gel/SDSC+TG-4 gel group. Hence, rhTG-4 treatment might have recruited host cells by modulating the stiffness of the ECM.

For the past several decades, TGs crosslinking activity has been used in the food industry to improve the quality and nutritional attributes of food (8, 53). TGs are included in various commonly consumed foods including bakery products, meat, and seafood. Recently, there has been an upsurge in interest of TGs for biomedical applications (8). In addition to the crosslinking ability, TGs are considered as safer substitutes for chemical agents widely used to produce scaffold materials, including formaldehyde or glutaraldehyde, which generate toxic byproducts. In skin tissue engineering, TGs are exploited to overcome the limitation of hydrogels such as soft tissue properties and in vivo biodegradation (54). However, there has been limited interest in approaches of TGs to cartilage regeneration. Few studies on the application of TGs to

hydrogel and stem cells treatment for damaged cartilage have been reported, and those have lack in vivo evidence to support the occurrence of chondrogenesis (55, 56). To the best of our knowledge, this is first study which demonstrates the efficacy of TGs on MSCs engraftment and differentiation in vivo. In specific, the present study used TG-4 due to its potential superiority over other TGs in terms of cell adhesion and chondrocyte safety. The results demonstrate that TG-4 could be helpful to cartilage regeneration. Although there is still much to be learned about the mechanism and function of TG-4, with additional study, clinical applications of TG-4 may be realized like the food industry and skin tissue engineering.

There are some limitations in this study. First, determining the major mediator between integrin α and β in the intracellular signal transduction pathway was challenging due to the poor expression of integrin α in the present study after treatment of rhTG-4 (Fig. 17). However, the chondrogenesis was well conducted through integrin β, while locking it with siRNA interrupted chondrogenesis, suggesting that integrin β was an important mediator in the intracellular signal transduction pathway. Akimov et al also reported that TGs interact directly with integrin β1 and β3 and complexes of TGs with integrins are formed inside the cell during biosynthesis (51). Future studies should investigate the association between many different mediators in the cell wall and intracellular signal transduction pathway of cartilage regeneration. Second, the effects of transplanted SDSCs on regenerated cartilage after transplantation was not determined since the fate of transplanted SDSCs was not fully investigated. Therefore, further investigation on the biomechanical roles of these stem cells is warranted. Only

macroscopic and histological evaluations with the objective scoring of the repaired tissue 12 weeks after plantation were performed; hence, further biomechanical and biochemical analyses of the cartilage regeneration over longer periods should be conducted.

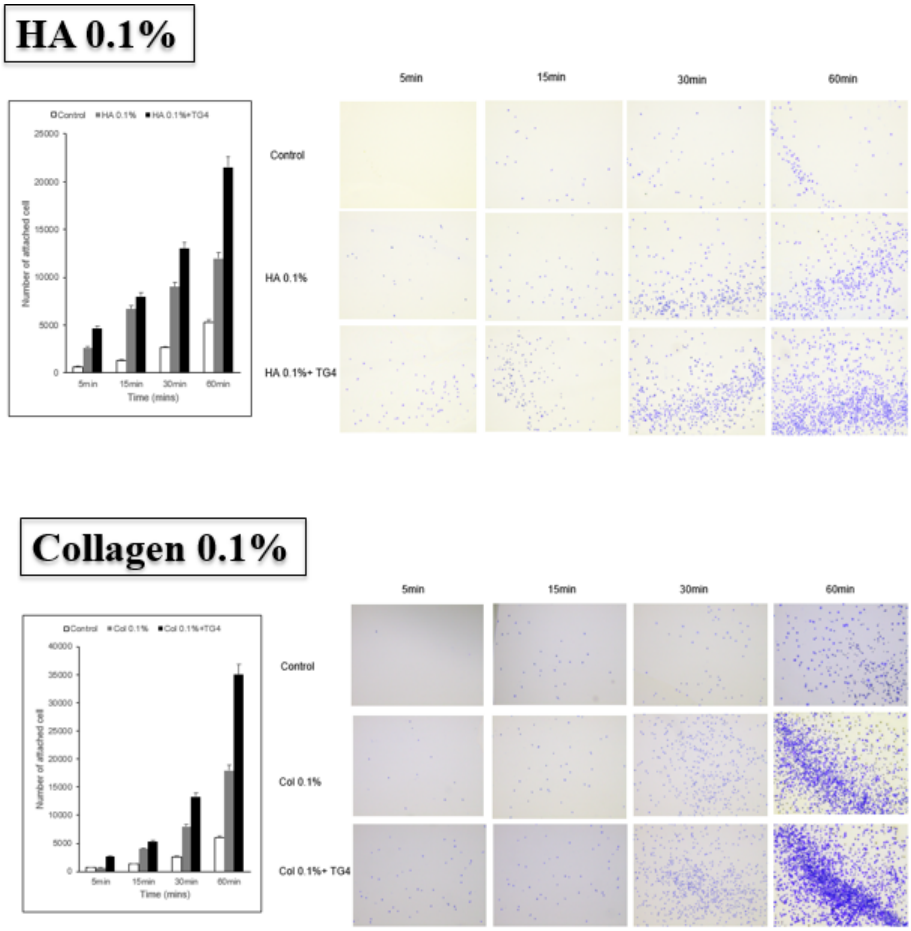

Figure 15. **Adhesion assay of SDSCs treated with TG-4 for hyaluronic acid and collagen.** SDSCs treated with TG-4 (400 μg/mL) in a hydrogel were seeded to the plate with hyaluronic acid and collagen. Direct cell counting and crystal violet staining were performed. TG−4 treatment also enhanced the SDSCs adhesion to hyaluronic acid and collagen. SDSCs, synovium-derived mesenchymal stem cells; TG, transglutaminase.

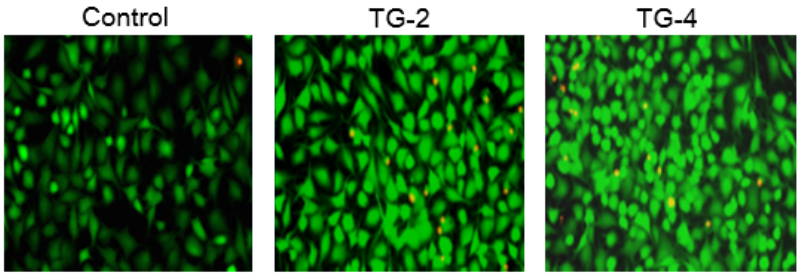

Figure 16. Comparison of adhesion and viability between TG-2 and TG-4. Chondrocyte were treated with 200 μg/mL TG-2 and TG-4 for Live/Dead Assay. TG-4 treatment showed more improved adhesion and viability of chondrocytes compared to the TG-2 treatment. TG, transglutaminase.

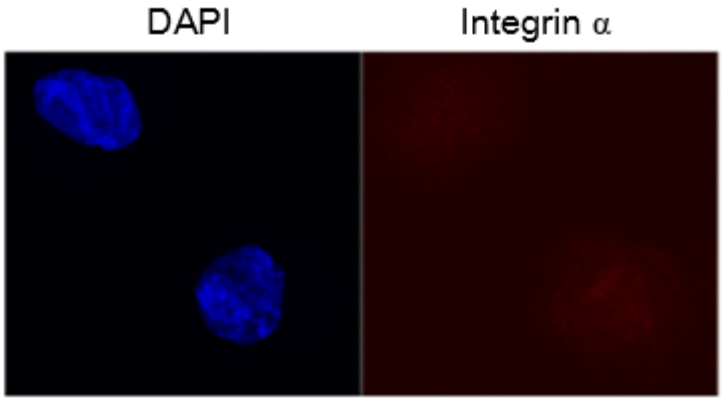

Figure 17. Immunofluorescence assay of integrin α treated with TG-4. The expression of integrin α was poor after treatment of TG-4 in SDSCs encapsulated hydrogle. Blue: DAPI, Red: DiI. SDSCs, synovium-derived mesenchymal stem cells; TG, transglutaminase.

Conclusion

The present results showed that rhTG−4 supplementation in the SDSC−encapsulated HA/COL/FG composite gel mediate successful cartilage regeneration by enhancing the engraftment and differentiation of SDSCs. Therefore, this combination can be an effective therapeutic strategy for the restoration of damaged or diseased articular cartilage.

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국문 초록

목 적: 손상된 연골을 재생시키기 위해 하이드로젤을 지지체로 한 중간엽 줄기세포를 이용한 치료는 그 효용성을 인정받아 임상적으로 많이 적용되고 있다. 하지만, 이러한 치료 방법도 줄기세포의 생착 및 지지대의 불안정성의 문제로 인해, 연골세포로 분화하는데 한계를 보이고 있다. 트랜스글루타미나제는 단백질 가교 역할 및 세포와 세포 외 기질과의 결합을 매개하는 능력을 인정받아, 세포의 유착 및 지지체의 안정화를 유도하기 위해 임상적으로 활용되고 있다. 따라서 본 연구에서는 관절연골 재생 치료에 있어 하이드로젤을 지지체로 사용시 줄기세포의 생착 및 분화능 향상을 위해 트랜스글루타미나제-4의 이용 가능성에 대해 검증하고자 한다.

대상 및 방법: 줄기세포는 슬관절의 인공관절 수술 시 확보한 활액막 조직으로부터 추출 하였다. 지지체로서 하이드로젤은 히알루론산, 콜라젠, 피브리노젠을 0.5:0.5:2.0 비율로 혼합하여 사용하였다. 트랜스글루타미나제-4는 사람의 생체에서 추출하여 baculo 바이러스를 이용한 과발현 시스템을 통해 재조합하여 사용하였다. 줄기세포의 유착능 향상을 위해 트랜스글루타미나제-4를 용량별로 처리한 후, 피브로넥틴으로 덮힌 판에 도포하여 세포를 직접세고, crystal violet 염색을 이용하여 분포를 확인하였다. 면역형광염색을 통해 부착된 세포의 형태 및 세포질내의 변화 여부를 확인하고 시스타민을

이용하여 트랜스글루타미나제를 억제하였을 경우와 비교 하였다. 하이드로젤 안에서 줄기세포를 4주간 배양하여 트랜스글루타미나제-4를 용량 별로 처리한 후 증식이 잘 되는지 확인하였고, 세포의 활성도를 확인하기 위해 Live/Dead 분석을 시행하였다. 하이드로젤의 경도를 측정하기 위해 트랜스글루타미나제-4를 용량 별로 처리한 후 작은 덩어리 형태로 만들어 그 경도를 측정하였고 하이드로젤 내에서 트랜스글루타미나제의 활성도를 확인하였다.

줄기세포의 분화능 향상을 확인하기 위해 트랜스글루타미나제-4를 용량 별로 처리하여 배양 한 후, RT-PCR을 통해 연골재생과 관련된 유전자들의 발현을 확인하였다. 인테그린 베타의 작용을 억제 할 수 있는 RNA을 사용하여, 세포체의 변화, 세포 내 신호전달체계 활성화 및 연골재생과 관련된 유전자들이 발현의 변화 유무를 확인하였다. 토끼의 연골을 이용한 생체 내 검사를 통해 연골 재생 정도를 육안으로 확인하고, 조직학적 분석을 시행하였다. 이를 객관화하기 위해 ICRS score와 Modified O'Driscoll score를 이용해 점수화 하였다. 이식된 세포의 생존여부를 하기 위해 DiI 염색을 이용하였다.

결 과: 트랜스글루타미나제-4의 용량에 따라 유착 된 줄기 세포의 수가 증가 하였으며, 전체적인 분포 양상도 더욱 조밀해졌다. 면역형광염색에서는 트랜스글루타미나제-4에 의해 인테그린 베타가 발현 되었고, 액틴의 모양이 줄기를 이루는 모양으로 변하면서 더욱 선명하고 진해졌다. 이는 트랜스글루타미나제를 통해 인테그린 베타의 활성화 및 세포내의 구조체의

변화를 일으킨 것으로 보인다. 트랜스글루타미나제-4의 용량에 따라 하이드로젤 내에서 줄기세포가 더 많이 증식되었으며, 생생하고 활성화되어 있는 세포의 비율이 더 높아졌다. 하이드로젤의 경도도 트랜스글루타미나제-4의 용량에 따라 증가하여, 트랜스글루타미나제가 가교 결합을 통해 하이드로젤의 세포 외 기질을 단단하게 하여 세포 성장의 안정화에 기여하는 것으로 보인다. 트랜스글루타미나제-4는 하이드로젤 내에서 10-20분 정도에 가장 높은 활성을 보였으며, 2시간 정도 지속되었다.

트랜스글루타미나제-4의 용량에 따라 연골재생과 관련된 유전자 (type II collagen, aggrecan) 발현이 증가 하였다. 인테그린 베타의 작용을 억제하는 RNA를 처리 하였을 때, 트랜스글루타미나제-4에 의해 활성화 되었던 세포 내 구조체의 변화 및 ERK 가 억제 되었다. 또한 연골재생과 관련된 유전자 (type II collagen, aggrecan) 발현도 다시 감소 하였다. 이는 트랜스글루타미나제가 인테그린 베타를 매개로 하여 세포내의 구조체의 변화 및 신호전달 체계를 활성화 시키고, 나아가 연골재생과 관련된 유전자 발현에도 영향을 미치는 것으로 보인다. 생체 내 실험에서는 육안적으로 트랜스글루타미나제-4가 처리 되었을 때, 연골 결손 부위가 잘 채워졌고, 조직학적 검사에도 조금 더 초자연골에 가깝고, GAG가 많이 함유 된 것을 확인할 수 있었다. 점수화하여 비교한 평가에서 트랜스글루타미나제-4가 투여된 군에서 통계학적으로 유의하게 높게 나타났으며, DiI 염색 검사에서도 넣어준 줄기 세포가 더 많이 살아있었던 것을 확인 할 수 있었다..

결 론: 기존의 연골 손상 시 사용되어 왔던 하이드로젤을 지지체로 한 줄기

세포를 이용하는 치료 방법에 트랜스글루타미나제-4를 보충한다면, 줄기 세포의 생착과 분화를 증진 시켜 연골재생이 더 잘 이루어 지게 할 것으로 사료 된다.

색인 단어: 트랜스글루타미나제, 가교 결합, 활액막 줄기세포, 하이드로젤, 줄기세포 생착, 줄기세포 분화, 연골 재생, 신호전달체계

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