Histological and biomechanical properties of regenerated articular cartilage using chondrogenic bone marrow stromal cells with a PLGA scaffold in vivo

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Abstract: The properties of regenerated cartilage using bone marrow-derived mesenchymal stem cells (MSCs) and poly lactic-co-glycolic acid (PLGA) scaffold composites pretreated with TGF-β3 were investigated and compared to the non-TGF-β3 treated MSCs/PLGA composites in a rabbit model. We prepared MSCs/PLGA scaffold composites and pretreated it with TGF-β3 for 3 weeks prior to transplantation. Then, composites were transplanted to the osteochondral defect in the rabbit knee. After 12 weeks of transplantation, 10 of the 12 rabbits in which TGF-β3 pretreated MSCs/PLGA scaffold composites were transplanted showed cartilaginous regeneration. In gross morphology, regenerated cartilage showed smooth, flush, and transparent features. In indentation test, this had about 80% of Young’s modulus of normal articular cartilage. Histological examination demonstrated hyaline like cartilage structures with glycosaminoglycan and type II collagen expression. Histological scores were not statistically different to the normal articular cartilage. These results showed improvement of cartilage regeneration compared to the non-TGF-β3 pretreated MSCs/PLGA scaffold composite transplanted group. Thus, we have successfully regenerated improved hyaline-like cartilage and determined the feasibility of treating damaged articular cartilage using MSCs/PLGA scaffold composite pretreated with TGF-β3. Also, we suggest this treatment modality as another concept of cartilage tissue engineering. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 87A: 850–861, 2008

Key words: bone marrow-derived mesenchymal stem cells; poly lactic-co-glycolic acid; cartilage; cartilage tissue engineering

INTRODUCTION

Focal cartilage defects frequently occur in secondary to trauma or to the idiopathic condition such as osteochondritis dissecans. Cartilage has a limited capacity to heal and the treatment of cartilage defects remains a challenge. Moreover, although many methods have been developed to treat cartilage defect, the results are unsatisfactory.
Recently the newly developed treatment modality of autologous chondrocytes transplantation (ACT) has shown the great progress, and is now widely used for the treatment of cartilage defects. However, some problems such as uneven distribution of grafted chondrocytes through the cartilage defects, high risk of leakage of grafted chondrocytes, and overgrowth of regenerated cartilage have been reported, and other methods are being experimented to assess the problems of ACT. Among these methods, transplantation of chondrocyte with various type of scaffold has been developed to promote even cell distribution and to prevent cell leakage, and these scaffolds have shown new progress in cartilage tissue engineering and even used clinically.2–4

However, use of autologous chondrocyte as a cell source still has additional problems of dedifferentiation with increased passage for cell expansion, donor site limitations, and morbidity.5,6 These problems could disturb the production of sufficiently available cells for transplantation which is an important factor for successful cartilage tissue engineering.7 Therefore, many researchers have searched for new cell sources that are easily expanded and handled for tissue engineering. The progenitor cells such as bone marrow stromal cells (MSCs), embryonic stem cells, and umbilical cord blood stem cells have been studied for this reason. Furthermore, because these cells can differentiate into chondrocyte like cells under defined conditions, they can be used as another cell source for cartilage regeneration in addition to chondrocytes.8–11 The chondrogenic differentiation of these pluripotent progenitor cells seems to be mediated by numerous cytokines most of which belong to the transforming growth factor (TGF)-β superfamily. The TGF-β superfamily has been detected in the developing skeleton including in areas where mesenchymal tissue are undergoing condensation and cartilage formation.12,13

In this study, we prepared MSCs/scaffold composites pretreated with TGF-β3, one of the TGF-β superfamily, and transplanted into the cartilage defect in rabbit knee. Then, we analyzed its biochemical, histological, and mechanical properties in vitro and in vivo. This study demonstrates the feasibility of treating the damaged articular cartilage with chondrogenic MSCs/scaffold composite and, therefore, may provide another concept for cartilage tissue engineering.

**MATERIALS AND METHODS**

**Polymer preparation**

Poly lactic-co-glycolic acid (PLGA) copolymer was synthesized using a conventional method. Its 1H-NMR (MERCURY plus 400 MHz, Varian, Palo Alto, CA) lactide-glycolide peak area ratio was determined (75:25). Its average molecular weight as determined using a gas permeation chromatography system equipped with 515 HPLC pump, 717 plus autosampler and 419 differential refractometer (Waters, Milford, MA) was approximately 120,000 (PDI, 1.33). PLGA scaffolds were fabricated by using a salt leaching method using a mixture ratio of 1:15 (Polymer: Salt). Briefly, 5 wt % PLGA solution was prepared by dissolving it in chloroform and then mixed with sodium. A Teflon mold, 3 mm in height and 6 mm in diameter was then filled with the polymer/salt paste. The paste was subsequently removed from the mold and placed in distilled water for 2 days. Finally, samples were lyophilized for several days. The overall porosity was analyzed by using mercury porosimeter.

**Cell culture and chondrogenic differentiation**

Rabbit MSCs were obtained from twelve 3-month-old New Zealand White rabbits according to the previously reported method.15 Briefly, a syringe containing 3000 U of heparin was used to aspirate 2–8 mL of the bone marrow from the posterior iliac crest of the rabbit. Dulbecco’s modified Eagle’s medium-low glucose (DMEM-LG, Gibco BRL, Grand Island, NY) was added to the aspirate, and cell pellets were produced by centrifugation. The cell pellet was washed once with basal medium, consisting of DMEM-LG, 10% (v/v) of FBS, 100 U/mL of Penicillin G and 0.1 mg/mL of Streptomycin, followed by culture within a T-75 cm² tissue culture flask at 37°C for 4 days. Subsequently, the attached cells in suspension were discarded, while the adherent cells were further cultured up to 10 days with media change every 3 days. Isolated MSCs were fully expanded and passage 2 cells were used in all experiments.

MSCs (Passage 2, 2 × 10⁶ cells) were incubated into prepared PLGA scaffolds (diameter 6 mm, depth 3 mm, disk type) in 2 mL of the defined DMEM medium supplemented with 1× ITS-A, 10 ng/mL of TGF-β3 (R&D System, Minneapolis, MN), and 30 µg/mL of ascorbic acid. The MSCs/scaffold composites were then cultured for 21 days prior to transplantation and were compared to the control group without TGF-β3 to observe chondrogenic potential.

**Reverse transcription-polymerase chain reaction**

The total RNA was isolated using a Trizol® (Invitrogen, Carlsbad, CA). Total RNA was reverse transcribed using an Omniscript kit (Qiagen, Valencia, CA). Aliquots (2 µL) of the resulting cDNA were amplified to a total volume of 50 µL containing PCR buffer, 0.2M dinucleotide triphosphate mixture, 1.5 mM MgCl₂, 0.5M of each primer,16,17 and 1 U of Taq DNA polymerase (Qiagen) using 25 to 40 cycles. The following primers, used in reverse transcription-polymerase chain reaction (RT-PCR), were synthesized according to the sequences reported in Genbank databases. Collagen type II: sense, 5'GCA CCC ATG GAC ATT GGA C for 4 days. Subsequently, the unattached cells in suspension were discarded, while the adherent cells were further cultured up to 10 days with media change every 3 days. Isolated MSCs were fully expanded and passage 2 cells were used in all experiments.

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by adding 200 µl of fresh MTT solution (0.5 mg/mL) was then added per well and the culture plates were incubated at 37°C for 4 h. The upper medium was then carefully removed, and the intracellular formazan was solubilized by adding 200 µL of dimethyl sulphoxide (Sigma) into each well. The absorbance of produced formazan was measured photometrically at 540 nm versus GADPH values.

Viability test

Rabbit MSCs (5 × 10^5) were inoculated evenly into the scaffolds and the MSCs/scaffold composites were maintained at 37°C under 5% CO₂ for 2 h to allow optimal attachment. The composites were incubated for 3 weeks in chondrogenic medium (150 µL/well) with or without TGF-β3. MSC viabilities were determined by methylthiazoletetrazolium (MTT, Sigma, St. Louis, MO) assay. Original culture medium was removed with a micropipette and 150 µL of fresh medium was added per well. One hundred microliters of fresh MTT solution (0.5 mg/mL) was then added per well and the culture plates were incubated at 37°C for 4 h. The upper medium was then carefully removed, and the intracellular formazan was solubilized by adding 200 µL of dimethyl sulphoxide (Sigma) into each well. The absorbance of produced formazan was measured at 570 nm three times on the 1st, 2nd, and 3rd week.

Total collagen and glycosaminoglycan secretion assay in vitro

During three-dimensional (3D) cultures for chondrogenesis, the total amount of secreted collagen and glycosaminoglycan (GAG) from medium was analyzed in accordance with a previously described method. Collagen in the medium treated with or without TGF-β3 was determined using a Sircol collagen assay (Biocolor, Newtownabbey, Northern Ireland, UK), a dye-binding method. In detail, chondrogenic culture medium (100 µL) was mixed with 1 mL of Sircol dye reagent by shaking for 30 min to complete collagen-dye binding. After centrifugation at 10,000g for 10 min, the dye bound to the collagen pellet was then dissolved in an alkali reagent. The recovered dye concentration was measured photometrically at 540 nm and rat tail collagen (Biocolor) was used as standard curves.

The amount of sulfated GAGs in the medium was determined by a 1,9-dimethyl-methylene blue method using Blyscan kit (Biocolor) according to the manufacturer’s instruction. The same amount of medium (100 µL) was mixed with 1 mL of Blyscan dye reagent by shaking for 30 min to complete the GAG-dye binding. After centrifugation, the dye bound to GAG was dissolved in dissociation reagent. The recovered dye concentration was determined photometrically at 656 nm and chondroitin 4-sulfate standard solution (Biocolor) was used to generate standard curves. All samples and standards were tested in triplicate.

Scanning electron microscopy

The morphology of the PLGA scaffold surface and cell attachment were examined by scanning electron microscopy. MSCs/scaffold composites after being cultured for 3 weeks were taken out of the culture well and washed three times with phosphate-buffered saline (PBS). Thereafter, the samples were fixed with 3% glutaraldehyde in PBS for 24 h at 4°C. After being thoroughly washed with PBS, the samples were dehydrated sequentially in 50, 70, 95, and 100% ethanol each for 2–10 min. The fixed samples were freeze-dried, sputter-coated with gold, and examined under a scanning electron microscope (SEM, s-800, HITACHI, Tokyo, Japan).

Transplantation of rabbit MSCs/scaffold composites into rabbit knee joint cartilage defect

Seventeen New Zealand adult male rabbits (3 ± 0.5 kg body weight) aged 12 month were used. Twelve rabbits operated with TGF-β3 pretreated MSCs/scaffold composites were designated as the test group. Another five rabbits were operated using the same method but non-TGF-β3 pretreated MSCs/scaffold composites were implanted as a control group. The contralateral knee was separated as normal control knees (6 knees) and defect control knees (6 knees). On the patella groove of right femoral condyle, 6-mm diameter and 3-mm depth of osteochondral defect was made with the sharp scalp and hand drill. Having prepared lesions, prepared chondrogenic MSCs/scaffold composites were transplanted with fibrin glue (Tissed, kit, Baxter, Vienna, Austria) and inserted by press fitting method. After confirming a firm fixation, the patella was repositioned and the capsule was strictly repaired with 4-0 Vicryl while checking the graft position. Finally, the subcutaneous tissues and skin were closed with a 5-0 nylon suture. After surgery all the animals were allowed to walk freely inside the cages without a splint. All animal studies were regulated under Korea Food and Drug Administration (KFDA) animal welfare committee.

The evaluation of gross morphology

Twelve weeks after operation, all rabbits were euthanized in a CO₂ chamber. At necropsy, the left and right distal femurs were photographed, closely examined, and harvested. After harvesting, the cartilage defect sites and adjacent tissues were evaluated and scored for edge integration, surface roughness, defect fill, and color/opacity using the Brun and Moran scoring system by three blinded observers. Scores for each category were summed giving a total maximum score of 8.

Mechanical analyses

Biomechanical properties of the regenerated articular cartilage were compared with that of normal appearing
cartilage from the patella groove using an indentation test. Indentation tests were performed on whole intact joint surface using a nondestructive in situ method previously described.23,24 The distal femur of 4 cm were harvested from the experimental rabbits, and placed in PBS, pH 7.4 containing 10% protease inhibitor (Sigma) and stored at −70°C until required for testing. Tests were performed using a self-designed computer controlled soft tissue test apparatus. Specimens were placed with the articular surface facing up into a metal sample holder and bathed in PBS containing protease inhibitors to prevent any digestion of the cartilage tissue during testing.

A 0.5-mm diameter conically shaped indenter was fixed to the load cell assembly, and five consecutive displacements of 20 µm were performed on normal control samples and 30 µm on test samples. Data were recorded over a time increment of 200 s; for each step, the criterion for a complete relaxation has a relaxation rate < 100 Pa over the final 60 s. After acquisition of data, equilibrium load was measured after each stress relaxation. The Young’s modulus, E of the regenerated cartilage was determined using the equation derived by Hayes et al. (1972).24

\[ E = \frac{P(1 - v^2)}{2\pi k m} \]

where \( P \) is the measured load at equilibrium, \( v \) is the Poisson’s ratio, \( a \) is the radius of the indenter, \( u \) is the applied displacement, and \( k \) the theoretical scaling factor. Because we used a conical indenter, \( a \) was determined using the reference equation.25 Because we had insufficient tissue to measure \( v \) directly, this parameter was assigned the value 0.20, as previously reported for rabbit cartilage.26 Young’s moduli were then calculated according to the above equation using a \( k \) value derived from previously published correlations.27

Histological scoring and immunohistochemical staining

Paraffin sections of 5–6 µm were stained with hematoxylin and eosiin for morphological evaluation of tissue and with safranin-O (Sigma) to assess the proteoglycans. Specimens were scored according to a histological grading scale (O’Dricoll’s scoring system),28 composed of nine categories that allocated a total score range of 24.

Immunohistochemical reactions using mouse anticallogen type II (ICN Biomedical, Costa Mesa, CA) as the primary antibody (dilution 1:500) were performed as previously described.29 The specificity of the polyclonal antibodies was verified by staining native articular cartilage from sham operated rabbit distal femur. Negative controls were prepared by excluding the primary antibody.

Statistical method

All data are represented as mean ± SD. Experimental groups were compared with each other. Viability test, total collagen, and GAG assay test results were analyzed by repeated measures of analysis of variance. Morphological, histological, and mechanical data were analyzed using one way analysis of variance. Significance was accepted for \( p \) values < 0.05.

RESULTS

Viability during chondrogenesis in vitro

The cell cultures in the PLGA scaffold were examined weekly from the first seeding until 3 weeks of culture. Week 0 data refers to the initial number of cells attached to the PLGA scaffold during the seeding procedure, and cell viability during the culture was determined assuming the initial number of cells as 100%. Percentage of cell viability ranged from 90 to 100%, and showed no significant difference in respect to culture time (\( p = 0.8160 \)) and two groups (\( p > 0.05 \)); ➲: (+) TGF-β, ➲: (−) TGF-β.

Collagen and GAG secretion in chondrogenesis in vitro

We observed the in vitro production of cartilage specific extracellular matrix (ECM) by MSCs/scaffold composites in chondrogenic medium. We measured the total amount of collagen and GAG in 1 mL of culture medium, and compared the variance in the amount during the culture. Collagen secretion from the medium of MSCs/scaffold composites was detected after 9 days of 3D culture. Collagen levels increased until 21 days and then plateaued [\( p < 0.05 \), Fig. 2(A)]. GAG appeared later than collagen
after 18 days and reached a maximum after 24 days \([p < 0.05, \text{Fig. } 2(\text{B})]\). Thus we found that GAG and collagen production peaked after 3 weeks of 3D culture \(\textit{in vitro}\), while the amount of GAG under the control medium was below the detectable range.

**Gene expression in chondrogenesis \(\textit{in vitro}\)**

RT-PCR was used to quantify the amounts of collagen type II and aggregan expression by the TGF-\(\beta\)3 after 5, 7, 11, 14, and 21 days of 3D culture. The RT-PCR results showed that collagen type II and aggregan mRNAs were well expressed after 5, 7, 11, 14, and 21 days in chondrogenic MSCs (Fig. 3). Aggre
can mRNA was up regulated after 5 days in PLGA scaffold culture. The type II collagen gene showed a more gradual increase and was readily detected after 5 days in PLGA scaffold culture. As in Figure 3, due to the lack of aggregan and collagen type II mRNA expression, chondrogenesis was not initiated under the control medium without TGF-\(\beta\)3. Therefore, based on \(\textit{in vitro}\) results, MSCs/scaffold composites leading to chondrogenesis by pretreatment with TGF-\(\beta\)3 for 3 weeks were transplanted for \(\textit{in vivo}\) study.

**Scanning electron microscope**

The pore size of 75:25 PLGA-scaffold was 300–500 \(\mu\)m with a final measured porosity of 98–99% [Fig. 4(A)]. Prior to transplantation of the MSCs/scaffold composites to the rabbit knee, SEM was performed to observe even distribution of MSCs with the secretion of ECM. After 3 weeks of culture, MSCs were well attached and evenly distributed around the scaffold, and MSCs in scaffold pore with ECM were also observed [Fig. 4(B,C)].

**Gross morphology after transplantation**

Twelve weeks after operation, all of the 16 transplanted rabbits survived. One of the nontreated control group rabbits died 2 weeks after operation. In TGF-\(\beta\)3 treated group, 10 of the 12 rabbits showed cartilaginous regeneration (success rate = 83.4%), while the other two rabbits showed no cartilaginous regeneration. In the non-TGF-\(\beta\)3 treated group, all survived rabbits showed cartilaginous like regeneration. All rabbits were euthanized and distal femurs of 4 cm including knee joint were obtained. The following analysis was conducted on knees showing successful cartilage regeneration. The gross morphology for the successfully regenerated tissues was evaluated. The score of TGF-\(\beta\)3 pretreated group averaged 7.3 ± 0.6 and non-TGF-\(\beta\)3 treated group averaged 5.5 ± 1.7 out of a total of 8.0 ± 0 in normal cartilage \((p = 0.1274)\). Grossly, both groups showed similar articular cartilage appearance like the normal group and showed no statistical significance in gross morphology score [Fig. 5(A,B)].

The defect control group had a score of 1.6 ± 0.6, which was significantly inferior to that of the regenerated cartilage group \((p < 0.0001)\) [Fig. 5(C)]. The regenerated cartilage by MSCs/scaffold composites showed smooth, flush, and transparent hyaline-like cartilage 12 weeks after operation. Continuity with neighboring cartilage was intact in most cases. But, in the defect control group, arthritic changes were evident and defects remained depressed and covered with fibrous tissue.
Histological scoring and immunohistochemical staining

In most cases of TGF-β3 treated group, regenerated cartilage showed longitudinal cell arrangement with lacunae formation without inflammatory changes (Fig. 6). In addition, they healed continuously at the interface with the surrounding normal cartilage. Attachment to the subchondral bone was also intact. However, the regenerated cartilage showed some defect of detachment from subchondral bone and cell clustering in some particular portions. In special staining, the regenerated cartilage was homogeneously stained by safranin-O and showed expressions of type II collagen in immunohistochemical staining similar to normal rabbit articular cartilage.

In most cases of non-TGF-β3 treated group, regenerated cartilage showed lacunae formation but arrangement of cells was not longitudinal like TGF-β3 treated group (Fig. 7). The interface healing was as intact as TGF-β3 treated group. The safranin-O staining of regenerated cartilage was not as good as TGF-β3 pretreated group and type II collagen in immunohistochemical staining was heterogenous in regenerated cartilage compared to TGF-β3 treated group. Therefore, we assumed that defects were filled with hyaline like cartilage in TGF-β3 treated group. In the non-TGF-β3 treated group, defects were filled with mixture of hyaline and fibrous cartilage like tissue. Specificity of type II collagen antibodies was verified by staining native articular cartilage from the normal control group.

Defect control group showed noncartilaginous structure and was almost filled with fibrous tissue and inflammatory changes without expression of GAG and type II collagen.

The histological score of the TGF-β3 pretreated group was 21.6 ± 1.5, which was less than that of the normal cartilage (24.0 ± 0.0) but there was no statistical significance (p = 0.0572). In non-TGF-β3 treated group, the score was 20.5 ± 1.6, which was less than that of normal cartilage and was statistically significant (p < 0.05). Therefore, regenerated cartilage in the TGF-β3 pretreated group was more similar to normal cartilage in our experiment. But the defect control group scored an average of 14 ± 5.2, which was significantly less than the transplanted group (p < 0.0001).

Indentation test

Normal cartilage thickness of nonoperated rabbits in the patellofemoral groove area was measured under the microscope to determine the depth of maximal strain. Mean cartilage thickness of the normal patellofemoral groove was measured as 0.44 ± 0.01 mm. We decided to use a 20 μm of one step displacement (a depth of about 5% of cartilage thickness) and a 100 μm maximum displacement for testing the normal cartilage. In the case of the regenerated articular cartilage, we could obtain an average 0.7-mm thickness of regenerated cartilage 12 weeks after operation in the preliminary study. Therefore, we decided to use 30 μm of one step displacement and 150 μm maximum displacement.

The mean thickness of regenerated cartilage in the center and interface area was 0.83 ± 0.28 mm and 0.71 ± 0.10 mm, respectively. In TGF-β3 pretreated group, value of the calculated Young’s modulus was 0.73 ± 0.05 MPa for the central area and 0.52 ± 0.25 MPa for the interface area. In non-TGF-β3 treated group, value of calculated Young’s modulus was...
1.37 ± 0.45 MPa for the center area and 2.54 ± 1.37 MPa for the interface area. Young’s modulus of the normal cartilage of the patellofemoral groove was 0.89 ± 0.04 MPa. Comparing the mechanical property of regenerated cartilage of each group with normal cartilage group, no statistical significance was

Figure 5. Gross morphology of distal femoral joint harvested at 12 weeks after transplantation. In both groups (A: TGF-β3 pretreated group, B: non-TGF-β3 treated group), gross examination shows that cartilage defects were filled smooth and transparent cartilage like tissue. Edge integration to normal cartilage tissue was full and no defects were found between the normal and repaired cartilage (black arrow). No arthritic changes were found around the joint area. However, arthritic changes were dominant in defect control group (C). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 6. Histology of tissues harvested at cartilage defects after 12 weeks posttransplantation in TGF-β3 pretreated group. A typical structure of hyaline cartilage was examined with the lacunae formation and longitudinal arrangement of cells (A, B, C). Type II collagen was well expressed in the regenerated cartilage comparing with the adjacent normal cartilage (D). Arrow demonstrated some fissures or defects in the repaired tissue (A) but, continuity to the subchondral bone was well preserved. N, normal cartilage; R, regenerated cartilage. H&E staining (A, B, C), and immunohistochemical staining for type II collagen (D); original magnification, ×50 (A, D), ×100 (B), ×400 (C). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
observed in the Young’s moduli of the center of the regenerated articular cartilage in TGF-β3 pretreated group \( (p = 0.2920) \). However, statistical significance was observed between the normal and non-TGF-β3 treated group \( (p < 0.05) \). In respect to the interface area, statistical significance was observed in both groups \( (p < 0.05, \text{for both}) \). Therefore, the TGF-β3 pretreated group is closer to normal cartilage in its mechanical property, even though interface healing was insufficient. Compared to the measured normal Young’s modulus of patellofemoral groove, the regenerated articular cartilage in the TGF-β3 pretreated group shows about 80% of mechanical property (Fig. 8).

**DISCUSSION**

Current tissue engineering procedures focused on the delivery or in situ mobilization of capable cells to restore pathologically altered architecture and the function of tissues. This approach comprises the interactive triad of responsive cells, a supportive matrix template, and bioactive molecules promoting differentiation and regeneration of the tissue structure.\(^{30,31}\) Based on these concepts, many studies have been conducted using MSCs, polymeric scaffold, and TGF-β superfamily in vitro. The transplantation of sufficient cells with or without scaffold is an important factor for tissue engineering because it affects the cell phenotype and the quality of the regenerated cartilage. But using chondrocyte as a cell source carries several problems such as the small number of available cells, the morbidity at the donor site, and the limited ability of the harvested cells to proliferate and undergo differentiation.\(^{32}\) However, MSCs are well characterized by their ability to proliferate in culture and can differentiate into multiple mesenchymal lineages under defined culture conditions. Also, MSCs are easily expanded while maintaining their phenotype and obtained from bone marrow of various sites.\(^{33}\)

Previous studies involving the transplantation of progenitor cells into a cartilage defect consistently failed to produce hyaline-like cartilage.\(^{34-36}\) The rationale for these studies was that the in vivo environment should provide appropriate signals for differentiation of transplanted cells; however, it is possible that the signals that are present during the development and growth of tissues such as articular cartilage are no longer present in adults. For successful production of articular cartilage, it may be necessary to provide these cells with signals such as TGF-β, or related bioactive factors, to promote their differentiation.\(^{36}\)

Although sufficient signals necessary for cartilage differentiation do not exist in adults, MSCs are able to differentiate into different kinds of mesenchymal cells in vitro. The transplantation of the pluripotent MSCs for cartilage regeneration in articular cartilage-subchondral bone defect can lead MSCs to differentiate into unnecessary cells such as osteoblast and adipocyte like cells which in turn leads to a relative decrease in chondrocyte-like cells needed for cartilage regeneration. We reasoned that the obstacle could be overcome by pretreating with TGF-β3 in vitro prior to the transplantation, which might commit the MSCs to prechondrogenitor cell or chondrocytes and yield adequate chondrocyte-like cell numbers in transplanted site. Therefore, improvement in the efficacy of MSCs differentiation into the chondrocyte like cells is necessary for successful cartilage regeneration. In our results, chondrogenesis under the control medium without TGF-β3 was not induced in vitro, and transplantation of undifferentiated cells to cartilage defect has not been shown to regenerate hyaline cartilage formation. This phenomenon was also found in the previous study.\(^{35}\) Even though some reports indicated the usefulness of transplantation with MSC alone for cartilage regeneration, we could not find such a well regenerated cartilage in the non-TGF-β3 treated group.

Therefore, many different methods have been suggested such as gene transfer, joint injection, and pretreatment for the delivery of chondrogenic factors to the cells.\(^{37-39}\) In our study, MSCs were pretreated with TGF-β3 in vitro and induced to chondrocytes like cells prior to transplantation. This was confirmed by checking the expression of type II collagen and aggrecan gene in RT-PCR and GAG secretion in the culture medium. However, the direct use of such cytokines in human is difficult at present because there are many safety issues remaining and its efficiency must be determined before clinical implementation. Therefore, for in vitro pretreatment, our method has its advantage in clinical application.

Another factor determining the success of cartilage regeneration is the selection of scaffolds. These scaffolds permit the 3D immobilization of cells and maintain the differentiated phenotype of cells.\(^{40}\) In the ACT without scaffold, the transplanted chondrocytes might accumulate on one side of the defect, mainly as a result of gravity, joint movement, and unequal distribution, even if a large number of cells did not leak from the grafted site. This may lead to uneven regeneration of cartilage defect and poor interface healing.\(^{2}\) However, in our study, even cell distribution around the center and boundary of scaffold was confirmed before transplantation under SEM examination and this might lead to good graft integration to the defect site. On gross examination, engineered cartilage was similar to the adjacent normal cartilage in color, transparency, and smoothness at
Figure 7. Histology of tissues harvested at cartilage defect after 12 weeks posttransplantation in non-TGF-β3 treated group. Regenerated cartilage showed lacunae formation but arrangement of cells was not longitudinal like TGF-β3 treated group (A, B, C). Type II collagen in immunohistochemical staining was heterogenous in regenerated cartilage compared to TGF-β3 treated group (D). Positive staining of Type II collagen was found only in peripheral area of transplanted region (black arrows). H&E staining (A, B, C), and immunohistochemical staining for type II collagen (D); Original magnification, ×50 (A, D), ×100 (B), ×400 (C). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 8. Young’s Modulus of regenerated cartilage. No statistical significance was observed in Young’s moduli of the center area of regenerated articular cartilage between the normal and TGF-β3 pretreated group. The regenerated articular cartilage showed about 80% of mechanical property of normal cartilage (A). The mechanical properties of interface region in both groups were inferior to the normal articular cartilage (B). Custom made micro indentational test equipment was used for measuring the Young’s modulus of regenerated articular cartilage (C). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
the joint surface with no arthritic change. On histological examination, good interface healing was observed between the engineered cartilage and the adjacent normal cartilage without remaining defects.

Alginate, hyaluronic acid, collagen, and chitosan have been used for cartilage regeneration\(^1\)\(^4\),\(^2\) however, their mechanical behavior was not sufficient for musculoskeletal tissue. For cartilage, a suitable scaffold should have adequate physical properties, enough to compensate for the initial mechanical qualities of cartilage during cell proliferation and differentiation.\(^3\) PLGA scaffold is one of the solid bioresorbable scaffolds for musculoskeletal tissue engineering, being used to achieve initial biomechanical stability.\(^4\)

Yet, it is hard to determine whether our PLGA scaffolds have the ideal properties for cartilage regeneration, because various physical and chemical properties of scaffolds should be determined to examine the growth of cells and the formation of ECM.\(^5\)

Factors for ideal scaffold are as follows: stable fixation, congruence between the scaffold and the cartilage defect, proper absorbability, and biocompatibility.\(^6\) The stability of the implanted scaffold was achieved by press fit and fibrin glue in this study. In some cases, gross examination showed excellent regenerated cartilage; however, microscopic examination revealed detachment from the subchondral bone, cartilage defects, cell clustering, and irregularity in the cartilage surface compared to normal cartilage. This might be caused by incongruence of the scaffold to cartilage defect, improper absorbability, and tissue incompatibility. These observations indicated that the determination of the most suitable scaffold should be continued.

For the evaluation of the mechanical property of the cartilage, the indentation test was used. A known standardized sample was tested prior to the experiment samples. In an unconfined indentation test, the cartilage depth is an important factor, because this determines the factor \(k\) and the depth of displacement. For exact calculations, the thickness of each indented point should be directly measured and the test performed according to this data. However, the thickness of the cartilage could not be measured before the indentation test in our experiment. Despite this obstacle, the preliminary data have shown that the repaired cartilage at 12 weeks after transplantation was about 0.7 mm; therefore, 150 \(\mu m\) and 20% were chosen as the values for the total thickness and displacement of the repaired cartilage. Also these values were recommended in other studies.\(^7\)\(^,\)\(^8\) Therefore, the variance was only small, even when the data of cartilage thickness were applied after measuring the load and displacement.

Hidaka et al. showed that Young’s modulus of the regenerated cartilage was about one tenth to a half of the normal cartilage in their repairing cartilage defect by the method of gene transfer to transplanted chondrocyte without scaffold.\(^9\) This inferiority in mechanical property might be due to not using the scaffold, one of the important factors for cartilage tissue engineering. However, both Schaefer et al.\(^10\) and our results showed the importance of scaffolds. The mechanical property of the regenerated cartilage was almost equal to normal tissue, in Schaefer et al.’s study, and 80% of the normal tissue, in our results. The factors responsible for our mechanical results such as cytokine, the type of scaffold and cell sources are under investigation.

**CONCLUSION**

We have successfully regenerated hyaline-like cartilage in knee joint osteochondral defect 12 weeks after transplantation of a chondrogenic MSCs/PLGA scaffold composite in vivo rabbit model. This regenerated cartilage showed similar histological and mechanical properties to normal cartilage. Histological examination demonstrated good interface healing and subchondral bone attachment. Longitudinal cell arrangement with lacunae formation was also demonstrated with the expression of GAG and type II collagen. Mechanically, it showed about 80% of Young’s modulus of normal cartilage in the indentation test. These might result from (1) transplantation of sufficient MSCs, (2) increased the efficacy of MSCs differentiation into the chondrocyte like cells by TGF-\(\beta\) prior to transplantation, and (3) usage of PLGA scaffold that provided even cell distribution and initial mechanical stability in the transplanted site.

Thus, we determined the feasibility of treating damaged articular cartilage using MSCs committed to chondrocyte by TGF-\(\beta\) with the PLGA scaffold and suggest this treatment modality as another concept of cartilage tissue engineering.

**References**


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