


Long-Term Results of Cartilage Repair after Allogeneic Transplantation of Cartilaginous Aggregates Formed from Bone Marrow-Derived Cells for Large Osteochondral Defects in Rabbit Knees

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Abstract

Objective: The purpose of this study was to evaluate the long-term results of cartilage repair after allogeneic transplantation of cartilaginous aggregates formed from bone marrow-derived cells. **Methods:** Bone marrow cells were harvested from 12-day-old rabbits. The cells were subjected to a monolayer culture, and the spindle-shaped cells attached to the flask surface were defined as bone marrow-derived mesenchymal cells. After the monolayer culture, a 3-dimensional cartilaginous aggregate was formed using a bioreactor with chondrogenesis. We created osteochondral defects, measuring 5 mm in diameter and 4 mm in depth, at the femoral trochlea of 10-week-old rabbits. Two groups were established, the transplanted group in which the cartilaginous aggregate was transplanted into the defect, and the control group in which the defect was left untreated. Twenty-six and 52 weeks after surgery, the rabbits were sacrificed and their tissue repair status was evaluated macroscopically (International Cartilage Repair Society [ICRS] score) and histologically (O'Driscoll score). **Results:** The ICRS scores were as follows: at week 26, 7.2 ± 0.5 and 7.6 ± 0.8 ; at week 52, 7.6 ± 1.1 and 9.7 ± 0.7 , for the transplanted and control groups, respectively. O'Driscoll scores were as follows: at week 26, 12.6 ± 1.9 and 10.1 ± 1.9 ; at week 52, 9.6 ± 3.0 and 14.0 ± 1.4 , each for transplanted and control groups, respectively. No significant differences were observed between the groups. **Conclusions:** This study demonstrates that allogeneic transplantation of cartilaginous aggregates formed from bone marrow-derived cells produces comparable long-term results based on macroscopic and histological outcome measures when compared with osteochondral defects that are left untreated.

Keywords

bone marrow-derived cells, cartilage repair, allogeneic transplantation, long-term study, animal model

Introduction

Articular cartilage injuries have a limited potential to heal because they are avascular¹ and, thus, over a period of time may lead to secondary osteoarthritis.² Mesenchymal stem cells (MSCs) are capable of differentiating into multiple cell lineages and are useful for regenerative medicine.³ Therefore the transplantation of MSCs is currently being investigated as a potential treatment for cartilage repair in animal and clinical studies.⁴ Previously, our research group established a 3-dimensional (3D) cell culture technique for the formation of cartilaginous aggregates from rabbit⁵ and human⁶ bone marrow-derived cells using a bioreactor without any scaffold. Using these aggregates, we successfully regenerated hyaline-like cartilage for up to 12 weeks after allogeneic transplantation for rabbit osteochondral defects.⁷ Furthermore, we tracked the transplanted cells in reparative

tissues for up to 26 weeks using our labeling technique.⁸ The results suggested that the transplanted cells indeed contributed to not only chondrogenesis but also osteogenesis and stromagenesis.

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Although this innovative approach to the repair of osteochondral defects has demonstrated positive results, the long-term outcomes are unknown. The objective of the present study was to evaluate the long-term results of cartilage regeneration following allogeneic transplantation of cartilaginous aggregates formed from bone marrow-derived cells into the osteochondral defects of rabbit knees.

Methods

Cell Culture and Formation of 3-Dimensional Cartilaginous Aggregates

This study was conducted under the protocol approved by the laboratory animal resource center, University of Tsukuba (ID: 07-323). Bone marrow-derived cells were collected from the femurs, tibias, and humeri of 12-day-old Japanese white rabbits. The femurs and tibias were removed aseptically, cleaned of soft tissues, and washed 3 times in phosphate-buffered saline with antibiotics. Then both ends were removed from the epiphyses, and the marrow was flushed out from the modularly cavity using 20 mL of standard medium. Next, the cell suspension was distributed through 30 T-75 culture flasks (BD Biosciences, Franklin Lakes, NJ) with 15 mL of a standard medium and then cultured in a humidified atmosphere containing 95% air and 5% CO₂. The standard medium consisted of Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (Sigma) and antibiotics (antibiotic-antimycotic; Gibco BRL, Gaithersburg, MD). Approximately 3 weeks after the seeding, the adherent cells grew confluent, at which time they were subcultured by trypsinization, and then resuspended in a rotating medium. The rotating medium consisted of Dulbecco's modified Eagle's medium containing 50 mg/mL of ascorbic acid (WAKO, Osaka, Japan), 40 mg/mL of L-proline, ITS culture supplement (BD), 10⁻⁷ M dexamethasone (Sigma), 10 ng/mL of TGF-β3 (Sigma), and antibiotics (antibiotic-antimycotic; Gibco BRL). Then, 10 mL of the cell suspension (1.5-3.0 × 10⁷ cells) was seeded into the rotating wall vessel bioreactor (RCCS-4D system with 10-mL disposable vessels; Synthecon Inc, Houston, TX) in a CO₂ incubator, and the rotator culture was performed for 1 week.³

Animal Model

All procedures were performed under general anesthesia induced by intramuscular injections of ketamine (1.0 mL/kg body weight) and xylazine (0.4 mL/kg body weight). Forty-nine female 10-week-old Japanese white rabbits (Tokyo Experimental Animals, Tokyo, Japan), weighing an average of 2.0 kg, were used. The left knee was opened via a medial parapatellar approach and the patella was dislocated laterally to expose the patellar groove of the femur. A

hand drill, with depth-controlled bits, was used to create cylindrical defects 5 mm × 5 mm in area and 4 mm in depth along the midline of the patella groove.⁷ The defects were randomized to the following 2 groups: (1) empty defects (control group: group C, *n* = 25) and (2) 3D cartilaginous aggregates into the defects without any flap covering (transplanted group: group T, *n* = 24). The animals were caged and allowed to move freely without any splinting after recovery before finally being sacrificed at 26 and 52 weeks after surgery.

Macroscopic Evaluation

The animals were sacrificed by injecting them with a lethal dose of pentobarbital sodium. At necropsy, the defects were examined macroscopically, and photographed. The International Cartilage Repair Society (ICRS) macroscopic scoring was used to assess the macroscopic appearance of the repair tissue. This score ranges from 0 to 12. The score included semiquantitative scales of defect filling, integration to native cartilage and repair tissue surface topography.⁹

Histological Evaluation

At sacrifice, the upper half of the reparative tissue was cut along the coronal plane from the distal femur and fixed by microwave in 4% paraformaldehyde and 1% glutaraldehyde in phosphate-buffered saline for 4 days. After decalcification in 0.5 M ethylenediamine tetraacetic acid and 0.1 M Tris and NaOH for 4 weeks, the samples were embedded in paraffin, and sectioned (5 mm) with a microtome. Using the sections obtained at 26 and 52 weeks in each group, we assessed the central one third of the reparative tissue. A histological evaluation was performed using the sections with safranin-O and fast green staining and graded using the O'Driscoll scoring system.⁷ All sections were observed under a BX51 microscope (Olympus, Tokyo, Japan) equipped with a CCD camera.

Statistical Analysis

The macroscopic and histological results were analyzed by 2 orthopedic surgeons who were blinded to the treatment groups. The data are presented as the mean and standard deviation. A plot of the data was indicated the need for non-parametric testing. Steel-Dwass tests were used to make statistical comparisons. *P* values <0.05 were considered to be significant.

Results

Seven rabbits in group C and 5 rabbits in group T died from pneumonia during the perioperative period. Therefore, we were able to study 18 rabbits in group C and 19 rabbits in

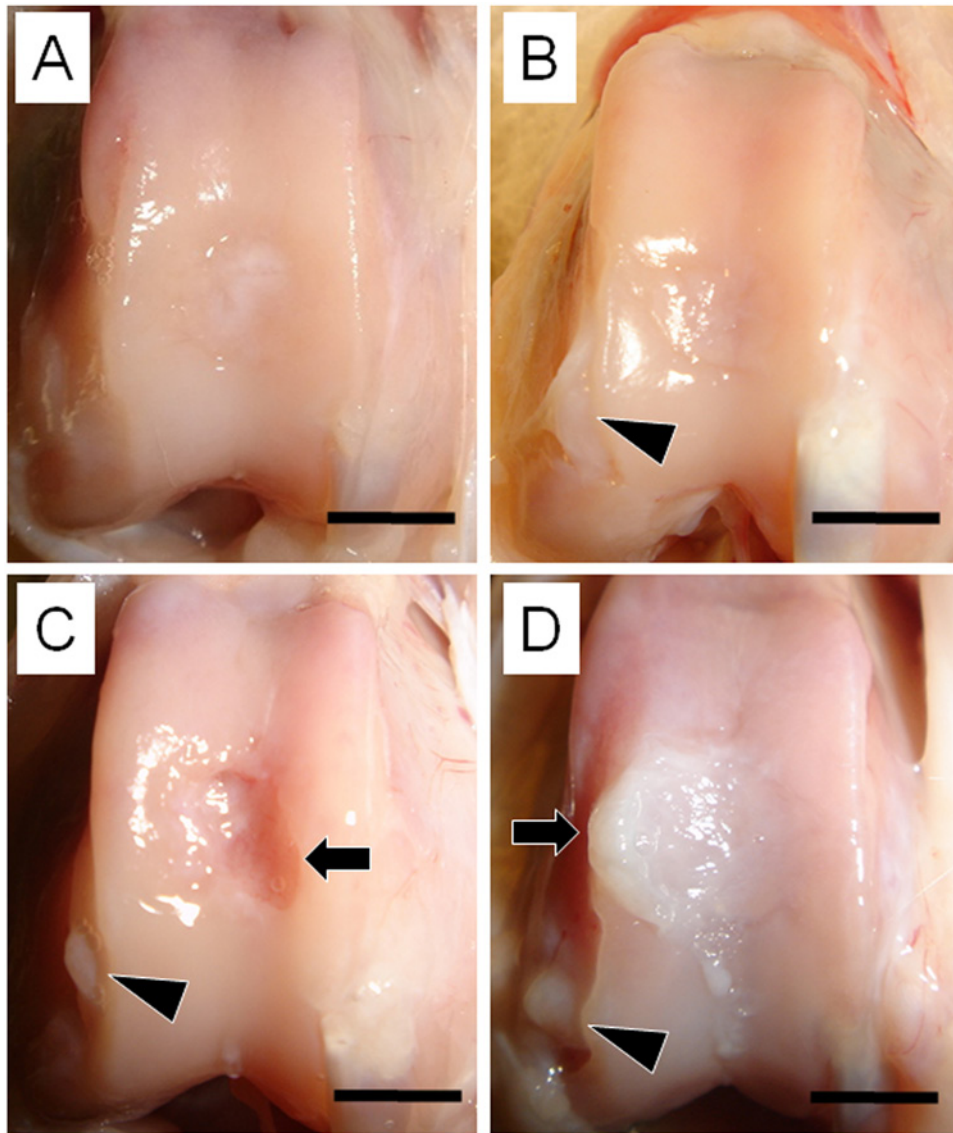


Figure 1. Representative macroscopic appearances of reparative tissue in Group C (**A, C, D**) and Group T (**B**) at 52 weeks after surgery. The arrowhead indicates osteophytes (**B, C, D**). The arrow indicates reparative tissue with a depression (**C**) and hypertrophy (**D**). Bar = 5 mm.

group T. Hence, the total number of rabbits used in this study was 37 (group C at 26 weeks, $n = 7$; 52 weeks, $n = 11$ and group T at 26 weeks, $n = 12$; 52 weeks, $n = 7$).

Macroscopic Findings

Neither joint contractures nor infection were observed in any of the rabbits. The typical gross appearance of the rabbits in groups C and T at 52 weeks is shown in **Figure 1A** and **B**. Osteophytes were present near the medial side of the

femoral condyle at the junction of the patellar and tibial articular area, in 1 (14%) of 7 knees at 26 weeks and 5 (45%) of 11 knees at 52 weeks in group C and 2 (25%) of 12 knees at 26 weeks and 3 (42%) of 7 knees at 52 weeks in group T. The regenerated tissue was irregular with depressions in 4 (57%) of 7 knees at 26 weeks and 3 (27%) of 11 knees at 52 weeks in group C (**Fig. 1C**) and 2 (18%) of 12 knees at 26 weeks and 3 (43%) of 7 knees at 52 weeks in group T. The regenerated tissue in the defects of the remaining knees was smooth and resembled articular cartilage. On

Table 1. International Cartilage Repair Society (ICRS) Macroscopic Scoring and the Histological Scores of the Reparative Tissue Using the O'Driscoll Grading Scale in Groups C and T.

Score/Group	26 Weeks		52 Weeks	
	Group C	Group T	Group C	Group T
Macroscopic	7.6 ± 0.8	7.2 ± 0.5	9.7 ± 0.7	7.6 ± 1.1
Histologic	10.1 ± 1.9	12.6 ± 1.9	14.0 ± 1.4	9.6 ± 3.0

^aThere were no statistically significant differences between the groups.

the other hand, hypertrophy of regenerated tissues was observed in 2 (18%) of 11 knees at 52 weeks in group C (**Fig. 1D**) and 1 (8%) of 12 knees at 26 weeks in group T. The ICRS macroscopic scores were as follows: at 26 weeks, 7.6 ± 0.8 ($n = 7$) and 7.2 ± 0.5 ($n = 12$) for groups C and T, respectively; at 52 weeks, 9.7 ± 0.7 ($n = 11$) and 7.6 ± 1.1 ($n = 7$) for groups C and T, respectively (**Table 1**). No significant differences were found between the groups.

Histological Findings

Despite the fact that the macroscopic appearance of the regenerated tissue resembled articular cartilage, the histology showed fibrous cartilage and fibrous tissue in both groups (**Fig. 2A** and **B**). Interestingly, there was a decrease in safranin-O staining and degenerative changes at the medial side (**Fig. 2C**) of the adjacent normal cartilage compared with that observed at the lateral side (**Fig. 2D**) in almost all the knees in both groups.

The O'Driscoll scores were as follows: at 26 weeks, 10.1 ± 1.9 and 12.6 ± 1.9 for groups C and T, respectively; at 52 weeks, 14.0 ± 1.4 and 9.6 ± 3.0 for groups C and T, respectively (**Table 1**). No significant differences were found between the groups.

Discussion

The most important finding of this study is that in the evaluation of macroscopic and histologic scoring, there were no significant differences in cartilage repair between the nontreated and transplanted group up to 52 weeks. Previously, we demonstrated that this therapeutic strategy induces hyaline-like cartilage repair up to 12 weeks. The results showed that the histological scoring was significantly better in the transplanted group than in the nontreated group at 4 and 8 weeks, whereas at 12 weeks, the mean score of the transplanted group was not significantly different from that of the nontreated group. This long-term study showed that the repaired hyaline-like cartilage gradually degenerated up to 52 weeks.

There are 3 possible explanations for this result. First, this study is based on allogeneic transplantation without

the use of immunosuppressive therapy. Although there was no obvious evidence of inflammation in the whole knee joints or mononuclear cell infiltrates suggestive of host-versus-graft reaction during these evaluation periods, there is a possibility that transplanted cartilaginous aggregates are gradually rejected by the host because of chronic immunogenic reactions. Recently, MSCs have been shown to modulate major histocompatibility complex-mediated immune responses through several modalities¹⁰⁻¹²; however, in this study, we did not purify MSCs prospectively but collected heterogeneous populations of bone marrow-derived cells. Consequently, cartilaginous aggregates were formed using a 3D culture with chondrogenesis from proliferated adherent spindle-shaped cells and self-produced extracellular matrix (ECM). It is unclear how much the phenotype of the cells constituting the cartilaginous aggregates differentiated into chondrocytes from MSCs or how much major histocompatibility complex class II antigens they contained during the transplantation and repair process. Furthermore, a large amount of clinical and experimental evidence exists suggesting that an immune response is elicited when isolated allogeneic cells are implanted into cartilage defects in the absence of any ECM.¹²⁻¹⁴ The presence of ECM is believed to form a protective barrier around the allogeneic cells that blocks both the infiltration of host immune cells into the graft and the escape of immunogenic allogeneic cells out of the graft. On the other hand, according to previous allogeneic chondrocyte transplantation studies, both chondrocytes and their embedded ECM contain antigens that can be immunogenic.^{12,15,16} In this study, we transplanted not only allogeneic cells but also self-produced abundant ECM as cartilaginous aggregate. While it is also uncertain how much the self-produced ECM exhibits antigenicity, the repaired tissue might be rejected from the host by immune reactions, not acutely but chronically. Nonetheless, future studies are needed to fully understand the immunogenic characteristics of the cells and ECM of transplanted cartilaginous aggregates and to optimize exactly how much chondrogenesis of MSCs and matrix accumulation is necessary to impede this immunogenicity. Certainly, there is another option to use immunosuppressive therapy in the same protocol. Second, the cartilage repair used in this study is based on an osteochondral defect model measuring 5 mm in diameter and 4 mm in depth. Hyaline articular cartilage has only limited self-regenerative abilities. This ability for self-repair has been shown to be somewhat modulated by the size, location, and depth of the cartilage lesion.¹⁷ Cartilage defects are classified into 3 types as follows: (1) a partial-thickness cartilage defect is a defect that does not fully penetrate through the cartilage layer, (2) a full-thickness defect penetrates through the entire cartilage thickness but not into the underlying subchondral bone, and (3) an osteochondral defect penetrates through the entire cartilage thickness and into the underlying bone. Any size defect, especially the one penetrating

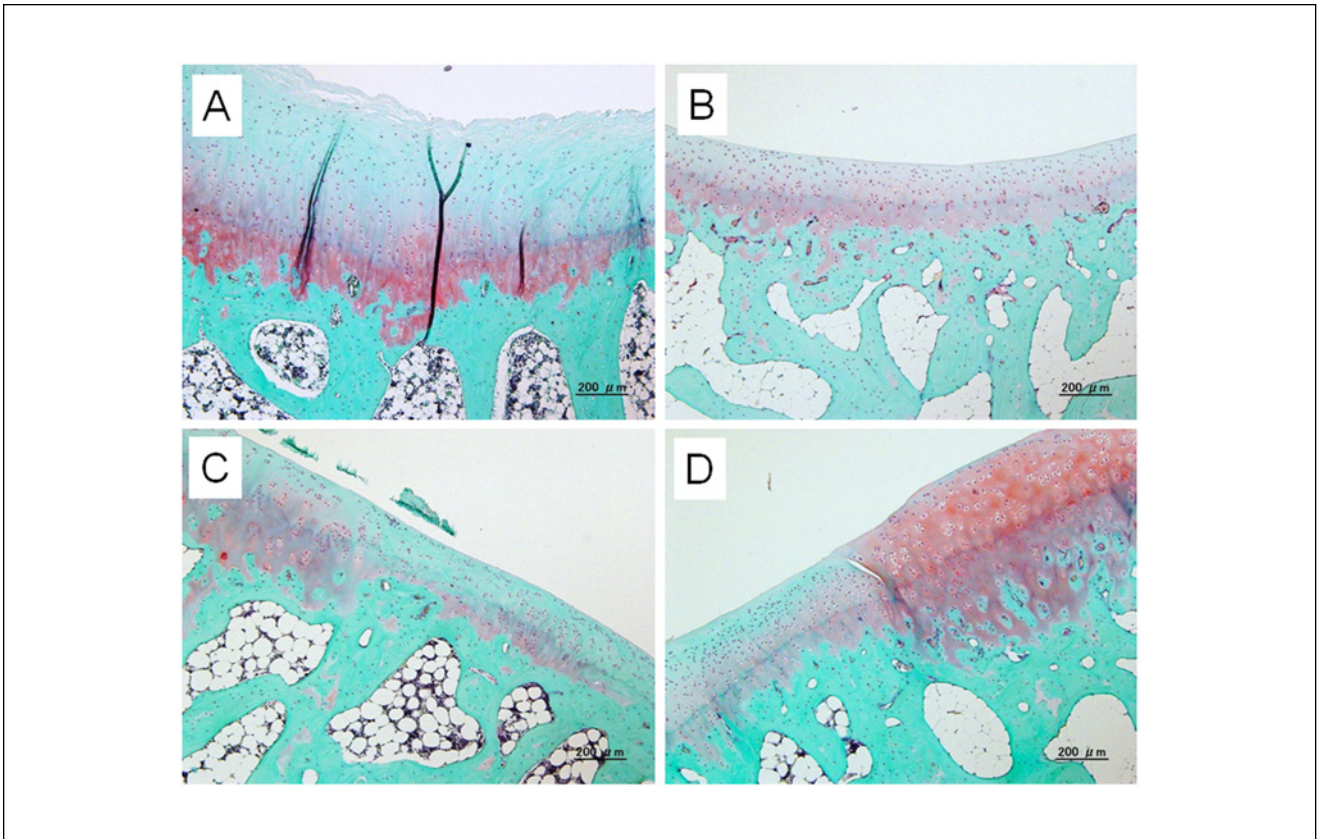


Figure 2. Histological photomicrographs of reparative tissue in Group C (**A**) and Group T (**B, C, D**). safranin-O staining was weaker or absent, indicating the absence of glycosaminoglycan (**A-D**) and decreased at the medial side of the adjacent normal cartilage compared to that observed at the lateral side.

into the subchondral bone, is repaired with fibrous tissue that is inferior to normal cartilage.¹² According to this definition, it is suitable to use not osteochondral defects but full-thickness cartilage defects for the next step to achieve clinical relevance. Third, it is important to ensure the phenotypic stability of cartilaginous aggregates before and after transplantation for the long-term survival of the reparative tissue as hyaline cartilage. O'Driscoll¹⁸ demonstrated that the hyaline cartilage that is produced by free autogenous periosteal grafts in full-thickness osteochondral defects is capable of withstanding a full year of the articular function without exhibiting marked deterioration, in rabbits that are treated with continuous passive motion postoperatively. Following this result, it is necessary not only to transplant cartilage aggregates but also to control the biomechanical environment of the knee joint postoperatively in order to maintain the characteristics of the repaired hyaline-like cartilage over a long period of time. In addition, interestingly, there was a formation of osteophytes near the medial side of the femoral condyle at the junction of the patellar and tibial articular area and a decrease in safranin-O staining and degenerative changes at the medial side of the

adjacent normal cartilage in the knees. These observations might be related to medial parapatellar capsular incision exposure in the knee joint.¹⁹ This result suggests that it would be better to treat cartilage lesions using arthroscopy as much as possible in the clinical setting.

In conclusion, the allogeneic transplantation of cartilaginous aggregates formed from bone marrow-derived cells for large osteochondral defects in rabbit knees contribute to moderately successful short-term cartilage repair, whereas the long-term results may be less promising. Further studies are needed to verify the efficacy of this cartilage repair modality in an autologous model, focusing on larger animals with more clinical relevance, and to optimize the phenotypic characteristics of the cells and ECM constituting the cartilaginous aggregates in order to achieve desired cartilage repair outcomes.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

This study was approved by our institutional review board.

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