［原著］Repair of Osteochondral Defects in Rat Knees with HumanBone Marrow Mesenchymal Stem Cell

Repair of Osteochondral Defects in Rat Knees with Human Bone Marrow Mesenchymal Stem Cells

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ABSTRACT

Articular cartilage (hyaline cartilage) has only a limited capacity for intrinsic healing. Human mesenchymal stem cells (MSCs) from bone marrow are useful candidate for cell sources in tissue engineering, since MSCs have the capacity to differentiate into various tissues. In this study, the pellets containing hyaline like cartilage differentiated from MSCs were transplanted in rat knee joints in an attempt to repair full-thickness osteochondral defect. MSCs, which were obtained by isolating from human bone marrow, were centrifuged and the resultant pellet was cultured in a medium containing various concentration of TGF-beta1 in the presence or absence of fetal bovine serum (FBS) for 2 weeks. As the results, the pellets cultured under a condition with 10 ng/ml TGF-beta1 and absence of FBS contained significant hyaline like cartilage that were evaluated histologically. The pellets cultured under this condition were transplanted into the osteochondral defect in the patellar groove of athymic rat knee joints. The transplanted pellets were covered with fibrin glue (pellet group), whereas the osteochondral defect filled only with fibrin glue was used as a control (control group). At 4, 8, 16, 24 weeks after the treatment, we performed macroscopic comparison, histological evaluation (toluidine blue, Mankin's score) and fluorescence in situ hybridization (FISH) of human X and Y chromosomes. The macroscopic findings of the pellet group at 24 weeks showed shiny and smooth surface, whereas the control group showed rough surface and subsided. After the treatment of osteochondral defect with pellets, the depth of the defect became thinner sequentially but surface layer consisted of hyaline like cartilage histologically. On the other hands, the surface of the defect treated without pellets was fibrous tissues. The Mankin's score showed no significant differences among weeks, however, the score of the pellet group at 24 weeks was significantly lower than that of the control group, suggesting that the histological repair of the pellet group was better than that of the control group. At 2 and 8 weeks after the transplantation, human cells were detected in the transplanted place but were not observed at 16 weeks by FISH. Although we could not explain the role of the transplanted cells, the full-thickness cartilage defect treated with pellets showed macroscopically nearly normal, and the surface of the defect was covered with hyaline like cartilage. These results suggest that the transplantation of pellets containing hyaline like cartilage differentiated from MSCs may have potential to restore full-thickness osteochondral defect. *Ryukyu Med. J., 27(3,4) 115~122, 2008*

Key words: mesenchymal stem cell, osteochondral defect, cartilage repair, pellet culture, transplantation

INTRODUCTION

It has been well recognized that articular cartilage (hyaline cartilage) has limited intrinsic healing. Cartilage acts as a cushion that controls and absorbs external pressure and provides lifetime painless joint motion. However, once damaged, cartilage has little capacity for regeneration. The defect that extended into the subchondral bone resulted in the formation of fibrous or fibrocartilaginous tissue[1-3]. Although

several clinical treatments, such as drilling of articular defects, abrasion chondroplasty, mosaicplasty and autologous chondrocyte transplantation have been attempted, the repaired tissues in most cases formed fibrocartilage[4-10]. Thus, the ability to restore the tissue to its original state is limited.

Mesenchymal stem cells (MSCs) are useful candidates for cell sources in tissue engineering[11]. MSCs from bone marrow have the capacity to differentiate into various tissues, such as bone, cartilage and adipose tissue with appropriate growth factors and culture conditions[12]. To facilitate the repair of hyaline cartilage, the transforming growth factor-beta1 (TGF-beta1) has been used to manipulate the host healing response at the site of injury. It increases the synthesis of collagen, proteoglycan and inhibitors of matrix breakdown, and also stimulates cell proliferation[13-18].

In addition, a three-dimensional aggregate culture system has been developed, in which bone marrow derived mesenchymal progenitor cells terminally differentiate into hypertrophic chondrocyte effectively. This system maintains the cells in an aggregate, and thereby more closely approximates the three-dimensional environment of articular cartilage in vivo. Recent works indicated that the aggregate culture system allowed the growth factors to stimulate the progression of chondrogenesis from progenitor cells[19,20]. Therefore, implantation of these cells into osteochondral defects could repair hyaline articular cartilage properly.

This study was conducted to test the hypothesis that the transplantation of pellets containing hyaline like cartilage differentiated from adult human MSCs improves the repair of osteochondral defect in the rat knee. The athymic nude rats were used as experimental animals for the transplantation. The pellets, that were three-dimensional like aggregates, were transplanted into an osteochondral defect.

MATERIALS AND METHODS

Isolation of MSCs from adult human bone marrow

This study was approved by the institutional review board of University of Pittsburgh (Pennsylvania, USA) and in accordance with the Helsinki Declaration. A human bone marrow sample was obtained from a 40-year-old adult male under sterile conditions. MSCs were isolated from marrow of the iliac crest by a modification of the procedure of Pittenger and others[12]. According to their procedure, the isolated cultured MSCs compromised a single phenotypic population more than 95% by flow cytometric analysis of expressed surface antigens. Briefly, the bone marrow aspirate was washed with Hanks' Balanced Salt solution (HBSS; GIBCO, Grand Island, NY, USA) and centrifuged at 900g for 10 minutes at 20℃. The samples were diluted 1:1 with HBSS and layered carefully onto Ficoll-Paque PLUS (density 1.077 g/ml; Amersham Biosciences, Piscataway, NJ, USA), then centrifuged at 900g for 30 minutes at 20℃. After centrifugation, the mononuclear cells at the gradient interface were washed with HBSS and centrifuged again. The cells were resuspended in Dulbecco's Modified Eagle Medium-Low Glucose (DMEM-LG; GIBCO) with 20% Fetal Bovine Serum (FBS; GIBCO), 1 % (v/v) Penicillin/Streptomycin (P/S; Cambrex Bioscience, Baltimore, MD, USA) and 50 μ g/ml Ascorbic acid (SIGMA, St. Louis, MO, USA) at T-75 flasks. Cell cultures were grown to confluence in 5%CO_2/ 95%air at 37℃ and media was changed twice a week. When the culture was almost confluent, the cells were detached by Trypsin-EDTA (GIBCO) and washed twice with HBSS[21-23]. These cells have an adherent, fibroblast-like morphology. We utilized these cells as MSCs.

Pellet cultures

The cells were trypsinized, counted and 2.5 x 10^5 cells were placed in 15ml conical tubes containing a defined medium and centrifuged at 900g for 5 minutes. The resulting pellet was resuspended DMEM-High Glucose (DMEM- HG, GIBCO) containing 37.5 μ g/ml ascorbic acid 2-phosphate (SIGMA), 10-7 M Dexamethasone (SIGMA), ITS (insulin, human transferrin, and selenous acid) +Premix (BD Biosciences, San Diego, CA, USA) and 1 mM Sodium Pyruvate (SIGMA) in 5% CO_2/ 95% air at 37℃. The pellet was cultured with or without 10% FBS and different concentrations of rhTGF-beta1 (recombinant human Transforming Growth Factor-beta1; 0, 10, 20 ng/ml, R&D Systems, MN, USA) in the defined medium[26-29]. The aggregate was maintained in 0.5 ml-culture medium in a 15ml-tube, respectively. The media was changed every 2-3 days. After 1-2 days, the cell aggregate was detached from the bottom of the tube and assumed a globular shape. All pellets were harvested at two weeks after the culture.

Histological evaluation of the pellet

The aggregates were washed with HBSS and fixed in 10% buffered formalin for an hour then placed in 70% ethanol. The each pellet was embedded in paraffin overnight. The embedded pellet was cut into 6 μ m sections and deparaffinized. The sections were stained with Safranin O.

Transplantation of the pellets to the rat knee

The pellets were made using above procedure for rat transplantation. Following the results of the pellet culture, the aggregates were cultured in 10 ng/ml of TGF-beta1 without FBS in defined medium. Forty-two male, 10 to 12-week-old, athymic nude rats were used. Animal experiments were carried out in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. The rats were placed on the supine position and they were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight), then surgery was conducted under sterile conditions. The knee joint was opened using a medial parapatellar incision and the patella was dislocated laterally to expose the patellar groove of the distal femur. A cylindrical defect of 1.5 mm in diameter and 1.5 mm in depth was made using a handdrill in the femoral articular surface of the patellofemoral joints. Two to three pellets containing hyaline like cartilage differentiated from MSCs were transplanted into the defect of the right knee and covered with fibrin glue (pellet group). The osteochondral defect of the left knee was filled only with fibrin glue (control knee). After irrigating the joint with sterile saline, the capsule and wound were closed with nylon sutures. The rats were housed 2 per cage and were returned to unrestrained cage activity. Each 5 rat was sacrificed at 4, 8, 16, and 24 weeks after surgery.

Histological evaluation of the rat knee

These rats were killed at 4, 8, 16, 24 weeks and the specimens from the patellar groove were harvested and fixed in 10% buffered formalin, then decalcified in Decalcifier II (Surgipath) solution and embedded in paraffin. Paraffin sections (6 μ m thick) were prepared for microscopic analysis as described above. The histological sections were stained with toluidine blue. Histological evaluation how osteochondral defect was repaired was performed with the Mankin's score[30,31]. The grading

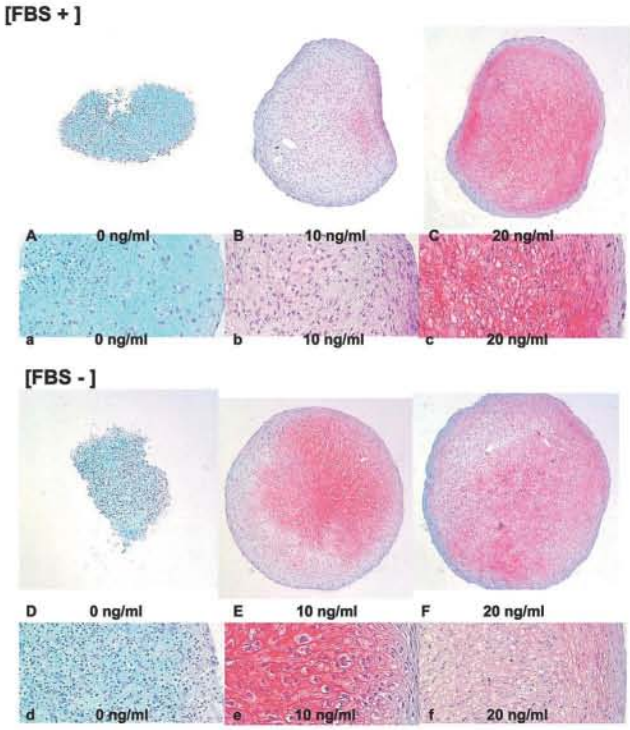

Fig. 1 Safranin O staining in the pellet.
Effect of the concentration of TGF-β 1 and fetal bovine serum (FBS) on the differentiation of the pellet. (A-F: 100x; a-f: 200x)

system was composed four categories - structure (0-6 points), cells (0-3 points), staining (0-4 points) and tidemark integrity (0 or 1 point) - with the highest score of 14 points; normal cartilage scored 0.

FISH (fluorescence in situ hybridization) for detection of human X-Y chromosome

Additional sections underwent FISH for detection of cells with human X and Y chromosome within the transplanted pellets and surrounding tissue at 2, 8 and 16 weeks. The components of the FISH procedure include the probe of interest and the target sequence to which the probe hybridizes. A fluorescent tag was attached to the probe so that it could be visualized. Fluorescent probes for the human X and Y chromosomes were used to localize human cells. Visualization was performed using a fluorescent microscope.

RESULTS

Aggregate formation

The cell aggregates adhered to the bottom of the tube following centrifugation and the tube was placed in the incubator for a few days. The aggregate

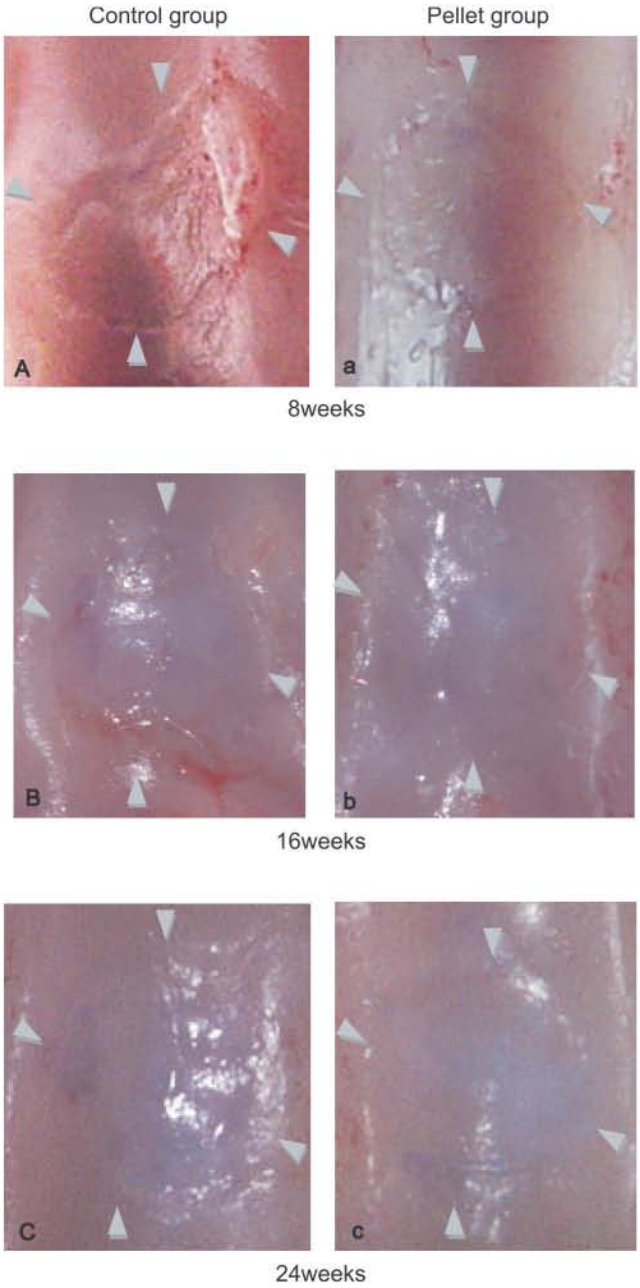

Fig. 2 Macroscopic evaluation
In the control group, the surface of the defect at 8 weeks appeared rough and less integrated. The margin was easily distinguishable. At 16 and 24 weeks, the surface of the defect appeared irregular, less integrated and subsided. After 8 weeks, the surface of the defects in the pellet group remained smooth and appeared integrated. At 16 weeks, the pellet transplanted region became shiny and acquired an elastic consistency. At 24 weeks, the region had a glossy smooth surface and almost the same hardness as the surrounding cartilage. (A-a: 8 weeks, B-b: 16 weeks, C-c: 24 weeks after surgery)

detached from the bottom of tube and formed a pellet.

Histological findings of the pellets

The cells in the pellets cultured in medium with FBS for 14 days showed an irregular shape. The differentiation of cells into chondrogenic cells was observed in a part of pellet only at the highest concentration (20 ng/ml) of TGF-beta1. However, the cells in the pellets cultured without FBS were uniformly round and showed chondrogenic morphology especially in 10 ng/ml TGF-beta1 (Fig.1).

Macroscopic findings

All of rats run normally after surgery and had a normal range of motion in the both knees. In the control group, the surface of the defect at 8 weeks appeared rough and less integrated. The margin was easily distinguishable (Fig. 2A). At 16 and 24 weeks, the surface of the defect appeared irregular, less integrated and subsided. There were a few osteophytes on the femoral groove and the surface was not well burnished but also irregular (Fig. 2B, 2C).

After 8 weeks, the surface of the defects in the pellet group remained smooth and appeared integrated (Fig. 2a). At 16 weeks, the pellet transplanted region became shiny and acquired an elastic consistency (Fig. 2b). At 24 weeks, the region had a glossy smooth surface and almost the same hardness as the surrounding cartilage (Fig. 2c).

Histological findings of the rat knees

No pellets were detached from the transplanted site during entire periods of experiments. At 4 weeks, in the pellet group, the cells displayed the characteristics of chondrogenic cells. The pellets kept its thickness and the surface was covered with hyaline like cartilage. In the control group, the surface of the defect was covered with fibrous tissue and there were less chondrogenic cells (Fig. 3A-a. Fig. 3B-b). At 8 weeks, in the pellet group, the transplanted pellets became thinner and replaced with host subchondral bone. However, the surface of the transplanted pellet was kept cartilage-like structure. In the control group, surface of the defect was covered with fibrous tissues (Fig. 3C-c. Fig. 3D-d). At 16 weeks, the surface in the pellet group was still covered with hyaline like cartilage cells. In the control group, surface layer was fibrous.

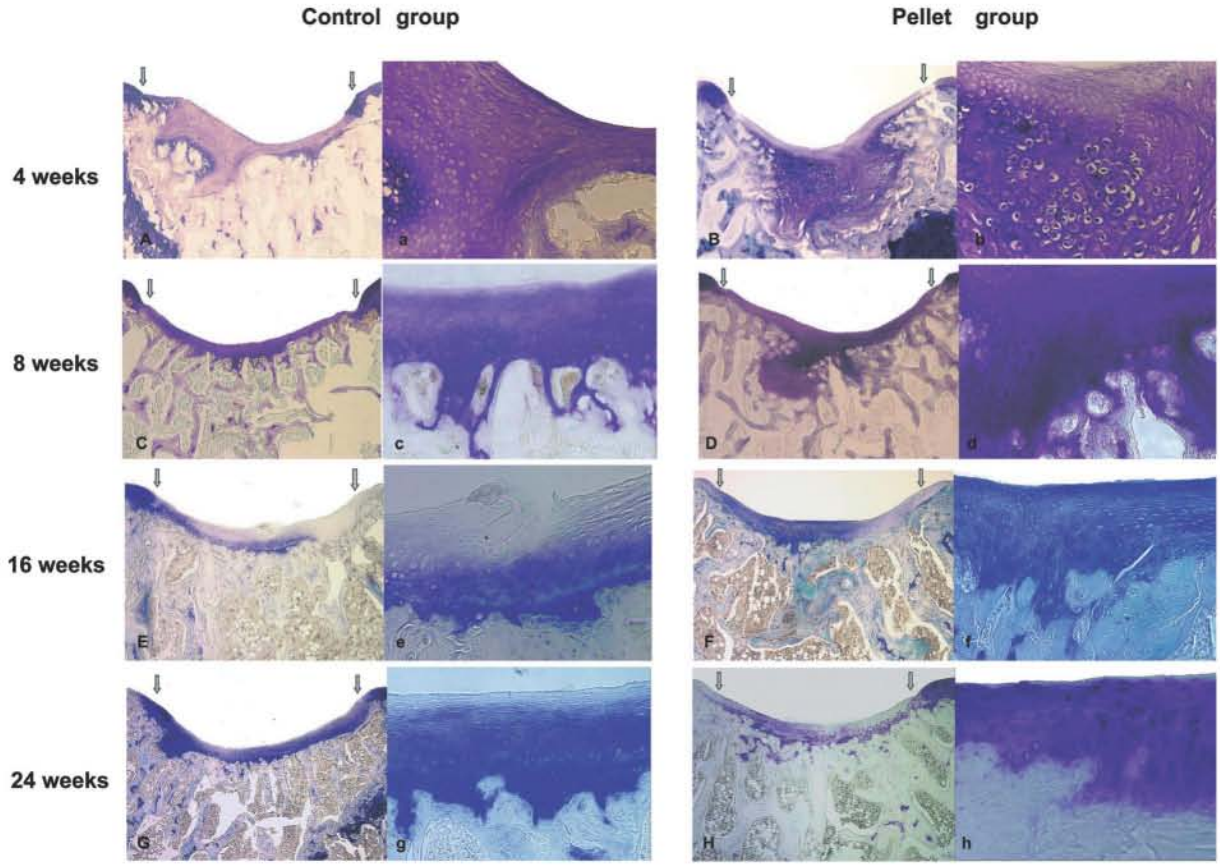

Fig. 3 Histological results (Toluidine blue staining)
Control group: At 4 weeks, the surface of the defect was covered with fibrous tissue and there were less chondrogenic cells. At 8 weeks, surface of the defect was covered with fibrous tissues. At 16 weeks, surface layer was fibrous. Chondrogenic cells were observed only in the deep layer. At 24 weeks, the surface was covered with fibrous tissue. Pellet group: At 4 weeks, the cells displayed the characteristics of chondrogenic cells. The pellets kept its thickness and the surface was covered with hyaline like cartilage. At 8 weeks, the transplanted pellets became thinner and replaced with host subchondral bone. However, the surface of the transplanted pellet was kept cartilage-like structure. At 16 weeks, the surface was still covered with hyaline like cartilage cells. At 24 weeks, transplanted pellets progressively thinner, but the surface kept hyaline like cartilage. (A-H: 40x; a-h: 200x)

Chondrogenic cells were observed only in the deep layer (Fig. 3E-e. Fig3F-f). At 24 weeks, transplanted pellets progressively thinner, but the surface kept hyaline like cartilage in the pellet group. In the control group, the surface was covered with fibrous tissue. The chondrogenic cells were observed in the deeply layer (Fig. 3G-g. Fig. 3H-h).

Histological score (Mankin's score) of repaired tissues

The mean Mankin's scores (mean± SD) for MSCs transplanted defects at 4,8,16 and 24 weeks postoperatively were 6.3± 0.9, 5.0± 0.7, 3.5± 0.5 and 3.6± 0.9, respectively. On the other hand, those of control were 9.5± 0.5, 9.0± 1.0, 9.0± 1.0 and 7.3± 1.2, respectively. Although no significant differences were observed among weeks in each group, scores between the pellet and control group at 24 weeks were significantly different ($P < 0.05$).

FISH findings

The FISH probes showed orange (human X-chromosome) and green (human Y-chromosome) dots in the cells if there were human X and Y chromosome in the tissue. Before transplantation, the pellets contained human X and Y chromosomes (Fig. 4A), indicating the pellet was made of human male cells. The fluorescent probes for human X and Y chromosomes revealed human male cells existed within the repaired tissues at 2 and 8 weeks after the transplantation (Fig. 4B, C). However, no human cells were observed in the repaired tissues at 16 weeks, suggesting disappearance of transplanted MSCs.

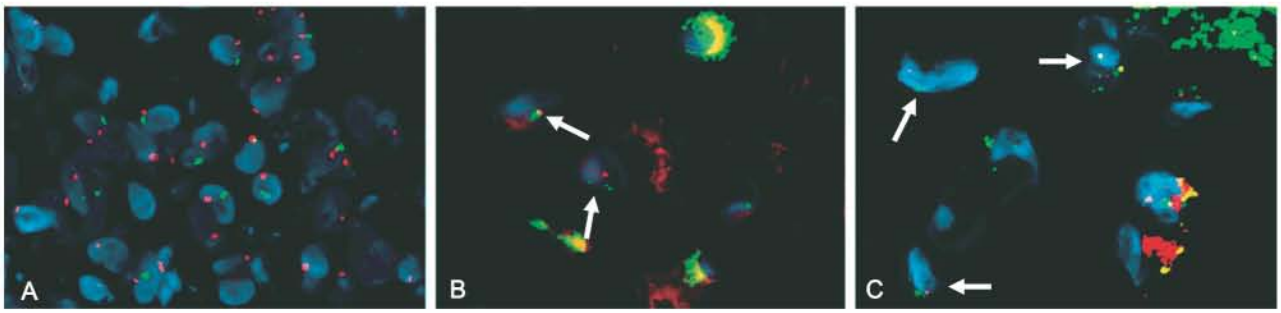

Fig. 4 FISH (Fluorescence in situ hybridization) for human X-Y chromosomes revealed human male cells (orange: human X chromosome, green: human Y chromosome). (A) The pellet that cultured for 2 weeks, (B) The transplanted pellet at 2 weeks after surgery, (C) The transplanted pellet at 8 weeks after surgery. The arrows show the X-Y chromosome.

DISCUSSION

We produced pellets containing hyaline like cartilage differentiated from human MSCs. In this study, we did not perform the characterization of the MSCs but we isolated MSCs from bone marrow by a modification of Pittenger's procedure. According to their procedure, the isolated cultured MSCs comprised a single phenotypic population more than 95% by flow cytometric analysis of expressed surface antigens. These expanded cells were uniformly positive for CD29, CD44, CD71, CD90, CD106 and many other surface proteins and negative for CD14, CD34, and CD45[12]. Although some researcher have reported as for the surface proteins of MSCs, the markers of define as MSCs remain incompletely clarified at this point[24,25].

This study demonstrated that the pellets transplanted into an osteochondral defect maintained their conformation, stimulated chondrogenesis, and full-thickness osteochondral defect was repaired more effectively in comparison to the defect treated without pellets.

Optimal ability of pellets for tissue repair was established by culture before transplantation in this study. Histological specimens of pellets without TGF-beta1 showed variable morphology containing irregular shape and fibrous tissue, and could not synthesize the adequate matrices for cartilage. Several studies indicated that TGF-beta increases the synthesis of type II collagen and proteoglycan[13-18]. In this study, we demonstrated that 10 ng/ml of TGF-beta1 without FBS in culture medium effectively stimulated the pellets to form hyaline like cartilage.

MSCs have the potential to form cartilage in vivo and easy to harvest from bone marrow[19,32,33]. Wakitani *et al.* reported the successful repair of a large full-thickness defect in the rabbit with the implantation of cultured mesenchymal osteochondral progenitor cells isolated from their periosteum and bone marrow[34,35]. Transplantation of mesenchymal stem cells could be one of the promising methods to enhance the repair of hyaline cartilage defects. We clearly showed transplanted chondrocyte derived from human MSCs were detected by FISH in the pellets transplanted into osteochondral defects until 8 weeks, and cartilage surface was repaired more smoothly compared to control rats.

It is important to evaluate how transplanted MSCs enhance the repair of osteochondral defects. Although transplanted human MSCs could not be detected at 16 weeks, conformation of repaired tissue was maintained to a certain extent during the entire experiments. These results demonstrated that human MSCs survived at least 8 weeks after transplantation and participated in cartilage repair by producing cartilaginous proteins in athymic rat knees.

The presence of bone marrow human MSCs were beneficial to the cartilage repairs, histological analyses did not reveal entire recovery. The histological differences between pellet group and control group were obviously, however, it was less than we expected. The transplanted cartilage like cell in the pellets reduced in number over time and they might be replaced with host cells. These findings indicated that even the use of TGF-beta1 regenerated cartilage was not identical with normal cartilage.

The reason why the cells derived from MSCs were disappeared after 8 weeks was unclear. These cells may have been replaced by new bone marrow

cells from the athymic nude rat. Alternatively, the transplanted cells might have stimulated the host cells to differentiate into chondrocytes to repair the defect. With respect to the cell characteristics, viability and differentiation, further studies are necessary to evaluate the role of transplanted cells. Furthermore, because of apparent differences in the healing potential to osteochondral defects between rats and human beings, these results may therefore not translate directly to human studies. However, in this experiment the full-thickness cartilage defect packed with pellets showed macroscopically nearly normal appearance, and the surface of the defect was covered with hyaline like cartilage.

This experiment may offer clue to treat full thickness cartilage defect with pellets containing hyaline like cartilage tissue differentiated from human MSCs.

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