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In vivo cell tracking by bioluminescence imaging after transplantation of bioengineered cell sheets to the knee joint

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ABSTRACT

In our previous studies, we have demonstrated effective regeneration of cartilage through the creation and application of layered cell sheets that combine both chondrocytes and synovial cells. In this study, we were able to demonstrate that cells derived from cell sheets can survive for long periods after transplantation into rat knee joints having osteochondral defects. We established a method for generating cell sheets from firefly luciferase-expressing chondrocytes obtained from transgenic Lewis rats, and carried out allogenic transplantation of these cell sheets into wild-type Lewis rats. We then administered luciferin and monitored the survival of the transplanted cells by using bioluminescence imaging (BLI). Our data showed that the transplanted cells survived and could be detected for more than 21 months, which was longer than expected. Furthermore, the BLI data showed that the transplanted cells remained in the knee joint and did not migrate to other parts of the body, thus confirming the safety of the cell sheets. In this study, we monitored the duration of survival of cell sheets composed of only chondrocytes, only synovial cells, or both chondrocytes and synovial cells, and found that all three types of cell sheets survived for an extended period of time.

1. Introduction

Articular cartilage tissue lacks blood vessels and has a poor ability to self-renew. Self-renewal is particularly difficult if the defect is partial and localized, and does not reach the subchondral bone. If the defect encompasses the entire articular cartilage layer, and has reached the subchondral bone, mesenchymal stem cells are introduced from the bone marrow. If the defect is small in size, regenerative repair may be possible [1]. In actual pathological instances of osteoarthritis, there is a mixture of partial- and total-thickness defects. Knee cartilage damage caused by aging or trauma easily converts to osteoarthritis and is accompanied by sharp pain.

Fortunately, the lack of blood vessels in articular cartilage results in a lower rate of rejection after grafting. Therefore, the use of allografts is more widely studied for cartilage regeneration than for the regeneration of other tissues and organs.

To date, many animal experiments and clinical studies have been carried out to evaluate strategies for improving self-renewal in articular cartilage. Recently, research involving tissue-engineered cartilage grafts has become increasingly common. In a clinical study, Ochi et al. [2] reported improvements in the response to mechanical loads and sensory innervation using tissue-engineered cartilage grafts created in atelocollagen gels. A wide variety of synthetic polymers and biomaterials are used as scaffolds to create bioengineered cartilage grafts. Consequently, biocompatibility risks associated with the application of such substances over the long term have been reported [3]. The creation of tissue-engineered cartilage tissue that does not utilize scaffolds would significantly mitigate these risks, making their application more feasible and safer for patients. Mainil-Varlet et al. [4] successfully created scaffold-free tissue-engineered cartilage using a static bioreactor system. Furthermore, Nagai et al. [5], using their rotation

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culture method, reported that it is possible to create tissue-engineered cartilage from a lower number of chondrocytes than previously reported.

In the field of cartilage regeneration, previous research has focused on finding therapeutic methods to treat cartilage defects by using grafts made from synovial cells. Synovial cells have a critical role in the repair of cartilage defects [3]. However, in the treatment of cartilage defects using grafts derived from synovial cells, fibrous tissue is more likely to be formed on the surface of the regenerated cartilage tissue [6].

To treat osteoarthritis, our group has carried out some basic studies concerning the repair and regeneration of articular cartilage using tissue engineering. We have focused on the importance of the interaction between the cells of the recipient and the donor in the repair and regeneration of articular cartilage. If there are a minimum number of bioengineered chondrocytes at or near the surface of the cartilage layer, we have found that the recipient's cells drive the regeneration of cartilage [7–9].

Chondrocyte sheets, generated using the bioengineered cell sheet technology developed originally in Japan, can be harvested on a temperature-responsive culture dish without damaging the cells and extracellular matrix [10,11]. Layered chondrocyte sheets fabricated using temperature-responsive culture dishes have characteristics that differ from those of cultured chondrocytes using normal culture dishes. These characteristics allow them to exert regenerative effects to the same degree as other bioengineered cartilage tissue [12]. Chondrocyte sheets have already proven more effective in promoting regeneration of both partial- and full-thickness defects [12,13].

Cell sheets exhibit superior adhesion to the surface of damaged cartilage having partial- or full-thickness defects. In addition, cell sheets have superior barrier functionality, which protects the site from the catabolic factors present in the joint fluid, and also prevents leakage of the extracellular matrix of cartilage tissue into the joint fluid. Moreover, cell sheets might contribute to the repair and regeneration of cartilage by continuously providing growth factors derived from cell sheets. Layered cell sheets maintain their three-dimensional structure. In our most recent clinical studies that focused on humans, we found that multilayered sheets express a significantly larger amount of humoral factors such as transforming growth factor β, prostaglandin E2, and melanoma inhibitor activity compared with single-layered sheets and cultured chondrocyte as the same number. These data indicate that multilayered sheets will probably have higher efficacy with respect to cartilage regeneration than single-layered sheets [14].

The synovial cells existing in the knee joint secrete the synovial fluid that nourishes chondrocytes. We have developed a method of harvesting chondrocyte sheets at a faster pace and with greater certainty by co-culturing chondrocytes and synovial cells in an environment that resembles the inside of the knee joint [15]. Furthermore, in experiments on Japanese white rabbits, the transplantation of a combination of chondrocyte sheets and synovial cells led to improved repair of osteochondral tissue defects compared with the transplantation of chondrocyte sheets or synovial cells alone [16].

We sought to determine the duration for which cell sheets comprising chondrocytes and synovial cells survive and continue to secrete growth factors at defect sites within the knee joint. We hypothesized that mature chondrocytes and synovial cells, as part of cell sheets, would gradually decrease in number from the moment of transplantation, and disappear after 3–4 weeks. We also expected that grafts consisting of a combination of chondrocytes and synovial cells within an environment resembling the inside of the knee joint would survive longer than grafts of each type of cell, by enhancing the regenerative effect exerted by the recipient's own chondrocytes.

Bioluminescent reporter genes are increasingly being used to image engineered cells *in vivo*. Of these, the firefly luciferase (*luc*) gene is the most commonly used. The enzymatic reaction between luciferase and its substrate, luciferin, results in photon emission, which can be detected using a cooled charge-coupled device (CCD) camera. In recent years, transgenic animals carrying specific marker genes have been generated [17–19]. Cells derived from them can be transplanted into other animals and then used to investigate survival time, cell migration, and so forth, in those animals. These cells are also frequently used in research on the regeneration of organs and tissues [20–24]. In this study, we transplanted luciferase-expressing transgenic rat cells into nontransgenic rats and then attempted to effectively track those transplanted cells.

The aim of this study was to determine the survival time of bioengineered chondrocyte sheets and synovial cell sheets after transplantation to cartilage defects in the knee joint of rats by using *in vivo* bioluminescence imaging (BLI).

2. Materials and methods

Animal experiments were performed in accordance with the guidelines of the Institutional Regulation for Animal Experiments as well as the Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology for animal handling and care. The study was approved by the Institutional Animal Experiment Committee of Jichi Medical University.

2.1. Luciferase-expressing transgenic rats

Hakamata et al. generated transgenic rats that express the luciferase gene (Rosa/luciferase transgenic Lewis rats) [25]. This gene contains the ROSA26 promoter, which allows the stable expression of luciferase ubiquitously throughout the entire body of the rats. When the substrate luciferin is injected *in vivo*, light is emitted through oxidation catalyzed by luciferase, a reaction that uses ATP. The strength of light emission differs according to the cell type, as emission strength is affected by endogenous ATP, Mg^{2+}, the amount of expression of the luciferase enzyme, and other elements that differ across cell types.

2.2. Harvest of chondrocytes and synovial cells from luciferase-expressing transgenic rats

Luciferase transgenic (Tg) 16-week-old male Lewis rats, obtained as described previously [25], were treated with general anesthesia using 2% isoflurane in O_2. An incision was made on the inner part of the lower extremities, exteriorizing the knee joint, and severing collateral ligaments as well as anterior and posterior cruciate ligaments. Then, the synovial and cartilaginous tissue was scraped with a scalpel. The tissue cells were isolated from the tissue samples using an enzymatic procedure. Luciferase-expressing synovial cells and chondrocytes were seeded in temperature-responsive culture dishes. Co-culture of the cells on these inserts permitted a shorter proliferation time than needed with single-cell-type cultures [15].

2.3. Cell culture using temperature-responsive culture dishes

After isolating synovial cells and chondrocytes from tissues using an enzymatic procedure, the synovial cells were seeded in temperature-responsive culture dishes, while the chondrocytes were seeded in temperature-responsive inserts. Co-culture via use of the inserts reduced the proliferation time compared with that required to culture each type of cell independently.

The luciferase-expressing cartilaginous and synovial tissues harvested from Tg rats were subjected to proteolytic digestion at 37 °C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM)/F12 adjusted with 0.016% collagenase (Worthington, NJ, USA), over a period of 4 h, with continuous stirring with a strainer. Subsequently, the luciferase-expressing chondrocytes and synovial cells were separated using a cell strainer with a pore size of 100 μm (BD, NJ, USA), centrifuged, and harvested. The synovial cells were incubated in DMEM/F12 containing 10% fetal bovine serum (FBS; GIBCO, NY, USA) and 1% mycotic antibody (Wako Pure Chemical Industries, Ltd., Japan). All cells were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% air.

The chondrocytes were seeded in temperature-responsive inserts (5.0 cm^2; CellSeed Inc., Tokyo, Japan). The synovial cells were evenly seeded in temperature-responsive culture dishes (9.6 cm^2; CellSeed Inc.) so that the cells were distributed at 10,000 cells/cm^2, and then co-cultured with the chondrocytes in DMEM/F12 supplemented with 20% FBS for a period of about 14 days.

After confirming that the cells were confluent after about 14 days of culture, the temperature-responsive culture dishes were transferred to a lower temperature (25 °C) incubator for an additional 30 min.