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「要約」

1. 論文タイトルページ

東海大学大学院平成25年博士論文

Study of articular cartilage regeneration using scaffold-free spheroids composed of synovium-derived cells and chondrocytes as the minimal therapeutic unit for transplants

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2. Introduction

Osteoarthritis (OA) is a degenerative and costly chronic bone disorder that affects more women than men. Brittberg and colleagues were among the first to use autologous chondrocyte implantation (ACI) as a treatment for OA in 1994. Since then, various clinical studies have been conducted, and ACI is now the preferred treatment for OA among other treatments, such as periosteal patch application, debridement operation, microfracture, and mosaicplasty. Although promising results have been obtained, ACI is not constraint free because the procedure requires vast numbers of cells from the patient and therefore induces donor site morbidity. Despite advances in cartilage regeneration techniques through the use of scaffolds, scaffold-free constructs, and cell transplantation therapy, ACI complications such as periosteal hypertrophy limit the size of the lesion that can be treated and changes in chondrogenic phenotype associated with lengthy ex vivo cell expansion remain to be resolved. In view of the transplantation process, early studies demonstrated the limitations of a pure cell suspension delivery system. Artificial scaffolds have emerged as a means to aid the delivery of therapeutic cells to the defect area, to provide mechanical support to the chondral grafts, and to increase extracellular matrix (ECM) development. However, questions remain about the biocompatibility of scaffolds and their effects on cell viability, including cell attachment, proliferation, and distribution. On the other hand,

biological and synthetic scaffolds continue to cause immunological problems such as acute rejection, foreign body reaction, and fibroblastic overgrowth, thereby delimiting their therapeutic use as a treatment for OA.

Articular cartilage defects caused by degenerative process or injury may not return to the normal state because mature chondrocytes have a limited life span and proliferative capacity. For this reason, in our previous study, we used transfection of human telomerase reverse transcriptase (hTERT) or glucose-regulated protein 78 (GRP78) into chondrocytes during the culture period to prolong their life span and to facilitate their therapeutic use. Alternative stem cells, such as bone marrow-derived mesenchymal stem cells (MSCs) and synovium-derived stem cells, have been investigated for their ability to repair cartilage defects. In cartilage regenerative medicine, MSCs are regarded as superior cell sources because they can be harvested in a minimally invasive manner, can be isolated simply and expanded, and are multipotent. Synovial MSCs are especially promising because of their high proliferative capacity and chondrogenic potential. Recent studies have focused on the use of SYs because of their similarities with MSCs including high replicative potency regardless of the donor's age, capacity for chondrogenesis, and ready accessibility. Stem cell biology, tissue engineering, and regenerative medicine are interrelated fields that contribute to progress in treatment for OA.

We have previously characterized the properties of bioengineered chondrocytes manufactured through cell sheet engineering for cartilage regeneration. Our data revealed that chondrocyte sheets contain specific phenotypic markers and adhesion molecules that lead to the production of a 3-D scaffold-free construct with good adhesive properties for both cells and the injured cartilage site. Similarly, in another study, we developed a 3-D scaffold-free cartilaginous-engineered tissue, which we transplanted into an osteochondral defect to stimulate *in vivo* cartilage differentiation and obtained remarkable results. However, one of the limitations of this method was the extensive culture period (>1 month). In the present study, we describe our new culture system, which we call a high-density suspension-shaking (HDSS) method. Using this system, we can expand the cells in a shorter period while promoting an equivalent mass of cells for transfer and the formation of cell aggregates (scaffold-free spheroids).

This research was designed intentionally to assess the effectiveness of HDSS, as an original method, in the construction of scaffold-free spheroids (spheroidal cell aggregates) comprising a mixture of two types of cells. We investigated whether SYs and articular chondrocytes (ACs) can form transplantable spheroids and how they promote cartilage regeneration in a rabbit model. In this study, we successfully constructed the scaffold-free spheroids using the HDSS culture method with different ratios of SYs and ACs, and we subsequently implanted the spheroids into injured knees in rabbits. The transplanted knee site was assessed anatomically on days 8 and 16 after the operation, and the recovered (explanted) spheroids were evaluated using

morphological, genetic, and histochemical analyses.

To evaluate the regenerative capacity of the representative cellular aggregate (75% SYs, 25% ACs), we performed macroscopic and microscopic assessments. The spheroid sections were analyzed histologically with Toluidine blue and Safranin O to characterize the location and distribution of cells and ECM proteoglycans and glycosaminoglycans. Immunohistochemistry for collagen ECM was performed to provide an index for comparing the spheroidmatrix with normal cartilaginous tissue. Gene expression was also analyzed using real-time PCR (RT-PCR) to quantify the levels of collagen I and collagen II in spheroids produced from different cell ratios.

3. Summary (総括)

Osteoarthritis (OA) is a degenerative disease that causes morbidity especially among the aged population. Among the established treatments, the preferred mode of treatment is autologous chondrocyte implantation (ACI), which requires harvesting healthy articular cartilage cells from the patient and consequently causes donor site morbidity. Despite the promising results brought about by advances in tissue engineering, various limitations of ACI remain unsolved, including immune response complications, a lengthy cell expansion period, and the cell delivery system. Improving ACI effectiveness and efficiency as a lifelong therapy for OA requires modifications of the current techniques and identification of other candidate cells for cartilage regeneration. In this study, we investigated whether synovium-derived cells (SYs) can be used as substitutes for articular chondrocytes (ACs) in ACI. ACs and SYs were isolated from the knees of rabbits and cultured, and their growth rates were assessed. Here, we describe our new culture system, the high-density suspension-shaking culture method (HDSS), in which a mixture of cells is cocultured in a circular movement, thereby allowing for the self-assembly of scaffold-free cellular aggregates. ACs and SYs were mixed in various ratios and expanded *in vitro* by HDSS. We successfully manufactured vast numbers of cellular spheroids in a short time using mixed SYs and ACs regardless of the different cell composition ratios. These injectable cellular spheroids were transplanted into full-thickness osteochondral defects in a rabbit model, and their *in vivo* chondrogenic regeneration was monitored 8 and 16 weeks later. Simultaneous histological evaluation using Toluidine blue and Safranin O, and immunohistochemistry to identify collagen types I and II, were performed and gene expression was evaluated to assess chondrocyte-specific differentiation. Our data showed that the minimal therapeutic unit for the transplants contributed to *in situ* regeneration of cartilage. In summary, SYs can be used as a replacement for ACs in clinical applications of ACI in patients with osteoarthritic lesions. HDSS is an effective coculture method for promoting self-assembled, scaffold-free implantable spheroids.

4. Discussion (考察と展望)

Synovium-derived mesenchymal stem cells (SY-MSCs) may be a new cell source for cartilage regeneration

Since synovium-derived mesenchymal stem cells (SY-MSCs) were first identified

and successfully isolated in 2001 as a new origin among MSC families, they have been increasingly regarded as a versatile therapeutic cell species for musculoskeletal regeneration, particularly for reconstruction of cartilage, bone, adipose tissue, tendons, and muscles. In addition to having general multipotency in common with the MSC community, SY-MSCs are superior to other-sourced MSCs in their greater capacity for proliferation and chondrogenesis. In addition, their multipotent capacity is not influenced by donor age, cell passage, or cryopreservation.

SY-MSCs appear to have limited senescence and can be expanded to large numbers under monolayer *in vitro* culture conditions, although they show a progressive age-associated decline in growth rate. Several reports have indicated that human MSCs derived from synovial tissues retain their proliferative ability even after passage 10. SY-MSCs from a variety of adult human donors retained a linear growth curve over at least 30 population doublings. Further, when passaged to 14 days using appropriate cell densities, the number of colonies per 10^3 nucleated SY-MSCs was more than 100-fold higher than for bone marrow-derived MSCs.

In this study, we chose SYs as a partial replacement for ACs in the mixtures of cells, and we were able to fabricate mixed SY-AC constructs as spheroid aggregates. SYs appear to be a promising cell source for cartilage regeneration. Compared with other sources of MSCs, SY-MSCs have greater proliferative and chondrogenic abilities, and large amounts of SY-MSCs can be obtained easily from small amounts of synovial tissue.

SYs displayed greater proliferative capacity than did ACs (Fig. 2B), which is consistent with the results of previous studies. We called the cells SYs used in the HDSS rather than SY-MSCs because we did not prove that our SYs were SY-MSCs because we did not perform differentiation assays such as osteogenesis, adipogenesis and chondrogenesis. However, we isolated the SYs according to methods described previously these reports indicated that such SYs had the characteristics of MSC stemness. In our study, the PGR of SYs was about double that of the ACs, indicating greater proliferation potency, and demonstrated chondrogenesis in *in vivo* animal experiments. Thus, our SYs also displayed some of the properties of SY-MSCs.

Although we did not perform extended cultures of the SY/AC spheroids for more than 3 days, we expect that the spheroids exhibit a significant chondrogenic pattern because of the shear stress induced during the culture period. Despite the similar chondrogenic matrix synthesis in all SY/AC combinations, we also expect that the SY-MSCs are capable of cartilage regeneration *in situ* and *in vivo*. This is supported by the immature state of the cartilage grafts, which may eventually develop into normal cartilaginous tissue *in vivo*. In future studies, we plan to extend the culture time for cocultures of SYs and ACs in the different SY:AC ratios, to examine their abilities for ECM production and spheroid formation, and to compare these abilities with those of conventionally cultured 3-D constructs. We will measure the diffusion of matrix components within the spheroids using our previously devised technique-

fluorescence correlation spectroscopy (FCS)—to characterize further the ECM of the implanted scaffold-free spheroids. Our prior data indicated that FCS can measure the diffusion of cartilaginous ECM directly both in normal and degenerating cartilage, and can be used to evaluate bioengineered cartilage for ACI.

HDSS system can rapidly generate scaffold-free spheroids

In clinical applications, a limited number of cells can be obtained from biopsies, and there are disadvantages of proliferating ACs only. The HDSS system rapidly generates scaffold-free artificial tissue from fewer cells than with other systems. Synovial tissue has a high self-regenerative ability. Using SYs as one cell component for clinical ACI should minimize the need to sacrifice undamaged cartilage within the same joint.

Coculturing ACs and MSCs has gained considerable attention recently. Monolayer cocultures of immortalized human ACs with immortalized human MSCs in the absence of exogenous TGF- β induces chondrocytic differentiation of the MSCs, which is based on collagen type II protein expression. These MSCs are committed to chondrocytes, whose differentiation is influenced by the cellular microenvironment and the paracrine signals of chondrocytes. The HDSS system provides this unique microenvironment to differentiate MSCs from ACs. However, our scaffold-free spheroids are still immature *de novo* cartilage grafts, which should regenerate into normal cartilaginous tissue *in situ* and *in vivo* within full-thickness osteochondral defects as soon as they are manufactured through HDSS.

Our previous studies showed that scaffold-free constructs can also be manufactured through cell sheet engineering. However, this technique requires a longer culture period than does the HDSS used in the present study, which required 2-3 days. This finding and the previous literature suggest that the duration of the 3-D cultures was too short to redifferentiate from the continuously passaged monolayer culture. However, we suggest that our HDSS culture system shortens the preparation time for fabricating a considerable quantity of spheroids and can thus substantially shorten the total time required for an *ex vivo* procedure.

A high-density culture of chondrocytes, which promotes cell-to-cell contact, increases ECM biosynthesis. In the present study, ACs and SYs were cultured in a high-density suspension state in a nonadhesive culture plate while maintaining continuous suspension conditions. The cells adhered to each other to develop spheroidal cell mixtures and produced a limited amount of ECM because of the short culture time. However, in general, a high-density culture may limit the amount of mass available for transfer. At present, our system, with a product size small enough for injection, is superior to other methods for delivering transplants. We have also used HDSS in our previous and more recent studies to produce scaffold-free constructs as treatment for musculoskeletal diseases, vascular diseases and genetic or metabolic disorders such as diabetes mellitus. These studies showed that the HDSS system can be used to manufacture a variety of spheroids with different cell compositions,

which can serve both as scaffold-free constructs and as a delivery system that may be useful for evaluating the effects of drugs or chemicals *in vivo*.

SY/AC spheroids regenerated articular cartilage defects

In the present study, PKH fluorescent dyes were used to label SYs and ACs for visualization under confocal microscopy and fluorescence microscopy. The fluorescent dye staining was stable enough to distinguish the cell distribution patterns. Delivering the spheroids using micropipette aspiration was straightforward because their size range is small enough to place injectable units through the syringe or micropipette tip.

The results of *in vitro* morphological analysis by confocal microscopy, fluorescence microscopy, and histology indicated that the SYs and ACs were located irregularly and randomly within the spheroids. The phenotype of the interior of the spheroids was very different from the normal cartilage phenotype regardless of the cellular component ratio. The ECM was defective and stained sparsely with Safranin O and Toluidine blue. The staining intensity for type I and type II collagen was low in immunohistochemistry, suggesting the presence of small amounts of glycosaminoglycans, proteoglycans, and collagen fibers. The same pattern of results was observed for spheroids comprising ACs alone and SYs alone.

Gene expression of collagen ECM (type I and type II) showed similar patterns to those shown in the histological analyses. Chondrocyte 3-D culture techniques such as pellet culturing have been developed to obtain a large amount of cultured cells with a well-maintained cartilage phenotype. Manipulation of the culturing environment for chondrocytes presents the most feasible mechanism for optimizing cell behavior and phenotype. In this pellet culture system, after 2 weeks, the ECM in the pellets was similar to that of normal cartilage. Chondrocyte redifferentiation from monolayer-cultured chondrocytes has been demonstrated within 14 days in neocartilage.

In the animal experiments, articular cartilage defects in which SY-AC spheroids were implanted *in vivo* showed improved histological findings (Figs 5C and 7). The allografted spheroidal aggregates differentiated slowly to chondrocytes in the articular cartilage defects and produced an ECM similar to that of hyaline cartilage, thereby maintaining the chondrocyte phenotype. The implanted spheroids exhibited proliferation and differentiation activities in the articular cartilage defects, resulting in the formation of hyaline cartilage.

No signs were observed of immunological rejection or degeneration of the reparative tissue during the observation period. One possible limitation of the present study is that we did not perform detailed laboratory investigations to detect specific immunological reactions such as development of antibodies or cell-mediated responses.

Our preliminary results showed that 75:25 SY:AC spheroids can produce hyaline cartilage *in vivo* therefore, we applied this ratio in the present implantation study using rabbits. Interestingly, numerous cluster formations of chondrocytes were observed near the interface with the regenerative tissue.

This clustering phenomenon is a sign of repair in early OA. This result indicates that scaffold-free spheroids inserted into osteochondral defects show high regenerative capacity. Koga described the viscosity and adhesive properties of SYs in an *ex vivo* analysis in rabbits and demonstrated that the number of attached cells increased in a time-dependent manner; more than 60% of the cells attached within 10 min under normal gravity. Similarly, we held the defects stationary after implantation for 15 min without additional fixation such as a periosteal patch. Accordingly, dislocation of spheroids and leakage from the implanted sites were minimal. The present results are consistent to some extent with the earlier report. However, the mechanical strength properties of regenerated articular cartilage remain to be investigated through *in vivo* studies in larger animals (i.e., sheep or pigs). Previous observations of the intrinsic healing capacity of osteochondral defects in rabbits identified a benchmark for healing time. Shapiro et al. reported impressive positive results for early repair (12 weeks) of 3-mm osteochondral defects, although increasing degenerative changes were observed after 24 weeks in their animal model. Therefore, a reasonable follow-up period of at least 24 weeks is necessary. In the present study, we evaluated cartilage regeneration at 8 and 16 weeks. We plan to perform a 24-week follow-up study of cartilage regeneration using the SY-AC spheroids.

We recently constructed 3-D, scaffold-free, tissue-engineered cartilage [12] and transplanted this cartilage into the superficial layer region of osteochondral defects as an initiator of cartilage differentiation in reparative cells and achieved good restoration effects over the long term. We confirmed that in the early stage of transplantation, effective restoration of articular cartilage is seen with reparative mixed cells derived from MSCs and ACs, which can induce the differentiation of recruited MSCs into ACs and trigger chondrogenesis. We hypothesized that good cartilage repair may be achieved by providing sufficient quantities of an initiator (ACs) and materials (SY-MSCs) to induce differentiation into ACs in the osteochondral defect. It does not seem to matter whether a full-thickness or partial defect is used. In a full-thickness defect, cartilage repair is much more complicated and requires a similar quantity of materials (SY-MSCs and bone marrow-derived MSCs). Therefore, our system has potential use in the treatment of partial-thickness defects of articular cartilage when it is impossible to recruit MSCs from the bone marrow.

This study has some limitations because we did not investigate all possible factors. For example, we did not compare our method with the conventional 3-D culture methods. We did not investigate the effects of an extended culture period (>3 days) on matrix production and compare these between the different ratios of SY/AC cells. We did not perform immunohistochemistry to analyze collagen X. Further experiments are under way to address these limitations. Overall, our data show that the HDSS culture system is an effective and fast technique for scaffold-free construction and therefore make ACI more clinically practical because of the shorter time needed for implant

preparation.

To our knowledge, no previous studies have demonstrated the feasibility of cellular transplants comprising different cells or origins of cells. The HDSS system maintains ACs and SYs in a high-density suspension for a short time and yields 3-D minimal building units of spheroids. Frequent cell-cell contact, as permitted by growth at high density, stimulates the formation of aggregates. This technology may have an advantage in the clinical setting because it is scaffold free and uses no artificial substances. With this new technique, broad lesions can be treated without the need for scaffolds and artificial biomaterials.

5. Conclusion (結論)

In tissue engineering and regenerative medicine, cell spheroid production, culture system, medium component modification, and monitoring system are just a few of the aspects that should be considered for translating certain therapeutic techniques to clinical application. Our study offers significant improvements for the application of ACI in treatments for patients with broad areas of osteoarthritic lesions.

Our results show that our newly developed HDSS culture system is a novel method to rapidly expand a cell population in a short time and to manufacture implantable spheroids as the therapeutic minimal building unit. The scaffold-free spheroids fabricated from the cocultured SYs and ACs through HDSS have a demonstrated capacity to generate ECM efficiently and to promote articular cartilage regeneration. The application of ACI as a treatment for osteoarthritis has been enhanced by HDSS. Interestingly, our findings show that SY-MSCs, whose proliferation and differentiation potencies remain unaltered during aging, can effectively replace ACs in ACI.