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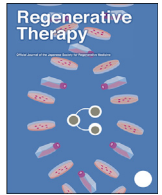
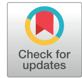


Original Article

Electron microscopic observation of human auricular chondrocytes transplanted into peritoneal cavity of nude mice for cartilage regeneration



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A B S T R A C T

Restoration of damaged cartilage tissue has been deemed futile with current treatments. Although there have been many studies on cartilage regeneration thus far, there is no report that chondrocytes were completely re-differentiated in vitro. The clarification of cellular composition and matrix production during cartilage regeneration must be elucidated to fabricate viable mature cartilage in vitro. In order to achieve this aim, the chondrocytes cultured on coverslips were transplanted into the peritoneal cavities of mice. At different time points post-transplantation, the cartilage maturation progression and cells composing the regeneration were examined. Cartilage regeneration was confirmed by hematoxylin & eosin (HE) and toluidine blue staining. The maturation progression was carefully examined further by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). At the first and second week time points, various cell shapes were observed using SEM. Chronologically, by the third week, the number of fibers increased, suggesting the progression of extracellular matrix (ECM) maturation. Observation through TEM revealed the chondrocytes located in close proximity to various cells including macrophage-like cells. On the second week, infiltration of lymphocytes and capillary vessels were observed, and after the third week, the chondrocytes had matured and were abundantly releasing extracellular matrix. Chronological observation of transplanted chondrocytes by electron microscopy revealed maturation of chondrocytes and accumulation of matrix during the re-differentiation process. Emerging patterns of host-derived cells such as macrophage-like cells and subsequent appearance of lymphocytes-like cells and angiogenesis were documented, providing crucial context for the identification of the cells responsible for in vivo cartilage regeneration.

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1. Introduction

Cartilage tissue is a promising target for regenerative medicine, because it is difficult to be repaired once damaged. With an abundant demand for the clinical application of regenerative cartilages, the topic has been one of great interest. In 1994, Brittberg et al. first reported autologous chondrocyte implantation (ACI) [1] as a viable reconstructive effort of cartilage, and since then, many have reported the success of this therapy to ameliorate knee defects [2,3]. The preparation of ACI involves the injection of cultured chondrocyte suspension, resulting in a soft consistency at the time of transplantation which is expected to harden as maturation progresses in vivo. In recent clinical studies, some groups performed dorsal nasal augmentation procedures using autologous cultured chondrocytes. One group reported a two-stage

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transplantation method in which regenerative cartilage was injection-transplanted subcutaneously in the patient's lower abdomen, and the matured cartilage tissue was then removed and transplanted into the nose for successful nasal reconstruction [4]. However, the two-step surgery requires multiple operations, resulting in increased potential for complications and burdens for the patient. Hoshi et al. reported the transplantation of regenerative cartilage into a scaffold before implantation into the target site [5]. The scaffold technique circumvented the disadvantages associated with the two-step surgery method. Yet, the cartilage implant was not fully matured pre-transplantation, thus creating a potential for composition deformities such as volume changes after transplantation. To form the desired physiologically and structurally stable regenerative cartilage, the cartilage must be matured in vitro. There are a number of factors, not previously applied for in vitro chondrogenesis, that has been reported to be involved in the proliferation or re-differentiation process of chondrocytes, including bone morphologic protein-2 (BMP-2) [6–8], transforming growth factor-β (TGF-β) [9,10], insulin-like growth factor (IGF) [11,12], fibroblast growth factor (FGF) [13–15] and hypoxia inducible factor-1α (HIF-1α) [16].

Production of mature cartilage in vitro necessitates a thorough comprehension of cartilage regeneration in vivo. The involvement of the host cells in cartilage regeneration has been shown in several reports. Fujihara et al. [17] showed that the interactions between chondrocytes and macrophages may increase granulocyte-colony stimulating factor secretion by macrophages and induce the expression of Fas ligand (FasL) on chondrocytes. FasL in turn induces the apoptosis of macrophages and suppresses tissue reactions, promoting the maturation of tissue-engineered cartilage. Additionally, they reported that the constructs containing Mif+/+ chondrocytes showed greater accumulation of cartilage matrix on the second week after transplantation than those containing Mif−/− chondrocytes [18]. Takebe et al. [19] showed that transient vascularization is important for cartilage regeneration. With accumulating evidence for the involvement of host cells, there are still many unclear points such as the mode of appearance of host-derived cells and temporal changes in the interaction between host and donor cells.

Along with host-derived cells, chondrocytes can also change their morphology. Electron microscopy is a technique commonly used in the field of regenerative medicine with which cell morphology and minute structures in and around cells can be analyzed. This imaging technique was utilized in past studies to examine the influence of scaffolds or extracellular matrixes (ECM) on the morphology of chondrocytes [20–22]. S. Nürnberger and colleagues provided evidence for differing chondrocyte morphology of horse cartilage in matrix-associated autologous chondrocyte transplantation (MACT) compared to horse cartilage chondrocytes in a collagen scaffold [23]. However, there have not been any reports on the temporal changes of the chondrocyte morphology or association of chondrocytes with host cells after transplantation.

In this study, we transplanted human auricular chondrocytes into the peritoneal cavities of nude mice and examined the emerging host cells in detail using electron microscopy at several time points in the re-differentiation process of chondrocytes.

2. Materials and methods

2.1. Cell preparation

All procedures were approved by the Research Ethics Committee of the University of Tokyo Hospital (ethical permission number 622). Human auricular chondrocytes from microtia patients were obtained from NAGATA Microtia and Reconstructive Plastic Surgery Clinic (Saitama, Japan). The patients provided the excess cartilage tissue voluntarily and agreed to the informed consent prescribed by the ethics committee. Human auricular chondrocytes were collected from the cartilage tissues as previously described [24]. The soft tissues and perichondria were removed from the specimen using a scalpel and scissors, and the auricular cartilage was finely minced. Cartilage fragments were incubated in 0.3% collagenase solution for 18 h at 37 °C in a shaking water bath. The solution was filtered through a cell strainer (100 μm pore size, BD Falcon), and the filtrate containing the human auricular chondrocytes were centrifuged at 400 g for 5 min. Cells were seeded at a density of 2.0×10^5 cells/dish onto φ100 mm collagen Type I Coated dish (AGC Techno Glass Co., Ltd.), and cultured for 10 days in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Sigma–Aldrich Co.) supplemented with 5% human serum (Sigma–Aldrich Co.), 100 ng/mL FGF-2 (Kaken Pharmaceutical Co, Ltd.), 5 μg/mL insulin (Novo Nordisk Pharma Ltd.) and 1% penicillin/streptomycin (Sigma–Aldrich Co.) (cartilage growth medium: HFI) at 37 °C in a humidified atmosphere containing 5% CO_2. Once the chondrocytes had reached complete confluency, the cells were collected from the dishes using Trypsin–EDTA (Sigma–Aldrich Co.), centrifuged, re-suspended in CELLBANKER (Nippon Zenyaku Kogyo Co., Ltd.), and stored at −80 °C.

2.2. Transplantation of chondrocytes cultured on the coverslips

Thermanox Coverslips (Thermo Scientific NuncTM) were cut into squares the size of 5 mm × 5 mm. Frozen stocks of P0 chondrocytes were thawed and cultured with HFI (described above) for a week. Then, a cover slip was placed on the bottom of a 48-well plate (Falcon), where 5×10^4 cells of P2 chondrocytes were seeded and cultured in HFI for a week.

The human chondrocytes along with the coverslip were transplanted into peritoneal cavities of 6-week-old male Balb/c nu/nu mice (CLEA Japan, Inc.). Five milliliters of DMEM/F12 (Sigma–Aldrich Co.) was injected into the peritoneal cavity, gently pumped in the abdomen for 3 min, and the washing solution was collected every week for 4 weeks. At specific time points, the coverslips were harvested and used for histological analyses. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

2.3. Hematoxylin & eosin (HE) and toluidine blue staining

The collected tissues were fixed with 4% paraformaldehyde at 4 °C overnight. The samples were dehydrated and embedded in paraffin. 5 μm thick sections were prepared and stained with HE and toluidine blue stainings. Images of each histological section were taken with the Kompaktes Fluoreszenz-Mikroskop HS-Modellreihe BZ-9000 and BZ-II Analyzer (KEYENCE Corp.).

2.4. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

The tissue samples attached to the cover slips were harvested on week 1, 2, 3 and 4 after transplantation, fixed with 2% glutaraldehyde in phosphate buffered saline, and subsequently post-fixed in 2% osmium tetra-oxide for 2 h in an ice bath. Thereafter, the specimens were dehydrated in a graded ethanol series.

To prepare the sample for SEM, specimens were dried using t-butyl alcohol freeze-drying, coated by osmium plasma ion coater and observed by SEM66 (JEM-6320F, JEOL). For TEM, dehydrated specimens were embedded in the epoxy resin. Ultrathin sections were obtained using the ultramicrotome technique. The ultrathin sections were stained with uranyl acetate for 10 min and then

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