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Original Article

Human auricular chondrocytes with high proliferation rate show high production of cartilage matrix

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ABSTRACT

Cartilage has a poor capacity for healing due to its avascular nature. Therefore, cartilage regenerative medicine including autologous chondrocyte implantation (ACI) could be a promising approach. Previous research has proposed various methods to enrich the cultured chondrocytes for ACI, yet it has been difficult to regenerate homogeneous native-like cartilage in vivo. The cell populations with an increased ability to produce cartilage matrix can show somatic stem cells-like characteristics. Stem cells, especially somatic stem cells are able to grow rapidly in vitro yet the growth rate is drastically reduced when placed in in vivo conditions [14]. Thus, in this study we investigated whether proliferation rate has an impact on in vivo regeneration of cartilage constructs by sorting human chondrocytes. The human chondrocytes were fluorescently labeled with CFSE and then cultured in vitro; once analyzed, the histogram showed a widening of fluorescence level, indicating that the cells with various division rates were included in the cell population. To compare the characteristics of the cell groups with different division rates, the chondrocytes were sorted into groups according to the fluorescence intensity (30 or 45 percent of cells plotted in the left and right sides of histogram). Then the cells of the rapid cell group and slow cell group were seeded into PLLA scaffolds respectively, and were transplanted into nude mice. Metachromatic regions stained with toluidine blue were larger in the rapid cell group compared to the slow cell group, indicating that the former had higher chondrogenic ability. We proposed a new method to enrich cell population with high matrix production, using proliferation rate alone.
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1. Introduction

Due to its avascular nature, cartilage has a poor capacity for healing once it has been damaged. Therefore, autologous chondrocyte implantation (ACI) could be a promising approach in the field of cartilage regenerative medicine. Human ACI was first reported in 1994 by Brittberg et al., in which autologous chondrocytes from a healthy non-bearing site of cartilage in a knee joint were cultured in vitro, and then transplanted into the defective sites [1,2]. While transplantation of autologous cells is advantageous in terms of controlling immune response, it requires the process of isolating and expanding the cells to the amounts that would suffice for transplantation. Meanwhile, changes in cell morphology or dedifferentiation could occur during culture, leading to reduced matrix production [3,4].

It is difficult to make native cartilage-like tissue with three-dimensional structure and uniform cartilaginous properties, however cartilaginous tissue has been regenerated by transplanting the dedifferentiated chondrocytes into the body [5]. A probable explanation for the nonuniform cartilaginous properties seen in previous research could be the inconstancy of cells used for transplantation. If the primary chondrocytes taken from cartilage contain multiple cell populations, the regenerated tissue may also

Abbreviation: CFSE, Carboxyfluorescein diacetate succinimidyl ester; PLLA, poly-L-lactic acid scaffolds.

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become heterogeneous. As a breakthrough for this issue, methods should be established to enrich the cells that have more potential to produce cartilage matrix. There have been studies on enriching the cells, in which culture conditions or cell surface markers have been examined [6–12]. Culture conditions have been regulated in which the cells should be seeded at low density, and cultured with low glucose medium or under hypoxia [6–9]. These in vitro studies have proved to be effective, however the efficacy has not been sufficiently replicated for in vivo transplantation thus far. There have been reports in which cell populations with high cartilage matrix capacity were identified, focusing on the enrichment of MSC-like and progenitor cells [10–13]. Yet the expression of cell surface markers was not consistent due to changes in culture conditions and passage numbers. Therefore, thus far, it has not been feasible to regenerate homogenous tissue-engineered cartilage in vivo.

The population with high ability to produce cartilage matrix shows somatic stem cell-like characteristics. Stem cells, especially somatic stem cells are able to grow rapidly in vitro yet the growth rate is drastically reduced when placed in in vivo conditions [14]. Thus, in this study it was imperative to investigate whether proliferation rate has an impact on in vivo regeneration of cartilage constructs. To concentrate the chondrocytes according to the proliferation rate, we sorted human chondrocytes with the high proliferation rate and then evaluated the regeneration of cartilage constructs in mice.

2. Materials and methods

2.1. Isolation of human auricular chondrocytes

This study was approved by the Research Ethics Committee of the University of Tokyo Hospital. Auricular cartilages were provided as excised remnant auricular cartilage tissue from the surgery of microtia patients in NAGATA Microtia and Reconstructive Plastic Surgery Clinic. We obtained informed consent from all patients. After the excision of soft tissues and perichondria by scalpel and scissors, auricular cartilage was minced, and digested by shaking with 0.3% collagenase solution for 18 h at 37 °C. The solution was filtered with a cell strainer (100 μm pore size, BD Falcon), centrifuged at 1500 rpm for 5 min and the supernatant was removed to obtain human auricular chondrocytes. Cells were seeded at 2.0×10^5 cells/dish to φ100 mm collagen Type I Coated dich (AGC Techno Glass Co., Ltd.), and cultured in the cartilage growth medium (HFI; 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.), 1% Penicillin/Streptomycin (Sigma–Aldrich Co.)) at 37°C in 5% CO_2. After 10 days, cells reached the confluence, and they were detached by Trypsin-EDTA (Sigma–Aldrich Co.), recovered by centrifugation, and stored frozen at −80°C in CELLBANKER (Nippon Zenyaku Kogyo Co., Ltd.).

2.2. Cell labeling

The stored cells were thawed and cultured under the conditions described above (passage 1: P1). The cells were collected using Trypsin-EDTA at the confluence on day 7, and stained by CellTrace™ CFSE Cell Proliferation Kit (CFSE; Thermo Fisher Scientific Inc.) or the CellTrace™ Violet Cell Proliferation Kit (Thermo Fisher Scientific Inc.), according to the protocols provided by the supplier. The cells were observed by Leica DM IL (Leica Microsystems K.K.). Viability of cells was examined by NucleoCounter (Chemometec A/S). The labeling of cells was confirmed with BD™ LSR II (Becton, Dickinson and Co.) or BD FACSAria™ Fusion (Becton, Dickinson and Co.). After labeling, the cells were seeded again as P2 and cultured after which they were analyzed or transplanted.

2.3. Flow cytometry for cell surface antigens

On day 4 of the P2 culture, cells stained with CFSE were collected and labeled with allophycocyanin-conjugated antibodies against human epitopes in accordance with the protocol provided by each manufacturer. Epitopes and the manufacturers of antibodies were as follows: CD14, CD90, CD166, STRO-1 (BioLegend Japan Inc.), CD29, TRA-1-60, TRA-1-81 (Affymetrix Inc.), CD44, CD73, CD105, SSEA-4 (Miltenyi Biotec K.K.), CD31, CD45 (Cymbus Biotechnology Ltd.), CD34, SSEA-1 (Sony Biotechnology Inc.). The cells were examined by BD™ LSR II, and collected data were analyzed by BD FACSDiva™ software (Becton, Dickinson and Co.) or FlowJo (Tomy Digital Biology Co., Ltd.).

2.4. Cell sorting

On day 4 of the P2 culture, cells stained with CFSE were collected and sorted using BD FACSAria™ (Becton, Dickinson and Co.). The gates were set to 45 percent (left spaces 10 percent between both sides, rapid and slow group) or 30 percent of both ends in fluorescence histogram. For longer observation, the cells collected on day 7 of the P1 culture were labeled with Violet, and cultured until day 4 of the P2 culture. Then, the cells were sorted using BD FACSAria™ Fusion, with the gates set to 45 percent of both ends in fluorescence histogram. The sorted cells were seeded again, and the cells in P3 were analyzed by flow cytometry.

2.5. Preparation of cartilage regenerative constructs and transplantation

The cells collected on day 7 of the P1 culture were labeled with CFSE, and cultured until day 4 of the P2 culture. Then, the cells were sorted into 2 groups (rapid and slow cells) according to their fluorescence intensities with the gates set to 30 or 45%. The sorted cells were mixed with 1% atelocollagen solution respectively at the concentration of 1×10^7 cells/mL, and 3×10^6 cells/300 μL and were embedded in poly-L-lactic acid scaffolds (PLLA; KRI) ($5 \times 5 \times 3$ mm) to make the cartilage regenerative constructs. After 2 h of incubation, constructs were implanted subcutaneously into 6-week-old male BALB/cA-nu nude mice (Nippon Bio-Supp. Center). The mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Then a small incision was made on the back at the midline, and cartilage regenerative constructs, were implanted subcutaneously. The grafts were harvested 4 and 8 weeks post-implantation.

2.6. Histology

The recovered grafts were split in half by a scalpel, and fixed for 2 h with 4% paraformaldehyde solution. The samples were dehydrated and embedded in paraffin. Sections were sliced to a thickness of 5 μm, and stained with Toluidine blue. Positive area of staining was quantified by the Kompaktes Fluoreszenz-Mikroskop HS-Modellreihe BZ-9000 and BZ-II Analyzer (KEYENCE Corp.).

2.7. Statistics

Data were expressed as mean ± SD, and statistically analyzed using Student's t test. A value of $p < 0.05$ was used to indicate statistical significance.

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