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Original Article

Optimal conditions of collagenase treatment for isolation of articular chondrocytes from aged human tissues

Kazumichi Yonenaga [a, b], Satoru Nishizawa [a], Takumi Nakagawa [b], Yuko Fujihara [a], Yukiyo Asawa [a], Atsuhiko Hikita [a], Tsuyoshi Takato [b], Kazuto Hoshi [a, b, *]

[a] Department of Cartilage & Bone Regeneration (Fujisoft), Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8655, Japan
[b] Department of Sensory & Motor System, Graduate School of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8655, Japan

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ABSTRACT

Introduction: There are various types of cartilage, including the auricular and articular cartilages. These cartilages have different functions, and their matrix volume and density of chondrocytes may differ. Thus, different protocols may be required to digest different types of cartilage.
Methods: In this study, we examined protocols for the digestion of articular and auricular cartilages and determined the optimal conditions for articular cartilage digestion.
Results: Our histological findings showed that the articular cartilage has a larger matrix area and fewer cells than the auricular cartilage. In 1-mm^2 areas of articular and auricular cartilages, the average numbers of cells were 44 and 380, respectively, and the average matrix areas were 0.94 and 0.77 mm^2, respectively. The maximum numbers of viable cells (approximately 1×10^5 cells/0.1 g of tissue) were obtained after digestion in 0.15, 0.3, or 0.6% collagenase for 24 h, in 1.2% collagenase for 6 h, or in 2.4% collagenase for 4 h. In tissues incubated in 0.15 or 0.3% collagenase, the cell numbers were lower than 1×10^5, even at 24 h, possibly reflecting incomplete digestion of cartilage. No significant differences were observed in the results of apoptosis assays for all collagenase exposure times and concentrations. However, cell damage appeared to be greater when collagenase concentrations were high. When cells obtained after digestion with different concentrations of collagenase were seeded at a density of 3000 cells/cm^2, they yielded the maximum cell numbers after 1 week.
Conclusions: We recommend a 24-h incubation in 0.6% collagenase as the optimal condition for chondrocyte isolation from articular cartilage. Moreover, we found that the optimum cell-seeding density is approximately 3000 cells/cm^2. Conditions determined in this study would maximize the yield of isolated articular chondrocytes and enable the generation of a large quantity of cultured cells.

1. Introduction

Collagenase digestion is the first step in cartilage tissue engineering [1–11]. Incomplete digestion reduces the efficiency of tissue engineering and affects the safety of the process and stability of grafts. Therefore, complete digestion is an important process in cartilage tissue engineering. We believe that complete digestion can be achieved by varying the digestion protocol according to the type of cartilage matrix. In a previous study, we optimized the conditions for isolating and seeding human chondrocytes from the auricular cartilage [1]. Specifically, we showed that 24 h of incubation in 0.3% collagenase or 6 h of incubation in 0.6% collagenase were optimal conditions for isolating chondrocytes from cartilage fragments ranging in size from 250 to 1000 μm. We also showed

* Corresponding author. Department of Cartilage & Bone Regeneration (Fujisoft), Graduate School of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8655, Japan. Fax: +81 3 5800 9891.
E-mail addresses: yonenaga-tky@umin.ac.jp (K. Yonenaga), pnishizawa-tky@umin.ac.jp (S. Nishizawa), takumin-tky@umin.ac.jp (T. Nakagawa), fujiharay-ora@umin.ac.jp (Y. Fujihara), asawasawa-tky@umin.net (Y. Asawa), ahikita-tky@umin.ac.jp (A. Hikita), takato-ora@h.u-tokyo.ac.jp (T. Takato), pochi-tky@umin.net (K. Hoshi).
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that the cell seeding density should be in the range of 3000–10,000 cells/cm^2. However, other reports described widely different collagenase concentrations (range, 0.04–0.25%; <960 U/mL) and incubation times (range, 4–24 h) for the isolation of human articular chondrocytes [12–16]. There are various types of cartilage besides the auricular cartilage, including the articular cartilage, and each type has a different function [17–20]. Therefore, the volume of the cartilage matrix and density of chondrocytes may differ among cartilage types.

These differences in the matrix necessitate the use of different protocols for digesting different cartilage types, such as the articular and auricular cartilages. In this study, we compared the two aforementioned types of cartilage, which have different density, matrix types, and chondrocytes. We determined the optimal conditions for digesting articular cartilage, following a protocol that we established previously for auricular cartilage digestion, as a reference.

2. Methods

2.1. Histology

The cartilage was fixed with 4% paraformaldehyde, embedded in optimum cutting temperature (OCT) compound (Sakura, Tokyo, Japan), and cryosectioned into 10-μm slices. The sections were stained with Toluidine Blue O. The cell number and matrix area were analyzed using EasyAccess (AD Science Co., Tokyo, Japan).

2.2. Chondrocyte isolation

All procedures were approved by the Ethics Committee of the University of Tokyo Hospital (ethics permission number 622). Remnant articular cartilages from three patients with osteoarthritis were obtained during surgery. Sampling was performed in accordance with the principles of the Declaration of Helsinki. The three patients were women aged 74–84 years (mean age, 80 years). The cartilage tissues were thoroughly minced with scissors and tweezers into fragments of 250–1000 μm in size [1]. Five different concentrations (0.15, 0.3, 0.6, 1.2, and 2.4%) of collagenase from *Clostridium histolyticum* (product catalog number: 038-10531; Wako Pure Chemical Industries, Osaka, Japan) were used. Collagenase solution from a single lot was used throughout the study (291 U/mL). Since this was a typical lot, no information regarding other types of collagenase was provided by the manufacturer. Approximately 3 mL of the collagenase solution was transferred into a 5-mL tube (BD Falcon, Bedford, MA, USA), and 20 tubes were prepared (four tubes for each concentration). A cartilage fragment weighing approximately 0.1 g was added into each tube, and the tubes were incubated in a 37 °C water bath, with agitation at 150 cycles/min. For each collagenase concentration, we measured the total number of cells, number of viable cells, as well as cell viability, using a NucleoCounter (ChemoMetec, Allerod, Denmark) after 2, 4, 6, and 24 h [1].

2.3. Chondrocyte culture

Viable cells were seeded into 6.4-mm plastic culture dishes (96-well plate) coated with collagen type 1. Cells were seeded at densities of 30,000, 10,000, 3000, 1000, 300, and 100 cells/cm^2, and the optimal cell-seeding density for primary cultures was determined. For apoptosis analysis, cells digested under specific conditions were cultured in 35-mm plastic culture dishes coated with collagen type 1. The culture medium was Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (Sigma Chemical Co., St. Louis, MO, USA) containing 5% human serum (Sigma Chemical Co.), 100 ng/mL fibroblast growth factor-2 (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan), and 5 μg/mL insulin (MP Biomedicals, Irvine, CA, USA) [21].

2.4. Photometric analysis of ssDNA in apoptotic cells by enzyme-linked immunosorbent assay (ELISA)

We transferred 5000 cells into each well of a 96-well microplate, and the microplate was centrifuged at 200 *g* for 5 min. The medium was removed, and 200 μL of fixative (80% methanol in phosphate-buffered saline) was added into each well. The microplate was incubated at room temperature for 30 min, and the fixative was then removed. Subsequently, the microplate was incubated at room temperature for 1–2 h to allow for cell attachment to the plate. Cell apoptosis was determined using the ssDNA apoptosis ELISA Kit (CHEMICON® International Inc., Billerica, MA, USA), according to the manufacturer's instructions. Absorbance was measured at 405 nm, using a standard microplate reader (ARVO SX 1420 Multilabel Counter; Perkin Elmer, Waltham, MA, USA).

2.5. Statistics

Data obtained from three replicate samples per group were analyzed by t-tests in MS Excel (Microsoft Co., Bellevue, WA, USA). The results are expressed as means ± standard deviation.

3. Results

3.1. Histology

Histological examination revealed a larger matrix area and fewer numbers of cells in the articular cartilage than in the auricular cartilage (Fig. 1). The average number of cells in a 1-mm^2 area of articular and auricular cartilages was 44 and 380 cells, respectively; the average matrix area in a 1-mm^2 area of articular and auricular cartilages was 0.94 and 0.77 mm^2, respectively (Fig. 2).

3.2. Cartilage digestion

We examined the effects of collagenase concentration and incubation time on cartilage digestion. Cartilage fragments were not completely digested after 24 h of incubation in 0.15 or 0.3% collagenase; however, the fragments were digested after incubation for the same duration in 0.6% or higher concentrations of collagenase (Table 1). The times required for the digestion of cartilage fragments were 6 h and 4 h in 1.2% and 2.4% collagenase, respectively (Table 1).

For all collagenase concentrations, except for the 24 h incubation with 2.4% collagenase, both the total number of cells and the number of viable cells appeared to increase with incubation time (Fig. 3A and B). The number of viable cells from approximately 0.1 g of tissue was close to the maximum number (1×10^5 cells) following incubation in 2.4, 1.2, and 0.6% collagenase at 4, 6, and 24 h, respectively. However, tissues incubated in 0.3% and 0.15% collagenase yielded less than 8.4×10^4 cells and 6.3×10^4 cells, respectively, even after 24 h (Fig. 3A and B). In other words, the yields were less than those obtained by digestion in 0.6, 1.2, and 2.4% collagenase, which may be due to the incomplete digestion of cartilage fragments incubated in 0.3% and 0.15% collagenase. The total number of cells and number of viable cells obtained from cartilage fragments incubated in 0.15, 0.3, and 0.6% collagenase for 24 h and in 1.2% collagenase for 6 h were significantly higher than those obtained from cartilage fragments incubated at all four concentrations of collagenase for 2 h (Fig. 3A and B). The total and viable cell counts decreased for tissue digested in 2.4% collagenase after 24 h of incubation as a result of reduced viability (Fig. 3C).