Application of floating cells for improved harvest in human chondrocyte culture

Kazumichi Yonenaga1, 2, Satoru Nishizawa1, Yuko Fujihara1, Yukiyo Asawa1, Sanshiro Kanazawa1, Satoru Nagata3, Tsuyoshi Takato2, and Kazuto Hoshi1
Departments of 1Cartilage & Bone Regeneration (Fujisoft), and 2Sensory & Motor System, Graduate School of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8655, Japan; 3Nagata Microtia and Reconstractive Plastic Surgery Clinic, Sasameminamichio 22-1 Totsuka, Saitama 335-0035, Japan

(Received 22 June 2012; and accepted 30 July 2012)

ABSTRACT
Cell culture medium, which must be discarded during medium change, may contain many cells that do not attach to culture plates. In the present study, we focused on these floating cells and attempted to determine their usefulness for cartilage regeneration. We counted the number of floating cells discarded during medium change and compared the proliferation and differentiation between floating cells and their adherent counterparts. Chondrocyte monolayer culture at a density of $5 \times 10^3$ cells/cm$^2$ produced viable floating cells at a rate of $2.7–3.2 \times 10^3$ cells/cm$^2$ per primary culture. When only the floating cells from one dish were harvested and replated in another dish, the number of cells was $2.8 \times 10^4$ cells/cm$^2$ (approximately half confluency) on culture day 7. The number of cells was half of that obtained by culturing only adherent cells ($5 \times 10^4$ cells/cm$^2$). The floating and adherent cells showed similar proliferation and differentiation properties. The recovery of floating cells from the culture medium could provide an approximately 1.5-fold increase in cell number over conventional monolayer culture. Thus, the collection of floating cells may be regarded as a simple, easy, and reliable method to increase the cell harvest for chondrocytes.

Research on cartilage regeneration is relatively advanced compared with that on other tissues, and some protocols have already been applied in clinical settings. Autologous chondrocytes have been implanted in patients with focal cartilage defects in their joints since as early as the 1990s (4), and these cells have been used for nasal augmentation by injection into subcutaneous pockets (31). It is important for further progress in cartilage regeneration to increase the number of chondrocytes that can be harvested.

Physiologically, chondrocytes proliferate and multiply even when surrounded by solid extracellular matrix in all directions. Thus, cartilage expands by means of interstitial growth in vivo (2, 5). However, once chondrocytes are isolated from native tissue and begin to multiply in culture, they inevitably lose their ability to produce cartilaginous matrix components such as glycosaminoglycans (GAG) and type II collagen (COL2), and they begin to produce type I collagen (COL1) in a process termed dedifferentiation (3). The prevention of dedifferentiation in cultured chondrocytes and the redifferentiation of dedifferentiated cells are issues to be solved in the cytological fields (17).

To prevent excessive dedifferentiation, a sufficient number of chondrocytes should be obtained to permit minimal multiplication of cells because an increase in the number of cell passages performed reduces the capacity of the chondrocytes for differentiation or cartilage regeneration in vivo (6, 30).
Long-term culture with repeated passaging also increases the risk of bacterial infection or cross-contamination of cells from other donors. Shortening the culture period may benefit patients by reducing the therapy period, thereby improving quality of life and health (8).

We attempted to improve the efficacy of collagenase digestion for chondrocyte isolation from cartilage and to harvest the maximum possible number of viable cells (34). With this method, we successfully obtained $1 \times 10^7$ viable cells/g from the tissue, which was approximately 10-fold higher than the value previously reported ($1 \times 10^6$ cells/g) (15). We seeded cells at a low cell density (5,000 cells/cm²), which improved the efficacy of cell expansion and suggests that the actual gain would be increased by more than 1 order of magnitude. However, we often observed cells that did not adhere to culture plates and continued to float over a certain time period after cell seeding. In general, we changed the culture medium every 2 or 3 days (23, 24), at which time these floating cells were generally discarded.

We reasoned that if adequate space is provided for the attachment of cells, the cells floating within the medium may effectively adhere to the plate and begin to proliferate. Thus, in the present study, we focused on the non-adherent cells in the cell culture medium. We examined the characteristics of these floating cells and discuss the possibility of using them in clinical applications.

MATERIALS AND METHODS

Cell isolation and monolayer culture. All procedures were approved by the Ethics Committee of the University of Tokyo Hospital (ethics permission number 622). Remnant auricular cartilage from 3 microtia patients was obtained during surgery in compliance with the Helsinki Principles. The chondrocytes were isolated by digestion with 0.3% collagenase over 24 h (Wako Pure Chemical Industries, Osaka, Japan). The primary auricular chondrocytes were seeded in 35-mm collagen type I-coated plastic culture dishes ($n = 36$) at a density of $5 \times 10^4$ cells/cm² and cultured in 2 mL of Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12) containing 5% human serum supplemented with fibroblast growth factor-2 (100 ng/mL) and insulin (5 μg/mL) as previously described (10, 22).

We divided the 36 dishes into 3 groups ($n = 12$ per group). The media were changed at different times (day 1, 2, or 3 of culture) in each group. Floating cells, which are usually discarded, were collected from the media (Fig. 1). The samples from each day were further divided into 2 subgroups: centrifugation and non-centrifugation. In the centrifugation group ($430 \times g$ for 5 min), the supernatants were discarded before seeding. The cells were counted using a NucleoCounter (ChemoMetec, Allerod, Denmark) (35). In contrast, the entire cell suspension was used for seeding in the non-centrifugation group. The cells obtained from 1, 2, or 3 dishes of primary cultures were combined into another, separate dish. Floating cells were cultured in a monolayer and then harvested at day 7, at which time the adherent cells had also reached confluence. Harvested cells were divided into three groups: adherent cells only (A), floating cells only (F), and mixed cell culture, in which the numbers of adherent and floating cells were equal (F + A) (Fig. 1). These cells were reseeded at a density of $2.5 \times 10^5$ cells/cm² and then incubated for 1 week, at which time the gene expression of type I collagen αI chain (COL1A1) and type II collagen αI chain (COL2A1) was evaluated. We used trypsin-EDTA solution to lift the cells during subculturing.

Pellet culture. For the pellet culture, cultured chondrocytes were suspended in 0.8% atelocollagen solution (Kawaken Fine Chemicals, Tokyo, Japan) at a density of $10^7$ cells/mL. The atelocollagen pellets were cultured in DMEM/F12 medium with soluble factors for 3 weeks. We used recombinant human bone morphogenetic protein-2 (rhBMP-2, kindly provided by Astellas Pharma, Tokyo, Japan), rh-insulin (MP Biomedicals, Irvine, CA), and L-3, 3’, 5-triiodothyronine (T3, EMD Bioscience). The dosage of each factor was determined to be 200 ng/mL BMP-2, 5 μg/mL insulin, and 100 nmol/L T3 on the basis of our and other previous reports (7, 9, 11, 12, 14, 16, 18, 21, 28, 36).

Real-time reverse transcription-polymerase chain reaction analysis. Total RNA was isolated from the chondrocytes with ISOGEN (Wako Pure Chemical Industries) according to the manufacturer’s protocol. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA). The cDNA of the target genes, including the PCR amplicon sequences, was amplified by PCR and used as standard templates after linearization. A QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) was used, and SYBR Green PCR amplification and real-time fluorescence detection were performed with the ABI 7700 sequence detection
system. All reactions were run in quadruplicate. The sequences of the primers were 5'-CTCCTCGCTT TCCCTTCCTC-3' and 5'-GTGCTAAAGGTGCCCAG TTGT-3' for COL1A1; 5'-GAGTCAAGGTTGACGT GTGTT-3' and 5'-CACCTTGGTCTCAGAAG GA-3' for COL2A1; and 5'-GAAG GTGAAGGTC GGAGTCA-3' and 5'-GAAGATGTTGATGGGAT TTC-3' for glyceraldehyde-3-phosphate dehydroge- nase (GAPDH).

GAG measurement. The sulfated GAG content was measured using an Alcian blue binding assay (Wieslab AB, Lund, Sweden). After digestion of the chondrocyte-containing atelocollagen pellet in 0.3% collagenase for 1 h at 37°C, the cell debris and insoluble material were removed by centrifugation at 6,000 × g for 30 min. GAG in the supernatant was precipitated using Alcian blue solution, and the sediments were redissolved in 4 mol/L GuHCl-33% propanol solution by centrifugation at 6,000 × g for 15 min. The spectrophotometric absorbance of the mixture was measured at 600 nm.

Enzyme-linked immunosorbent assay for COL1 and COL2. Collagen protein levels in the pellets were quantified by an enzyme-linked immunosorbent assay using a human type 1 and type 2 collagen detection kit (Chondrex, Redmond, WA). The pellets were dissolved in 10 mg/mL pepsin and 0.05 M acetic acid at 4°C for 48 h and then incubated in 1 mg/mL pancreatic elastase, 0.1 mmol/L Tris, 0.02 mol/L NaCl, and 5 mmol/L CaCl₂ (pH 7.8–8.0) at 4°C overnight. The samples were centrifuged at 9,100 × g for 5 min to remove the residue. The collagen proteins were bound by polyclonal anti-human COL1 or COL2 antibodies (Chondrex) and detected by biotinylated secondary antibodies and streptavidin peroxi- dase. o-Phenylenediamine and H₂O₂ were added to the mixture, and the spectrophotometric absorbance of the mixture was measured at 490 nm.

Fig. 1 Experimental design. (Upper) In conventional culture, we discarded the floating cells. (Lower) In this experiment, floating cells were collected from the media for reseeding culture. On day 7 of culture, we subcultured the cells under three conditions: only floating cells (F), both floating cells and adherent cells (F + A), and only adherent cells (A).
Histology. The regenerated cartilage was fixed with 4% paraformaldehyde, embedded in optimal cutting temperature (OCT) compound (Sakura, Tokyo, Japan), and cryosectioned into 10-μm-thick slices. The sections were stained with toluidine blue O.

RESULTS

Viable floating cells from day 1, 2, or 3 of culture were harvested in amounts ranging from $2.7 \times 10^3$ to $3.2 \times 10^3$ cells/cm$^2$, with no significant differences observed among the groups. The floating cells were counted constantly regardless of the harvested period within 3 days (Fig. 2). We harvested the floating cells on day 1, 2, or 3 and transferred them to new dishes with or without centrifugation (Fig. 3). Regardless of whether the floating cells were removed from the primary culture on day 1, 2, or 3, the number of adherent cells remaining in the primary cultures on day 7 was almost identical (approximately $5 \times 10^4$ cells/cm$^2$) in each group (Fig. 3 and Fig. 4A). For the reseeding of floating cells, centrifugation before seeding appeared to improve cell adhesion and proliferation (Fig. 3).

On day 7 of culture, the number of floating cells harvested from one dish on day 1 and reseeded on one culture dish reached approximately $2.8 \times 10^4$ cells/cm$^2$, whereas the number of floating cells combined from two dishes reached $4.2 \times 10^4$ cells/cm$^2$, and that from three dishes reached $4.8 \times 10^4$ cells/cm$^2$. These values were similar when the floating cells were harvested and reseeded on day 2, but they clearly decreased and were below the limit of detection for counting when the cells were harvested and reseeded on day 3 of culture (Fig. 4B). Thus, the number of cells reached a maximum when the cells harvested from one original dish on day 1 of culture were reseeded onto a new dish (Fig. 4C).

We next examined the properties of the regenerated cartilage containing floating cells and/or adherent cells (Fig. 5). Chondrocytes were embedded in 0.8% atelocollagen gel at a density of $10^7$ cells/mL. We used RT-PCR to examine the expression of COL1A1 and COL2A1 in cultured human auricular chondrocytes embedded in these three-dimensional matrices after 1 week. No significant differences were observed among groups containing floating cells and adherent cells in different ratios (Fig. 5). At 3 weeks, the sizes of the pellets were almost identical in all of the groups (Fig. 6A, Upper), and histological analyses using toluidine blue O staining revealed similar accumulation of proteoglycan (Fig. 6A, Lower). The levels of COL2 and GAG, both of which are specific components of cartilaginous tissues, were equivalent in regenerated cartilage produced by floating cells and adherent cells, and the levels of COL1 were comparable across groups (Fig. 6B).

DISCUSSION

The results presented here indicate that the proliferation capacity of floating cells is the same as that of adherent cells. We postulate that the initially non-adherent cells represent the population of cells that did not have adequate space to adhere in primary cultures. Cells may also become less adherent during cell division (19). During this period, some cells detach from the culture dishes, possibly becoming floating cells. The floating cells may be continuously produced, even after primary culture.

With centrifugation before reseeding, i.e., before the medium change, the cells strongly adhered to the plates and began to proliferate, as shown in Fig. 3. This may have been caused by some collagenase remaining in the medium of the primary culture when the washing step performed after enzymatic digestion was not sufficient. High concentrations and long incubation periods with collagenase are cytotoxic, as shown in a previous study (34). Another explanation for our observation is that a shortage of nutrition or growth factors may have occurred because of the decrease in the number of medium changes, which may have reduced the cell viability and adhesion of the chondrocytes. Therefore, although frequent medium changes with centrifugation may increase the risk of cell loss during

![Floating cell numbers](image-url)
Fig. 3  Phase-contrast images obtained upon reseeding of floating cells. The floating cells harvested from 1, 2, or 3 dishes were plated on a new dish with or without centrifugation. Regardless of whether floating cells were removed from the primary culture on day 1, 2, or 3, the number of adherent cells remaining in the primary culture dish on day 7 was almost identical across groups (red squares). Centrifugation before reseeding improved the adhesion and proliferation of floating cells (blue square).
Aggrecan is a cartilage-specific proteoglycan whose levels are elevated in cartilaginous regions where COL2 is also present (27). Because repeated passaging decreased the COL2 level and cell proliferation (26), it is preferable to minimize the passaging of the cells to preserve their physiological function.

Every laboratory engaged in the research and development of biomedical applications utilizes various unique protocols to provide three-dimensional structures (13, 20, 25). Future directions include the cell passaging, a medium change is required before the reseeding of floating cells.

The number of cultured floating cells prominently decreased when the cells were harvested and reseeded on day 3 of culture (Fig. 4B), possibly because a culture time of only 4 days was insufficient. Thus, we could not accurately count the cells using the NucleoCounter (35).

The COL2 evaluated in this study is a specific cartilage component. COL2 forms fibrils that permit cartilage to entrap proteoglycan aggregates and impart tensile strength to the tissue. Aggrecan is a cartilage-specific proteoglycan whose levels are elevated in cartilaginous regions where COL2 is also present (27). Because repeated passaging decreased the COL2 level and cell proliferation (26), it is preferable to minimize the passaging of the cells to preserve their physiological function.

Fig. 4  The number of cells on day 7 of culture. (A) The conventionally cultured chondrocytes reached a density of approximately $5 \times 10^5$ cells/cm$^2$ regardless of whether the floating cells were harvested on day 1, 2, or 3 of culture. (B) On day 7 of culture, cells from a dish that had been reseeded on day 1 numbered approximately $2.8 \times 10^5$ cells/cm$^2$; cells combined from two dishes numbered $4.2 \times 10^5$ cells/cm$^2$; and cells combined from three dishes numbered $4.8 \times 10^5$ cells/cm$^2$; the numbers were similar for cells reseeded on day 2. However, cells reseeded on day 3 were markedly reduced in number on day 7 of culture. (C) The maximum number of cells was recovered when the floating cells obtained from one dish on day 1 of culture were reseeded on one new dish. All values are presented as the mean and standard deviation of three samples per group.

Fig. 5 COL1A1 and COL2A1 mRNA levels measured by real-time RT-PCR after 1 week of pellet culture did not significantly differ among pellets consisting of only floating cells (F), both floating cells and adherent cells (F + A), and only adherent cells (A). All values are presented as the mean and standard deviation of three samples per group.
Floating cells in cell culture

The surgical invasiveness of the biopsy and the physical strain on patients (22, 29, 31, 32).

Minimal invasiveness is one of the major goals of biomedical techniques, and our method may increase the advantages of this approach (33). Moreover, if this culture method can be used not only for primary cultures but also for subcultures, it may permit large quantities of cultured cells to be obtained in a short time. This method could be applied to various cell culture systems and may be commonly used as a key biomedical technique.

Acknowledgements

We thank Mr. Takashi Nakamoto, Ms. Miki Akizawa, Mr. Motoki Yagi, Mr. Tomoaki Sakamoto, and Mr. Makoto Watanabe for technical support. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture,
Sports, Science and Technology of Japan (MEXT, No. 21390532 and 21659462), and Research and Development Programs for Three-dimensional Complex Organ Structures from the New Energy and Industrial Technology Development Organization and for Resolving Critical Issues from Special Coordination Funds for Promoting Science and Technology (SCF) commissioned by MEXT.

REFERENCES


