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Comprehension of terminal differentiation and dedifferentiation of chondrocytes during passage cultures

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A high density collagen type I coated substrate (CL substrate) was used to evaluate the chondrocyte phenotypes in passaged cultures. With increasing age of cell population (population doubling (*PD*) = 0–14.5), the frequency of non-dividing spindle shaped cells without ALP activity increased, accompanied with an increase in gene expression of collagen type I, meaning the senescence of dedifferentiated cells. At the middle age of cell population (*PD* = 5.1 and 6.6), the high frequency of polygonal shaped cells with ALP activity existed on the CL substrate together with up-regulated expressions of collagen types II and X, indicating the terminal differentiation of chondrocytes. When the chondrocytes passaged up to the middle age were embedded in collagen gel, the high frequency of single hypertrophic cells with collagen type II formation was recognized, which supports the thought that the high gene expression of collagen type II was attributed to terminal differentiation rather than redifferentiation. These results show that the CL substrate can draw out the potential of terminal differentiation in chondrocytes, which is unattainable by a polystyrene surface, and that the CL substrate can be a tool to evaluate cell quality in three-dimensional culture with the collagen gel.

[**Key words:** Tissue engineering; Rabbit chondrocytes; Terminal differentiation; Passage cultures; Collagen-coated substrate; Cultured cartilage]

In reconstructive surgery for repairing articular cartilage defects, serial monolayer cultures of isolated chondrocytes are performed for expanding cells so as to be sufficient for subsequent tissue cultures. One of the drawbacks in this approach is the partial or complete loss of proliferative ability with increasing passage number of subculture (1), which leads to hindering spatial growth in a scaffold typically employed for tissue reconstruction.

The decrease in proliferating vitality of chondrocytes in vitro has been associated with cellular senescence due to aging toward terminal differentiation. The progression of chondrocytes toward terminal differentiation is characterized by prolonged state of cell cycle arrest with significant increase in apparent cellular volume as well as with enhancement of collagen type X synthesis and alkaline phosphatase activity (2–4). However, the transition toward terminal differentiation in serial monolayer cultures for cell expansion of chondrocytes is still unclear and the effort to study its progression in three-dimensional (3-D) cultures has been hampered by lack of a suitable in vitro model.

An alternative is to use a two-dimensional (2-D) culture system for the evaluation of cell behavior. However, it is well-known that chondrocytes dedifferentiate when grown in vitro on a traditional tissue culture polystyrene (PS) surface in a way of monolayer manner, acquiring a fibroblastic-like morphology, and that instead of the cartilage-specific collagen (collagen type II), they synthesize the collagen type I (5,6). Despite the fact that chondrocytes in suspension and pellet cultures have potential to undergo terminal differentiation (7–9), the limitation for single cell analysis in these systems prevents thorough understanding of the cell phenotypes and the heterogeneity in the cell population.

The previous findings have resolved this dilemma by modifying a conventional PS surface by coating with high-density collagen type I (CL substrate). The CL substrate provides a 3-D mimicking environment to chondrocytes, and enabled to evaluate the cell states for dedifferentiation in a quantitative manner (10). Recent studies have demonstrated that the cell morphology indicates the states of rabbit chondrocytes, and on the CL substrate the morphological change from round to stretch shape was observed during serial subcultures with higher mRNA expression of collagen type I, suggesting that the cell morphology can offer an indicator for chondrogenic potency during dedifferentiation process (11).

The time-lapse observation of each single cell yields a wealth of quantifiable data on cellular properties such as changes in cell morphology, adhesion, migration and cell division pattern. Another feature for time-lapse experiment allows the movie to be rewound, thus one can survey the morphological and behavioral properties of the cells which were targeted at the end of culture, in a backward

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manner. In the current study, the CL substrate was employed for the evaluation of chondrocyte phenotypes by morphological analysis of time-lapse images of individual cells. Furthermore, the cells passaged at various population doubling levels were used to understand the progression of chondrocytes towards terminal differentiation as well as dedifferentiation in the heterogeneous population on the CL substrate and in the collagen gel (CL gel).

MATERIALS AND METHODS

Preparation of cells with varied population doubling levels Articular cartilage slices were harvested from humeri, femora and tibiae of Japanese white rabbits (approximately one month old) as described elsewhere (12). The isolated chondrocytes were subcultured using a 75 cm^2 T-flask (Corning Inc., Corning, NY, USA). The inoculum size was set at 1.0×10^4 cells/cm^2, and Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY, USA), antibiotics (100 U/cm^3 penicillin G, 0.1 mg/cm^3 streptomycin and 0.25 mg/cm^3 amphotericin B, each obtained from Gibco, Invitrogen) and 0.17 μmol/cm^3 L-ascorbic acid (Wako Pure Chemical Industries, Osaka) was supplied at a depth of 0.4 cm in the flask placed under a 5% CO_2 atm at 37°C. The spent medium was renewed every 3 days, and the cells in the flask were detached for subsequent passage by using 2.0 ml of 0.25% trypsin solution when they reached about 80% confluence.

The number of viable cells (n_c) in the flask was determined by the trypan blue exclusion test through direct counting of the detached cells on a hemocytometer under an optical microscope. The differential value of population doubling (ΔPD) was calculated as follows:

$$\Delta PD = log_2\left(\frac{n_c + \Delta n_c}{n_c}\right) \quad (1)$$

where Δn_c is the differential in the number of viable cells in each passage. The value of population doubling (PD) was obtained by summation of ΔPD with respect to each passage conducted as mentioned above. The freshly isolated chondrocytes at 3 days after seeding in the primary culture, during which the living cells could attach on the PS surface without multiplying, were defined as a cell population with $PD=0$. In the subsequent subcultures, the cell populations at different PD levels were prepared according to the procedure reported in our previous study (10). In this work, three groups of the cell populations, namely $PD=0$, $PD=5.1$, 6.6, 7.2 and 9.7, and $PD=12.5$ and 14.5, were prepared as young, middle and old age states, respectively.

Analysis of cell behaviors on collagen type I coated substrate and on tissue culture polystyrene surface An acidic solution of bovine collagen type I (0.5% collagen I-AC; Koken Co., Ltd., Tokyo) was used to prepare the CL substrate as described elsewhere (10). Under aseptic conditions, the collagen solution (5.25 ml) was poured into a 25 cm^2 T-flask (Nunc; Nalge Nunc International, Rochester, NY, USA) so as to yield 1.05 mg of collagen per cm^2 of surface area of the flask bottom. After water was evaporated in a vacuum chamber for 2 days, the surface was rinsed twice with sterile phosphate buffered saline (PBS) (Sigma), giving a collagen coating with approximately 70 μm thickness. Cell population at a given PD value was incubated on the CL substrate and PS surface for 12, 24, 48, 72 and 96 h. The cells were subjected to the detection of alkaline phosphatase (ALP) activity using Fast Red Substrate System® (Dako, Carpinteria, CA, USA) at the end of each culture and the frequency of ALP-positive cells was estimated as a ratio of them to the total cells.

To investigate the cell behaviors, the time-lapse observation of cells incubated on the CL substrate and PS surface was carried out for 72 h, as described previously (13), and images were captured every 10 min at 6 random positions or more. The cells on the CL substrate and PS surface were subjected at 72 h of culture time (t) to the detection of ALP activity and the backtracking of more than 100 cells was then conducted to evaluate cell behaviors. The time course of morphology was obtained to estimate cell roundness by the following equation:

$$R_C = \frac{2(\pi A_C)^{1/2}}{l_C} \quad (2)$$

where A_C and l_C denote cell area and peripheral length, respectively, and the cell which possesses $R_C>0.9$ was regarded to be a round shape in the present study.

Incubation of cells embedded in collagen gel and observation of cell behaviors The chondrocytes at a prescribed PD value were suspended in the culture medium and then mixed with a 4-fold volume of 3% Atelocollagen solution (Koken Co., Ltd.) as described elsewhere (10,14). The mixture (0.1 cm^3) was transferred to a 6-well plate (Nunc) and subjected to gelation at 37°C for 1.5 h, yielding a dome-shaped gel of approximately 0.8 cm diameter and 0.2 cm top height. To examine the cell growth and extracellular matrix (ECM) formation, the triplicate CL gels incubated for 14 days were subjected to the staining of cytoplasm and collagen type II respectively, according to procedures described in our previous study (15), and the specimen of CL gel was mounted on a glass-bottomed dish (Asahi Glass Co., Ltd., Tokyo) for 3-D observation of cell morphology and ECM parameter using a confocal laser scanning microscope (model FV-300; Olympus, Tokyo). Here, more than 60 cells were provided for the semi-quantitative analysis.

Total RNA extraction and real-time RT-PCR analysis

Total RNA was extracted from the cells using an RNeasy mini kit (Qiagen, Hilden, Germany), and RNA sample was subjected to DNase-I (Qiagen) treatment according to the manufacturer's protocol. Reverse transcription from RNA was carried out as indicated previously (15). Gene expressions were examined by means of quantitative real time PCR with a Chromo4™ detector and furnished program (Bio-Rad Laboratories, Hercules, CA, USA) according to procedures indicated in the previous report (15). Specific primers for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene and the target genes (collagen types I, II and X) were designed as indicated in Table S1. PCR was performed using 0.2 μM of selected primers and SYBR Premix ExTaq™ (Takara Bio Inc., Shiga) under the conditions of 10 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. The cycle threshold value (Ct) for each gene was determined as cycle time when fluorescence of given sample became distinct from a base signal. The Ct value of GAPDH was subtracted from that of target gene to obtain the ΔCt value, and the expression level was calculated in terms of $2^{-\Delta Ct}$.

RESULTS

Morphological and proliferative characteristics of cells To investigate the influence of culture surfaces on terminal differentiation of the chondrocytes, the time profiles of cells density, frequency of ALP-positive cells and gene expression of collagen type X were estimated in the cultures of passaged cells (middle age state at $PD=8.5$) on the CL substrate and PS surface. Appreciable growth of the cells was not observed on the CL substrate, suggesting that the cells are in a prolonged cell cycle arrest (Fig. 1A). In addition, the

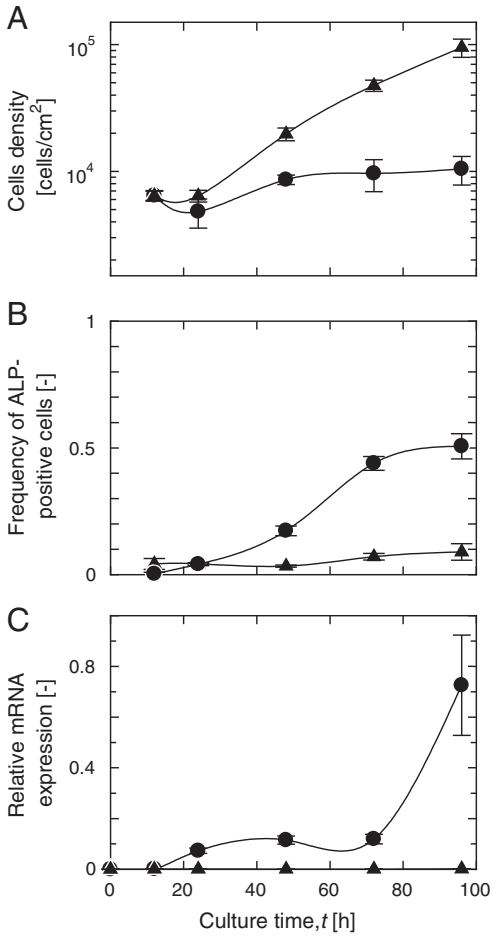

FIG. 1. Time profiles of cells density (A), frequency of ALP-positive cells (B) and gene expression of collagen type X (C) in cultures of chondrocytes at $PD=8.5$ on PS surface and CL substrate. The data represent the average values with standard deviation determined from triplicate independent experiments. Symbol: Triangle; PS surface, and circle; CL substrate.