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SURFACE MARKERS AND GENE EXPRESSION TO CHARACTERIZE THE DIFFERENTIATION OF MONOLAYER EXPANDED HUMAN ARTICULAR CHONDROCYTES

TAKASHI HAMADA[1], TADAHIRO SAKAI[1], HIDEKI HIRAIWA[1], MOTOSHIGE NAKASHIMA[1], YOHEI ONO[1,2], HIROHITO MITSUYAMA[3] and NAOKI ISHIGURO[1]

[1]*Department of Orthopaedic Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[2]*Department of Anatomy and Cell Biology, Brody School of Medicine, East Carolina University, 600 Moye Blvd. Greenville, NC 27834, USA*
[3]*Nagoya Grampus Clinic, Toyota, Japan*

ABSTRACT

Autologous chondrocyte implantation (ACI) is a method of cartilage repair. To improve the quality of regenerated tissue by ACI, it is essential to identify surface marker expression correlated with the differentiation status of monolayer expanded human articular chondrocytes and to define the index for discriminating dedifferentiated cells from monolayer expanded human articular chondrocytes. Normal human articular chondrocytes were cultured in monolayer until passage 4. At each passage, mRNA expression of collagen type I, II, and X and aggrecan was analyzed by real-time quantitative PCR, and the surface marker expression of CD14, CD26, CD44, CD49a, CD49c, CD54, and CD151 was analyzed by fluorescence-activated cell sorting (FACS). The ratios of mRNA levels of collagen type II to I (Col II/Col I) represented the differentiation status of chondrocytes more appropriately during monolayer culture. The surface marker expression of CD44, CD49c, and CD151 was upregulated according to the dedifferentiation status, whereas that of CD14, CD49a, and CD54 was downregulated. The most appropriate combination of the ratio of Col II/Col I was CD54 and CD44. Cell sorting was performed using a magnetic cell sorting system (MACS) according to CD54 and CD44, and real-time quantitative PCR was performed for the cell subpopulations before and after cell sorting. The expression of collagen type II and aggrecan of the chondrocytes after MACS was higher than that before sorting, but not significantly. The mean fluorescence intensity (MFI) ratio of CD54 to CD44 could be an adequate candidate as the index of the differentiation status.

Key Words: Autologous chondrocyte implantation, surface marker, differentiation index, CD44, ICAM-1

INTRODUCTION

The treatment of cartilage defects in younger patients is a major challenge for surgeons because articular cartilage has limited potential of intrinsic repair.[1)] Two strategies have been considered for the restoration of the joint surface. The first approach is to enhance the intrinsic healing capacity of both the cartilage and subchondral bone through the release of mesenchymal stem

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Corresponding author: Tadahiro Sakai
Department of Orthopaedic Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan
Tel: +81-52-741-2111 (ext. 5095), Fax: +81-52-744-2260, E-mail: tadsakai@med.nagoya-u.ac.jp

cells involving procedures such as subchondral drilling,[2] microfracture,[3] abrasion arthroplasty,[4,5] electrical stimulation,[6] and laser stimulation.[7] These methods have generally led to the formation of fibrocartilage, which has limited functionality than hyaline articular cartilage. An alternative approach is to repair the hyaline articular cartilage, particularly suited to younger patients. This approach involves the use of tissue engineering strategies to elicit a biological repair that has long-term functionality. Meanwhile, cartilage tissue engineering relies on in vitro expansion of primary chondrocytes. Monolayer expansion of primary chondrocytes from small biopsies is very important in cartilage repair strategies such as autologous chondrocyte implantation (ACI).

There have been many reports of successful cartilage defect repair by ACI.[8-11] In the US and Europe, variants of ACI have also been tried in approximately 10,000 patients.[12] This method requires an in vitro cell expansion process to obtain a large number of transplantable chondrocytes for fulfilling the cartilage defect. However, cell expansion using monolayer cell culture causes chondrocytes to lose their phenotype, e.g., switching their gene expression from the cartilage-specific collagen type II to collagen type I[13] or decreasing the production of proteoglycans such as aggrecan.[14] Normal adult hyaline articular cartilage usually does not contain collagen type X, markers of chondrocyte hypertrophy[15]; however, the expression of collagen type X is observed during monolayer culture.[16] In addition, it is thought that during monolayer expansion, human articular chondrocytes change with respect to their cell surface marker expression.[17] Human articular chondrocytes reportedly express surface molecules belonging to different categories such as integrins, other adhesion molecules, tetraspanins, receptors, and ectoenzymes that induce changes in the expression levels during monolayer expansion.[17] Otherwise, graft hypertrophy is a major complication observed in ACI.[18] It has been strongly suggested that dedifferentiation of chondrocytes affects the quality of the cartilage regenerated by ACI. Although midterm clinical results of ACI are satisfactory, hyaline cartilage that should be related to long-term results is not regenerated thoroughly till date. To improve the quality of the regenerated tissue by ACI, it could be essential to discriminate dedifferentiated cells from monolayer expanded human articular chondrocytes by using some index related to the surface markers.

On the other hand, the technique of cell sorting using antibodies to cell surface markers can be reportedly used in various fields of medicine.[19-21] However, the technique of sorting was hardly put for the practical use in the field of the cartilage tissue regeneration till date.[22]

This study aimed to identify surface marker expression correlated with the differentiation status of monolayer expanded human articular chondrocytes and to define the index for discriminating dedifferentiated cells from monolayer expanded human articular chondrocytes.

MATERIALS AND METHODS

Cell culture

Human articular cartilage (HAC) samples were obtained at the time of joint surgery for loose body removal from patients who suffered from osteochondritis dissecans or notchplasty of the intercondylar roof of the femur from patients who underwent anterior cruciate ligament reconstruction. The patients included three women and two men, with a mean age of 29.4 years (range 17–40 years). None of them showed any symptoms related to osteoarthritis. These experiments were conducted in accordance with a protocol approved by the Ethics Committee of Nagoya University, and all the patients gave written consent for the use of their tissues for this research.

Cartilage samples were minced and digested at 37°C with trypsin–EDTA solution (Sigma, MO, USA) for 15 min, with collagenase type XI (2 mg/ml) (Sigma) in F-12 nutrient mixture medium (Gibco-BRL, Belgium) for 12 h, filtered through a 70-μm nylon mesh, and washed

extensively. Following this, the isolated chondrocytes were seeded in 75-cm^2 culture flasks and were incubated in F-12 nutrient mixture medium containing 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 mg/ml), amphotericin B (2.5 μg/ml), and ascorbic acid (0.2 mM) at 37°C under an atmosphere of 5% CO2. The medium was changed every second day. At 80% confluence, the cells [considered passage zero (P0) cells] were detached and expanded in monolayer culture by replating the cells in a 10-cm tissue culture dish with culture medium. The cells were passaged at subconfluence until passage 4 (P4). The cells from each passage were used in subsequent experiments.

Real-time quantitative polymerase chain reaction

Total RNA was extracted using the RNeasy Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time polymerase chain reaction (PCR) was performed in a LightCycler (Roche, Basel, Switzerland) using the FastStart DNA Master^{PLUS} SYBR Green Kit (Roche). The primers used are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Standard samples were prepared from the PCR amplicon extracted using the QIAquick Gel Extraction Kit (Qiagen Inc.). The standard curve was imported to each PCR run by the software to determine the concentration of the samples. All data were normalized, and the value for the sample at P0 was 100%.

Immunofluorescence staining of chondrocytes and fluorescence-activated cell sorting

All monoclonal antibodies (mAbs) used in this study (Table 2) were directly conjugated antibodies to fluorescein isothiocyanate (mAb-FITC) or to phycoerythrin (mAb-PE).

The cells were analyzed on a fluorescence-activated cell sorter, FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Data analysis was performed with CellQuest software (Becton-Dickinson).

The level of expression of each marker was the analyzed geometric mean fluorescence intensity (MFI) of sample cells. To determine changes in surface markers during monolayer culture, fold changes of the levels of expression of a marker at different time points (P1–P4) were calculated in relation to P0 (beginning of the culture), and a two-tailed, unpaired Student's t-test was performed for the same data. A significant change in expression was defined as a fold change

Table 1 Primer sequence
(GAPDH, glyceraldehyde-3-phosphate dehydrogenase)

GAPDH	Sense	5′-TGCACCACCAACTGCTTAGC-3′
	Antisense	5′-GGCATGGACTGTGGTCATGAG-3′
collagen type I	Sense	5′-CCTCAAGGGCTCCAACGAG-3′
	Antisense	5′-TCAATCACTGTCTTGCCCCA-3′
collagen type II	Sense	5′-TGGAGACTGGCGAGACTT-3′
	Antisense	5′-TGGGAGCCAGATTGTCATC-3′
aggrecan	Sense	5′-CCAGGAGGTATGTGAGGA-3′
	Antisense	5′-CGATCCACTGGTAGTCTTG-3′
collagen type X	Sense	5′-ATGCATATGGAGGTAGGCT-3′
	Antisense	5′-AGAGAGGCTTCACATACGTT-3′

Table 2 Antibodys used in the present study

Specificity	Isotype	cat.#	Fluochrome	Source
CD14	IgG2a	555397	FITC	BD Pharmingen
CD26	IgG2a′	340426	FITC	BD Pharmingen
CD44	IgG1	ab27285	FITC	abcam
CD49a	IgG1	559596	PE	BD Pharmingen
CD49c	IgG1	556025	PE	BD Pharmingen
CD54	IgG1	555511	PE	BD Pharmingen
CD151	IgG1	556057	PE	BD Pharmingen

of greater than two with a P value < 0.05.

Cell sorting

Cell sorting was performed using a magnetic cell sorting system (MACS) according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany).

P2 monolayer expanded human articular chondrocytes were prepared as single-cell suspensions. To obtain $CD44^{-}CD54^{+}$ cells, the suspensions were incubated with FITC-anti-CD44 (Abcam, Tokyo, Japan), followed by incubation with anti-FITC microbeads (Miltenyi Biotec). $CD44^{-}$ cells were negatively selected using MACS. The flow-through cells (considered $CD44^{-}$ cells) were secondary incubated with PE-anti-CD54 antibodies (BD Pharmingen, San Jose, CA, USA), followed by incubation with anti-PE microbeads (Miltenyi Biotec). Subsequently, $CD44^{-}CD54^{+}$ cells were positively selected using MACS. Purity of the isolated fraction was verified by FACS analysis.

RESULTS

The ratios of mRNA levels of collagen type II to I (Col II/Col I) represented the differentiation status of chondrocytes more appropriately during monolayer culture

To investigate the change in the phenotype of human articular chondrocytes, real-time quantitative PCR was performed at each time point during monolayer culture.

The expression of collagen type II and aggrecan decreased in a passage-dependent manner (Figure 1a, b). In contrast, the expression of collagen type I increased (Figure 1c). However, a significant change was not observed in the expression of collagen type X (Figure 1d). To examine which combination of changes in gene expression of the extracellular matrix components will represent the differentiation status of HAC more clearly, we calculated the ratios of mRNA levels of collagen type II to I (Col II/Col I) and of aggrecan to collagen type I (Agg/Col I) (Figure 2). These results showed that the ratio of collagen type II to type I seemed to represent the dedifferentiation status of chondrocytes more appropriately than that of aggrecan to collagen type I as an extracellular matrix-based differentiation index. It was also revealed that the ratio of Col II/Col I showed the maximum change at the beginning of the culture period (P0–P1).

Immunophenotyping of HAC during monolayer culture

To investigate changes in cell surface markers during chondrocyte monolayer culture, we analyzed cell surface molecule expression levels at different time points by flow cytometry.

CD44, CD49c, and CD151 were up-regulated according to the dedifferentiation status, whereas CD14, CD49a, and CD54 were down-regulated. Most surface markers showed major changes at

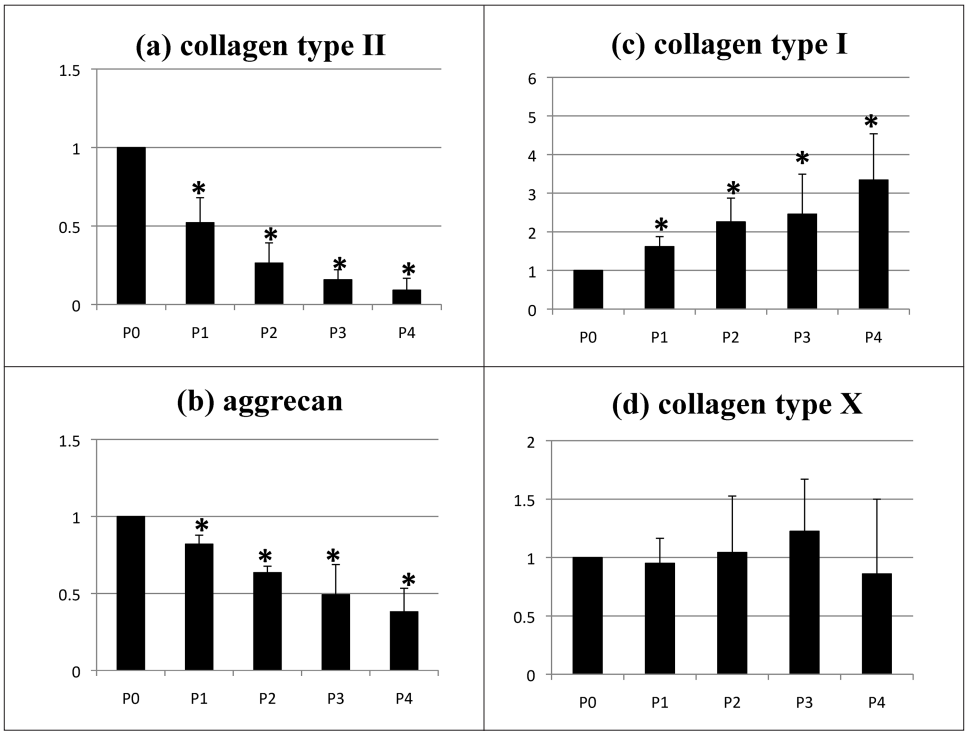

Fig. 1 Chondrocyte mRNA levels for collagen type II (a), aggrecan (b), collagen type I (c) and collagen type X (d) at different time points in monolayer culture, as measured by real-time, quantitative RT-PCR. Values are expressed as fold differences compared to levels in P0. The results are shown as mean ± SEM (error bars) from three donors for each passage. Differences when compared to P0 considered statistically significant ($P < 0.05$) are indicated (*).

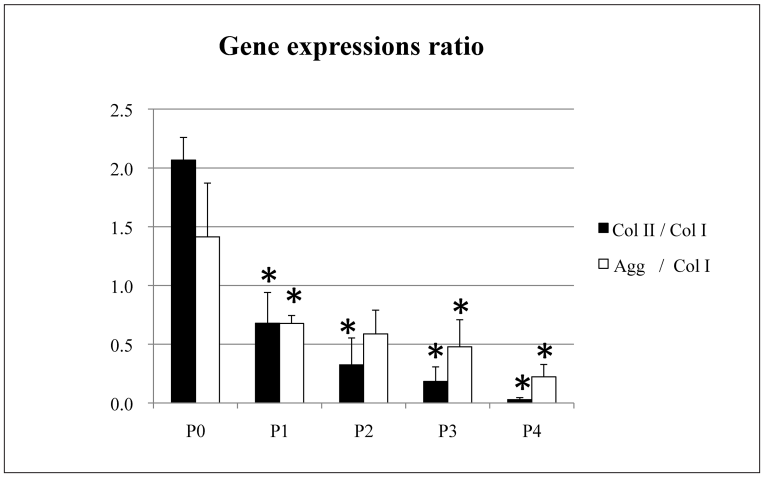

Fig. 2 Ratios for collagen type II to I (Col II/Col I) and aggrecan to collagen type I (Agg/Col I) were obtained from chondrocyte mRNA levels at different time points in monolayer culture as measured by real-time, quantitative RT-PCR (qRT-PCR). The results are shown as mean ± SEM (error bars) from three donors for each passage. Differences when compared to P0 considered statistically significant ($P < 0.05$) are indicated (*).

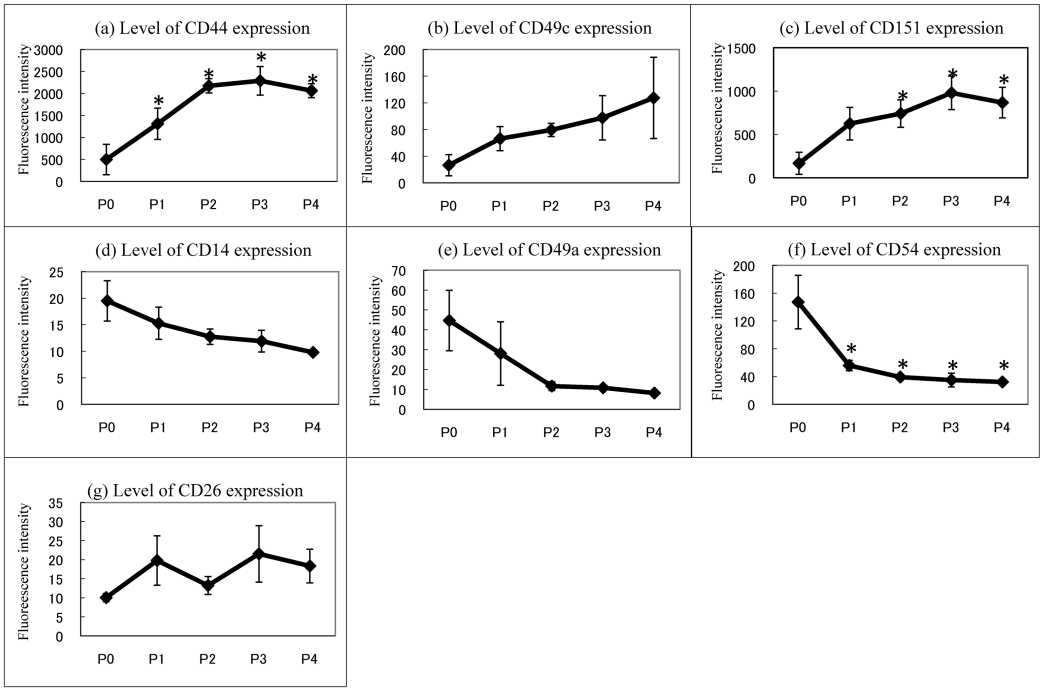

Fig. 3 Chondrocyte surface marker expressions. MFI levels for CD44 (a), CD49c (b), CD151 (c), CD14 (d), CD49a (e), CD54 (f) and CD26 (g) at different time points in monolayer culture. The results are shown as mean ± SEM (error bars) from three donors for each passage. Differences when compared to P0 considered statistically significant ($P < 0.05$) are indicated (*).

the beginning of the culture period (P0–P1), with small variations for the remaining time course. No significant change was observed in the expression of CD26 (Figure 3a-g).

Interrelationship between gene expression and surface marker expression

To correlate changes in gene expression of the extracellular matrix components with the established changes in cell surface markers during chondrocyte monolayer culture, we calculated the MFI ratio of each down-regulated cell surface marker (CD14, CD49a, and CD54) to each up-regulated marker (CD44, CD49c, and CD151). Subsequently, we compared the extracellular matrix-based differentiation index (Col II/Col I) with a differentiation index based on the cell surface markers. As a result, the most appropriate combination of the ratio of Col II/Col I was CD54 and CD44 (CD54/CD44) (Figure 4).

The results revealed dramatic changes occurring as early as the first passage. The Col II/Col I ratio steadily declined until the end of the culture. A similar pattern was observed for the ratio of CD54/CD44. Therefore, it was suggested that a positive interrelationship was present between the expression of surface markers (CD54/CD44) and the dedifferentiation status of monolayer expanded human articular chondrocytes.

Flow cytometric analysis of HAC sorted by MACS

Next, we conducted cell sorting experiments according to CD54 and CD44 using MACS. Figure 5a, b and Figure c, d show the results of flow cytometry of the chondrocytes after MACS according to CD54 and CD44, respectively. Purity of isolated $CD54^{+}CD44^{-}$ fraction was 79.9%.

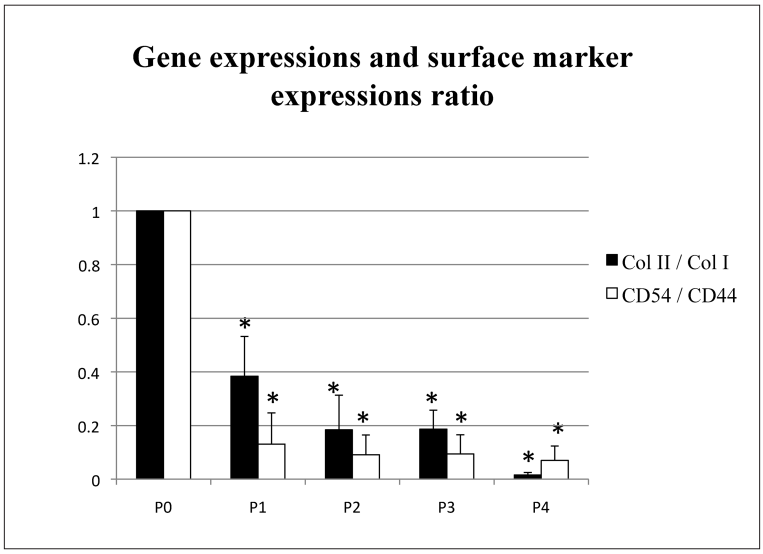

Fig. 4 Interrelationship between the gene expressions and the surface marker expressions. The results are shown as mean ± SEM (error bars) from three donors for each passage.
Differences when compared to P0 considered statistically significant ($P < 0.05$) are indicated (*).

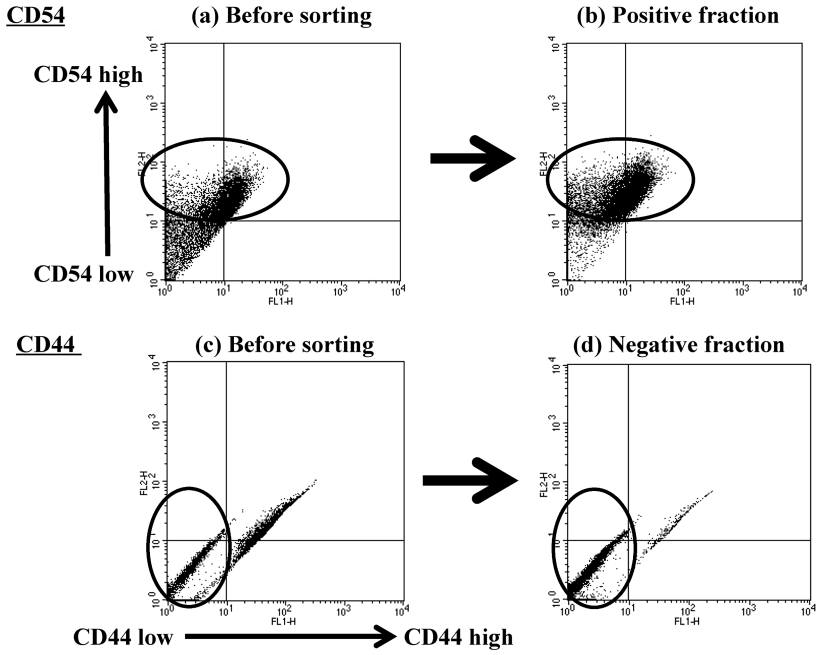

Fig. 5 Flow cytometric analysis of HAC sorted by MACS technology. (a) Dot plot before sorting according to CD54. (b) CD54 positive fraction after sorting. (c) Dot plot before sorting according to CD44. (d) CD44 negative fraction after sorting.

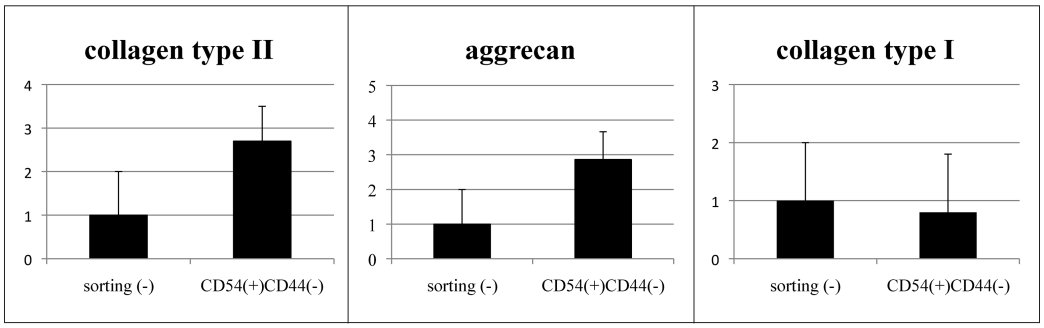

Fig. 6 Chondrocyte mRNA levels for collagen type II, aggrecan and collagen type I, before and after cell sorting, as measured by real-time, quantitative RT-PCR. Values are expressed as fold differences compared to levels before sorting. The results are shown as mean ± SEM (error bars) from three donors for each passage. No significant differences are observed, respectively.

These results indicated the possibility of discriminating the $CD54^{+}CD44^{-}$ cell subpopulations from monolayer expanded human articular chondrocytes using MACS.

Gene expression analysis of HAC comparing before and after cell sorting

To confirm whether the distinguished chondrocytes could have a more advantaged phenotype for forming neo-cartilage tissue, we performed real-time quantitative PCR for the cell subpopulations before and after cell sorting. The expression of collagen type II and aggrecan of the chondrocytes after MACS tended to be higher than that before sorting, but not significantly (Figure 6).

DISCUSSION

Articular cartilage consists of only one cell type: round chondrocytes characterized by their ability to produce the main constituents of the hyaline extracellular matrix, namely collagen types II, IX, and XI and proteoglycans.[23] Chondrocytes can be released from cartilage by enzymatic digestion and be expanded in culture, an important step for their clinical application in cell-based cartilage repair therapies.[24,25] However, during serial monolayer culture, the chondrocyte phenotype is lost, and cells alter their morphology and metabolism in a process termed dedifferentiation.[13,26] While a clear link between chondrocyte dedifferentiation and the differential production of extracellular matrix components has been established, the cell surface protein composition of chondrocytes during the dedifferentiation process remains poorly understood. Uncovering chondrocyte surface proteins are attractive because some of these proteins could be used as markers to discriminate between differentiated and dedifferentiated cells or even different stages of dedifferentiation.[27] The identification of new markers to characterize chondrocyte differentiation status based on cell surface marker expression would contribute to cartilage tissue engineering, from quality control for cell expansion to the optimization of culture conditions. Further validation of these surface molecules as markers of the differentiation status will require determination of whether the profile of cell surface proteins induced by monolayer culture can be reversed using redifferentiation procedures.

Therefore, in this study, we investigated changes in gene expression and surface marker expression correlated with the dedifferentiation of monolayer expanded human articular chondrocytes

to define the index for cell sorting related to the surface markers.

We compared the extracellular matrix-based differentiation index (Col II/Col I) with a differentiation index based on the cell surface markers. As a result, it was suggested that single surface marker expression is not appropriate as the index of differentiation because of insufficiency in the amplitude of changes, whereas the ratio of CD54 to CD44 demonstrates the state of differentiation more clearly.

Hyaluronan (HA) is a large glycosaminoglycan composed of repeating disaccharides of D-glucuronic acid and N-acetyl-glucosamine. Articular chondrocytes constitutively express CD44[28] and ICAM-1,[29] two specific HA receptors.[30] There is evidence that those HA receptors biologically mediate the effects of HA. In contrast, CD44 reportedly has multiple functions beside the HA receptor, such as HA endocytosis, cell proliferation, or migration.[31-33] While the principal HA receptor is CD44,[34] ICAM-1 is induced during the inflammatory response and by cytokines such as IL-1.[35] ICAM-1 is a glycosylated protein of 80–114 KDa with a core polypeptide of 55 KDa, and it has an important role in leukocyte trafficking and cell–cell adherence in immunological response.[36,37] We speculated that the switching of HA receptors on monolayer cultured chondrocytes continues along the line of the dedifferentiation status. During monolayer culture, it is speculated that chondrocytes tend to be more proliferative instead of losing their specific phenotype, resulting in the increased expression of CD44. Simultaneously, the expression of ICAM-1 is decreased because the chondrocytes are spreading out to advance their cell–cell contact. Consequently, the ratio of ICAM-1 (CD54) and CD44 could be decreased in the dedifferentiation status during monolayer culture.

Then, we performed the cell sorting according to CD54 and CD44 using MACS. In our results of real-time quantitative PCR, the expression of collagen type II and aggrecan of the chondrocytes after MACS tended to be higher than that before sorting, but not significantly. It is speculated that in the cell subpopulations before and after cell sorting that were cultured by the technique of redifferentiation such as pellet culture or the other three dimensional culture, significant differences could be observed in the expression of the chondrocyte-specific phenotype. We are studying whether the distinguished cells from monolayer expanded chondrocytes could form better regenerated cartilage tissue in pellet culture or in vivo experiments and by the ACI procedure.

There are three problems of cell sorting using MACS technology. First, the number of chondrocytes is decreased in comparison after sorting using MACS. Second, whether the magnetic beads used in MACS may affect the human body during the ACI procedure remains unknown. Third, purity of isolated fraction is not so high. There might be nonspecific binding, because we performed the cell sorting in indirect method. Therefore, purity might become relatively low. It is necessary to establish the techniques to obtain a higher recovery rate and purity, and further experiments are required to investigate the influence of the magnetic beads on the human body in vivo.

There are some limitations in this study. Because it is difficult to obtain normal human cartilage in Japan mainly on account of religious reasons, the number of samples is small and the amount of cartilage tissue is very small. Therefore, we could not conduct sufficient experiments. We speculated that the small number of samples ($n = 3$) was one of the reasons why no significant difference was observed in this experiment (Figure 6). If the number of samples would increase, a significant difference could be observed.

In summary, the present study indicates that the ratio of mRNA expression of collagen type II to type I represented the differentiation status of human articular chondrocytes. Surface marker expression also changed according to the differentiation status. The MFI ratio of CD54 to CD44 could be an adequate candidate as the index of the differentiation status. Further studies are

required to evaluate distinguished chondrocytes could form better cartilage tissue and to confirm the usefulness for the ACI procedure.

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