Rapid phenotypic changes in passed articular chondrocyte subpopulations

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Abstract

Articular chondrocytes are often expanded in vitro and then used to assist in healing articular cartilage defects. We investigated the extent of dedifferentiation in monolayer-passaged, zonal articular chondrocytes by using quantitative, real-time PCR. The relative gene expressions for collagen type I and II, aggrecan, and superficial zone protein were analyzed for relevant passage numbers (P0–P4) to determine how the expansion of chondrocytes affects the expression of cartilage extracellular matrix proteins. Results reveal that dramatic changes occur as early as first passage. Furthermore, these changes are shown to persist even when the expanded cells are encapsulated in 3D, alginate beads. Successful tissue engineering and autologous cell transplantation procedures rely heavily on having a cell source that expresses the chondrocytic phenotype. The results of this study suggest that major problems exist at the front-end of cartilage regeneration efforts.

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Introduction

Natural repair in articular cartilage defects is limited to the formation of a mechanically inferior tissue termed fibrocartilage, which eventually breaks down after extended usage [5,16]. Recognizing that chondrocytes do not sustain a sufficient healing response on their own, tissue engineering is a possible solution to assist the healing of articular cartilage [7]. The goal of tissue engineering is to regenerate a tissue's native functionality, commonly through the use of a biodegradable scaffold seeded with primary or passaged cells and bioactive agents such as growth factors and peptides. The cell-seeded scaffold is then either immediately implanted or cultured for a period in vitro, where it is mechanically stimulated, and then implanted. In general, articular cartilage is a prime candidate for tissue engineering because this tissue is avascular, aneural, and has a relatively simple composition. The major extracellular components are water, collagen type II, and proteoglycans. Fully differentiated, articular chondrocytes secrete these proteins in vivo or when cultured under favorable conditions. However, the in vivo natural repair process, as well as environmental changes due to physical or biochemical damage, can change the chondrocytes' phenotype to a more fibroblastic state. The process in which a chondrocyte's phenotype changes from chondrocytic to fibroblastic is termed dedifferentiation. This process also occurs when harvested cells are grown in monolayer culture [3].

A new trend in tissue engineering is directed at recreating the zonal arrangement of articular cartilage [15,17,18,31], since the distinct zones present in cartilage vary in their structure and function [20]. It is expected that each type of zonal subpopulation of chondrocytes may respond differently to mechanical stimuli, and the
cell products secreted may be specific to that zone’s function [11,23]. Simply put, the best way to reproduce the function of articular cartilage may be to mimic its structure. Therefore, instead of using a mixed population of chondrocytes to seed a scaffold, specific subpopulations should be used to replicate each zone.

Beginning with the articulating surface, mature cartilage can be separated into the superficial, middle, deep, and calcified zones. The first 10–20% of the full thickness contains densely packed collagen type II fibrils and flattened, elongated cells oriented in the direction of shear stress and is classified as the superficial zone [2]. The superficial zone secretes a zone-specific protein, superficial zone protein (SZP), which is hypothesized to contribute to the lubrication properties of the joint [11]. The middle zone (40–60% of full thickness) contains round cells and collagen fibers that are randomly arranged. It serves as a transition between the superficial and deep zones and is characterized by a higher concentration of proteoglycans. The deep zone is characterized by columns of ellipsoid cells distributed between radially oriented collagen fibers. The collagen fibrils extend into the calcified zone to reinforce the bond between cartilage and bone [24]. The deep zone is separated from the calcified zone by a distinct tidemark, which is often considered the boundary between cartilage and bone. The calcified zone contains cells trapped within a calcified matrix. Through the depth of cartilage, water content falls linearly, from approximately 84% wet weight (ww) to 40–60% ww. The collagen content falls from 86% dry weight (dw) in the superficial zone to 67% dw in the deep zone. The proteoglycan content increases from around 15% dw in the superficial zone to a peak of 25% dw in the middle zone, then falls to 20% in the deep zone [24]. The natural variations in biochemical composition between zones undoubtedly play an important role in cartilage’s functionality, so it makes sense to wish to replicate this zonal structure.

Current articular cartilage engineering studies use homogeneous cell mixtures from immature animals (1–6 weeks old) that include chondrocytes from all the zones. This cell source, while excellent at producing large amounts of extracellular matrix, is not clinically relevant or even practical. Furthermore, fetal cartilage does not exhibit the zonal structure present in mature articular cartilage. While the development of the tissue’s zonal structure has not been examined in depth, a basic progression has been documented [10]. The zones develop incrementally, with the superficial zone forming first, followed by the middle and deep zones. In 8 month-old goats, the superficial zone (100–200μm thick) has been formed, while the remaining depth of cartilage (≤800μm thick) is not fully differentiated. This zone is termed the ‘growth zone’ and will eventually develop into the middle and deep zones of mature articular cartilage [8,10]. The presence of a distinct superficial zone is an advantage of using young cartilage over fetal tissue for zonal tissue engineering and is also relevant for chondrocyte transplantation procedures in younger patients. Mature tissue has distinct zones, but the chondrocytes’ synthetic abilities are less than those of immature cells. Using young chondrocytes is a compromise that allows zonal cartilage studies while still having increased synthesis levels in the harvested cells. Although at this time there is no universally acceptable animal model for investigating the repair processes in articular cartilage, it is becoming increasingly evident that the goat may fulfill this role [1]. Young goat cartilage, which exhibits partial zonal organization along with enhanced biosynthetic abilities, is used in this study to determine the effect of passing on the gene expression of articular chondrocytes.

Normally in tissues with low cellularity a small number of cells are harvested from a patient and then expanded as necessary. With chondrocytes, however, monolayer culture causes rapid dedifferentiation and the subsequent inability to produce functional cartilage tissue. Autologous chondrocyte transplantation studies, in which the patient’s own cells are removed, expanded, then sealed in a defect with a periosteal patch, have had occurrences of fibrous tissue formation that is indicative of dedifferentiated chondrocytes [27]. The eventual result may be a breakdown in the functionality of the tissue. Besides losing the ability to secrete the major articular cartilage proteins, the current study shows that dedifferentiated cells also lose their zonal differences.

Methods

Tissue harvesting and cell culture

Tissue was harvested from the knees of 8 month old, female Spanish goats, obtained through a local abattoir. Mixed-population samples were comprised of chondrocytes from both the superficial and growth zones. Zonal abrasion was conducted to obtain separate populations of superficial and growth zone chondrocytes. This procedure, described previously [8], involves drawing a scalpel blade firmly across the cartilage surface to remove the top 10–20% of the tissue. All chondrocytes were released from the tissue using standard digestion techniques [8]. Briefly, the tissue was minced using a scalpel and digested overnight in 2mg/ml type II collagenase (Worthington, Lakewood, NJ). The resulting cell suspensions were pelleted, and the supernatants removed. Upon re-suspension in phosphate buffered saline, superficial and growth zone cells were plated separately and cultured with DMEM containing 10% FBS, 100mM HEPES, 0.4 mM L-proline, 1% NEAA, 50μg/ml ascorbic acid, 1mM L-glutamate, 100U/ml penicillin-streptomycin, and 0.25μg/ml fungizone (Sigma-Aldrich, St. Louis, Missouri). Initial seeding densities were approximately 16,000 cells/cm². Near confluence (~50,000 cells/cm²), cells were passed by trypsinization using standard procedures. Samples for gene expression analysis were taken before re-plating the cells at one-quarter confluence levels. Additionally, zonal chondrocytes were encapsulated in alginate to determine whether this 3D environment helps cells re-express the chondrocytic phenotype. For this portion of the study, 50,000–100,000 zonal cells/ml (low density seeding) were gelled in 2% alginate beads after the initial harvest (P0) and after each passage (P1–P4) using standard procedures [13]. Low cell densities in alginate have been
shown to increase the expression of cartilage extracellular matrix proteins [14]. Previous work in our laboratory has shown good cell viability within alginate, and media color changes during the experiment as well as transcribable RNA indicated that cells were alive during algin
cate culture. The beads were cultured for two weeks after encapsula
tion to allow for sufficient re-differentiation [22], at which point the
cell-alginate beads were dissolved using sodium citrate. The resulting
solution was centrifuged to pellet the released cells, and the superna
tant was subsequently removed. The remaining cells were lysed and
processed as described in the RNA isolation section.

Primer design

Primer sequences for all genes were designed using bovine and
human mRNA information published on the NCBi website. Software
used for primer design included Primer3 and NCBi's standard and
pairwise BLAST programs. The genes examined in this study were
glyceraldehyde-3-phosphate dehydrogenase (GAPDH), type II collagen
(Col2), type I collagen (Col1), superficial zone protein (SZP), and
aggrecan (AGC). Primer efficiency was found to be constant,
although lower (70-80%) than optimal levels. However, the correct pri-
mer products were produced for all genes examined, as determined
using gel electrophoresis and DNA sequencing. Table 1 lists the se-
quence, accession number, product size, and fluorescent dye and quen-
cher for each primer/probe combination.

RNA Isolation and real-time RT-PCR

Sample RNA was obtained from the cells after initial harvest (P0)
and after each passage (P1, P2, P3, P4). The chondrocytes were lysed,
and their RNA isolated using an RNAqueous kit (Ambion, Austin,
Texas). RNA was subjected to DNase-I treatment prior to the reverse
transcription (RT) reaction, in which 12.5 μl of RNA was incubated
with 1 mM dNTPs, 1 mM random hexamers, and Stratagene Strata-
script RT enzyme (La Jolla, CA) for 90 min at 37°C. After cDNA syn-
thesis, real-time PCR for GAPDH, Col1, Col2, SZP, and AGC was
performed using the Rotor-gene 3000 real-time PCR machine (Corbett
Research, Sydney, AU). Multiplex PCR master mix (Qiagen, Valencia,
CA) was used in conjunction with the proper primers (0.2 μM) for each
reaction. A 15 min denaturing step was used, followed by 45 cycles of
15 s at 95°C and 30 s at 60°C. Fluorescence measurements (on FAM,
ROX, and Quasar 670) were taken every cycle at 60°C to provide a
quantitative, real-time analysis of the genes analyzed. Each test run
consisted of measuring either Col1/Coll2/GAPDH or AGC/SZP/
GAPDH for all portions of the study (mixed population, zonal popu-
lations, alginate encapsulated). Direct comparisons of relative gene
expression values cannot be made across test runs, although qualitative
comparisons are valid.

Relative expression

Gene expressions for Col1, Col2, SZP, and AGC were normalized
using the housekeeping gene, GAPDH. By analyzing gene expression
in this manner, variations in the number of cells per sample were ren-
dered insignificant. Relative expression values (R) were calculated
using the take-off cycle (Ct) of the gene of interest (GOI) and house-
keeping gene (HKG) and their respective primer efficiencies (E). Effi-
ciences were determined using standard curves run during the PCR
testing. The relative expression equation (adapted from Pfaffl [28])
used is:

\[ R_{GOI} = \frac{(1 + E_{HKG})^{Ct_{HKG}}}{(1 + E_{GOI})^{Ct_{GOI}}} \]

High relative expression values indicate that the gene of interest is
expressed to a greater extent than the housekeeping gene. Since
the expression of GAPDH is assumed to be constant from chondrocyte
to chondrocyte, the gene of interest's relative expression can be quan-
tified in a way that is not dependent on the number of cells in the
sample.

Statistics

A sample size of n = 3 was used for each group indicating three re-
lications stemming from an initial harvest pool, and PCR was run in
duplicate or triplicate for each sample. For the mixed-zone experiment,
the initial harvest population consisted of cells from three different ani-
mals. The zonal subpopulation experiment stemmed from one initial
animal. Graphs are depicted as mean ± standard error. A single-factor
ANOVA with repeated measures was used to determine if signifi-
cance existed for the mixed-population experiment, and a two-factor
ANOVA with repeated measures, subgrouped by culture environment,

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primer sequences used for real-time PCR analysis</th>
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<tr>
<td>Primer name (abbreviation, accession number, product size)</td>
<td>Forward sequence (5’ to 3’)</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, U85042, 86bp)</td>
<td>ACCCTCAAGATTTGTCAGC6A</td>
</tr>
<tr>
<td>Type 1 collagen (Col1, NM_174520, 97bp)</td>
<td>CATTACGGCGAATGTC6</td>
</tr>
<tr>
<td>Type II collagen (Col2, X20240, 69bp)</td>
<td>AAGGGCTTCTCAGCTTC</td>
</tr>
<tr>
<td>Superficial zone protein (SZP, AF056218, 77bp)</td>
<td>CACACACTAGGTCACTACA</td>
</tr>
<tr>
<td>Aggrecan (AGC, U76615, 76bp)</td>
<td>GCTACCTGACCCCTCATC</td>
</tr>
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GAPDH is a housekeeping gene used to determine the relative expression of the genes of interest. Type I collagen is indicative of the fibroblastic phenotype, while type II collagen and aggrecan are positive markers for the chondrocytic phenotype. SZP is a marker for the superficial zone and indicates whether zonal distinctions within the two populations still exist.
was used to determine whether significance existed among passage groups and between zones for the zonal population experiment. A p-value less than 0.05 was considered significant for the ANOVAs. If significance existed, a post-hoc analysis was performed using the Tukey-Kramer Honestly Significant Difference to test significance for all comparisons.

Results

Mixed-zone population

Gene expression changes for this portion showed rapid dedifferentiation as evidenced by an increase in Col1 expression and decreases in Col2 and SZP expression. Fig. 1 depicts the results of the mixed-zone passage experiment. Col2 expression decreased approximately 10-fold \((p < 0.0001)\) over four passages (Fig. 1a), while Col1 expression increased 20-fold \((p < 0.0001)\) over the same time frame (Fig. 1b). SZP expression was negligible after only two passages (Fig. 1c). While AGC expression did not significantly change over the course of the experiment (Fig. 1d), its expression levels did appear to decrease with each successive passage in monolayer.

Superficial and growth zone populations

To further investigate the effects of passaging chondrocytes, we isolated zonal populations from articular cartilage and examined their gene expressions for signs of dedifferentiation. Initially, significant differences existed between the two populations in terms of Col2 and SZP gene expression (Fig. 2a and c). We observed that SZP expression was 2.3 times greater in the superficial population than in the growth \((p < 0.0001)\), while the growth population expressed Col2 20 times more than the superficial population \((p < 0.0001)\). However, after only three passages these differences no longer existed (Fig. 2a and c). Additionally, after four passages the Col1 expression (Fig. 2b) in both the superficial and growth populations increased dramatically \((p < 0.0001)\) from their initial values (1200 and 8000-fold increase, respectively). As with the mixed-zone samples, AGC expression did not change markedly over four passages (Fig. 2d).

Alginate encapsulation

Contrary to expectation, alginate encapsulation proved inadequate as a means to fully re-differentiate expanded chondrocytes. The gene expression for cells at all passage points never returned to initial levels. Overall, chondrocytes encapsulated in alginate had more phenotypically correct gene expressions when passaged less than three times. When compared to initial levels, however, all passage groups showed major phenotypic changes in gene expression (Fig. 3). Most apparent was
Fig. 2. Zonal population passage results. Significant differences \((p < 0.0001)\) in gene expression existed between the superficial and growth zone chondrocytes. The growth zone expressed Col2 20 times more than the superficial zone (a), while the superficial zone expressed SZP 2.3 times more than the growth zone (c). These differences disappeared progressively over four passages. Both Col2 and SZP expression dropped to a miniscule fraction of initial values. In contrast, Col1 expression increased over the course of the study, a sign of dedifferentiation for both cell populations (b). As in the mixed-zone populations, AGC did not change dramatically for most passage points (d).

Fig. 3. Alginate encapsulation of passed cells. The gene expression of chondrocytes encapsulated in alginate for two weeks showed that full redifferentiation was not achieved. Initial, P0, gene expression levels are indicated for both zones by the dashed lines (\(- -\), \(- -\)). The expression levels for Col2 (a) and SZP (c) were a fraction of initial values, and Col1 (b) was expressed strongly for all passage groups \((p < 0.05)\). AGC expression showed no significant changes (d). However, expression characteristics for Col2 (growth zone) and SZP (superficial zone) were better in alginate than in monolayer from P1 to P4, as can be seen when compared with expression values in Fig. 2. This was most likely due to the forced, rounded morphology that is characteristic of native, and encapsulated, cells.
the average increase in Coll expression (Fig. 3b) for the passed superficial and growth zone populations (19,000- and 65,000-fold increase ($p < 0.05$), respectively, over initial levels). SZP expression in encapsulated chondrocytes dropped precipitously, with a 34- and 220-fold average decrease ($p < 0.0001$) for the superficial and growth populations (Fig. 3c). Average Col2 expression decreased 7.7-fold ($p < 0.0001$) for the growth zone cells but actually increased slightly, although not significantly, for the superficial zone cells (Fig. 3a).

Another method for analyzing the degree of dedifferentiation is to examine the ratio of Col2 to Coll for each passage (Fig. 4). A larger value indicates a more chondrocytic gene expression, while smaller values indicate a more fibroblastic gene expression. Over only two passages, the average Col2/Coll ratio decreased four orders of magnitude ($p < 0.0001$), indicating a rapid change in phenotype from chondrocytic to fibroblastic. Surprisingly, alginate encapsulation caused immediate dedifferentiation at the gene expression level of zonal chondrocytes. We observed this result for all passage groups and view it as another indication that major problems might exist with using alginate for two weeks as a means to regain the chondrocytic phenotype of cells previously cultured in monolayer.

Discussion

For most cartilage regeneration studies, the large quantities of chondrocytes needed would require at least four passages, if not many more. Zonal reconstruction requires even more passages due to the small amount of tissue present in the superficial zone. While the problem of dedifferentiation in expanded chondrocytes has been observed in the past [3], there was no evidence that the changes were occurring so early. As yet, there is no procedure that can successfully retain the native chondrocytic phenotype during expansion. Re-differentiation after expansion may be the best hope for returning chondrocytes to the proper mode of expression.

While past studies have used protein synthesis data to support their findings, this study utilizes gene expression to determine the degree of dedifferentiation. Gene expression is not a clear indicator of the products secreted by the cell but does reveal changes that are occurring at the messenger level. Previously, researchers accepted that chondrocytes could be expanded to four passages without permanent dedifferentiation [29]. However, the results of this study indicate that gene expression is affected much more quickly, and even one expansion might have a major detrimental effect on the chondrocytic phenotype. In the mixed-population of chondrocytes after one passage, expression of Col2 was two times lower, SZP was 2.4 times lower, and Coll was 2.6 times higher. These phenotypic changes are alarming for only one expansion. In particular, the rapid decrease in SZP expression might indicate that zone-specific proteins are the first to disappear during monolayer expansion. Furthermore, over the course of the study Col2 expression was 10 times lower, SZP was 600 times lower, and Coll was 20 times higher than at P0.

In the zonal populations, initial differences existed between the superficial and growth populations, specifically in Coll and SZP expression. The superficial zone expressed more SZP than the growth zone, as expected, and helps verify that the two zonal populations were separated successfully. We hypothesize that the higher expression of Coll in the growth zone exists because of active collagen remodeling in the developing growth zone. Similar gene expression differences have been noted previously [8,17].

The gene expression changes in passed, zonal populations showed similar trends to the mixed-zone experiment, although the changes were much more dramatic. This similarity occurs because the high proportion of growth zone cells (~80%) dominates the overall expression characteristics of the mixed-zone sample groups. Coll, AGC, and SZP expression decreased while Coll expression increased. Overall, the phenotypes of the two independent populations appeared to converge over several passages, indicating a homogenization of the zonal chondrocytes to a phenotype that is not zone-specific.

These findings have major implications for tissue engineering studies. Since chondrocytes dedifferentiate rapidly, expansion of cells in monolayer may not be a viable option for procedures requiring differentiated cells.
Therefore, an alternative solution must be found that enables retention of the chondrocytic phenotype for cell populations that cannot be harvested in large numbers.

Suspension of chondrocytes in 3D hydrogels has been shown to promote the chondrocytic phenotype [19,21,26]. Alginate and agarose are natural hydrogels that can be used to encapsulate the cells, thereby forcing a rounded morphology. The shape of the cell plays a major role in the genes that are expressed. If the cell is spread out, then it will proliferate, but if a rounded morphology is forced on the cell due to encapsulation or other means, then the cell expresses synthesis genes instead [12]. This study supports the finding that chondrocytes cultured in alginate retain limited chondrocytic gene expression. However, the level of expression is much reduced from the cells’ native characteristics.

This study’s results indicate that encapsulating pasaged chondrocytes in alginate might not be ideal for re-differentiating the cells. It appears that 3D culture in alginate does not help to fully regain the correct phenotype of pasaged chondrocytes. The ratio of Coll2/Col1 expression in alginate-encapsulated cells was approximately five orders of magnitude lower than primary chondrocytes, and the expression of S1P could not be recovered after two weeks in culture. However, the rounded morphology induced by encapsulation does appear to retain a certain level of gene expression in P0–P2 cells. Unfortunately, due to the dedifferentiation that occurs in monolayer, the cell continues to express undesirable genes such as Coll to a much greater degree. Coll expression is drastically higher for all passage points once placed in alginate. This result is most likely due to the low cell densities in the alginate beads. For monolayer cultures, the chondrocytes reach a high-density, confluent state before sampling that could help to expression the expression of the chondrocytic phenotype, as seen in Fig. 4. The lack of cell–cell and cell–matrix interactions could play a major role in the altered gene expression of the chondrocytes.

In other studies alginate has been used to re-differentiate chondrocytes with good success [26,30]. A major difference between alginate encapsulation in this study and ones that show a beneficial effect is the cell seeding density. The proximity of other chondrocytes within a matrix could influence whether cells express the proper genes, and if the cell density is too low then the cells would begin to express genes not normally associated with a healthy chondrocyte. Cell density has enough influence over chondrocyte phenotype that it can affect the secretion of cartilage macromolecules even for cells in monolayer [29]. Other variations that might affect alginate’s re-differentiation ability are oxygen tension levels in the culture environment and the overall time in alginate. Higher oxygen tensions (20%) have shown a positive effect on chondrocytic gene expression in experiments with four week encapsulation times [25]. However, even at the low cell densities and two week culture times used in this study, alginate appears capable of at least retaining the gene expression of pasaged cells without much further dedifferentiation. This is much more apparent for P1–P2 cells than later pasaged chondrocytes.

Another possible method for retaining the proper protein expression in expanded chondrocytes is to use growth factors. For example, applying TGF-β2 to chondrocytes in monolayer maintains the proliferation ability of chondrocytes. However, the expression of collagen type II is still lost after the third passage [9]. A plethora of growth factor studies are currently underway in the field of articular cartilage engineering. The results when using primary chondrocytes appear promising, showing increases in matrix production and proliferation, while retaining the chondrocytic phenotype. Whether zonal proteins remain after these treatments has yet to be determined.

While the best results in articular cartilage engineering today appear encouraging in their progress [6], they unfortunately use a clinically irrelevant cell source. While immature, animal chondrocytes are great for producing large amounts of extracellular matrix in vitro, we cannot readily translate this cell source to humans. If articular chondrocytes are to be used, they will most likely be adult chondrocytes. Mature cartilage tissue has a low cell density, so expanding the harvested cells will be required before obtaining sufficient numbers. The results of this study indicate that current methods for expanding cells are not conducive to producing cell populations capable of expressing the proper matrix molecules. These findings may provide reasoning for the inherent problems associated with cell transplantation procedures, in which clinicians passage autologous chondrocytes multiple times in vitro before implanting them in a defect site [4,27]. The solution to retaining the chondrocytic phenotype during cell expansion may lie with defining a specific growth factor treatment or creating a novel expansion environment. Once researchers discover a method for obtaining sufficient chondrocyte numbers, the field of articular cartilage regeneration can advance to the point of clinical feasibility.

Acknowledgement

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References


