GENE EXPRESSION OF SINGLE CHONDROCYTES

*Eleswarapu, SV; *Shieh, AC; +*Athanasiou, KA
+*Rice University, Houston, TX

INTRODUCTION

Articular cartilage is organized into several zones with differing properties, which as a whole contribute to the overall function of the tissue. These distinct properties arise from zonal differences in chondrocyte phenotype, as previously described [1,2]. To date, however, no studies have addressed zonal variations among single chondrocytes. The goal of this study was to quantify zonal variations in phenotype at the level of a single cell.

In this study, the expression of collagen type II and aggrecan in single articular chondrocytes was quantified as a function of zone and seeding time using a novel single cell real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Seeding time is an important factor since the process of adhesion is known to have a fundamental effect on cell behavior. The ability to measure gene expression in single cells will be crucial to future experiments studying mechanobiology of single chondrocytes, making it possible to establish relationships between mechanical forces and biosynthetic responses.

METHODS

Tissue harvest and cell seeding: Articular chondrocytes were isolated from the distal metatarsal of a six-month-old heifer. Using a scalpel, the surface of the joint was abraded several times to separate superficial zone tissue, and the remaining uncalcified cartilage was set aside as middle/deep zone tissue. Harvested tissue was minced up into small fragments and placed in DMEM containing 10% FBS and 560 U/ml collagenase overnight at 37ºC and 5% CO_2. Each collagenase suspension was centrifuged to form a cell pellet, which was resuspended in DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, and 0.25 μg/ml fungizone. The cell suspension was incubated in a petri dish at 37ºC and 10% CO_2 for 3 hours or 18 hours at an areal cell density of 10^4 cells/cm^2.

Single chondrocyte isolation: Chondrocytes were captured using a 30 μm inner diameter micropipette and CellTram Vario (Brinkmann) oil hydraulic microaspirator. At the end of each seeding period, individual cells from the seeding group were collected by gentle suction pressure (Fig. 1). Captured cells were ejected into 100 μl of lysis buffer.

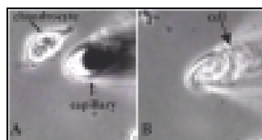

Fig. 1: Single cell isolation

Real-time RT-PCR: Total RNA was extracted from each cell lysate using the Absolutely RNA Nanoprep procedure (Stratagene) with DNase I treatment to eliminate genomic DNA. The purified total RNA was eluted into a volume of 9 μl, and first-strand cDNA was synthesized using the SuperScript III reverse transcriptase (Invitrogen) protocol with oligo(dT)$_{20}$ primers. Multiplex real-time PCR was performed on a Rotor-Gene 3000 (Corbett Research) with HotStarTaq polymerase (Qiagen) and 5 mM $MgCl_2$. Primers and probes were designed using Primer3 software (Whitehead Institute, MIT). Primers were synthesized by Sigma-Genosys, and probes were synthesized by Biosearch Technologies. Sequences and concentrations for primers and probes are provided in Table I. The HotStarTaq polymerase was activated at 95ºC for 15 min, followed by 60 cycles of 95ºC for 15 s and 60ºC for 30 s.

Table I. Oligonucleotide Information

Gene name (abbreviation, accession no., product size)	*Forward primer, concentration* *Reverse primer, concentration* *Probe, concentration, dye/quencher*
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, U85042, 86 bp)	ACCCTCAAGATTGTCAGCAA, 50 μM ACGATGCCAAAGTGGTCA, 50 μM CCTCCTGCACCACCAACTGCTT, 100 μM, FAM/BHQ-1
Collagen type II (COL2, X02420, 69 bp)	AACGGTGGCTTCCACTTC, 100 μM GCAGGAAGGTCATCTGGA, 100 μM ATGACAACCTGGCTCCCAACACC, 50 μM, ROX/BHQ-2
Aggrecan (AGC, U76615, 76 bp)	GCTACCCTGACCCTTCATC, 50 μM AAGCTTTCTGGGATGTCCAC, 50 μM TGACGCCATCTGCTACACAGGTGA, 100 μM, Quasar 670/BHQ-2

Data analysis: The threshold cycle (C_T) for each sample was calculated based on the cycle number at which the peak of the second derivative of the fluorescence vs. cycle number occurred. Efficiency of the PCR was calculated by running a standard curve for serially diluted cDNA from bovine chondrocytes. The relative abundances of COL2 and AGC were calculated using a method adapted from Pfaffl [3]. Briefly, the relative abundance, R, was determined by Eq. 1, where E is reaction efficiency, HKG denotes the housekeeping gene (GAPDH), and GOI refers to the gene of interest.

$$R_{GOI} = \frac{(1+E_{HKG})^{C_{T,HKG}}}{(1+E_{GOI})^{C_{T,GOI}}} \quad \textbf{Eq. 1}$$

A two-way ANOVA was used to determine the effects of zone and seeding time on gene expression. Results were considered statistically significant for $p < 0.05$. Results are presented as means ± standard errors.

RESULTS

Despite the difficulty involved in quantifying gene expression at such a fundamental level, this study was successful in detecting COL2 and AGC in single cells using multiplex real-time RT-PCR. Qualitatively, a number of trends were observed. Most notably, the middle/deep cells appear to express both COL2 and AGC at levels higher than superficial cells, expression of matrix genes seems to increase with increased seeding time, and COL2 is more abundant than AGC. Also of note was that the rate of detection of gene expression was higher in the 18 hour seeding groups. However, despite these trends, no significant differences were found due to zone or seeding time. A summary of results appears in Figs. 2 and 3.

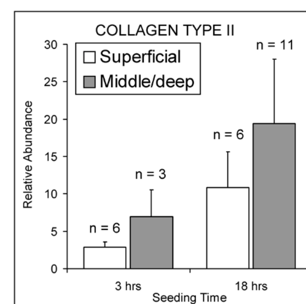

Fig. 2

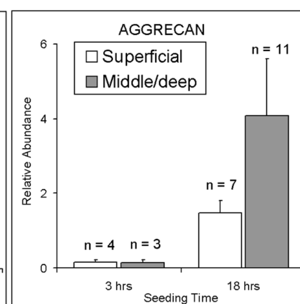

Fig. 3

DISCUSSION

Middle/deep cells appear to express COL2 and AGC at greater levels than superficial cells. Previous work has shown that cells from different zones exhibit differing biosynthetic activity [1,2]. The data also indicate that a longer seeding time allows for increased expression of matrix genes. This may result from the fact that the cells are recovering from the tissue isolation process. The greater expression of COL2 compared to AGC agrees with previous work on human articular chondrocytes [4]. This result is not surprising, given that COL2 is more prevalent in native tissue than AGC. The absence of a significant difference may be attributed to the small sample sizes and substantial cell-to-cell variability. No previous work was available as a guide to choosing values for n. Based upon post-test power analysis (α=0.05, β=0.2), a sample size of n=35 would be necessary to detect a 100% difference, using the results for COL2 from middle/deep cells at 18 hrs.

Furthermore, this study demonstrates the feasibility of examining gene expression at the level of a single chondrocyte. The technique described can be applied in future studies on the effects of specific stimuli, such as mechanical forces or growth factors, on single cells [5].

ACKNOWLEDGMENTS

This research was supported by the Arthritis Foundation.

REFERENCES

[1] Aydelotte MB & Kuettner KE, *Connect Tissue Res*, 18(3):205-22, 1988.
[2] Aydelotte MB et al., *Connect Tissue Res*, 18(3):223-34, 1988.
[3] Pfaffl MW, *Nucleic Acids Res* 29(9):2002-7, 2001.
[4] Martin I et al., *Osteoarthritis Cartilage*, 9(2):112-8, 2001.
[5] Shieh AC & Athanasiou KA, *Ann Biomed Eng* 31(1):1-11, 2003.