Video Article

A 3D System for Culturing Human Articular Chondrocytes in Synovial Fluid

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Abstract

Cartilage destruction is a central pathological feature of osteoarthritis, a leading cause of disability in the US. Cartilage in the adult does not regenerate very efficiently *in vivo*; and as a result, osteoarthritis leads to irreversible cartilage loss and is accompanied by chronic pain and immobility^{1,2}. Cartilage tissue engineering offers promising potential to regenerate and restore tissue function. This technology typically involves seeding chondrocytes into natural or synthetic scaffolds and culturing the resulting 3D construct in a balanced medium over a period of time with a goal of engineering a biochemically and biomechanically mature tissue that can be transplanted into a defect site *in vivo*³⁻⁶. Achieving an optimal condition for chondrocyte growth and matrix deposition is essential for the success of cartilage tissue engineering.

In the native joint cavity, cartilage at the articular surface of the bone is bathed in synovial fluid. This clear and viscous fluid provides nutrients to the avascular articular cartilage and contains growth factors, cytokines and enzymes that are important for chondrocyte metabolism^{7,8}. Furthermore, synovial fluid facilitates low-friction movement between cartilaginous surfaces mainly through secreting two key components, hyaluronan and lubricin^{9,10}. In contrast, tissue engineered cartilage is most often cultured in artificial media. While these media are likely able to provide more defined conditions for studying chondrocyte metabolism, synovial fluid most accurately reflects the natural environment of which articular chondrocytes reside in.

Indeed, synovial fluid has the advantage of being easy to obtain and store, and can often be regularly replenished by the body. Several groups have supplemented the culture medium with synovial fluid in growing human, bovine, rabbit and dog chondrocytes, but mostly used only low levels of synovial fluid (below 20%)¹¹⁻²⁵. While chicken, horse and human chondrocytes have been cultured in the medium with higher percentage of synovial fluid, these culture systems were two-dimensional²⁶⁻²⁸. Here we present our method of culturing human articular chondrocytes in a 3D system with a high percentage of synovial fluid (up to 100%) over a period of 21 days. In doing so, we overcame a major hurdle presented by the high viscosity of the synovial fluid. This system provides the possibility of studying human chondrocytes in synovial fluid in a 3D setting, which can be further combined with two other important factors (oxygen tension and mechanical loading)^{29,30} that constitute the natural environment for cartilage to mimic the natural milieu for cartilage growth. Furthermore, This system may also be used for assaying synovial fluid activity on chondrocytes and provide a platform for developing cartilage regeneration technologies and therapeutic options for arthritis.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3587/>

Protocol

A 3D system for culturing human articular chondrocytes in synovial fluid

In this work, we encapsulated human articular chondrocytes in alginate beads using a modified manufacture-suggested encapsulation protocol (Lonza, and³¹). Using these 3D constructs, we have developed a system for culturing cells in a culture medium containing varied percentages of human synovial fluid and have assessed these 3D constructs for cartilage gene expression.

1. Prepare human articular chondrocytes (HAC) for three-dimensional (3D) encapsulation

1. Thaw a vial (1 ml) of human articular chondrocytes (HAC) (Lonza) (Passage 2) in 37°C water bath for 1 min.
2. Mix with 1ml of chondrocyte growth media (Lonza) and centrifuge at 3000 rpm for 3-5 min to collect the cells. Discard the supernatant.
3. Resuspend cell pellet in chondrocyte growth media (Lonza).
4. Plate cells in a 10 cm tissue culture plate (BD Biosciences), and culture in chondrocyte growth media (Lonza) until the cells are confluent. HACs should be used at a passage number no higher than 3 or 4 (P3 or P4).
5. Wash HACs on the plate with 155mM NaCl instead of the standard PBS, followed by trypsinization to collect cells for alginate bead encapsulation. Once cells are detached, spin down the cells at 3000rpm for 5min. The cells are now ready for 3D encapsulation.

2. Encapsulate HACs into 3D beads

Resuspend the HACs in 1.2% alginate solution (Sigma) at a density of 8×10^5 cells/ml. Cell numbers were determined prior to encapsulation, using a standard cell counter. It is very important to mix well to ensure even distribution of the cells in the beads.

1. Pipette the HAC/Alginate solution mixture into a 12 ml syringe attached to a 22-gauge needle (Tyco healthcare, Inc.).
2. Meanwhile, prepare a 50 mL beaker with 102 mM CaCl_2 at a volume 5 times that of the HAC/alginate solution. Place a stir bar inside the beaker and stir the 102 mM CaCl_2 solution slowly (at approximately 150 rpm).
3. Hold the syringe tip about 6 inches above the surface of the CaCl_2 solution and add the HAC/alginate mixture into the CaCl_2 solution dropwise. In general, the resulting alginate beads are 2mm in diameter with an encapsulation density of approximately 10^4 cells/bead. Note: The height of the syringe over the CaCl_2 solution is important. Shorter distance will result in tear-drop shaped instead of spherical shaped beads, causing uneven mechanical integrity and cell encapsulation.
4. Let the beads stir in the CaCl_2 solution for 20-25 min. While other alginate encapsulation protocols indicate a much shorter incubation time of 10min, we found that longer incubation time with the CaCl_2 solution enhanced the integrity of the beads.
5. Remove the CaCl_2 solution, wash the beads 2-3 times in 2-3 volumes of NaCl, and then once in chondrocyte differentiation medium (Lonza). While other alginate bead encapsulation protocols involve using a filter for the above washing procedures, we found that the alginate beads often become trapped in the filter and dry out, thus reducing the efficiency of encapsulation. We found it most efficient to allow the beads to settle to the bottom of the beaker before discarding the solution.
6. Use a standard spatula to transfer the beads into culture dishes. We usually culture 12 beads per 3 cm well for 2 days in chondrocyte growth medium to allow chondrocytes to adjust to the encapsulation process in the medium they were grown in before switching to chondrocyte differentiation medium (Lonza) with synovial fluid.

3. Culture chondrocytes in synovial fluid culture medium

1. Fresh synovial fluid can be obtained from an outpatient clinic (We obtained synovial fluid from Tufts Medical Center). The synovial fluid can be transferred in 15ml falcon tubes to the laboratory, and immediately centrifuged at 3000 rpm for 15min to remove cell debris. Aliquot cell-free synovial fluid into 1.5 mL microcentrifuge tubes, to avoid repeated freeze-thawing. Supernatant can be stored at -80°C until use.
2. Prior to culturing, mix the synovial fluid with chondrocyte differentiation medium (CDM, Lonza) at varying volumetric ratios, with a constant concentration of 100mM ascorbic acid and 9 mM CaCl_2 (this trace amount of CaCl_2 is to ensure the integrity of the alginate beads).
3. Keep the plate on a rocking platform in a 37°C incubator (rocking frequency: about 75 times/min) to help nutrient distribution within the alginate beads, and to reduce clumping of the beads. This is especially important as we culture these chondrocytes for an extended period of time (up to 4 weeks). Note: While chondrocyte growth and differentiation media (Lonza) contain two commonly used antibiotics gentamycin and amphotericin, we did not supplement any antibiotics when we used different percentage of synovial fluid for chondrocyte culturing. No contamination was ever observed.
4. Change media mixtures every 2-3 days. The viscosity of synovial fluid has been a major hurdle in culturing chondrocytes encapsulated in alginate beads during long-term cultures. We found it most effective to dilute synovial fluid-supplemented medium with 102mM CaCl_2 (50% V/V), which also strengthens the integrity of the alginate beads. In this way, the medium can slowly be removed with a pipet, without damaging the beads.

4. Harvest HACs in alginate beads for gene expression analysis

Special care must be taken when harvesting the HACs from the alginate beads for gene expression analysis.

1. Wash the alginate beads 3 times in 102 mM CaCl_2 for about 5 min each time.
2. Retrieve the chondrocytes by adding 55 mM NaCitrate at a volume 4-5 times that of the beads. It is important that the alginate beads are totally immersed in the NaCitrate solution. Shake or rock for 20-30 min.
3. Spin down the cells and discard the supernatant.
4. For RT-PCR analysis, resuspend the cell pellet in cell lysis buffer (Qiagen RNA isolation kit), and proceed with RNA purification.

5. Fix HACs in alginate beads for histological analysis

Special care has to be taken to harvest the HACs from the 3D beads for histological analysis.

1. Wash the alginate beads 3 times in 102 mM CaCl_2 for about 5 min each time. To completely wash away the viscous synovial fluid, we found it most effective to wash with chondrocyte differentiation medium (Lonza) overnight, rocking in a 37°C incubator (rocking frequency: about 75 times/min). The chondrocyte growth medium was used to ensure chondrocyte survival in this prolonged wash and to allow maximum penetration of the fixing agent.
2. Fix the beads in 70% ethanol overnight, and proceed with histological analysis. To perform Dapi staining, incubate alginate beads with Dapi solution (500ng/ml) for 1hr under gentle rocking, and wash for 3 times with 102mM CaCl_2 for 15 min each time. Dapi images can then be viewed under a fluorescent microscope. The beads can also be sectioned for alcian blue and H&E staining.

6. Representative Results

Our 3D culturing method for human chondrocytes in high percentages of synovial fluid is depicted in the schematic diagram shown in Fig.1. After human chondrocytes were encapsulated in alginate beads, they were allowed to grow in medium supplemented with varying ratios of synovial fluid. Because of the viscosity of the synovial fluid, it is essential to culture chondrocytes under constant rocking conditions to prevent the clumping of the cartilage constructs and to ensure even distribution of the nutrients. It is also essential to wash the alginate beads extensively before retrieving the chondrocytes, so that the fixing or lysis buffer can penetrate the beads (Fig.1). Bright field (BF) images of chondrocytes in the alginate beads are shown in Fig.2. Dapi staining was performed to confirm uniform distribution of the cells within the beads (Fig. 2). At the end of the 21-day culture period, gene expression analysis was performed by qRT-PCR. The reference gene GAPDH was used for normalization for all PCRs, as it was determined to be one of the most reliable reference genes for qPCR analysis on chondrocytes³². An example is shown in Fig. 3, where we analyzed the results from human articular chondrocytes cultured in synovial fluid pooled from six patients with osteoarthritis. Consistent with the fact that the chondrocytes from Lonza have been expanded in 2D cultures, which would inevitably lead to de-differentiation³³, we found that Day 0 chondrocytes expressed minimal levels of cartilage matrix genes (Fig. 3). 3D culturing of chondrocytes with chondrocyte differentiation medium (Lonza) or medium supplemented with synovial fluid significantly increased cartilage gene expression of collagen,

aggrecan and MMP13, which indicates chondrocyte re-differentiation by Day 21 (Fig.3) ³⁴. Increasing the percentage of synovial fluid in the media resulted in comparable levels of cartilage matrix markers collagen II and aggrecan mRNA expression (Fig.3A and 3B). Furthermore, chondrocytes cultured in 100% synovial fluid even exhibited a decrease in cartilage degrading enzyme MMP13 mRNA expression as compared with those cultured in medium alone (Fig.3C). Interestingly, the expression level of cell death indicator caspase 3 gradually decreased with increasing ratios of synovial fluid, suggesting that synovial fluid culturing has led to decreased apoptosis levels (Fig. 3D). Therefore, our results show that culturing human articular chondrocytes in high levels of synovial fluid in a 3D setting is a feasible technology.

Tables and Figures

Figure 1. Schematic diagram of the method to culture human articular chondrocytes in high percentages of synovial fluid in 3D alginate beads. First, chondrocytes and alginate solution are mixed. When applied drop-wise to the CaCl₂ solution, chondrocytes are immobilized within the crosslinked Ca-alginate hydrogel beads. These 3D constructs are then cultured in the chondrocyte differentiation medium (Lonza) with varying ratios of human synovial fluid. After 21 days of culturing under a rocking condition, alginate beads containing cells are washed extensively after which the cells are retrieved for gene expression analysis.

Figure 2. Bright field (BF) and Dapi images of human articular chondrocytes encapsulated cultures with 0%, 30%, 50%, 70% and 100% synovial fluid. Chondrocytes were spherical in shape in all culture conditions. Images of bright field (BF) and Dapi were overlaid to confirm the location and distribution of chondrocytes. Insets, magnified images. Arrowheads, colocalization of chondrocytes in BF and Dapi staining images.

Figure 3. qRT-PCR analysis of encapsulated human articular chondrocytes at day 0 (D0) and after 21 days of culturing (D21) in medium supplemented with varying ratios of synovial fluid (SF) (0%, 30%, 50%, 70% and 100%). Results of four independent samples are shown here. GAPDH was used as internal reference for all PCRs. **A.** Collagen II mRNA expression. **B.** Aggrecan mRNA expression. **C.** MMP13 mRNA expression. **D.** Caspase 3 mRNA expression. Statistical significance was assessed for Day 0 samples and Day 21 samples of 0% and 100% synovial fluid (SF) culturing, using INSTAT software. * denotes $P < 0.05$.

Discussion

In this report, we developed a method that allows for the culture of human articular chondrocytes in a 3D environment in medium that contains high concentrations of human synovial fluid. Synovial fluid is one of the major components that constitute the natural environment in the joint cavity, where articular chondrocytes reside. However, the viscosity of the synovial fluid has been a major challenge for three-dimensional long-term culturing of chondrocytes. To overcome the challenge of maintaining even nutrient distribution in a 3D construct in a viscous environment and to prevent aggregation, we placed cartilage constructs under constant movement with gentle rocking. To enhance the structural integrity of the cartilage constructs, we modified the traditional procedure of seeding chondrocytes into an alginate hydrogel including optimization of the length of time for bead formation and the media-changing procedure³¹. Such procedures are essential when cartilage constructs are handled in the viscous synovial fluid during long-term cultures.

We believe this method will allow us to study the biology of human articular chondrocytes in their natural milieu, the synovial fluid. One potential concern is that reagent diffusion may not be ideal for chondrocytes encapsulated in alginate beads and grown in a viscous environment. Thus for studying the effect of certain reagents on engineered cartilage, better diffusion may be achieved by reducing the size of the alginate beads, and by using perfusion systems to facilitate reagent distribution in synovial fluid. While our representative result was obtained from culturing normal human chondrocytes in synovial fluid derived from patients with osteoarthritis, we believe this method can be extrapolated to use synovial fluid from an otherwise healthy person or other healthy vertebrate animals. Because synovial fluid activity can be correlated to the combinatorial activities of many biochemical factors present in the fluid, the net effect of the synovial fluid on chondrocyte gene expression in our system may be correlated with the severity of disease and will ultimately help us to understand the intraarticular milieu before and after treatments. Furthermore, our method may also lead to the possibility of using a patient's own synovial fluid for culturing autologous chondrocytes to regenerate articular cartilage that is individually tailored. To this end, this system may provide insights into studying the biomechanical forces acting through synovial fluid on engineered cartilage as well³⁵.

Disclosures

We have nothing to disclose.

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