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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**CARTILAGE TISSUE ENGINEERING WITH
HUMAN CHONDROCYTES FROM
OSTEOARTHRITIC KNEES
AND A SEMI-PERMEABLE MEMBRANE**

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Chemical Engineering

by

Nancy D. Hsieh-Bonassera

Committee in charge:

Professor Robert L. Sah, Chair
Professor William D. Bugbee
Professor Pao C. Chau
Professor David A. Gough
Professor Sungho Jin
Professor Jan B. Talbot

2009

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University of California, San Diego

2009

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Hsieh-Bonassera ND, Wu I, Lin JK, Schumacher BL, Chen AC, Masuda K, Bugbee WD, Sah RL: Expansion and redifferentiation of chondrocytes from osteoarthritic cartilage: cells for human cartilage tissue engineering. *Tissue Eng* (in press), 2009.

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ABSTRACT OF THE DISSERTATION

CARTILAGE TISSUE ENGINEERING WITH HUMAN CHONDROCYTES FROM OSTEOARTHRITIC KNEES AND A SEMI-PERMEABLE MEMBRANE

by

Nancy D. Hsieh-Bonassera

Doctor of Philosophy in Chemical Engineering

University of California, San Diego, 2009

Professor Robert L. Sah, Chair

Articular cartilage is a load-bearing tissue with a limited capacity for self-repair. One approach to cartilage repair is to use tissue engineered constructs to repair defects by replacement of the absent and damaged tissue. Current therapies only target focal defects and utilize chondrocytes from intact cartilage. In the case of larger defects, or an entire joint, a tissue engineered construct is a possible future therapeutic solution. Human chondrocytes from osteoarthritic (OA) knees are potentially an abundant cell source for creating large-scale human cartilaginous constructs. Another requirement for such constructs is attainment of mechanical function, which relates directly to the biochemical properties of the tissue. The application of a semi-

permeable membrane can modulate the matrix content within the construct. The aims of this dissertation were to determine the utility of chondrocytes from OA knees as a cell source for large-scale human cartilage tissue engineering and the effects of a semi-permeable membrane on the matrix properties of cartilaginous constructs.

Chondrocytes from OA cartilage were expanded with growth factors and serum to levels relevant to joint-scale cartilage tissue engineering. When chondrocytes were redifferentiated with human serum, they produced chondron-like structures and matrix molecules characteristic of native cartilage. Cohesive cartilaginous tissues were formed from these chondrocytes derived from OA cartilage. To study effect of a semi-permeable membrane on matrix properties, cartilaginous constructs were formed with calf chondrocytes in a perfusion bioreactor system. The content of matrix, in particular aggrecan, was dependent on membrane pore size and porosity, with larger amounts of matrix retained in the construct when a smaller pore size and lower porosity was utilized. A compartmental model was developed to describe the effects of the membrane on the distribution of matrix in the construct and culture medium.

These studies demonstrate that chondrocytes from OA cartilage are a feasible cell source for human cartilage tissue engineering, and that a semi-permeable membrane can be used to modulate matrix content in cartilaginous constructs. For clinical applications, both methods could be implemented in concert, to create large-scale cartilaginous constructs with enhanced biochemical properties, and, ultimately, a treatment solution for resurfacing of damaged or OA joints.

CHAPTER 1

INTRODUCTION

1.1 General Introduction to the Dissertation

Cartilage has a limited capacity for self repair, and when damaged, defects can progress to osteoarthritis without treatment. Surgical and biological treatments currently available have varying degrees of success, but none have approached the full restoration of tissue and function of the damaged cartilage. Tissue engineered cartilage is a potential solution, where a cartilaginous construct with specific properties can be created to replace the original tissue. For clinical applications, adequate cell source for tissue engineering is an important consideration, especially for creation of constructs to repair joint-scale defects. The matrix properties of the construct, which are directly related to its biomechanical strength, are crucial to the functionality of the engineered tissue. The overall motivation of this dissertation work was to utilize chondrocytes from OA cartilage as a cell source for human cartilage tissue engineering and implement a semi-permeable membrane for retention of matrix molecules in cartilaginous constructs.

This chapter gives an overview of articular cartilage and current repair strategies for cartilage defects. Methods in tissue engineering are discussed, with a focus on cell sources and matrix modulation.

Chapter 2 describes the expansion and redifferentiation of human chondrocytes derived from OA cartilage for use as a cell source for large-scale cartilage tissue engineering. Chondrocytes were isolated from tissue removed during total knee arthroplasty (TKA) surgery, expanded in monolayer, and redifferentiated in alginate beads. Different combinations of growth factors and serum were applied, and their effects were assessed via cell number at the expansion stage and matrix content and type at the redifferentiation stage.

Chapter 3 extends the findings in chapter 2 and focuses on the formation of cartilaginous constructs from the expanded and redifferentiated chondrocytes derived from OA cartilage. The chondrocytes were cultured in cell culture inserts under culture conditions containing growth factors GDF-5, IGF-I and TGF- β 1 in the presence or absence of human serum. Resulting constructs were evaluated by cell and matrix content, and biomechanical properties.

Chapter 4 examines the effect of a semi-permeable membrane on the formation of cartilaginous constructs in a perfusion bioreactor system. Constructs were formed from alginate-recovered chondrocytes (ARC) and freshly isolated chondrocytes, on membranes of pore size 0.05, 0.4 or 1.0 μ m with porosities of 1.2, 12.7 and 15.7% respectively. The constructs and corresponding media compartments upstream (bulk fluid) and downstream (permeate) of the membrane in the bioreactor system were assayed for cell and matrix content. In addition, proteoglycan monomer sizes and their ability to aggregate were examined via gel chromatography in the different compartments. A compartmental model was developed to describe the system and its effects.

Chapter 5 summarizes the findings of the dissertation and discusses future directions for this work.

1.2 Articular Cartilage Composition

Articular cartilage is an avascular, load-bearing tissue found at the ends of long bones [6, 9, 61] (Figure 1.1). In the knee, cartilage enables movement by providing a low friction surface for articulation [11, 61] and sustaining compressive and shear forces in the joint [6, 61]. The cells, termed chondrocytes, are sparsely distributed throughout the tissue and surrounded by fluid-filled extracellular matrix. Chondrocytes are very closely associated with their extracellular matrix and form structures consisting of one or more cells surrounded by a pericellular matrix capsule [67]. This surrounding matrix provides protective and signaling functions for the chondrocytes within the chondron [29, 33, 53, 67]. The two primary matrix molecules present in cartilage are proteoglycans and collagen, which contribute to the structure and load bearing properties of cartilage. The majority of proteoglycan molecules in cartilage are aggrecan, making up 90% of the total proteoglycans by weight [11]. The aggrecan macromolecule consists of negatively charged sulfated glycosaminoglycans (sGAG) attached to a core protein, and is mostly found in the form of aggregates that are non-covalently bound to hyaluronan via link protein [61, 62, 66]. The negative charge of the proteoglycans creates an osmotic swelling pressure that contributes to the compressive strength of cartilage [62, 66]. The swelling of the proteoglycan aggregates is constrained by a collagen network [61, 62, 66]. These collagen molecules (primarily type II) form covalent crosslinks and provide the tensile stiffness and strength to the tissue [61, 62, 66].

The tissue has a zonal architecture (Figure 1.2), where cells and matrix molecules vary in composition and organization with depth and are divided into the superficial, middle and deep zones [43, 48, 66]. In the superficial zone of cartilage, chondrocytes are organized in clusters parallel to the surface; in the deeper zones,

cells are organized in columns [43]. Cell density also decreases with depth, with the highest density of chondrocytes at the surface [66]. Proteoglycan content increases with depth and correspondingly, the compressive stiffness of the cartilage also increases with depth [15, 26, 46, 57, 66]. In the surface layers of cartilage, the collagen fibrils are oriented parallel to the surface and become more perpendicular to the surface with increasing depth [61, 62, 66]. Correspondingly, the tensile stiffness decreases with depth [45]. The amount of collagen also changes with depth with higher amounts of collagen in the surface layers and decreasing collagen content with increasing depth [62, 66]. Together, proteoglycans and collagen closely interact, to provide the structure and strength found in articular cartilage.

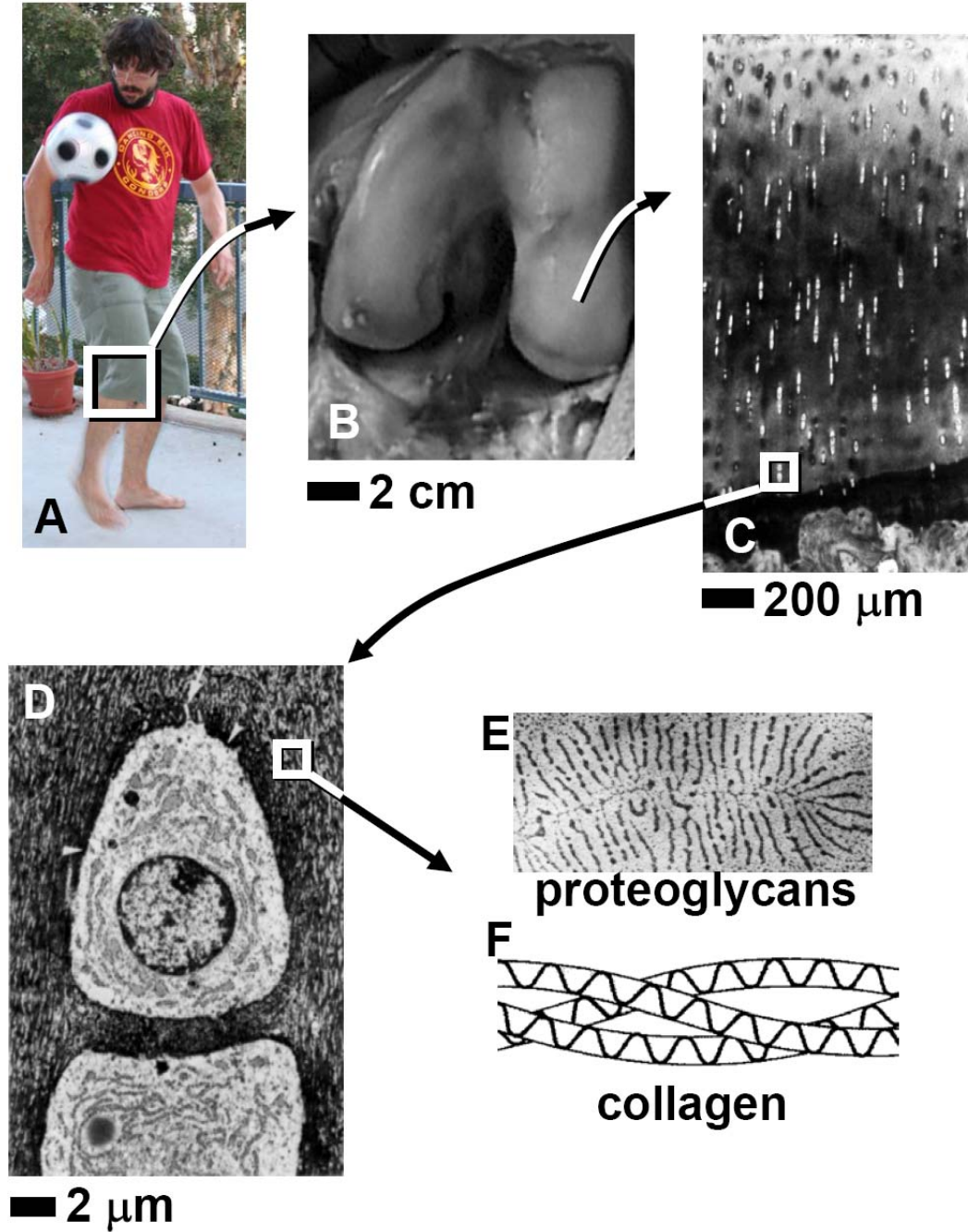


Figure 1.1: Articular cartilage structure and composition. (A) The knee joint is covered with (B) cartilage which is a (C) tissue sparsely populated with cells organized into (D) chondrons consisting of cell(s) surrounded by matrix consisting of (E) proteoglycans and (F) collagen. (Photo of Mark Bonassera. Micrographs and schematic from [13, 22, 40]. Adapted from [3].)

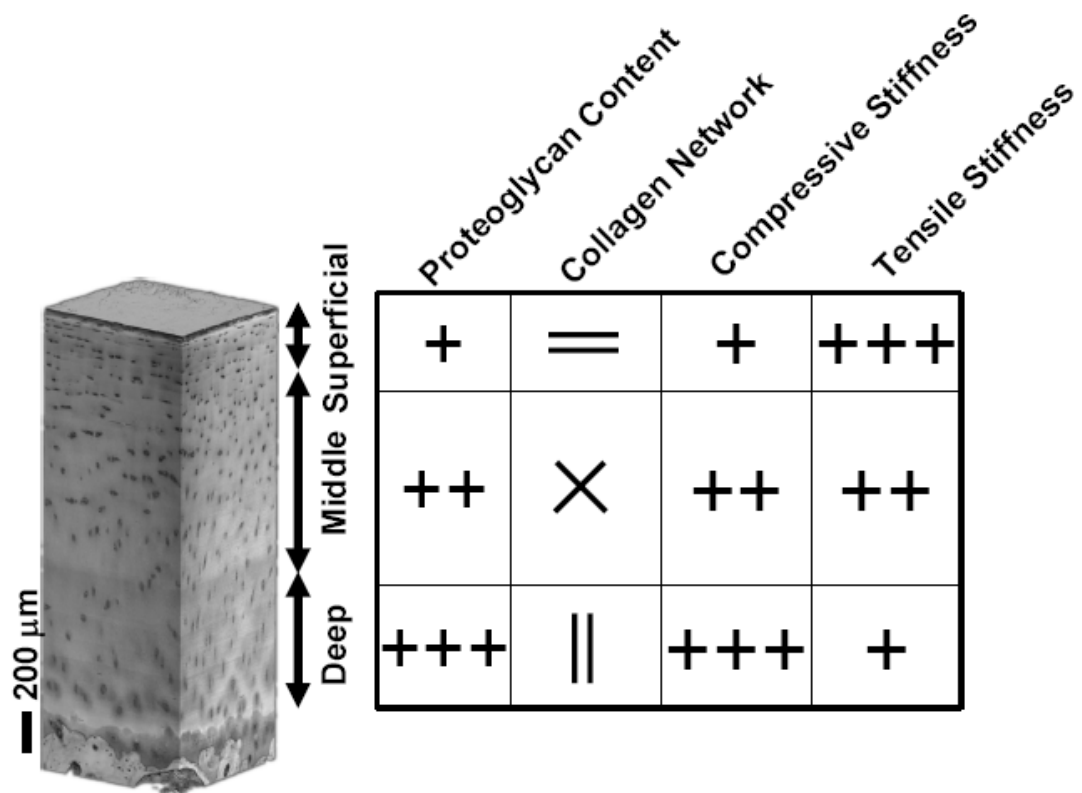


Figure 1.2: Articular cartilage zonal composition. Cartilage is divided in superficial, middle and deep zones, with cell organization and various matrix properties changing with depth. Adapted from [3].

1.3 Articular Cartilage Repair

Cartilage has a limited capacity for self-repair due to its avascular nature and slow matrix turnover rate [73]. Additionally, the cells are immobilized within their extracellular matrix, and are unable to initiate repair by migration to and proliferation within an injury site [73]. When lesions occur in cartilage, they may progress into osteoarthritis without treatment [38, 41, 82]. In osteoarthritis, the cartilage becomes fibrillated and can become eroded to the bone, resulting in pain and impaired joint function [12].

Surgical and biological therapies currently available have varying degrees of success for treatment of patients. These therapies (Figure 1.3) include microfracture, osteochondral grafts and autologous chondrocyte implantation (ACI) [5, 10, 12, 38, 41]. In the microfracture technique, holes are drilled into the defect site, penetrating into the subchondral bone, and thus stimulating a repair response from the bone marrow; however, the repair tissue formed using this technique is fibrocartilaginous [12, 41]. Damaged tissue can be replaced directly with osteochondral grafts, but the availability of donor tissue is limited [41]. With ACI, biopsies from non-load bearing portions of the patient's knee are removed, and cells are isolated and expanded, then injected into the defect site and covered with a periosteal flap [10]. There are questions about ACI's effectiveness; a study which compared ACI with microfracture also found the two therapies to be similar and not significantly different in terms of clinical, macroscopic or histological results [50], while another study comparing ACI with osteochondral allografts found that ACI did not provide as much improvement in function, and also resulted in fibrocartilage within the defect site [39]. These treatments are primarily for focal sized defects; for larger defects and severe osteoarthritis, in which the entire joint is damaged, total knee replacement surgery

may be required. Although they provide relief and restore some function, these plastic and metal joints are subject to wear. For an athletic patient, the prosthesis may not allow a full return to the original level of physical activity [36].

Tissue engineering therapies have not been widely implemented, but one such treatment is a modification of the ACI procedure, matrix-induced autologous chondrocyte implantation (MACI), which is clinically available in Europe. MACI consists of a collagen [5] or hyaluronic acid [38] scaffold component seeded with chondrocytes which is implanted into the osteochondral defect. In a study comparing ACI with MACI in effectiveness of treatment, ACI and MACI were found to be comparable in clinical, arthroscopic and histological outcomes [5]. Despite some clinical success, none of the currently available methods approach full restoration of the function and tissue of the damaged cartilage [41]. A cartilaginous construct engineered *in vitro* with properties tailored specifically for the defect that is to be repaired may be the solution [41, 73, 82].

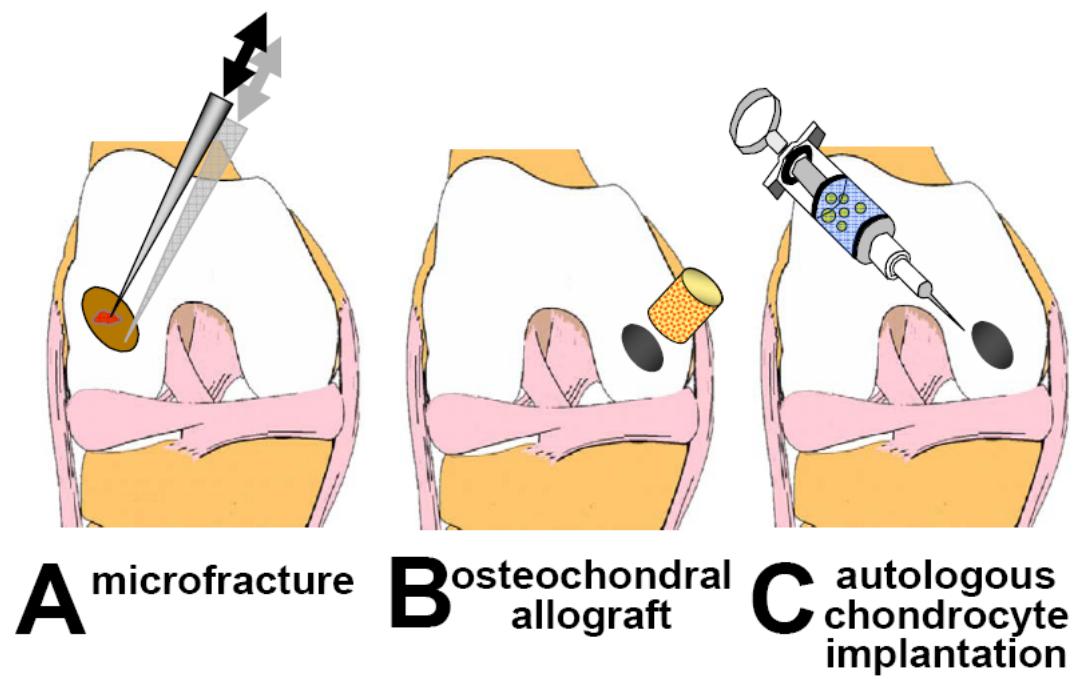


Figure 1.3: Surgical and biological cartilage repair therapies. A) microfracture B) osteochondral allograft C) autologous chondrocyte implantation. Adapted from [3].

1.4 Cartilage Tissue Engineering

Tissue engineering is described as “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ” [52]. The process of cartilage tissue engineering is essentially the combination of several components: cells, scaffolds, and chemical and mechanical stimuli, to form engineered cartilaginous tissue [72].

The growth and development of native articular cartilage is dependent on biochemical and mechanical factors present in its environment. In tissue engineered cartilage, this environment must be artificially recreated. Culture conditions can be chosen to modulate properties in an engineered cartilaginous construct; a construct with properties similar to immature cartilage may have more integration potential, whereas a construct with properties similar to mature cartilage may be useful for mechanical load bearing [49]. These conditions include cell type, scaffold, media supplements, and mechanical stimulation [30, 54]. Cell types that can be utilized include chondrocytes or mesenchymal stem cells. The geometry and materials in a scaffold can be chosen to increase proliferation, matrix synthesis and mass transfer [30, 54]. Media supplements such as growth factors and serum can also be used to enhance the proliferation and matrix synthesis in a cartilaginous construct [30, 54]. Mechanical stimulation can also result in increased proliferation and matrix synthesis [30, 54].

In this work, the culture conditions utilized for cartilage tissue engineering included: 1) cell type - chondrocytes from OA cartilage, 2) scaffold - scaffold-free culture system using the alginate recovered chondrocyte method, 3) media supplements - growth factors and serum, and 4) mechanical stimulus - perfusion.

1.5 Cell Sources for Human Cartilage Tissue Engineering

One of the challenges of engineering tissue constructs for clinical repair of cartilage defects is the limited availability of normal intact cartilage as a cell source for generating such constructs. The scale of defects found in a human femoral condyle could be on the range of 2-4 mm depth and 3-4 cm² in area, requiring a construct of 0.9 to 1.0 cm³ in volume for repair [41]. Based on the average density of normal adult human cartilage (~10 million cells/cm³) [42], that would be a requirement of 9-10 million cells per defect. In the case of resurfacing an entire joint which has a surface area of 120 cm² [24], the requirement of cells would be ~250 million cells. Cell yields from normal cartilage tissue ranges from approximately 6-8 million/gram of tissue for donors below the age of 40 to 2-4 million/gram of tissue for donors above the age of 40 [7]. To obtain enough cells from cartilage for large-scale constructs, cell expansion is required.

Expansion of cells in monolayer culture is commonly used when there is a limited source of cells. When put in a monolayer environment, chondrocytes will dedifferentiate and assume a fibroblastic morphology and produce collagen type I and versican which are typical of pre-chondrogenic mesenchymal cells [37, 44, 51, 78, 81]. Along with the decreased cell yield from cartilage tissue, the rate of expansion for adult human chondrocytes also decreases with age, most markedly after the age of 40 [4, 35, 44, 64]. Additionally, the ability of these cells to redifferentiate can also decrease with age [4]. With these limitations, in particular the scarcity of healthy donor cartilage, alternative cell sources may be necessary.

Stem cells have been researched as a cell source for cartilage tissue engineering. Stem cells have the ability to proliferate readily and differentiate into different cell types, including chondrocytes. Embryonic stem cells have the capacity to

differentiate into any cell type, but there are ethical concerns regarding their use; adult mesenchymal stem cells (MSCs) are more limited in their differentiation capacity, but are more readily available [31, 60, 68]. In particular, bone marrow derived MSC have been of interest for cartilage tissue engineering [31]. However, there are some limitations to using these stem cells. They have the tendency to become hypertrophic and produce collagen type X and alkaline phosphatase, indicating they may not have a stable chondrogenic phenotype [18, 60]. More research is also required to determine the proper signals for inducing chondrogenesis in MSCs. In addition, the harvesting procedure for obtaining MSCs can be invasive and the isolation of a pure population of MSCs can be difficult [68]. Thus, in this work, the focus is on the use of chondrocytes for cartilage tissue engineering.

One potential alternative source for chondrocytes is the tissue that is removed during TKA surgery, when a patient has his or her joint replaced with a synthetic prosthesis. The tissue from these joints is OA cartilage, and its articular surface varies from being completely eroded to mildly fibrillated. Previously, cartilage from TKA surgeries has been used as a source of chondrocytes for a variety of research studies [77, 84]. However, the amount of macroscopically normal tissue available from TKA is limited and may be as little as 1 gram of normal tissue per donor. Additionally, TKA patients are on average over the age of 40 [25, 55], which means that there is a lower cell yield and capacity for expansion and redifferentiation for cells derived from their cartilage. Cell expansion is still required to obtain numbers required for large-scale tissue engineering, but the tissue source is much more abundant and readily available.

There are several methods to accelerate expansion and minimize redifferentiation that have been applied to normal human chondrocytes, which may be

similarly applied to cells derived from OA cartilage. A combination of the growth factors transforming growth factor beta 1 (TGF- β 1), fibroblastic growth factor 2 (FGF-2) and platelet derived growth factor bb (PDGF-bb), referred to collectively as TFP, can be used to increase the rate of proliferation in monolayer for human articular chondrocytes up to 3.7-fold [4]. Use of a lower plating density in monolayer can also improve the rate of proliferation of human cells and reduce the number of passages (which can decrease redifferentiation ability) required to obtain the required number of cells [37].

The TFP growth factor cocktail has also been demonstrated to increase the chondrogenic capacity of the chondrocytes after expansion. In pellet cultures, post expansion cells produced 3.2-fold more sGAG than cells in control cultures expanded without TFP [4]. Human serum (HS) applied during the expansion stage has also been shown to also to improve the redifferentiation ability of expanded cells in pellet culture [78] but has not been widely applied in tissue engineering. Use of HS would eliminate the possibility of foreign protein contamination as well as viral protein transmission [78]. To eliminate any potential immune reactions from a tissue engineered construct implanted in a patient, the combination of autologous human serum and autologous cells could be used during culture.

For redifferentiation of expanded cells, a 3D culture system such as the alginate bead culture system can be used to redifferentiate these cells and restore their chondrogenic phenotype and ability to produce cartilaginous matrix protein, such as aggrecan and collagen type II [1, 17, 58]. In previous studies redifferentiation of normal human chondrocytes has been achieved by inclusion of growth factors such as TGF- β 1 [4, 28], insulin-like growth factor I (IGF-I) [83], and growth and

differentiation factor 5 (GDF-5) [2, 8]. Supplementation of expanded chondrocytes in alginate bead cultures with HS has also enhanced redifferentiation [1].

To form tissue from these cells, the alginate recovered chondrocyte (ARC) method can be applied. The ARC method is scaffold-free, eliminating potential concerns in a clinical application for degradation of non-biological scaffold material that has been implanted [58]. The first step of ARC method involves the encapsulation of chondrocytes in alginate gel; after a culture period of 1-2 weeks, the cells and their associated matrix (the pericellular matrix that has formed during culture) are released from the polymer with the application of a chelating agent [58]. These cells and their associated matrix can then be seeded into cell culture inserts containing membranes, and cultured for an additional period of 1-2 weeks to form cartilaginous tissue [58]. During the construct formation stage, serum and growth factors can also be added to culture medium to enhance construct properties.

Overall, there are four main steps (Figure 1.4) for utilization of OA cartilage from TKA surgeries as a cell source for human cartilage tissue engineering: 1) cell isolation 2) cell expansion 3) cell redifferentiation and 4) construct formation.

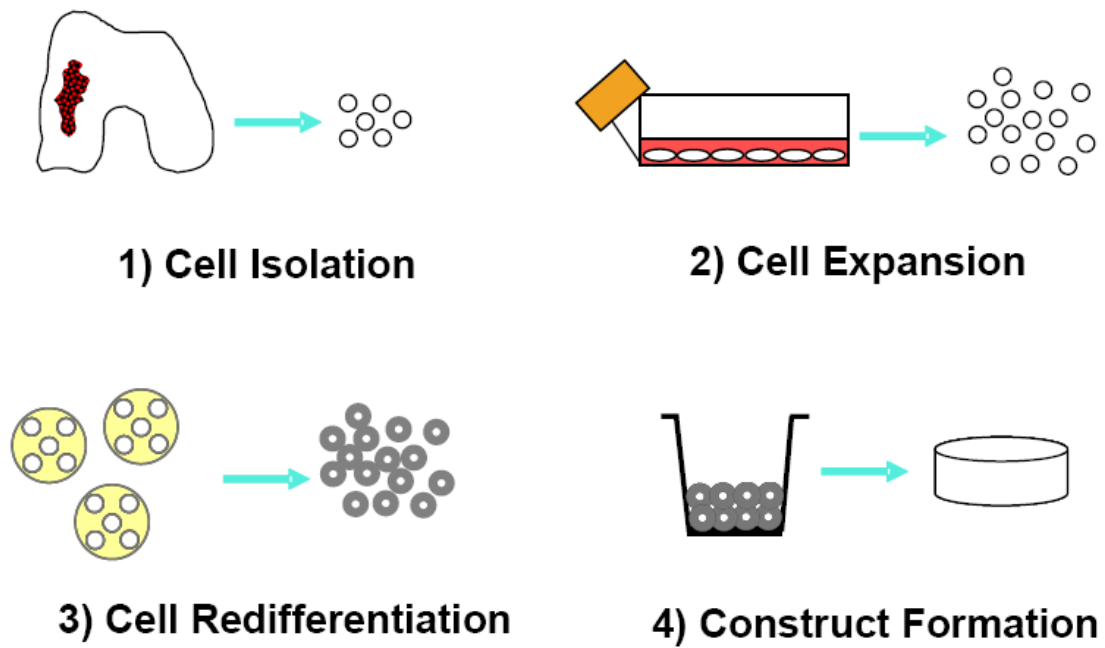


Figure 1.4: OA cartilage as a cell source for human cartilage tissue engineering. From surgical remnant tissue from TKA, chondrocytes are 1) isolated, 2) expanded, 3) redifferentiated and 4) utilized to form cartilaginous constructs.

1.6 Growth Factors for Cartilage Tissue Engineering

In the *in vivo* environment, growth factors provide signals to chondrocytes and their precursors to mediate cell processes during cartilage growth and development. In an analogous manner, these growth factors may be applied *in vitro* to modulate the formation of a cartilaginous construct. During embryogenesis, skeletal development is initiated with the condensation (cell adhesion in groups) of mesenchymal cells [14, 68] to form the cartilage anlagen [21]. Growth factors that regulate the condensation step include TGF- β s and FGFs [21, 32]. The cartilage anlagen is the precursor to the skeletal elements and subsequently the cells are differentiated into chondrocytes [14, 21]. The proliferation and differentiation of these chondroprogenitors is regulated by several growth factors, including IGF-I, FGF-2 and GDF-5 [32]. Bone formation occurs through the process termed endochondral ossification; first, chondrocytes within the center of the anlagen are terminally differentiated and become hypertrophic, then vascularization occurs, allowing osteoblasts to enter and replace the chondrocytes with mineralized bone [21, 32]. Growth factors that are present during the steps of endochondral ossification include FGFs, bone morphogenic proteins (BMPs) and vascular endothelial growth factor (VEGF) [32]. These growth factors have been applied individually or in various combinations to enhance cell proliferation and matrix production in cartilage tissue engineering.

Additionally, serum has been used as a media supplement during expansion and redifferentiation of chondrocytes and also during cartilaginous construct formation. Serum from both bovine and human origin has been applied in the culture of human chondrocytes. Serum has been shown to increase chondrocyte proliferation and matrix production and may have species specific effects [1, 78]. The components in serum have not been fully defined, but several have been identified, including the

following growth factors: TGF- β 1, FGF, IGF-I, PDGF-ab, epidermal growth factor (EGF) and VEGF [78, 79]. Serum can be used alone or in addition to growth factors for media supplementation for cartilage tissue engineering. With different growth factor and serum combinations, the properties of cartilaginous constructs can be modulated.

1.7 Bioreactors for Cartilage Tissue Engineering

Many types of bioreactors aim to provide mechanical stimuli or increased mass transfer of nutrients for culture of engineered cartilage tissue [30, 51, 54, 69, 76]. Bioreactors also provide a controlled environment of nutrients, gases, pH and waste removal for the culture of cells [19, 54, 63, 80]. There are many different types of mechanical stimulation that are relevant to cartilage development, including: shear, compression, hydrostatic pressure and fluid flow [19, 30, 54]. In perfusion bioreactors, fluid flow may act as a mechanical stimulus or enhance convective mass transfer of nutrients and other soluble factors [47, 56, 70, 71, 85].

Perfusion in bioreactors can increase cell proliferation and matrix production in cartilaginous constructs [20, 23, 56, 65]. Specifically, perfusion bioreactor systems have been shown to increase proteoglycan synthesis [20, 65]. Bovine chondrocytes seeded onto PLLA/PGA scaffolds and cultured under static and perfusion (1 μ m/sec) conditions have been compared, with perfusion conditions producing 184% more sGAG content than static conditions [65]. Ovine chondrocytes seeded onto PGA scaffolds and cultured under perfusion conditions (10-170 μ m/sec) also showed 40% increased sGAG content when compared to static conditions [20]. Additionally, perfusion can also increase collagen content, with chondrocytes cultured with

perfusion stimulating collagen synthesis by 155% [65]. However, with perfusion, especially in the early stages of culture, newly synthesized molecules may be lost into the medium due to the fluid flow and the high porosity of the developing tissue. Implementation of a semi-permeable membrane may aid in the retention of these molecules.

1.8 Membranes for Modulation of Cartilage Tissue Engineering

Bioseparation processes often involve the use of membranes with distinct pore sizes to selectively retain molecules of a particular size. By incorporating membranes of small pore sizes and lower porosity into a perfusion bioreactor system, matrix molecules, such as proteoglycans and collagen may be retained and improve the properties of these constructs. In previous studies, membranes have been used to modulate matrix content in constructs in static cultures of cartilaginous constructs [34] and cartilage explants [74]. A semi-permeable membrane may be implemented in bioreactors for retention of newly synthesized matrix molecules in cartilaginous constructs and enhance the early growth of such constructs [20, 47].

In addition to retention of molecules based on their size in relation to the membrane pores, and due to lower overall pore area with lower porosity, there are also effects that are characteristic of membranes in a flow filtration application that may increase the retention of molecules. Molecules can be retained by the membrane via pore plugging, pore adsorption, or hydrodynamic bridging [75] even if they are smaller than the membrane pore size. Another phenomena is concentration polarization, which occurs at the surface of the membrane when the retained molecules accumulate and a concentration gradient is created by molecules in the high

concentration layer attempting to diffuse back into the bulk fluid [59, 75]. If enough molecules are accumulated, a layer on top of the membrane forms, which differs in rheological and mechanical properties from the overlying solution; this layer is referred to as the cake, and the process is termed cake formation. The cake has a maximum concentration of solutes at the surface of the membrane [16, 27, 59, 75]. The occurrence of this layer can decrease the permeability of the membrane. It is possible that these effects can occur in the case of the formation of a cartilaginous construct on top of the membrane, in a sense; the construct tissue itself may be considered a “cake”. Due to these potential effects, the amount of molecules retained within the construct may not be solely dependent on pore size and porosity.

1.9 References

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CHAPTER 2

EXPANSION AND REDIFFERENTIATION OF CHONDROCYTES FROM OSTEOARTHRITIC CARTILAGE: CELLS FOR HUMAN CARTILAGE TISSUE ENGINEERING

2.1 Abstract

Objective: To determine if selected culture conditions enhance the expansion and redifferentiation of chondrocytes isolated from human osteoarthritic cartilage with yields appropriate for creation of constructs for treatment of joint-scale cartilage defects, damage or osteoarthritis.

Methods: Chondrocytes isolated from osteoarthritic cartilage were analyzed to determine the effects of medium supplement on cell-expansion in monolayer and then cell-redifferentiation in alginate beads. Expansion was assessed as cell number estimated from DNA, growth rate, and day of maximal growth. Redifferentiation was evaluated quantitatively from proteoglycan and collagen type II content, and qualitatively by histology and immunohistochemistry.

Results: Using either serum or a growth factor cocktail (TFP: TGF- β 1, FGF-2, PDGF-bb), cell growth rate in monolayer was increased to 5.5x that of corresponding conditions without TFP, and cell number increased 100-fold within 17 days. In subsequent alginate bead culture with human serum or TGF- β 1 and ITS+1, redifferentiation was enhanced with increased proteoglycan and collagen type II production. Effects of human serum were dose-dependent, and 5% or higher induced formation of chondron-like structures with abundant proteoglycan-rich matrix.

Conclusion: Chondrocytes from osteoarthritic cartilage can be stimulated to undergo 100-fold expansion and then redifferentiation, suggesting they may be useful as a cell source for joint-scale cartilage tissue engineering.

2.2 Introduction

The treatment of joints affected by osteoarthritis (OA) using tissue engineering presents challenges that are distinct and formidable compared to those for the treatment of focal cartilage defects. OA involves widespread damage to articular cartilage, resulting in decreased joint function and pain [20, 21, 47]. Cartilage does not undergo effective self-repair because of the limited intrinsic metabolism of chondrocytes or extrinsic influx of reparative cells [6, 20]; therefore surgical and biological therapies are attractive treatment options. Current therapies for focal cartilage defects include microfracture, osteochondral autografts, and autologous chondrocyte implantation (ACI) [5, 7, 20, 21]. In Europe, subsequent generations of ACI are in use and development, with cells being seeded onto scaffolds or other support structures and then implanted into defects [5, 50]. For larger defects and severe OA, in which the entire joint is damaged, allografts and total knee

replacements, respectively, are available treatments [21]. However, none of these therapies fully restore function to damaged cartilage [21]. A cartilaginous construct engineered *in vitro* may be a regenerative or repair solution providing normal function [21, 40, 47]. While animal studies have shown some success in repairing focal defects using constructs [21], currently, there are no tissue engineering treatments for OA, in which the entire articular surface would need to be replaced.

Creation of large tissue engineered constructs to resurface entire joints will require sufficiently large cell sources. Cell-based therapies such as ACI utilize autologous sources starting from ~100 mg of cartilage from a non-load bearing region [5, 7, 22]. These cells are isolated and expanded to obtain the prescribed number, 2 million cells per cm² of defect area [22]. For restorative treatment of the entire knee joint, with a surface area of 120 cm² [11], 240+ million cells are required. In an osteoarthritic joint, if an autologous source is to be used, the amount of cartilage available for cell isolation could be limited due to tissue damage; the yield of freshly isolated cells can be as low as 2 million per gram [4, 6, 13], and decreases with increasing age [4, 6]. Extensive chondrocyte expansion is thus required to obtain the number of cells needed for joint resurfacing.

However, longer expansion times and multiple passages in monolayer can lead to chondrocyte dedifferentiation [15, 45], with cells going through morphological and phenotypical changes, becoming fibroblastic and producing collagen type I, not collagen type II [15], which may be detrimental to repair. To minimize dedifferentiation and maximize the number of cells that can be obtained within a single passage, a growth factor cocktail (TFP) consisting of transforming growth factor beta 1 (TGF- β 1), fibroblast growth factor 2 (FGF-2), platelet derived growth factor type bb (PDGF-bb), which has previously been used to accelerate growth with

normal chondrocytes [4, 13, 15] may be applied to expand chondrocytes derived from OA cartilage. In the present study, the effect of TFP in combination with human serum (HS) and fetal bovine serum (FBS) on expansion of cells from OA cartilage was evaluated.

To utilize such cells for tissue engineering applications, redifferentiation would be required to restore their functional phenotype after expansion. Previously, redifferentiation of human chondrocytes has been enhanced by the application of growth factors and HS [4, 9, 13, 15, 45]. Alginate bead encapsulation was selected as the method for studying redifferentiation since the three-dimensional environment enhances retention of phenotype in human articular chondrocytes and encourages formation of matrix molecules, such as aggrecan and collagen type II [9, 17, 31]. The redifferentiation of expanded chondrocytes [3, 4, 9, 31, 45, 49] and chondrogenesis [23, 35] of stem cells is stimulated by the factors ITS+1 (insulin-transferrin-selenium-linoleic acid-bovine serum albumin), dexamethasone, TGF- β 1, insulin-like growth factor I (IGF-I), and bone morphogenic protein 14 (BMP-14). Serum may provide chondrogenic factors in a manner dependent on maturation, so that HS, FBS, and adult bovine serum (ABS) may have different effects on chondrogenesis. Chondrocytes from OA cartilage have not been previously studied to determine if the specific factors described above, alone or with serum, facilitate expansion and then redifferentiation in alginate.

The hypothesis of this study was that cells derived from OA cartilage can be expanded and redifferentiated at amplification levels relevant to cell sourcing for human cartilage tissue engineering at the whole-joint scale. The aims of this study were to determine the ability of selected serum and defined chemical factors to stimulate (1) proliferation of primary human chondrocytes from OA cartilage as

assessed by DNA and (2) redifferentiation as assessed by accumulation of proteoglycan, and types I, II, and VI collagen.

2.3 Materials and Methods

Materials

Materials were obtained as previously described [3, 14, 24], except for: bovine serum albumin (BSA), ITS+1 (10 mg/mL insulin, 5.5 mg/mL transferrin, 5 ng/mL selenium, 0.5 mg/mL bovine serum albumin, 4.7 mg/mL linoleic acid), dexamethasone (Sigma-Aldrich, St. Louis, MO); human serum (Gemini Bioproducts, Sacramento, CA); recombinant human BMP-14, FGF-2, IGF-I, PDGF-bb, TGF- β 1 (Peprotech, Rocky Hill, NJ).

Cell Isolation

Chondrocytes were isolated from the cartilage of osteochondral tissue fragments removed from patients during total knee arthroplasty (TKA) for OA. Tissue fragments included articular cartilage along with bone that was removed from the joint surface during surgery and usually discarded, in order to accept implantation of the artificial knee prosthesis. Patients (4 males and 5 females, 65 $\pm$ 9 years, range 56-79 years) were from Scripps Green Hospital (La Jolla, CA) and had consented according to an Institutional Review Board approved protocol. Macroscopically intact cartilage was harvested from lateral femoral condyles and the lateral portion of trochlea, and initially cultured in DMEM supplemented with 25 μ g/mL ascorbate, 0.1 mM MEM non-essential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, antimicrobials (100 U/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL Fungizone) and 10% FBS for

7 days. Subsequently, cartilage was digested sequentially with pronase and collagenase to obtain chondrocytes [27].

Experiment I: Chondrocyte Proliferation

To determine the ability of various growth factors and serum to stimulate proliferation, primary human chondrocytes were seeded at low density in monolayer and analyzed for DNA content after various durations of culture. Chondrocytes (from 2 males and 3 females, 67 ± 9 years, range 58-79 years) were seeded at 5,000 cells/cm² in 48-well tissue culture plates with duplicate cultures for each condition and patient, and incubated with basal medium and (1) 0.1% BSA, (2) 2% FBS, (3) 2% HS, (4) 5% FBS, or (5) 5% HS, alone or with additional supplementation with 1 ng/mL TGF- β 1, 5 ng/mL FGF-2, 10 ng/mL PDGF-bb (TFP), which stimulates proliferation of normal human chondrocytes [4, 13]. Media was changed every other day. On days 1, 5, 7, 9, 13, 17 and 21, one plate of cultures was terminated after recording a phase microscopy image of each culture, removing media and freezing samples at -20°C.

To quantify cell number, samples were solubilized (0.5 mL per well of 0.5 mg/mL proteinase K in phosphate buffered EDTA at 37°C for one hour and then overnight at 60°C), and portions analyzed for DNA using PicoGreen[®] and human DNA standards [34]. Cell number was calculated using a conversion factor of 7.7 pg of DNA per cell [34].

For each set of samples from the same patient and culture condition, cell growth was characterized by fitting cell number data to a logistic growth equation. The equation was: $Y = A + C / (1 - e^{-B(x-M)})$, where Y=number of cells, A=initial number seeded, C=maximum number of cells, B=growth rate, x=day of culture, and M=day of maximal growth [19]. C was assumed to be the average number of cells on day 21 in

cultures including serum and TFP. Values for B and M were determined by iterative minimization of sum of squared error with Excel (Microsoft Corporation, Redmond, WA).

Experiment II: Cell-redifferentiation

To determine the ability of selected growth factors and serum additives to stimulate redifferentiation of chondrocytes from OA cartilage after expansion, cells were expanded under a favorable condition from Experiment I, cultured in alginate beads, and analyzed. Chondrocytes (from 4 males and 4 females, 66 ± 9 years, range 56-79 years) were seeded in monolayer at a density of 5,000 cells/cm² in 175 cm² tissue culture flasks and incubated in medium supplemented with 2% HS and TFP for 14 days. Cells were released from monolayer with pronase and collagenase, and encapsulated in alginate beads at a density of 4×10^6 cells/mL of alginate [33]. Samples (10 beads each) were incubated in 24-well non-tissue culture-treated plates for 14 days with triplicate cultures for each condition and patient, with DMEM/F-12 supplemented with: (A) 0.01% BSA, (B) ITS+1, (C) ITS+1 and 10^{-7} M dexamethasone (dex), (D) ITS+1 and 5 ng/mL TGF- β 1, (E) ITS+1 and 10 ng/mL TGF- β 1, (F) ITS+1, 10^{-7} M dex and 5 ng/mL TGF- β 1, (G) ITS+1, 10^{-7} M dex and 10 ng/mL TGF- β 1, (H) 2% HS, (I) 2% HS, 200 ng/mL IGF-I, 100 ng/mL BMP-14, (J) 5% HS, (K) 10% HS, (L) 10% FBS, (M) 10% ABS, and (N) 20% HS. Media was changed every other day. Cells from all replicates were released from alginate using sodium citrate [33], and centrifuged to separate cells with their cell-associated matrix (CM, pellet) and further-removed matrix (FRM, supernatant).

Biochemical Analysis

To determine the extent of redifferentiation, the contents of sulfated glycosaminoglycan (sGAG) and type II collagen, which are markers of chondrogenesis [8, 38], were assayed. Portions of CM and FRM were digested with proteinase K overnight at 60°C. Digested samples were analyzed for DNA content as described above and for sGAG content using dimethylmethylene blue [12]. To assess levels of collagen type II, portions of the CM fractions were pooled from 4 patients, digested with 1 mg/mL pepsin in 0.05 M acetic acid at 4°C overnight, then with 0.1 mg/mL pancreatic elastase in 1.0 M Tris, 2.0 M NaCl, 50 mM CaCl₂, pH 8.0 at 4°C overnight, and processed according to manufacturer's instructions using an ELISA kit (Native Type II Collagen Detection Kit, Chondrex, Redmond, WA). FRM fractions were not analyzed for collagen type II because levels were below the sensitivity of the assay.

Histochemistry and Immunohistochemistry (IHC)

Histochemical staining was performed on cells and associated matrix released from alginate beads from each culture condition. To visualize the proteoglycan matrix surrounding the cells, the CM was fixed in 2.5% glutaraldehyde and stained with 1% alcian blue overnight [44]. Samples were destained with 3% acetic acid and observed using brightfield microscopy.

IHC staining for collagen types I, II and VI was performed on the CM portions of alginate bead cultures. The presence of collagen type I indicates dedifferentiation whereas that of collagen type II indicates redifferentiation [3, 15]. The presence of collagen type VI can also indicate redifferentiation, and is a marker for chondrons [14], which are structures consisting of groups of cells and matrix found in native

cartilage [39]. Collagen types I and II were localized using the Vectastain ABC Elite Kit and visualized using the Vector VIP substrate kit. The CM was resuspended in PBS and placed on poly-L lysine-coated slides and allowed to dry overnight. Slides were post-fixed in acetone, rinsed with ABC buffer, blocked with 2.5% normal horse serum, and incubated with the following primary antibodies: mouse monoclonal anti-collagen type I (1:2,000 dilution, Sigma-Aldrich), mouse monoclonal anti-collagen type II (1:1,500 dilution, Chondrex) and mouse nonspecific IgG (Pierce, Rockford, IL) at a concentration of 1 $\mu\text{g/mL}$ as a negative control. Slides were counterstained with methyl green nuclear stain and visualized using brightfield microscopy (when methyl green is used with the VIP chromagen, cell nuclei appear blue). Collagen type VI was localized with the same steps as described for collagen type I and II, with an additional digestion step of 5000 U/mL of bovine testicular hyaluronidase in buffer (0.1 M $\text{NaH}_2\text{PO}_4\text{-H}_2\text{O}$, 0.15 M NaCl, pH 5.3), using goat monoclonal anti-collagen type VI antibody (1:80 dilution, Southern Biotech, Birmingham, AL); as a negative control for collagen type VI, a goat non-specific IgG (1:80 dilution, Southern Biotech) was used, also with the additional hyaluronidase digestion step.

To perform correlations with biochemical data, collagen type II IHC slides were graded manually for the percent of positively stained cells, the intensity of stain and the total number of cells. IHC slides (2 males and 2 females, 62 ± 8 years, range 56-74 years) were graded by two independent observers for stain intensity using a quadratic scoring scale (in order to correspond to the area of staining). A grade of 0 indicated no staining, a grade of 1 indicated a light stain, and grades of 4, 9 and 16 indicated increasing intensity and area of staining. The number of positively stained cells per 100 cells was calculated by dividing the sum of the cells with grades of 1 through 16 by the total number of cells and multiplying by 100. The score per cell was

calculated by multiplying the number of grade 1 cells by 1, the number of grade 4 cells by 4, the number of grade 9 cells by 9 and the number of grade 16 cells by 16 and summing, then dividing by the total number of cells. Clusters were counted, with a cluster defined as a group of 3 or more cells surrounded by positively stained matrix, and normalized to the number of clusters per 100 cells for comparison. Collagen type I IHC slides were also graded manually for the percent of positively stained cells using the same grading system as for collagen type II. All positive collagen type I cells were graded as 1, and there were virtually no positive cells in clusters, so the score per cell and the number of clusters data are not presented.

Statistical Analysis

The data are expressed as the mean $\pm$ standard error of the mean (SEM), and were analyzed using Systat 10.2 (Systat Software, Point Richmond, CA). Experiment I. Effects on cell number, growth rate (B) and day of maximal growth (M) were assessed by two-way ANOVA with serum and TFP as independent factors. Experiment II. Effects on total sGAG per cell and collagen type II IHC grading parameters (number of positive cells per 100 cells, score per cell, and number of clusters per 100 cells) were assessed by ANOVA with media as an independent factor. When significant effects ($p < 0.05$) were found, Tukey post-hoc testing was performed.

2.4 Results

Experiment I: Chondrocyte Proliferation

TFP and serum supplementation of expansion cultures resulted in acceleration of cell growth rates and reduction of time to confluency. Phase micrographs (Figure

2.1) indicated differences in growth between conditions. In conditions 2-5 with serum and TFP, cells typically reached confluency by day 7 and hyperconfluency by day 13. Without serum, in condition 1, TFP stimulated cells to reach confluency by day 13. In contrast, cells without TFP with 2% serum, FBS and HS in conditions 2 and 3, respectively, did not reach confluency until day 21. However, with more serum (5%, conditions 4 and 5), cells were confluent by day 13. Without serum or TFP, in condition 1, cell proliferation appeared minimal during the 21 day culture period. Because cell growth was minimal, data from condition 1 without TFP was not curve-fit for logistical growth parameters.

These qualitative observations were consistent with cell number (Figure 2.2) and calculated growth rate and day of maximal growth (Table 2.1). With TFP, cell number increased ($p < 0.001$) as did growth rate ($p < 0.001$), and day of maximal growth shifted earlier ($p < 0.001$). Growth rate was approximately 5.5-fold faster for TFP conditions than corresponding non-TFP conditions. Serum supplementation affected cell number ($p < 0.05$) with significant differences between serum-containing conditions 2-5 and serum-free condition 1. There were no differences due to percentage or type of serum except with cell number on day 9 between condition 2 (2% FBS), condition 3 (2% HS), and condition 5 (5% HS) ($p < 0.01$). There was an interactive effect of TFP and media ($p < 0.05$) between days 7 and 21. An approximately 100-fold expansion was achieved with serum and TFP within a 17 day period. At day 21, the fold expansion varied between patients by 44% for conditions without TFP or serum, whereas it varied by only 8% with the addition of TFP and serum.

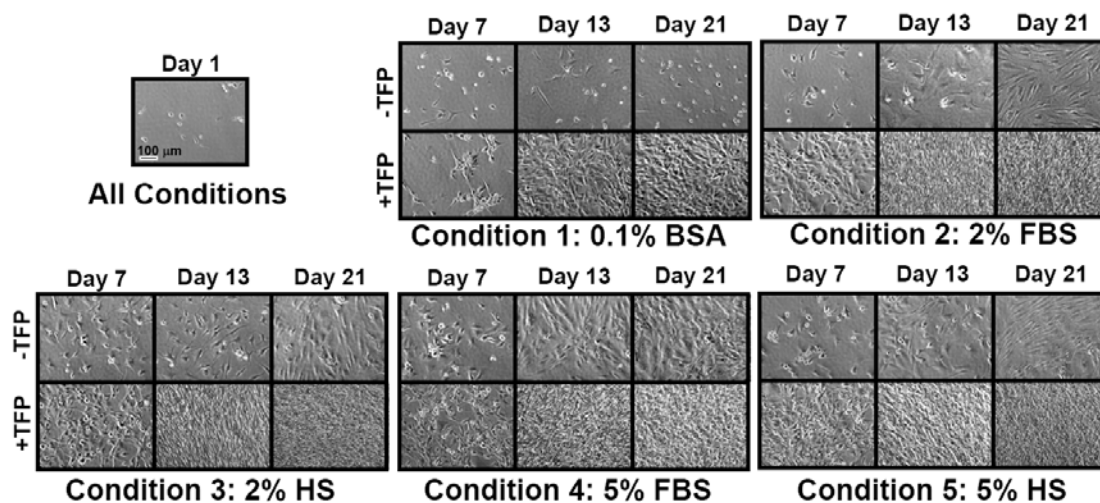


Figure 2.1: Cell-expansion in monolayer with and without growth factor cocktail (TFP: transforming growth factor beta 1 (TGF- β 1), fibroblast growth factor 2 (FGF-2), platelet derived growth factor bb (PDGF-bb)) supplementation. Phase micrographs of cells. Day 1 is shown as an example of seeding density, which is 5,000 cells/cm² for all conditions. BSA = bovine serum albumin, FBS = fetal bovine serum, HS = human serum.

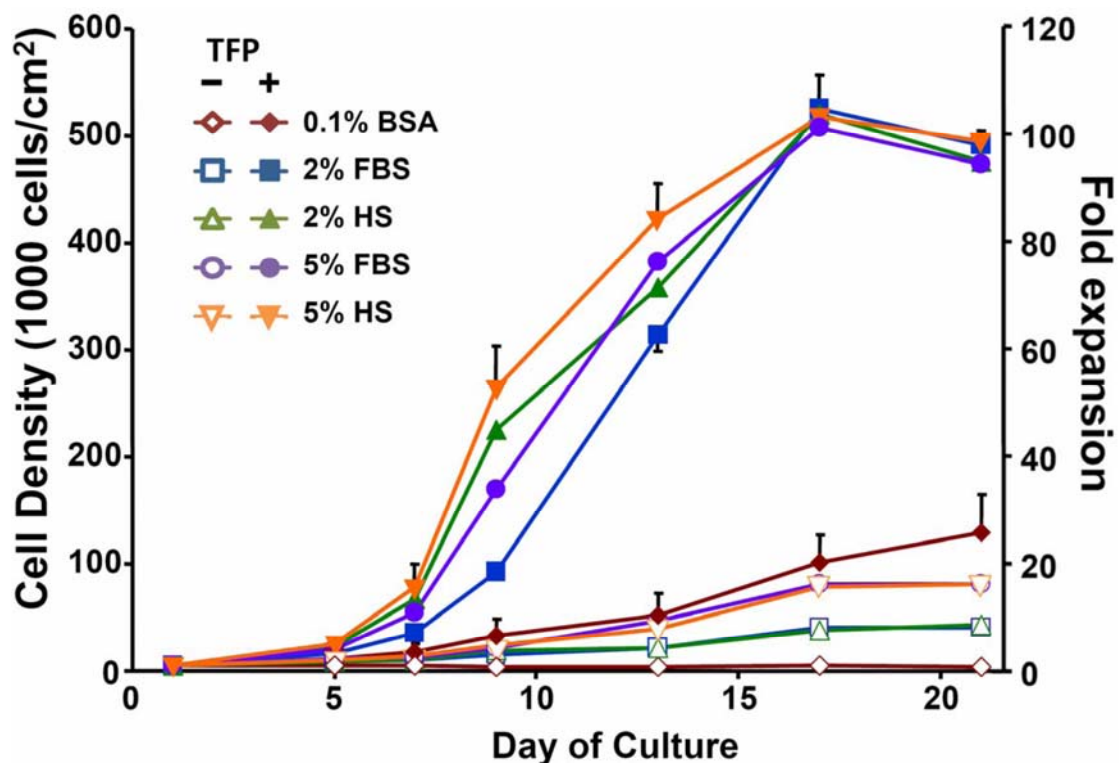


Figure 2.2: Time course of cell growth under different media conditions with serum $\pm$ TFP. $n = 4-5$ patients. Representative standard error bars are shown. TFP = growth factor cocktail as defined in the caption to Figure 2.1, BSA = bovine serum albumin, FBS = fetal bovine serum, HS = human serum.

Table 2.1: Cell-expansion model fit: Logistic growth curve. n=4, $\pm$ SEM. No values are included for condition 1 without the growth factor cocktail (TFP, as defined in the caption to Figure 2.1) because data could not be fit to a curve. Significant differences between + and - TFP are indicated: *, $p < 0.001$.

		B (Growth rate, day⁻¹)		M (Day of Maximal Growth)	
Media		-TFP	+TFP	-TFP	+TFP
1	0%+0.1% BSA		0.152 $\pm$ 0.016		28.5 $\pm$ 2.8
2	2% FBS	0.122 $\pm$ 0.018	0.738 $\pm$ 0.039*	41.9 $\pm$ 2.8	10.9 $\pm$ 0.6*
3	2% HS	0.121 $\pm$ 0.026	0.672 $\pm$ 0.130*	43.7 $\pm$ 4.7	9.9 $\pm$ 0.8*
4	5% FBS	0.131 $\pm$ 0.015	0.725 $\pm$ 0.011*	33.4 $\pm$ 2.1	10.1 $\pm$ 0.7*
5	5% HS	0.139 $\pm$ 0.011	0.815 $\pm$ 0.142*	34.4 $\pm$ 4.6	9.2 $\pm$ 0.6*

Experiment II: Cell-redifferentiation

Biochemical Analysis

Medium formulation had an effect on matrix production, assessed as sGAG/cell after incubation in alginate beads ($p < 0.001$, Figure 2.3a). sGAG/cell was similarly low for serum-free conditions, A-G. Compared to levels in basal medium, sGAG/cell was 3.6- to 5.7-fold higher for serum-containing conditions H-N ($p < 0.05$). For conditions without serum, A-G, variability in matrix production was 36% between patients and 49% for conditions, H-N, with serum. Collagen type II content on a per cell basis was low in all conditions except those with 5 and 10 ng/mL TGF- β 1 (D, E), and 10 and 20% HS (K, N) (Figure 2.3b).

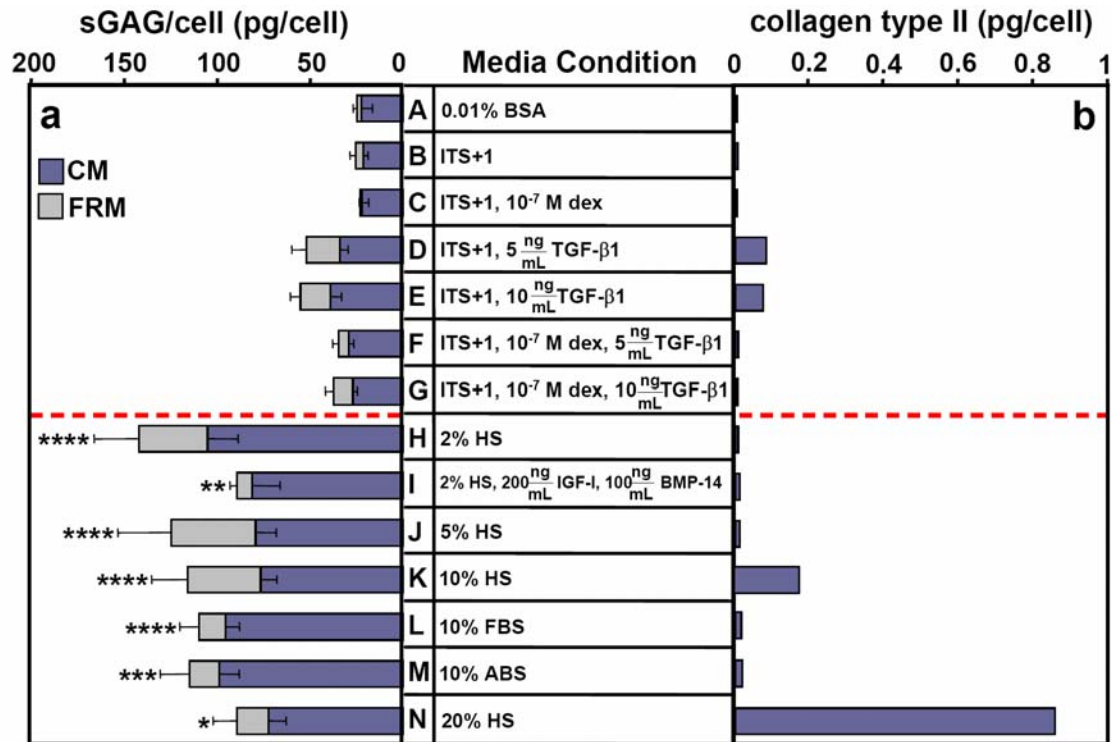


Figure 2.3: Cell redifferentiation. (a) Sulfated glycosaminoglycan (sGAG) and (b) collagen type II production on a per cell basis. sGAG samples are reported as pg/cell $\pm$ standard error of the mean (SEM) for $n=5-8$ patients. Collagen type II samples are the pooled results from $n = 4$ patients. The red dotted line separates non-serum from serum conditions. Significant differences from the basal control condition, A, are indicated: *, $p<0.05$, **, $p<0.01$, ***, $p<0.005$, ****, $p<0.001$. CM = cells with cell associated matrix, FRM = further removed matrix (panel a only), BSA = bovine serum albumin, dex = dexamethasone, TGF- β 1 = transforming growth factor beta 1, HS = human serum, IGF-I = insulin-like growth factor 1, BMP-14 = bone morphogenic protein 14, FBS = fetal bovine serum, ABS = adult bovine serum.

Histology and Immunohistochemistry

Alcian blue staining was consistent with quantitative values for proteoglycan content, with culture conditions without serum resulting in less staining than culture conditions with serum. However, alcian blue staining (Figure 2.4) also indicated variation among serum conditions in proteoglycan localization in the pericellular matrix even when sGAG/cell quantities were similar. In particular, conditions with 5% and higher HS (J, K, L) had structures where cells were surrounded by a “shell” of matrix.

Immunostaining for collagen types I, II and VI indicated differences and similarities between media conditions during redifferentiation. Collagen type I staining was light in all conditions (Figure 2.5). Staining patterns for collagen type II (Figure 2.6) indicated that most conditions exhibited minimal positive staining, except for serum-free conditions with TGF- β 1 (D, E) and higher concentration HS conditions (K, N), consistent with biochemical data. Similar to proteoglycan staining patterns, with 10 and 20% HS, collagen type II staining also had structures where cells were surrounded by a “shell” of matrix. Collagen type VI (Figure 2.7) was present in most conditions, with more staining intensity in conditions where clusters of cells were present. IgG controls (not shown) were negative.

Semi-quantitative analysis of staining for collagen type II indicated differences between medium conditions (Figure 2.8), consistent with the biochemical results. Media had an effect on number of positive cells per 100 cells (Figure 2.8B, $p < 0.001$), score per cell (Figure 2.8C, $p < 0.001$) and cluster number per 100 cells (Figure 2.8D, $p < 0.001$). Score per cell and cluster number were highest for 20% HS (N), followed by 10% HS (K), and 5 and 10 ng/mL TGF- β 1 (D, E), respectively. Percent positive for those groups (D, E, K, N) were similar. HS also appeared to have a dose-dependent

effect, with increasing percentages of HS resulting in an increasing number of positive cells per 100 cells, score per cell and cluster number per 100 cells. Semi-quantitative analysis of staining for collagen type I indicated that conditions were similar, with all conditions on average having less than or equal to 33 positive cells per 100 cells except for 10% ABS (M) (Figure 2.8A, $p < 0.05$, M vs. D, E, F, I; $p < 0.005$, M vs. H, J, K, L, N; $p < 0.001$, M vs. B, C).

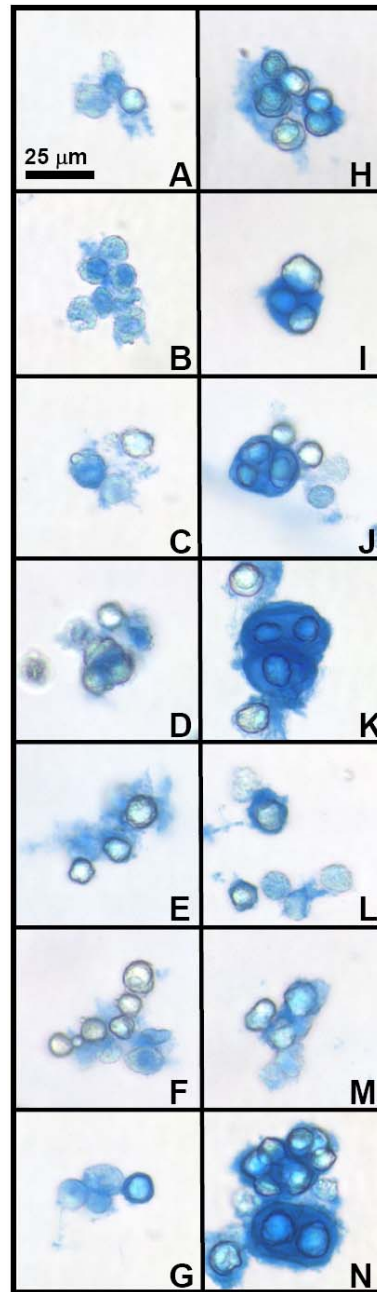


Figure 2.4: Effect of media condition on proteoglycan content of the cell-associated matrix. Typical alcian blue staining patterns for groups of cells are shown in micrographs, A-N. Letters correspond to media conditions shown in Figure 2.3.

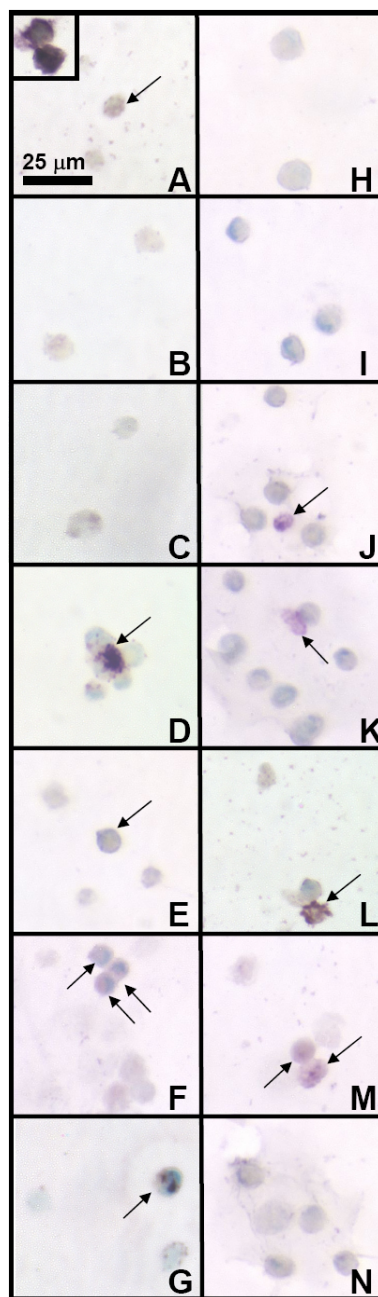


Figure 2.5: Effect of media condition on collagen type I content of the cell-associated matrix. Typical immunohistochemical staining patterns for groups of cells are shown in micrographs, A-N. Letters correspond to media conditions shown in Figure 2.3. Inset shows example of positive staining for collagen.

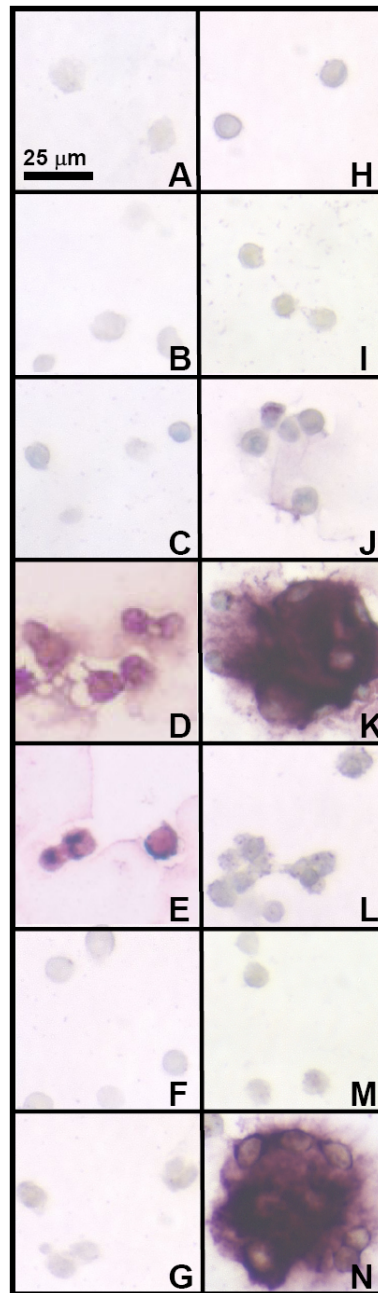


Figure 2.6: Effect of media condition on collagen type II content of the cell-associated matrix. Typical immunohistochemical staining patterns for groups of cells are shown in micrographs, A-N. Letters correspond to media conditions shown in Figure 2.3.

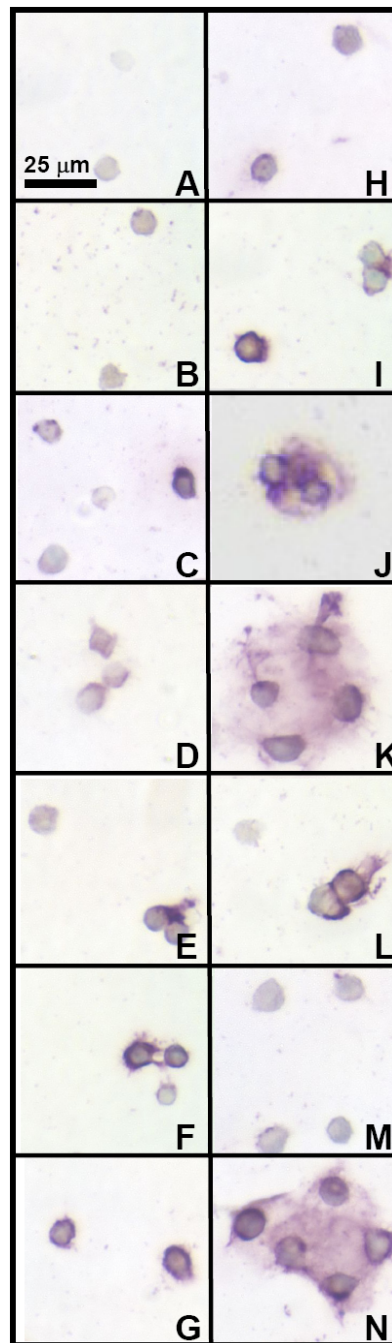


Figure 2.7: Effect of media condition on collagen type VI content of the cell-associated matrix. Typical immunohistochemical staining patterns for groups of cells are shown in micrographs, A-N. Letters correspond to media conditions shown in Figure 2.3.

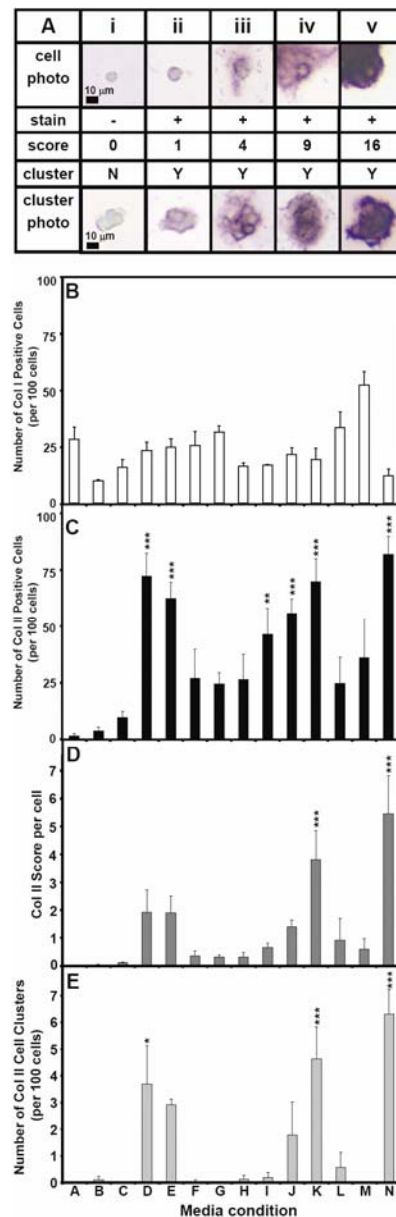


Figure 2.8: Effect of media conditions on grades of collagen types I and II expression. A) Example of semi-quantitative immunohistochemistry (IHC) grading of cells using a scale of 0-16. B) Percent positive collagen type I cells. C) Percent positive collagen type II cells. D) Score per cell. E) Number of clusters per 100 cells. $n = 4$ patients, $\pm$ standard error of the mean (SEM). Significant differences from condition A are indicated: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

2.5 Discussion

The results of this study demonstrate that OA cartilage is a source of chondrocytes that can both proliferate and redifferentiate at amplification levels necessary for engineering large cartilaginous constructs. The expansion of chondrocytes from OA cartilage was accelerated by TFP (Figures 2.1, 2.2; Table 2.1), with an increase of growth rate of 5.5-fold resulting in a 100-fold expansion within 17 days for serum-containing conditions. Expanded chondrocytes could be redifferentiated in alginate when incubated in certain types of medium (Figure 2.3). Histology and IHC indicated that the produced matrix, including proteoglycan and collagen type II, was localized to the pericellular environment and consistent with biochemical data (Figures 2.4-2.8). Conditions with high percentages of cells depositing collagen type II (Figure 2.8C), score per cell (Figure 2.8D) and collagen type II expressing cells in clusters (Figure 2.8E), generally had low percentages of cells expressing type I collagen (Figure 2.8B), consistent with a chondrogenic phenotype. Additionally, supplementation of medium with HS, but not a variety of other components including FBS, induced the formation of clusters of cells with large amounts of matrix, resembling chondrons (Figures 2.4J, K, N; 2.6K, N). Like chondrons present in native cartilage, the clusters exhibited a characteristic pericellular staining pattern for collagen type VI (Figures 2.7J, K, N). Collectively, these findings indicate that OA cartilage has the potential to be utilized as a cell source for creating large scale cartilage constructs.

A limitation to this study is that only chondrocytes from full thickness OA cartilage were analyzed. Such cartilage contains a mix of cells including clones and those from superficial, middle and deep zones. The presence of clones within the cell population could have affected the results. Clones, a feature of OA pathogenesis,

appear as clustered cells in lacunae under fibrillated surfaces of cartilage [2, 25, 26, 36, 41], and may result as part of a cellular attempt at matrix repair, although they can be present with or without matrix [2, 25, 26, 36]. The cartilage used in this study was macroscopically intact and contains a lower number of clones than fibrillated cartilage [26, 36]. Minimal preparation was needed to use cells from the full thickness of cartilage, thereby facilitating a high yield of isolated cells. Differences in cartilage zonal phenotype, which include cell morphology and matrix synthesis, could influence proliferative and redifferentiative properties, and may be of interest to investigate in future studies.

Additionally, cells in this study were not compared directly to chondrocytes derived from normal cartilage. During culture, OA and normal articular chondrocytes exhibit differences in gene expression [1, 45, 49] and OA chondrocytes synthesize less cartilage-specific matrix components under certain culture conditions [10, 45]. However, after prolonged incubation and expansion, gene expression profiles of cells derived from OA and from normal cartilage indicated little difference, suggesting that culture conditions rather than disease state may determine the biosynthetic profile of such chondrocytes [30]. Additionally, in the present studies on redifferentiation, the OA chondrocytes were expanded with TFP, which has been demonstrated to encourage redifferentiation [4]. Studies that have investigated the matrix synthesis of OA chondrocytes describe proteoglycan synthesis rates that are similar to those of normal chondrocytes [31, 45, 49], but decreased collagen synthesis rates [45]. However, as shown in this study, collagen synthesis may be markedly stimulated by certain supplements, such as 20% HS.

The present study extends prior analysis of proliferation of normal articular chondrocytes by defining the responses of chondrocytes from OA cartilage to a

variety of putative regulators. With normal human chondrocytes from the knee joint, the addition of TFP increased the growth rate 3.7-fold (for donors 40 and older) [4], whereas in the present study, TFP increased the growth rate 5.5-fold or higher than that of corresponding control medium conditions for chondrocytes from OA cartilage. With added TFP, serum, regardless of percentage or type, caused a similar increase in chondrocyte growth rate and total fold expansion. Minimal variation (8%) between patients for total fold expansion with the use of serum and TFP suggests that these supplements likely accelerate cell-expansion for all patients. The ability for cells to proliferate up to 100-fold in one passage also has the advantage of preservation of their chondrogenic potential, which appears to be decreased further with passaging [43, 46]. A low seeding density (5,000 cells/cm²), which has been studied for expansion of normal articular [13] as well as nasal septal [19] and auricular [32] chondrocytes, was utilized to allow higher fold expansion in one passage.

The effects of HS on redifferentiation, which have not been examined with articular chondrocytes, were demonstrated in this study. Redifferentiation with HS has been previously studied in a dose-dependent manner using human nasal septal chondrocytes [3], but, with normal or OA articular chondrocytes, only human serum albumin has been used as a supplement during redifferentiation culture [4, 45, 49]. In this study, different percentages of HS (2, 5, 10, 20) had similar effects on the sGAG content on a per cell basis, although alcian blue indicated variation in matrix localization, where some cells stained positively and others did not. Further analysis could be done on the alcian blue pattern of cell staining to clarify heterogeneity of cell responses, for example, that due to different cell types found within articular cartilage [24, 42]. For collagen type II on a per cell basis, only higher concentrations (10 and 20%) of HS resulted in measurable amounts (along with serum-free conditions of 5 or

10 ng/mL TGF- β 1), in contrast to human nasal septal chondrocytes, where low concentrations (2%) of HS have been adequate to stimulate production of collagen type II [3]. There was also a distinct and dose-dependent effect of HS on the formation of chondrons, which may also be important to the propensity of these cells to create functional tissue-engineered cartilage. Compared to chondrocytes, chondrons in pellet culture have an increased propensity to accumulate and produce cartilaginous matrix [16, 29]. Additionally, the pericellular matrix that is characteristic of chondrons has been postulated to have protective and signaling functions for chondrocytes [14, 16, 29, 39], which may also be crucial for the ability of chondrocytes from OA cartilage to form cartilaginous tissue.

While the results suggest the potential utility of cartilage from OA joints, an OA cell source for tissue engineering could be used in either an autogenic or allogeneic manner. Current clinical treatments for large cartilage defects include both autologous and allogeneic cell and tissue sources. OA cartilage from total joint surgeries would be an abundant source of cartilage, with more than a half million surgeries performed each year in the US [18]. Cartilage is considered to be immunoprivileged, because the chondrocytes are protected from an immune response by their surrounding matrix [28, 37, 48]; however, implantation of allogeneic chondrocytes without matrix can cause immune reactions [37]. Autologous HS could be used during both expansion and redifferentiation phases to eliminate the possibility of adverse immunological reaction or transmission of infectious diseases through HS. Some patient variability was noted for the redifferentiation portion of this study and will need to be assessed for specific treatment applications. If a particular patient's cells were to outperform other cell sources, those cells could potentially be used to create a bank of cells for creating a supply of tissue-engineered constructs. Again,

because the immune response should not be a critical issue with chondrocytes that have surrounding matrix, this would be a feasible therapy.

These cells could also potentially be used in a therapy analogous to ACI, except that the source would be osteoarthritic cartilage. Characterized chondrocyte implantation includes an additional step before implantation, where expanded chondrocytes are screened for gene expression levels of markers that indicate the capacity to form hyaline-like cartilage [42]. Similarly these chondrocytes could be screened to select for chondrocytes that may have higher capacity for cartilage formation. Another potential application would be to utilize these cells with the additional redifferentiation step, which is not included in the ACI procedure. Cells with matrix, or chondrons, would possibly have greater capacity for regenerating cartilage within the defect to be repaired [16, 29], and may also facilitate allogeneic applications since the immune response should be minimized [28, 37, 48]. Overall, OA cartilage may serve as a cell source for joint-scale engineering of cartilage tissue. Cells could be used for several different types of therapies in an autologous or allogeneic manner such as chondrocyte implantation with or without a scaffold or a cell-associated matrix, or construct formation (Figure 2.9). Ongoing studies are assessing whether cells from OA cartilage expanded and redifferentiated under select conditions presented in this study can generate cartilaginous tissue *in vitro*, a finding that would support the use of these cells as a cell source for tissue engineering and potential clinical applications.

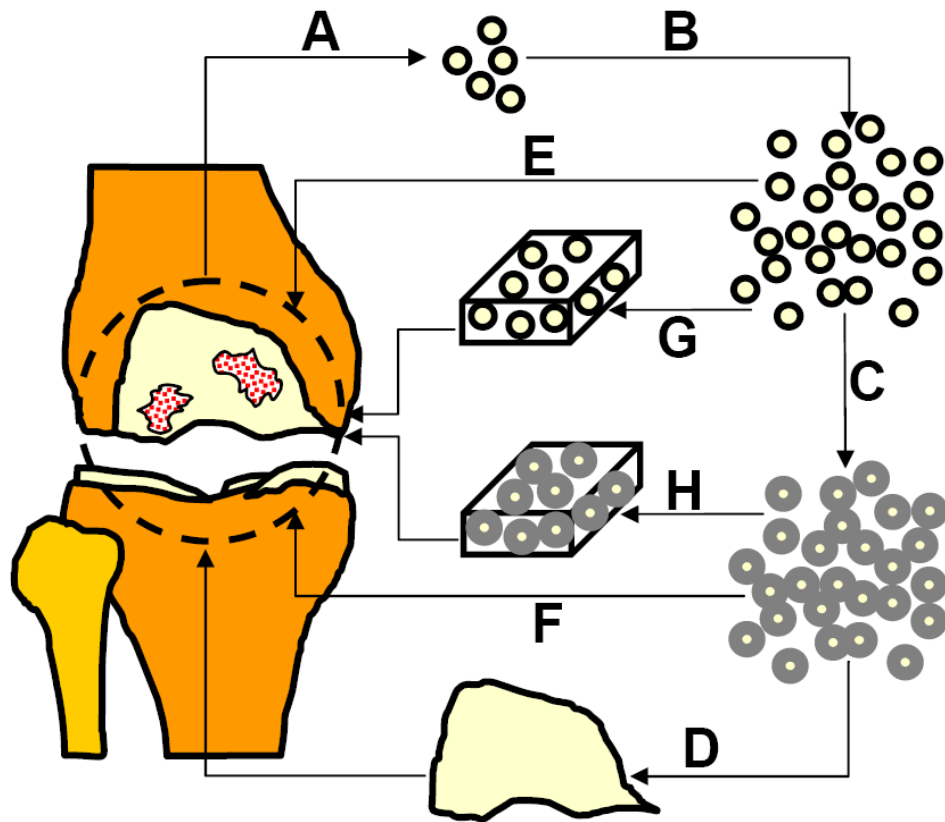


Figure 2.9: Potential therapeutic applications of culture-expanded and redifferentiated chondrocytes from osteoarthritic cartilage. From an osteoarthritic knee, cells are isolated (A), expanded (B), and then redifferentiated (C). The redifferentiated cells could be used to create a construct (D) for resurfacing the knee joint. The expanded or redifferentiated cells could be injected (E, F) or implanted in a scaffold (G, H) for focal defect repair.

2.6 Acknowledgements

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CHAPTER 3

HUMAN CARTILAGE TISSUE ENGINEERING USING CHONDROCYTES FROM OSTEOARTHRITIC CARTILAGE

3.1 Abstract

Objective: To determine the effects of GDF-5, TGF- β 1, and IGF-I, individually and in combination, on formation of cartilaginous constructs using human chondrocytes derived from osteoarthritic cartilage.

Methods: Chondrocytes were isolated from cartilage removed during total knee arthroplasty for osteoarthritis. Cells were expanded ~20X over 2 weeks in medium with 2% human serum (HS), TGF- β 1, FGF-2, and PDGF-bb, and then redifferentiated over 2 weeks in alginate beads in medium with 20% HS. Cells with associated matrix were then released from alginate and seeded into 3.2 mm and 6.5 mm diameter cell culture inserts and incubated for 2 weeks to form constructs. Medium was serum-free or with 10% HS, and $\pm$ GDF-5, $\pm$ IGF-I, $\pm$ TGF- β 1, individually and in combination. Constructs were evaluated by appearance, wet weight (WW), cell number, sulfated glycosaminoglycan (sGAG) and collagen content. Large constructs were tested mechanically to determine compressive modulus and hydraulic permeability.

Results: Construct formation was enhanced by inclusion of TGF- β 1 and IGF-I in culture medium relative to basal conditions in terms of WW (+38%), cells (+53%), sGAG (+147%) and collagen (+139%) per construct. Inclusion of 10% HS in culture medium led to higher densities of certain components, cells/WW (+83%) and collagen/WW (+62%), lower construct WW (−42%) and sGAG (−32%), and better load-carrying mechanical properties, with increased compressive modulus (+287%) and decreased hydraulic permeability (−80%).

Conclusion: During the stage of cartilaginous tissue formation from culture-expanded chondrocytes that originate from osteoarthritic knees, inclusion of IGF-I and TGF- β 1 stimulated deposition of cartilaginous matrix.

3.2 Introduction

Articular cartilage is an avascular, load-bearing tissue found at the ends of long bones [10, 15, 55] that enables movement by providing a low-friction low-wear surface to facilitate articulation [17, 55] while sustaining compressive and shear forces in synovial joints [10, 55]. Since adult articular cartilage has a limited capacity for spontaneous self-repair [13, 34], defects do not heal and, without treatment, may expand and progress to osteoarthritis [34, 36, 66]. Tissue engineering and regenerative medicine therapies currently available for patients are limited to those with focal cartilage defects. These therapies include induction of host cell repair (e.g., microfracture) and use of cells derived from healthy cartilage, either by injection (e.g., autologous chondrocyte implantation, ACI) or transplantation on a carrier scaffold (e.g., matrix-induced autologous chondrocyte implantation, MACI) [9, 16, 34, 36].

Despite some clinical success, these therapies do not address joints with advanced and extensive degeneration typically present in osteoarthritis (OA).

Human articular chondrocytes from OA joints are a potential cell source for cartilage tissue engineering [35, 61, 72]. In a joint afflicted by OA, the amount of undamaged cartilage available for tissue engineering is more limited, and the typical yield of chondrocytes is relatively low, ~2 million per gram cartilage tissue [7, 12, 26] and decreases with increasing age [7, 12]. However, such tissue is from the patients who need treatment and in abundant supply with >500,000 total joint replacement surgeries performed annually in the US [32]. Cartilaginous tissue constructs formed from such cells could be utilized in an autogenic or allogeneic manner. In an autologous application, cell expansion would be required for sufficient cell numbers to create replacement tissue similar to native cartilage.

With increasing expansion in monolayer culture, the propensity for chondrocyte dedifferentiation increases and the propensity for redifferentiation decreases [11]. However, during monolayer expansion, the inclusion of a combination of growth factors (TFP) consisting of transforming growth factor beta 1 (TGF- β 1), fibroblast growth factor 2 (FGF-2), and platelet derived growth factor type bb (PDGF-bb) accelerates chondrocyte expansion and also promotes redifferentiation, thus facilitating production of chondrogenic cells [7, 26]. Redifferentiation of monolayer-expanded cells can also be facilitated by encapsulation in alginate [21, 31, 49], which also allows recovery of cells with their associated matrix for subsequent use in forming scaffold-free cartilaginous constructs [52]. During culture of such human chondrocytes in alginate, inclusion of human serum (HS) in the medium promotes cartilaginous matrix production by culture-expanded human articular and septal chondrocytes [2, 35]. Whether OA-derived chondrocytes expanded and

redifferentiated under these conditions could be used to form cartilaginous tissue remains to be established and is the subject of the present study.

During cartilaginous tissue formation, the properties of the tissue formed are affected by media supplements including growth factors and serum [28]. One approach to *in vitro* tissue engineering is to recapitulate the *in vivo* microenvironment, such as by including the growth factors that are important for cartilage development and growth. Previous studies of human chondrocytes and stem cells have identified a number of chondrogenic factors including growth and differentiation factor 5 (GDF-5) [3, 5, 14, 22], insulin-like growth factor I (IGF-I) [21, 37, 38, 64, 70, 71] and TGF- β 1 [7, 8, 33, 40, 51, 61, 70]. GDF-5 is essential for normal joint formation [4, 18, 30, 45] and can initiate cell condensation and chondrogenesis [18, 30]. IGF-I is required for normal skeletal growth as evidenced by knockout mice studies [6, 65]. TGF- β 1 is expressed throughout embryonic development in cartilage and bone tissues [57]. Both IGF-I and TGF- β 1 are present in subsequent steps of chondrogenesis and cartilage development [4, 30, 50]. As these growth factors have distinct receptors and signaling pathways [20, 23, 24, 48], the present study examined effects of each factor alone and in combination. Since HS promotes cartilaginous matrix production by human chondrocytes [2, 35], these growth factors were also examined in combination with HS.

The hypothesis of this study was that culture-expanded and redifferentiated cells derived from OA cartilage can form cartilaginous constructs under media conditions with the growth factors GDF-5, IGF-I and TGF- β 1 individually and in combination, with or without the addition of 10% HS. The effect of these media components on construct formation was assessed based on gross appearance, wet

weight, thickness, cell number, sulfated glycosaminoglycan (sGAG), collagen, and compressive properties.

3.3 Materials and Methods

Materials

Materials were obtained as previously described [35], with cell culture inserts being Anopore™ (3.2 mm diameter, 0.2 µm pore size, Nunc, Rochester, NY) and Transwell® (6.5 mm diameter, 0.4 µm pore size, Corning, Lowell, MA).

Chondrocyte Isolation, Proliferation and Redifferentiation

Chondrocytes were isolated from osteochondral fragments removed routinely from the joints of patients during total knee arthroplasty for OA. Consent was obtained from the patients (2 males and 3 females, 57±12 years, range 42-76 years) at Scripps Green Hospital (La Jolla, CA) per a protocol approved by the Institutional Review Boards at Scripps and UCSD. Cartilage that appeared macroscopically intact was removed from the osteochondral fragments, typically from the lateral femoral condyles and the lateral portion of trochlea. The tissue was incubated in medium (DMEM supplemented with 25 µg/mL ascorbate, 0.1 mM MEM non-essential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL Fungizone, and 10% FBS) for 7 days before cell isolation by sequential digestion with pronase and then collagenase [46].

Chondrocytes were expanded and redifferentiated according to previously described methods [35]. The isolated chondrocytes were seeded in monolayer at a density of 5,000 cells/cm² in 175 cm² tissue culture flasks and incubated in medium

supplemented with 2% HS and TFP (1 ng/mL TGF- β 1, 5 ng/mL FGF-2, 10 ng/mL PDGF-bb) for 14 days. After expansion (~20X) to confluence, cells were released from monolayer with pronase and collagenase, and encapsulated in alginate beads at a density of 4×10^6 cells/mL of alginate [52]. Beads were incubated in non-tissue culture-treated plates for 14 days with DMEM/F-12 supplemented with 20% HS. Media was changed every other day. Cells were released from alginate using sodium citrate [52], and centrifuged to separate cells with their cell-associated matrix (CM, pellet) from their further-removed matrix (FRM, supernatant). Under these conditions, chondrocytes and their CM have a cartilaginous phenotype characterized by expression and deposition of primarily type II rather than type I collagen, as well as pericellular type VI collagen.

Construct Formation and Analysis

The effect of culture conditions on formation of cartilaginous constructs was analyzed using 3.2 mm and 6.5 mm diameter constructs. For the former, CM (from 1 male and 3 females, 58 ± 14 years, range 42-76 years) were seeded at a density of 3×10^6 cells/cm² into 3.2 mm diameter cell culture inserts and incubated in replicate cultures for 14 days with the following media formulations: DMEM/F-12 media supplemented with (1) 0.01% BSA (basal), (2) 100 ng/mL GDF-5, (3) 200 ng/mL IGF-I, (4) 10 ng/mL TGF- β 1, (5) 100 ng/mL GDF-5, 10 ng/mL TGF- β 1, (6) 200 ng/mL IGF-I, 10 ng/mL TGF- β 1, (7) 100 ng/mL GDF-5, 200 ng/mL IGF-I, 10 ng/mL TGF- β 1 with the addition of 10^{-7} M dexamethasone and ITS+1 for non-serum conditions (1-7), and addition of 10% HS for serum conditions (8-14).

For larger constructs, CM (from 1 male and 2 females, 51 ± 9 years, range 42-59 years) were seeded into 6.5 mm diameter cell culture inserts and incubated in

triplicate for 14 days with the following media formulations, selected based on results with the 3.2 mm diameter cell culture inserts: (1) 10 ng/mL TGF- β 1, (2) 200 ng/mL IGF-I, 10 ng/mL TGF- β 1, (3) 100 ng/mL GDF-5, 200 ng/mL IGF-I, 10 ng/mL TGF- β 1 with the addition of 10^{-7} M dexamethasone and ITS+1 for non-serum conditions (1-3), and addition of 10% HS for serum conditions (4-6).

For both sizes of constructs, media was changed every day. At the end of the culture period, constructs were removed from cell culture inserts, photographed in *en face* and vertical orientations for gross appearance and measured for wet weight (WW).

Biochemical Analysis

Both 3.2 mm diameter and 6.5 mm diameter constructs were solubilized in 0.5 mg/mL proteinase K in phosphate buffered EDTA at 37°C for one hour and then overnight at 60°C. Portions of digested samples were analyzed for DNA content using PicoGreen[®] and human DNA standards [53]. Cell number was calculated using a conversion factor of 7.7 pg of DNA per cell [53]. Additional portions of the sample were analyzed for sGAG content using dimethylmethylene blue [25] and for total collagen content as hydroxyproline [69], assuming 7.1 g of collagen/g hydroxyproline [39].

Mechanical Testing

The 6.5 mm diameter constructs were tested by confined compression as described previously [19]. In brief, constructs were placed in radially confining chambers between two titanium platens (2 μ m porosity) all within a PBS bath. Chambers were attached to a mechanical spectrometer (Dynastat, IMASS, Accord,

MA), interfaced with computer-controlled instrumentation. Constructs were tested by applying 0-15% ramp compression over 400 seconds and allowed to relax to equilibrium, and then subjected to a sequence of oscillatory displacements from 1% to 0.3% of the construct thickness, at frequencies of 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 Hz, with the resultant loads measured during testing. This testing sequence was repeated at ramp compression levels of 30% and 45%. The confined compression modulus, H_{A0} , was determined by fitting the equilibrium stress-strain data to a non-linear model [47]. Hydraulic permeability, k_p , was determined at each compressive strain level by fitting measured dynamic stiffnesses to a theoretical solution using computed values of modulus [27].

Statistical Analysis

The data are expressed as the mean $\pm$ standard error of the mean (SEM), and were analyzed using Systat 10.2 (Systat Software, Point Richmond, CA). Effects of medium formulations on WW, thickness, and biochemical content were assessed by two-way ANOVA with growth factor and serum as independent factors; effects on biochemical content normalized to wet weight were also assessed. Effects on mechanical properties were assessed by ANOVA with serum as an independent factor. When significant effects ($p < 0.05$) were found, Tukey post-hoc testing was performed. Since effects of medium formulations on gross and biochemical parameters were generally similar for 3.2 and 6.5 mm diameter constructs, only the data and statistical results of these parameters for the smaller constructs are presented. Data from the larger constructs tested mechanically was analyzed by univariate and multiple regression to determine if there were correlations between mechanical properties (H_{A0} and k_p) and biochemical properties (DNA/WW, sGAG/WW and collagen/WW).

3.4 Results

Gross Appearance

Constructs appeared circular, conforming to the circumferential geometry of the cell culture inserts (Figure 3.1). Overall, constructs grown under serum-free conditions appeared to be slightly larger than their counterparts grown under serum-containing conditions. For the basal or GDF-5 containing serum-free conditions, constructs were not cohesive enough to be handled so no gross photographs are shown.

Wet Weight

Consistent with their appearance, constructs grown under serum-free conditions had a higher wet weight than those grown under analogous but serum-containing conditions (Figure 3.2, $p < 0.001$). Additionally, constructs grown in medium with TGF- β 1 and IGF-I in combination had a higher wet weight ($p < 0.05$) than all other conditions except the combination of GDF-5, IGF-I and TGF- β 1.

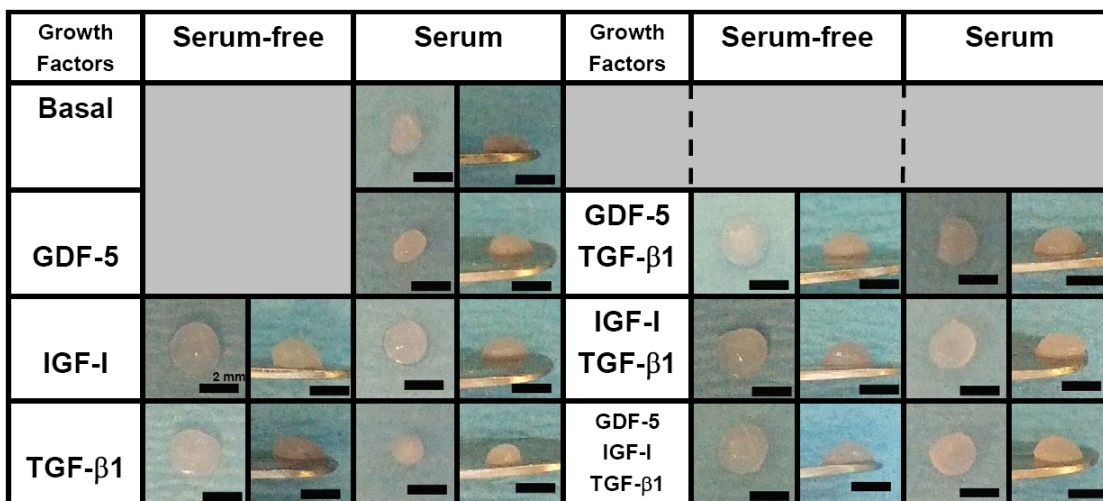


Figure 3.1: Gross appearance of constructs. 3.2 mm diameter constructs, *en face* and vertical appearance.

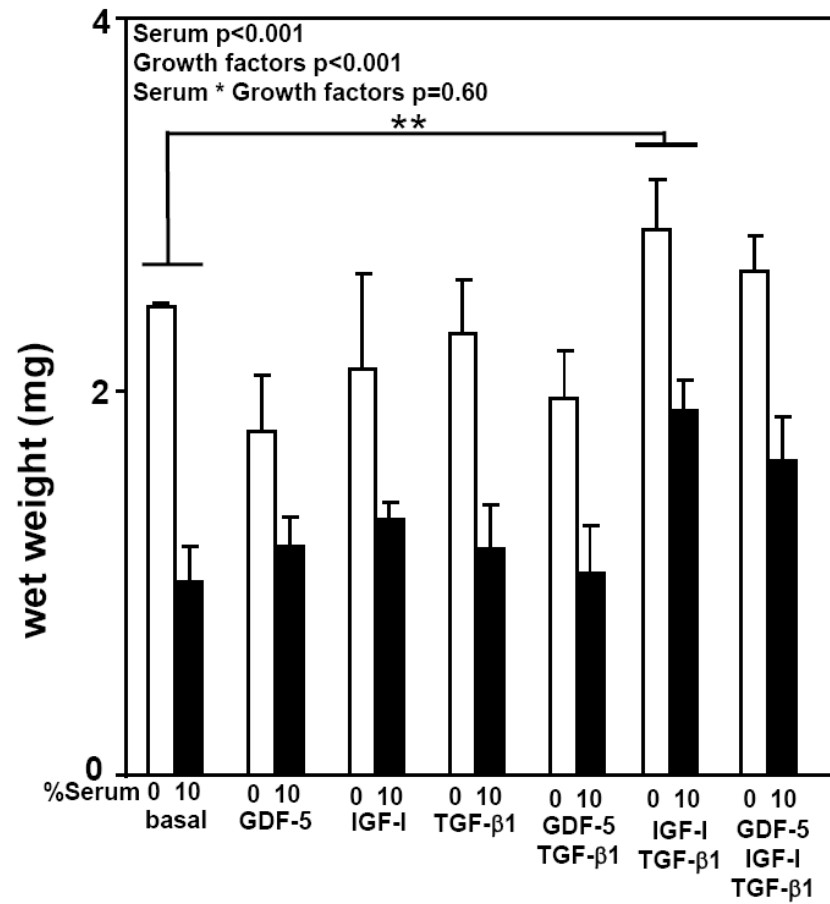


Figure 3.2: Wet weight of constructs. 3.2 mm diameter constructs. $n=8$ constructs per condition, 4 patients, mean $\pm$ SEM. Significant differences between growth factor conditions from basal condition are indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Cell Number

Cell number was generally similar between constructs, being slightly higher after incubation in medium containing added growth factors (Figure 3.3A, $p < 0.001$). On a wet weight basis, cell content was higher after culture with serum (Figure 3.3B, $p < 0.001$).

Biochemical Content

The sGAG content of constructs was affected by growth factors (Figure 3.4A, $p < 0.001$) and decreased by serum ($p < 0.001$) overall and for certain growth factor conditions (interaction $p < 0.001$). With the growth factors IGF-I and TGF- β 1 in combination, sGAG content was higher ($p < 0.05$) than all other growth factor conditions except the combination of GDF-5, IGF-I, TGF- β 1. With the inclusion of serum, the growth factor response was diminished for all conditions including TGF- β 1 ($p < 0.05$) in comparison to other growth factor conditions with serum. On a wet weight basis, sGAG content was similar between growth factor conditions with no significant effects except for the combination of TGF- β 1, IGF-I and GDF-5 versus the basal condition (Figure 3.4B, $p < 0.05$). There was no significant effect of serum ($p = 0.12$) on sGAG/WW.

Collagen content was affected by growth factors in a manner generally similar to sGAG content, whereas serum had somewhat different effects. Collagen content was affected by growth factors (Figure 3.5A, $p < 0.001$). Overall, collagen content was not affected by serum ($p = 0.20$), although there was an effect of serum under certain growth factor conditions (interaction $p < 0.05$). IGF-I and TGF- β 1 in combination resulted in deposition that was higher than all other growth factor conditions ($p < 0.05$) except for TGF- β 1 alone and the combination of GDF-5, IGF-I and TGF- β 1. When

serum was included, growth factor responses were similar, except for in the condition with TGF- β 1 and GDF-5, where inclusion of serum decreased growth factor response ($p=0.061$). Overall, collagen per wet weight was higher in serum-containing conditions (Figure 3.5B, $p<0.05$) and similar between growth factor conditions ($p=0.13$).

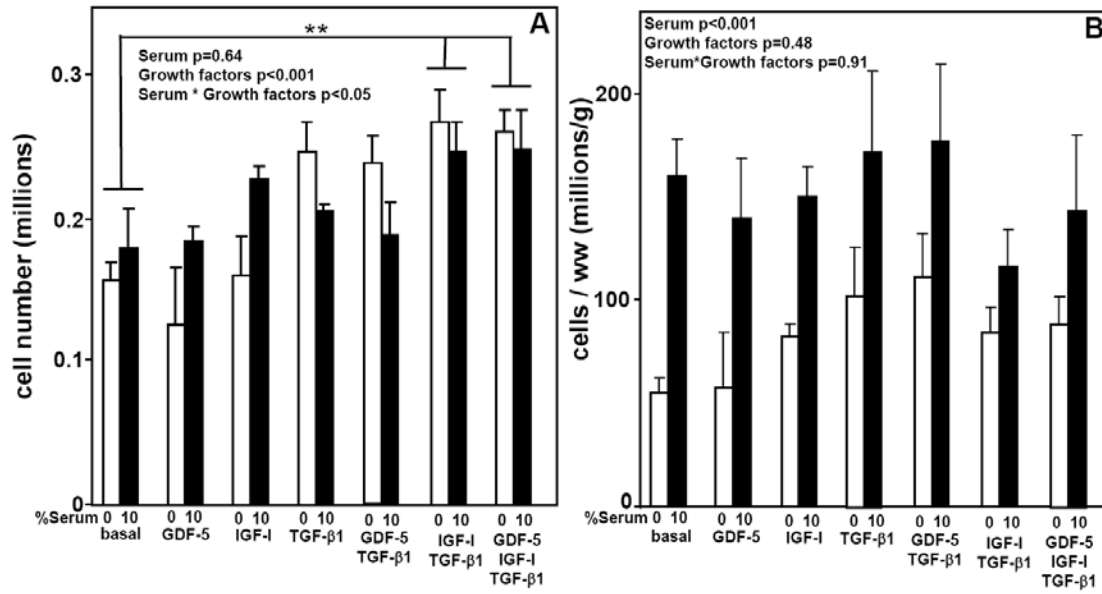


Figure 3.3: Cell content in constructs. 3.2 mm diameter constructs. A) Cell number. B) Cell number per wet weight. $n=8$ constructs per condition, 4 patients, mean $\pm$ SEM. Significant differences between growth factor conditions from basal condition are indicated: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

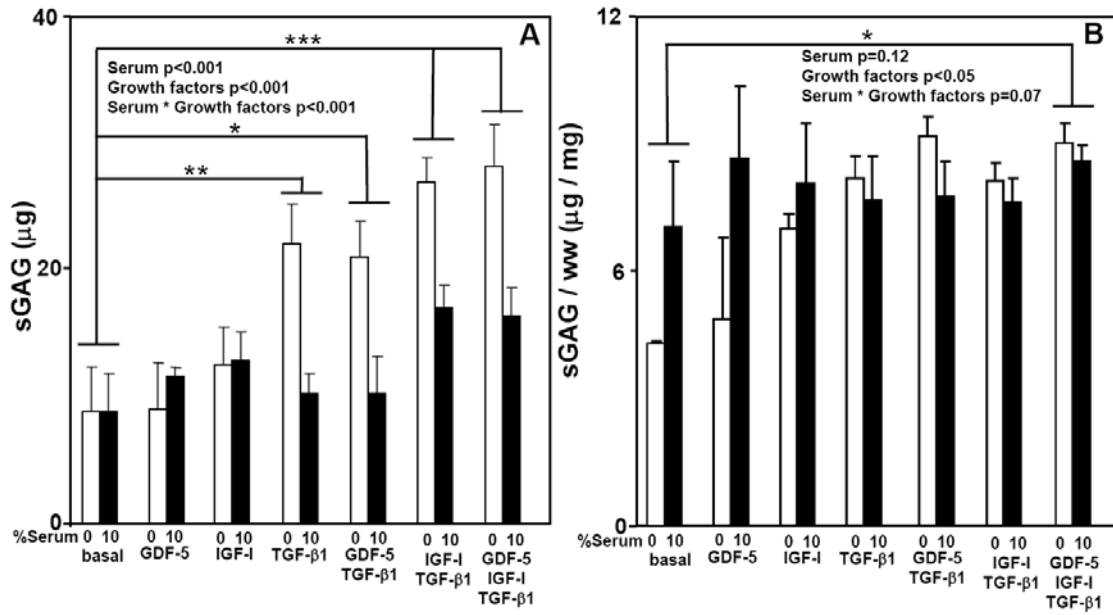


Figure 3.4: sGAG content in constructs. 3.2 mm diameter constructs. A) sGAG per construct. B) sGAG content per wet weight. n=8 constructs per condition, 4 patients, mean $\pm$ SEM. Significant differences between growth factor conditions from basal condition are indicated: * p<0.05, ** p<0.01, *** p<0.001.

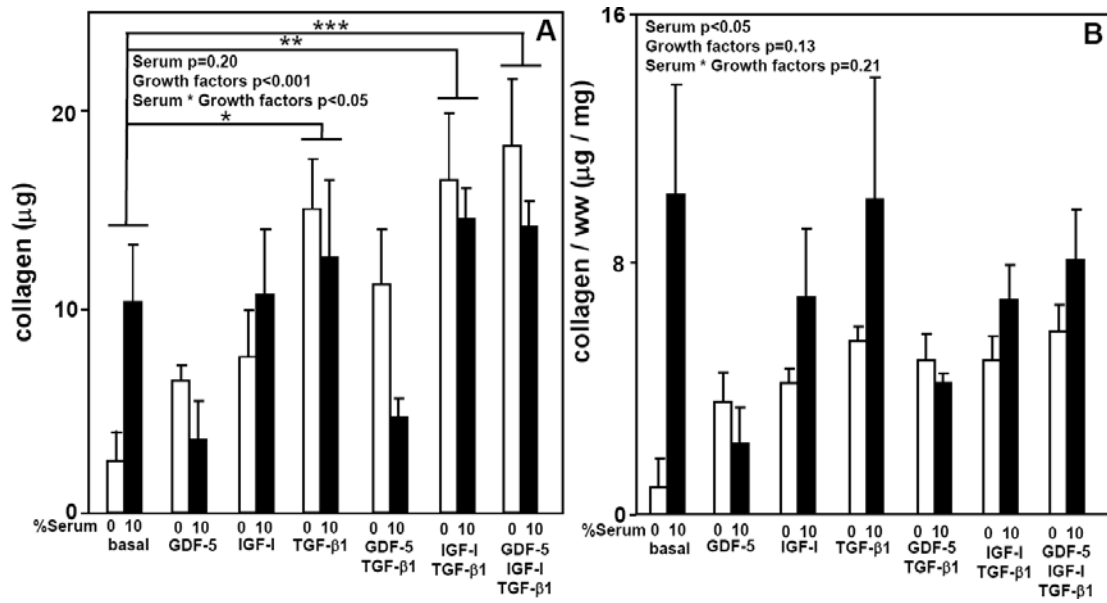


Figure 3.5: Collagen content in constructs. 3.2 mm diameter constructs. A) Collagen per construct. B) Collagen content per wet weight. n=8 constructs per condition, 4 patients, mean $\pm$ SEM. Significant differences between growth factor conditions from basal condition are indicated: * p<0.05, ** p<0.01, *** p<0.001.

Mechanical Properties

Mechanical properties tended to vary with culture conditions in a manner correlated with biochemical content. While there were no statistically significant effect of serum supplementation on the compressive modulus, H_{A0} , there was a trend for increased H_{A0} with serum (+287%, $p=0.16$, Figure 3.6A). Also, there was a trend toward permeability, k_p , being decreased with serum (−80%, $p=0.21$, Figure 3.6B).

Mechanical properties were related to certain biochemical properties. By univariate regression analysis, H_{A0} correlated positively with cell number, sGAG and collagen on a wet weight basis (Figure 3.7A-C, $r^2 = 0.34, 0.45, 0.61$ respectively). The log of k_p correlated negatively in a weak manner with cell number per wet weight (Figure 3.7D, $r^2 = 0.16$) and in a stronger manner with sGAG and collagen per wet weight (Figure 3.7E, F, $r^2 = 0.21$ and 0.34 , respectively). Multiple regression analysis yielded similar relationships, without detecting any combined effects of individual tissue components.

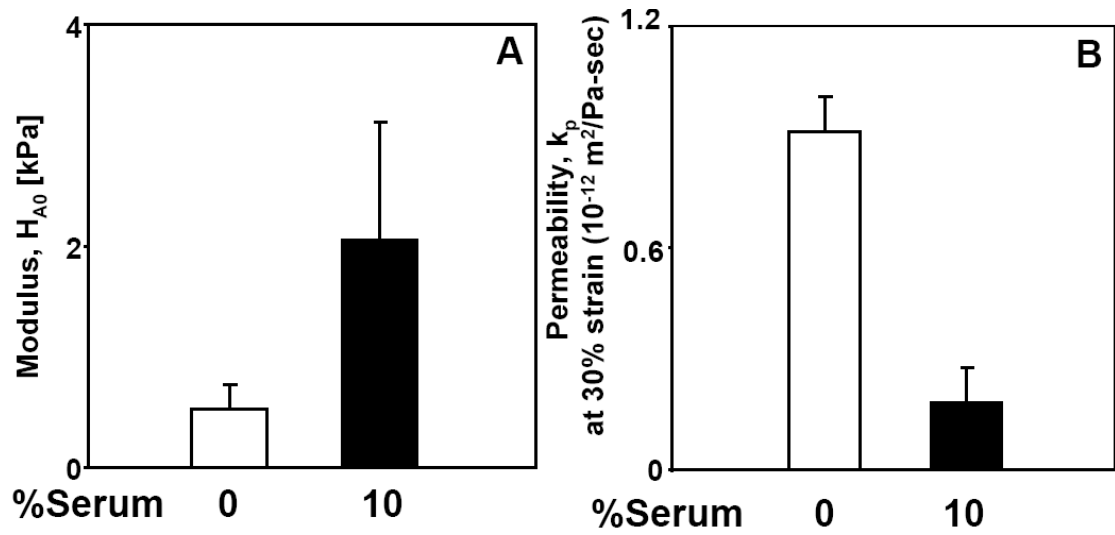


Figure 3.6: Mechanical properties. 6.5 mm diameter constructs. A) Compressive modulus H_{A0} (kPa). B) Hydraulic permeability k_p (m²/Pa-sec) at 30% strain. n=5-6 constructs per condition, 3 patients, mean $\pm$ SEM.

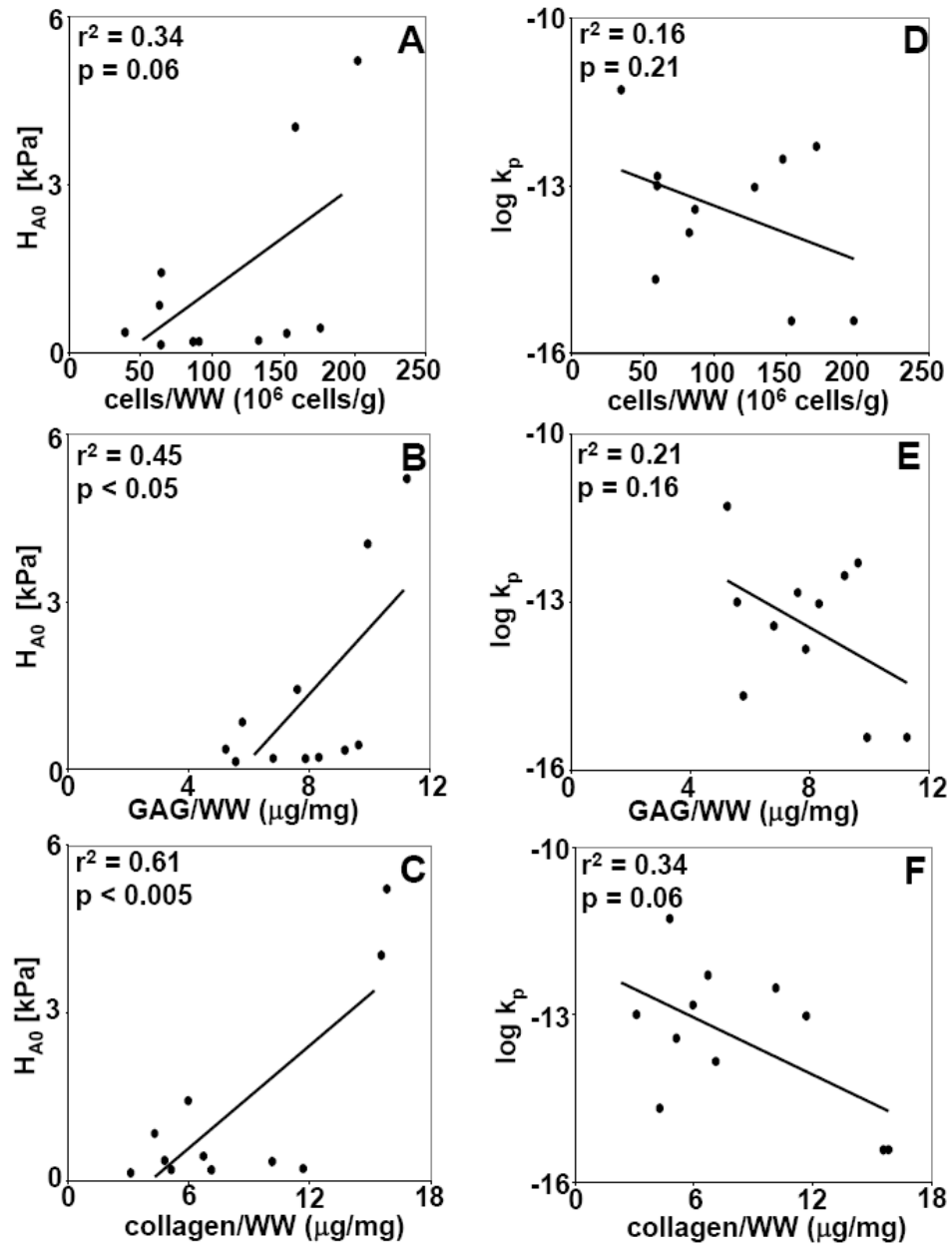


Figure 3.7: Compressive modulus H_{A0} correlated with biochemical properties. 6.5 mm diameter constructs. A) cells/WW. B) sGAG/WW. C) collagen/WW. Log of hydraulic permeability k_p at 30% strain correlated with biochemical properties. D) cells/WW. E) sGAG/WW. F) collagen/WW. $n=11$ constructs, 3 patients.

3.5 Discussion

The results of this study demonstrate that cartilaginous constructs can be formed from cells derived from OA cartilage, with biochemical and mechanical properties modulated by growth factors and HS. In general, the addition of growth factors, and in particular the combination of IGF-I and TGF- β 1, stimulated deposition of cartilaginous matrix in comparison to other conditions, including each growth factor individually. The HS component also stimulated deposition of collagen, when normalized to wet weight. Mechanical properties also tended to be influenced by the addition of HS, with constructs with increased load-carrying ability being formed under conditions including serum; compressive modulus correlated positively and hydraulic permeability correlated negatively with cells, sGAG and collagen on a wet weight basis. Thus, medium including IGF-I and TGF- β 1 together, and also with HS, were able to stimulate culture-expanded and redifferentiated human chondrocytes from OA knee cartilage to yield cartilaginous constructs which have biochemical components typical of normal cartilage and, although not nearly as functionally load-bearing as normal tissue, were robust enough to be handled. Taken together, these results indicate that OA cartilage may be a feasible cell source for tissue engineering of human articular cartilage.

One limitation to this study is that the constructs were formed from full thickness OA chondrocytes, and may have contained a heterogeneous population of cells. The cells were isolated from cartilage that was macroscopically intact, but still likely contained cells in brood clones, although less than the number of such clones in extensively fibrillated cartilage [44, 54]. Such clones are characteristic of OA cartilage, and may contain cells that complicate tissue formation; although such cells are a product of proliferation, they do not appear to be effective in repairing damaged

cartilage [1, 43, 44, 54, 59]. For clinical application, in a manner similar to “characterized chondrocyte implantation”, the population of cells could be screened to select those chondrocytes, based on the expression levels of certain markers, with the ability to form hyaline-like cartilage [60]. In addition, the cells were heterogeneous in terms of zonal phenotype since they were isolated from the full thickness of the cartilage. Articular cartilage has a distinct zonal architecture with phenotypic differences between cells from each zone. Recapitulating zonal tissue architecture using cells from different regions has been suggested as a way of creating a more physiological cartilaginous tissue [41, 42]. In the present study, the usage of cells from the full thickness of the OA cartilage was chosen to enable straightforward methods of cell isolation and construct formation.

In addition, the constructs formed from chondrocytes derived from OA cartilage were not compared directly to constructs formed from chondrocytes derived from normal cartilage in this study. It is difficult to obtain sufficient age-matched normal tissue for such studies. Those studies that have directly compared cartilaginous tissue formed from cells derived from OA versus normal joints [61, 72] have found that cells from OA cartilage are able to form tissue with aggrecan and type II collagen matrix; although of somewhat lower cartilaginous quality than cells from normal cartilage. Other studies focused on cells from OA joints, and their results support the ability of such cells to form tissue and produce cartilage-specific proteins.[29, 67] Such comparative studies, focused on distinct cell populations and phenotypes and with careful matching of donor age and harvest location, may be useful to further delineate similarities and differences between normal and OA cartilage.

This study builds upon previous ones that have examined the effects of TGF- β 1 [8, 33, 40, 56, 61], IGF-I [37, 38, 64, 71], and GDF-5 [3, 5] on human cartilaginous

tissue formation, not only in terms of effects on OA-derived chondrocytes but also for effects on construct formation of combinations of growth factors. The results indicated that the combination of TGF- β 1 and IGF-I stimulates an increase in wet weight, sGAG, collagen content over all other growth factor conditions, including each growth factor individually. These two growth factors have been shown to have synergistic and additive effects over the growth factors individually in promoting a chondrogenic gene expression profile in bone marrow stromal cells [50] and human articular chondrocytes [70]. The results of this study also showed that when GDF-5 was added to the TGF- β 1 and IGF-I combination, there was no significant additional effects. This could be due to GDF-5's role in regulating the differentiation of progenitor cells into the chondrocyte lineage [4, 30, 45], with GDF-5 not having a marked effect on a cell population derived from mature articular chondrocytes.

HS and its effects on construct formation are also evident in the present study, providing new information on the potential benefits of using HS in culture of human articular chondrocytes. Overall, there was a more marked effect on matrix content when growth factors were used under non-serum conditions. When HS was added, the effect of the growth factors appeared to be moderated, especially in the case of sGAG content, where levels of sGAG were similar between conditions containing serum. This could be due to the presence of TGF- β 1 in HS [63], which with 10% HS added to media, may be on the order of the concentration required for stimulation of matrix production. HS also contains other growth factors such as PDGF-ab, EGF and VEGF [62, 63], which may also have additional effects on the cells. HS also had the effect of increasing compressive modulus along with collagen per wet weight. The finding in the present study that compressive modulus is positively correlated with collagen content per wet weight is consistent with previous studies, identifying such a

relationship for immature and young adult articular cartilage [58, 68]. The effect of HS promoting collagen content without additional effects on sGAG deposition may be useful for modulation of tissue properties, as the proteoglycan to collagen ratio is usually higher in tissue engineered cartilage than that found in normal cartilage. Usage of HS could also be implemented for clinical applications, with an autologous source eliminating risk of immunological reaction or transmitted diseases.

The present study was able to utilize chondrocytes from OA cartilage to create constructs with media formulations, containing GDF-5, IGF-I, and TGF- β 1 along with HS, that may be useful for future clinical therapies. The results indicate that the properties of the constructs could be modulated by the growth factor and serum combinations they were grown under. Overall, this study demonstrates the feasibility of utilizing cells from OA cartilage after expansion and redifferentiation to create tissue engineered cartilaginous constructs, and provides initial evidence that these chondrocytes are a cell source to be considered for cartilage tissue engineering for clinical applications.

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CHAPTER 4

SELECTIVE ENHANCEMENT OF MATRIX RETENTION IN TISSUE ENGINEERED CARTILAGE WITH A SEMI-PERMEABLE MEMBRANE

4.1 Abstract

Objective: Perfusion stimulates anabolic responses in cartilaginous constructs, including increased matrix production. However, in early perfusion culture, newly synthesized matrix molecules may be lost due to convection of the molecules out from the developing porous tissue. A semi-permeable membrane may be implemented in bioreactors for retention of matrix molecules. The objectives of this study were to: (1) determine the effects of membranes of different pore sizes on the retention of matrix molecules in cartilaginous constructs formed using freshly isolated chondrocytes (FIC) or alginate recovered chondrocytes (ARC) in a perfusion bioreactor system, and (2) use a compartmental model to describe these effects.

Methods: FIC or ARC were seeded into bioreactors on top of membranes with pore size 0.05, 0.4, and 1 μm with porosities of 1.2, 12.7 and 15.7% respectively, and cultured for 7 days. Cultures were static during the initial 3 days, after which

perfusion was initiated at a flow rate of 0.25 $\mu\text{m}/\text{sec}$. Resulting constructs and medium upstream and downstream of the membrane were assayed for sulfated glycosaminoglycan (sGAG), collagen, and DNA contents. Relative sizes and aggregation ability of proteoglycan monomers within ARC constructs were determined using size exclusion chromatography. A compartmental model was formulated, with the compartments being: (1) bulk fluid, or media upstream of the membrane (2) cartilaginous construct atop the membrane (3) the permeate, or media downstream of the membrane. Model simulations were performed with parameter values that were estimated based on experiments and literature.

Results: For ARC constructs, decreasing pore size enhanced sGAG retention as evidenced by increased content within the construct (67 to 172 μg) and bulk fluid (38 to 56 μg), and decreased content in the permeate (169 to 37 μg). Collagen retention in the ARC constructs increased as well (20 to 31 μg) with decreasing pore size. Correspondingly, construct thickness also increased (0.33 to 0.62 mm). Cell retention in ARC constructs was similarly high for all membranes. In FIC constructs, pore size did not have an effect on sGAG retention, with content within the construct, bulk fluid and permeate similar between different membranes. Collagen content and thickness for FIC constructs also did not vary with pore size. The results were also affected by the porosity of the membranes, with the low porosity of the smallest pore size membrane adding to the retention effects.

Size analysis of proteoglycan monomers in ARC constructs indicated that the molecules retained in the membranes were smallest in constructs grown on the membrane with the smallest pore size and porosity ($p < 0.05$, $K_{av} \sim 0.268, 0.226, 0.258$ for 0.05, 0.4 and 1.0 μm pore sizes respectively, where larger K_{av} indicates smaller molecular size). Sizes of monomers were similar in bulk fluid and permeate and were

smaller than those found in the constructs, but did not vary much between different membranes. Results of aggregation ability studies showed that in constructs formed on the smallest pore size membranes, there was a lower percentage of aggregates (60, 89, 80% for 0.05, 0.4 and 1.0 μm pore sizes respectively), indicating higher retention of the smaller non-intact proteoglycan monomers, which was consistent with the monomer size analysis results.

The compartmental model described effects of the membrane, with decreasing pore size corresponding to increased proteoglycan content within the construct and bulk fluid compartments, and decreased content within the permeate compartment.

Conclusion: A semi-permeable membrane implemented in a perfusion bioreactor system can enhance matrix retention in cartilaginous constructs formed from alginate recovered chondrocytes.

4.2 Introduction

The growth and maturation of articular cartilage is dependent on biochemical and mechanical factors present in its environment. One approach to engineering functional cartilaginous constructs is to recreate the conditions that facilitate native cartilage development. Bioreactors provide a means to recapitulate elements of the *in vivo* environment in an *in vitro* setting to modulate the development of cartilaginous construct [13, 24, 42, 53]. Bioreactors have been designed to provide different types of mechanical stimulation for tissue engineering including compression, shear, hydrostatic pressure, and fluid flow [6, 13, 24]. Additionally, bioreactors provide a controlled biochemical environment of nutrients, gases, pH and waste removal for construct development [6, 24, 35, 57].

Cartilaginous constructs may be engineered to develop biochemical properties that approach and resemble those found in native articular cartilage. The two main matrix molecules present in cartilage are proteoglycan and collagen, which contribute to the load-bearing properties of the tissue. Proteoglycan molecules consist of sulfated glycosaminoglycans attached to a core protein. The negative charge of the glycosaminoglycans creates an osmotic swelling pressure that is constrained by the collagen network and that contributes to the compressive stiffness of the tissue [33, 40]. Aggrecan is the main type of proteoglycan present in cartilage, making up 90% of the total proteoglycan mass [1]. Aggrecan mostly exists in the form of aggregates in which the proteoglycan monomers are non-covalently bound to hyaluronan via link protein [33]. These monomers remain bound within the aggregates until they are degraded; after degradation these monomers lose their ability to bind to hyaluronan and are also smaller in size than intact monomers [50]. Collagen, which is primarily present as type II collagen in native cartilage, forms covalent crosslinks which provide the tensile strength to the tissue [32, 33, 40].

Direct perfusion bioreactors can stimulate anabolic responses in cartilaginous constructs. In these bioreactors, fluid flow may act as a mechanical stimulus or enhance convective mass transfer of nutrients and other soluble factors to the cells in a developing construct [21, 43, 45, 61]. Perfusion in bioreactors can increase cell proliferation and matrix production in cartilaginous constructs. Chondrocytes grown under perfusion conditions formed constructs with significantly higher cell, proteoglycan and collagen content when compared to those produced under static conditions [7, 10, 38]. The enhancing effects of perfusion on biosynthesis of developing cartilaginous tissue may be useful especially when the goal is to create constructs rapidly, or with cell and matrix content that is comparable to native

cartilage. However, especially in the early stages of culture, newly synthesized proteoglycans are lost into the medium due to the high porosity of the tissue and the additional influence of perfusion-induced convective transport [7].

A semi-permeable membrane may be implemented in bioreactors for retention of newly synthesized matrix molecules in cartilaginous constructs and enhance the early growth of such constructs [7, 21]. Bioseparation processes often involve the use of membranes with distinct pore sizes to selectively retain molecules of a particular size; however, this concept has not been widely applied to cartilage tissue engineering. Matrix molecules such as aggrecan monomers, are approximately 30 nm in diameter [5, 34] and 100-400 nm in length [5, 31, 33, 34]. Aggrecan aggregates, which can contain more than 100 aggrecan monomers [9, 29, 31, 33], are approximately 200-800 nm [5, 29, 31, 33, 34] in diameter (based on length of aggrecan monomers branching off either side of the hyaluronan backbone) and are on the order of microns in length [5, 33, 56]. Procollagen molecules, which are the precursors to collagen molecules, are approximately 1.5 nm in diameter [20, 39] and 300 nm in length [33, 39, 63]. Collagen molecules will self-assemble with each other to form fibrils which can range from 20-120 nm in diameter [41] and reach several millimeters in length [2]. By incorporating membranes of small pore sizes into the bioreactor system, newly synthesized matrix molecules, such as proteoglycan monomers and procollagen may be retained and improve the properties of these constructs. Additionally, the porosity, or overall pore area, can be chosen to modulate retention of matrix molecules, with lower porosities increasing retention. In previous studies, membranes have been used to modulate matrix content in static cultures of cartilaginous constructs [15] and cartilage explants [48]. The concept of a membrane may be applied to retain matrix molecules in cartilaginous constructs in a perfusion bioreactor system.

Such a membrane may also facilitate development of cartilaginous constructs in a bioreactor system without the use of a scaffold by allowing retention of the cells on top of the membrane. Alginate recovered chondrocytes have been used extensively to create cartilaginous constructs with high matrix content [4, 22, 25, 26]. Cells cultured in alginate maintain a stable phenotype and synthesize aggrecan and type II collagen while encapsulated within the gel [4, 22, 25, 26]. The culture period of the cells within the alginate beads is usually at least one week and can be as long as several weeks in duration. To further accelerate the process of creating cartilaginous constructs, freshly isolated chondrocytes may be cultured in a perfusion bioreactor system without the alginate step. It is unknown if the effects of perfusion may stimulate formation of cartilaginous constructs from cells which have not been cultured in alginate previously and do not contain pericellular matrix. The usage of freshly isolated chondrocytes may also be relevant to examining the efficacy of cartilage repair methods such as autologous chondrocyte implantation, where cells are implanted without pre-culturing.

A compartmental model may be useful to describe effects of a semi permeable membrane in this bioreactor system. Compartmental models have been utilized in many fields of research. These models have been traditionally used to study drug kinetics [44, 62]. They have also been used to describe the process of stem cell differentiation [37] and utilized in metabolic engineering applications [23, 55]. The underlying assumption of compartmental models is that each compartment is uniform and well-mixed [62]. Important parameters include volume of compartment, transfer rates of molecules between compartments and residence times of molecules in each compartment [44, 62]. A compartmental model has been used previously to describe proteoglycan metabolism in cartilage explants [16]. Similarly, a compartmental model

can be developed to describe the content and distribution of proteoglycans in a perfusion bioreactor system.

The objective of this study was to (1) determine the effects of membranes of different pore sizes on the retention of matrix molecules in cartilaginous constructs cultured in a perfusion bioreactor system and (2) formulate a compartmental model to describe these effects. The selective retention properties of three membranes of pore sizes: 0.05, 0.4, 1.0 μm , and corresponding porosities 1.2, 12.7 and 15.7%, were experimentally assessed for constructs formed using freshly isolated chondrocytes (FIC) and alginate recovered chondrocytes (ARC) in a perfusion bioreactor system.

4.3 Materials and Methods

Perfusion bioreactor system

A perfusion bioreactor system was implemented to study formation of cartilaginous constructs. System temperature control was maintained by a waterbath and pH control by an external gas source. The bioreactor consisted of a custom made polycarbonate chamber [10] containing a porous titanium frit (2 μm pore size, 36% porosity) on top of which the polycarbonate track-etch membrane (pore sizes 0.05, 0.4, and 1.0 μm with porosities of 1.2, 12.7 and 15.7% respectively, Whatman, Inc., Clifton, NJ) was placed. The membrane was secured onto the frit with silicone o-rings that were compressed by the threaded cap when the chamber was closed. The chamber was connected to a media reservoir that was supplied with humidified gas composed of 5% CO_2 balance air. A 12 channel peristaltic pump (Model 205U; Watson-Marlow, Inc., Falmouth, Cornwall, UK) controlled the rate of media flow, and was set up to follow a flow-through path without circulation (Figure 4.1A). All bioreactor

components were connected using C-Flex[®] and Tygon[®] tubing and polypropylene connectors. Construct, media upstream of the membrane, and media downstream of the membrane were considered to be divided into 3 compartments that were referred to respectively as construct, bulk fluid and permeate (Figure 4.1B).

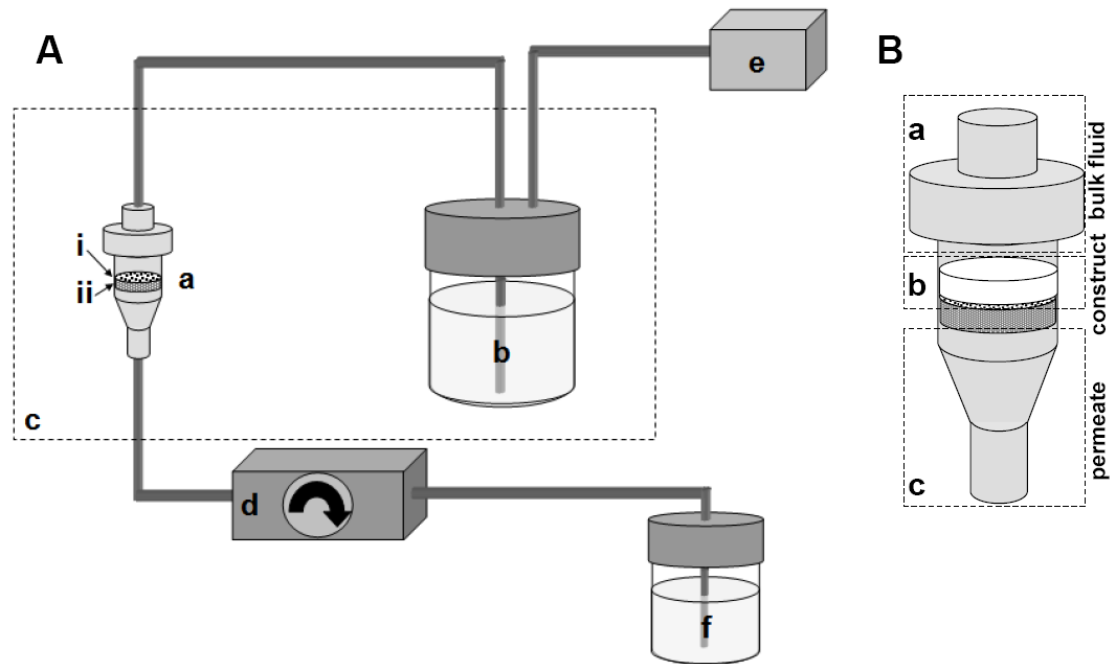


Figure 4.1: Schematic of perfusion bioreactor system. (A) The system consists of 6 main components: (a) the bioreactor chamber, in which the cells are seeded and the cartilaginous construct is formed on top of (i) the membrane which is placed on top of (ii) the frit, (b) the media reservoir, which provides the nutrients for the cells, (c) the waterbath, which provides temperature control, (d) the peristaltic pump, which controls the perfusion rates and drives the flow of the media through the system, and (e) the gas source, which provides pH control to the media, and (f) the collection reservoir, where the spent media is collected after it has passed through the bioreactor chamber. (B) For metabolic analysis, three compartments containing tissue and conditioned medium are analyzed: (a) bulk fluid, (b) construct, and (c) permeate. The bulk fluid compartment includes the medium in contact with the construct within the bioreactor chamber, while the permeate compartment encompasses all spent medium, including that downstream in the collection reservoir.

Chondrocyte isolation and preparation

Cells for construct formation were isolated from calf articular cartilage. Eight millimeter diameter osteochondral cores were harvested from the patellofemoral groove of 1-3 week old bovine calves (n=5) using the Osteochondral Autograft Transfer System (Arthrex, Naples, FL). Cores were sectioned to obtain 200 μm thick slices from the middle to deep zone cartilage (600-2800 μm from the articular surface). Chondrocytes were isolated from cartilage slices by sequential digestion with 0.20% pronase (Sigma-Alrich, St. Louis, MO) and 0.02% collagenase (Roche Diagnostics, Indianapolis, IN) [30]. Isolated cells were then either pre-cultured in alginate (Keltone LV, Kelco, Chicago, IL) for 1 week and released as described previously [22, 26] to form ARC or utilized immediately as FIC for seeding the perfusion bioreactor. Each batch of cells from a single animal was utilized as either all FIC or all ARC cells for a total of 5 separate experiments, 2 with FIC cells, 3 with ARC cells, where 1 experiment with ARC cells was radiolabeled with [^{35}S]sulfate throughout the culture during alginate bead and construct formation stages. The samples from the radiolabeled experiment were utilized for size exclusion chromatography portion of the study.

Construct formation

Cells were seeded into the bioreactor chambers and cultured for one week with perfusion to form cartilaginous constructs. FIC or ARC were seeded (in triplicate for each pore size condition) via injection using a 16 gauge pipetting needle at a density of 4×10^6 cells/ cm^2 for a total of 1.4×10^6 cells per bioreactor based on a membrane surface area of 0.36 cm^2 . Cells were incubated for 7 days in DMEM/F12 with additives (20% fetal bovine serum, 25 $\mu\text{g/mL}$ ascorbate, 100 U/mL penicillin, 100

μg/mL streptomycin, 0.25 μg/mL Fungizone, 0.1 mM MEM non-essential amino acids, 0.4 mM L-proline, 2 mM L-glutamine) at 37°C with 5% CO₂. Culture conditions were static during the initial 3 days, after which perfusion was initiated at a flowrate of 0.25 μm/sec in the flow-through configuration, and the spent media was collected in the collection reservoir. After 1 week of culture, constructs were harvested from bioreactors along with media components upstream (bulk fluid) and downstream (permeate) of the membrane. Upon termination of culture, constructs were measured for thickness using a contact sensing micrometer [52]. In the radiolabeled experiment, cultures were performed as described but with an additional media supplement of [³⁵S]sulfate (250-1000 mCi/mol, Perkin Elmer, Waltham, MA) at a concentration of 5 μCi/mL.

Biochemical Analysis

All samples were analyzed for cell and proteoglycan content, and construct samples were also analyzed for collagen content. The constructs and media components were digested with proteinase K (Roche Diagnostics, Indianapolis, IN) overnight and portions were analyzed for DNA, as a measure of cell content using the Picogreen[®] assay [28] and sGAG, as a measure of proteoglycan content using the DMMB assay [11]. Portions of construct digests were assayed for collagen by measuring hydroxyproline content and assuming a collagen to hydroxyproline ratio of 7.25 to calculate collagen content [36, 59].

Size Exclusion Chromatography

Dissociative Columns (Monomer Size Analysis)

³⁵S macromolecules in samples in construct, bulk fluid and permeate compartments were analyzed for size by gel filtration chromatography under dissociative conditions. Portions of radiolabeled constructs were extracted for 44 hours at 4°C with agitation in 4 M guanidinium chloride, 50 mM sodium acetate, 10 mM HEPES, with protease inhibitors at pH 7.0 at a volume ratio of 50:1 extract solution to tissue. After extraction, the tissue was removed and the solution was filtered over glass wool to remove any remaining tissue particles. Construct extract, bulk fluid and permeate samples were loaded onto Sephacryl S-500 (Pharmacia Biotech, Piscataway, NJ) columns (0.7 cm x 50 cm) and eluted under dissociative conditions to assess the size of proteoglycan monomers using an elution buffer of 4 M guanidinium chloride, 0.5 M sodium acetate, 0.1% Triton-X, pH 6.8. Samples loaded onto the columns contained approximately 100,000 counts per minute (CPM) of ³⁵S radioactivity as well as 4 µg DNA (calf thymus, Sigma-Aldrich) and 0.01 µCi ³H₂O (1 mCi/g, Perkin Elmer) as markers of void volume (V_0) and total volume (V_t), respectively, and were made up to be 0.30 mL in volume with elution buffer. The flow rate was 7.5 mL/hr, with 0.6 mL fractions collected over the course of 4 hours. Column fractions were assayed for DNA with Picogreen[®] using 0.050 mL of each fraction and ³⁵S and ³H radioactivity by addition of 2 mL of scintillation cocktail (Ecolume, ICN Pharmaceuticals, Inc., Costa Mesa, CA) and scintillation counting. Typical recovery of ³⁵S and ³H radioactivity from columns was 90-100%. Fraction ³⁵S CPM were scaled to original sample CPM, and graphed versus K_{av} , which was calculated from the ratio of the elution volume to the total volume of the column ($V_t - V_0$). K_{av} is the partition coefficient which describes the relative size of the molecule

based on its position of elution, where a K_{av} of 0 represents large molecules eluting at the beginning of the column that are excluded from the S-500 resin, and a K_{av} of 1 describes small molecules eluting at the end of the column, that completely enter the S-500 resin.

Associative Columns (Aggregation Ability Analysis)

^{35}S macromolecules in samples in construct, bulk fluid and permeate compartments were analyzed for ability to aggregate with hyaluronan and link protein by gel filtration chromatography under associative conditions. Radiolabeled construct extract, bulk fluid and permeate samples (triplicate) were pooled for each of the three membranes for a total of nine samples. Samples were lyophilized, and reconstituted in 4M guanidinium chloride with 0.15 M sodium acetate, with 5% hyaluronan (Healon, Advanced Medical Optics, Inc., Santa Ana, CA) and 5% link protein. Aggrecan was added to bring the total sGAG content to 200 μg per sample. Aggrecan and link protein were purified from bovine calf cartilage [18, 47, 56]. Samples were allowed to dissociate at 4°C for 2 hours and then dialyzed using dialysis membranes (6-8 kDa MWCO, Spectrum Laboratories, Inc., Rancho Dominguez, CA) contained in QuixSep micro dialyzers (Membrane Filtration Products, Inc., Seguin, TX) in 0.15M sodium acetate, pH 6.8 for 24 hours at 4°C to allow for aggregation. Samples containing 150 μg of sGAG, with 0.01 μCi $^3\text{H}_2\text{O}$ added as a marker of total volume, were loaded onto Sephacryl S-1000 (Pharmacia Biotech) columns (0.7 cm x 50 cm) and eluted under associative conditions to determine aggregation ability of proteoglycan monomers using 0.5M sodium acetate, 0.1% Triton X-100, 0.02% sodium azide, pH 6.8 as column buffer. The flow rate was 5 mL/hr, with 0.5 mL fractions collected over the course of 4 hours. Column fractions were assayed for sGAG with DMMB using 0.050

mL of each fraction as well as ^{35}S and ^3H . Typical recoveries of sGAG and radiolabel from columns were 90-100%. Counts or sGAG per fraction volume normalized to overall counts or sGAG and column volume were graphed versus K_{av} . The aggregability index of the newly synthesized proteoglycans was also calculated as the ratio of the percentage of radiolabeled aggregates (^{35}S) to the percentage of non-radiolabeled aggregates (sGAG).

Compartmental Model

To formulate the model of proteoglycan homeostasis, the perfusion bioreactor system was divided into three compartments: (1) medium from the bulk fluid, (2) the construct, and (3) medium from the permeate (Figure 4.2). Within the construct compartment, there were three subcompartments containing: (2) newly synthesized proteoglycan (monomers), (2b) bound proteoglycans (aggregates) and (2d) degraded proteoglycans (monomers) within the construct. A reflection coefficient (σ) was used to represent the membrane retention properties for the proteoglycans. Transfer coefficients (k_{ij}) between compartments represented flow and diffusion-related transport of proteoglycans between compartments. Construct metabolism of proteoglycans was described by the rate of formation (r_f), rate of binding (k_b) and rate of degradation (k_d). The compartments were assumed to be well-mixed, so the proteoglycan content was uniformly distributed within each compartment. The bulk fluid and construct compartment volumes were assumed to be constant, and the tubing volume was assumed to be negligible. The permeate compartment volume increased with perfusion. The transfer coefficients in the direction of flow were assumed to only be dependent on the volumetric flow rate and any other transport phenomena were assumed to be negligible. It was also assumed that once proteoglycans passed through

the membrane, they could not move back through the membrane and be transferred from the permeate into the construct. All proteoglycans, except those in aggregate form, were assumed to be mobile and therefore able to move between compartments.

Key Parameters

The key parameters and variables are shown in Table 4.1. The flow rate parameter is accounted for by the transfer coefficients, k_{ij} , which are calculated by dividing the volume of each compartment by the flow rate. The membrane pore size is represented by a nondimensional parameter, σ , the reflection coefficient, which describes the properties of the membrane in relation to the molecule of interest. A high reflection coefficient would represent a membrane with pores generally smaller in size than the molecule of interest and/or lower porosity, whereas a low reflection coefficient would represent a membrane with pores generally larger in size than the molecule of interest and/or higher porosity. The reflection coefficient ranges from a possible minimum value of 0 which indicates a freely permeable membrane (or no membrane) and a possible maximum value of 1 indicates an impermeable membrane [54]. It was also assumed that σ was the same for both soluble and degraded proteoglycans, and that the degraded proteoglycans only travel in the direction of flow, and move from the construct subcompartment to the permeate compartment. It is assumed that all parameters are time-invariant.

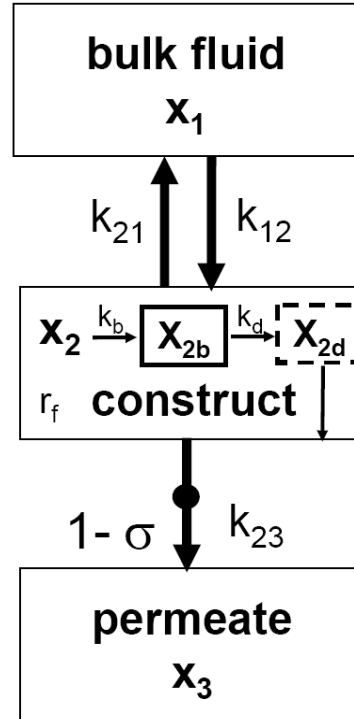


Figure 4.2: Compartmental model representation of bioreactor system. x_i represents the amount of proteoglycan in the compartment i , k_{ij} is the transfer coefficient between compartments i and j , r_f is the rate of formation of proteoglycan, k_b is the binding coefficient for newly synthesized proteoglycan, and k_d is the degradation coefficient for proteoglycan from the bound state. Within the construct, is proteoglycan in soluble, x_2 , bound, x_{2b} , and degraded, x_{2d} , form. The semi-permeable membrane is described by σ , the reflection coefficient.

Table 4.1: Key parameters and variables in the compartmental model.

Variables	Description	Units
t	time	days
x_1	amount of molecule in the bulk fluid compartment 1	μg
x_2	amount of molecule in the construct compartment 2	μg
x_{2b}	amount of molecule in the construct bound compartment 2b	μg
x_{2d}	amount of molecule in the construct degraded compartment 2d	μg
x_3	amount of molecule in the permeate compartment 3	μg
Parameters	Description	Units
k_{12}	rate of transfer of molecule from compartment 1 to 2	day^{-1}
k_{21}	rate of transfer of molecule from compartment 2 to 1	day^{-1}
k_{23}	rate of transfer of molecule from compartment 2 and 2d to 3	day^{-1}
r_f	rate of formation of molecule	$\mu\text{g}/\text{day}$
k_b	rate of binding of molecule (transfer from compartment 2 to 2b)	day^{-1}
k_d	rate of degradation of molecule (transfer from compartment 2b to 2d)	day^{-1}
σ	reflection coefficient of molecule (membrane pore size and porosity)	none

Governing equations

The equations that describe the rate of change of the amount of proteoglycan in each compartment are given in this section. Compartment 1 is the bulk fluid component of media that is present above the construct. There is loss of proteoglycan into the bulk fluid media from the surface of the developing construct. The bulk fluid proteoglycan content is governed by:

$$\frac{dx_1}{dt} = -k_{12}x_1 + k_{21}x_2 \quad (1)$$

Compartment 2 is the tissue construct. Proteoglycans are present initially in the construct compartment when the cells are seeded. Proteoglycans also enter the construct compartment from the bulk fluid compartment, and exit into the permeate compartment. A membrane below the construct reflects back some of the molecules that are exiting. Proteoglycans also enter the compartment at the rate of formation, r_f , since the cells contained within that compartment are metabolically active. A portion of the proteoglycans in the construct compartment is also bound at a rate of binding, k_b . These bound proteoglycans are part of a subcompartment, compartment 2b, which includes the proteoglycans that become bound the extracellular matrix network in the construct and will not be transferred into other compartments unless they are degraded [8, 21] at a rate of degradation, k_d . The degraded proteoglycans become part of subcompartment 2d, also within the construct compartment, and cannot be bound. The construct proteoglycan content is governed by the three equations for soluble, bound and degraded components:

$$\frac{dx_2}{dt} = k_{12}x_1 - k_{21}x_2 - (1 - \sigma)[k_{23}x_2] + r_f - k_b x_2 \quad (2)$$

$$\frac{dx_{2b}}{dt} = k_b x_2 - k_d x_{2b} \quad (3)$$

$$\frac{dx_{2d}}{dt} = k_d x_{2b} - (1 - \sigma)[k_{23} x_{2d}] \quad (4)$$

Compartment 3 is the permeate media component. Proteoglycans enter the permeate compartment from the construct (soluble and degraded compartments), and exit into the media reservoir. The permeate proteoglycan content is governed by:

$$\frac{dx_3}{dt} = (1 - \sigma)k_{23}(x_2 + x_{2d}) \quad (5)$$

Initial Conditions

Initially all compartments are empty except for the construct bound compartment, x_{2b} , into which the cells and associated matrix are seeded. The constant x_o represents the amount of proteoglycan present at the start of culture in the construct compartment.

$$x_1(0) = x_2(0) = x_{2d}(0) = x_3(0) = 0 \quad (6)$$

$$x_{2b}(0) = x_o \quad (7)$$

Parameter Estimation

Values for parameters were estimated based on experimental parameters and values from literature (Table 4.2). The transfer coefficient k_{12} and k_{23} were set equal to 0 during static culture, and during perfusion culture estimated by dividing the volumetric flow rate (0.8 mL/day) by the volume of the compartment. For compartment 1, the volume was the amount of headspace in the bioreactor above the membrane, which is 1 mL. For compartment 2, the construct was assumed to be equal

to the volume of a construct of thickness 0.6 mm, which is 0.22 mL. For transfer coefficient k_{21} , it was assumed that it was equal to 0.5 day^{-1} based on rates of secretion of newly synthesized proteoglycans into the media by constructs in previous studies with a similar bioreactor system [7]. Transfer coefficients were assumed to be constant. It was also assumed for the simulation that the reflection coefficient is time-independent for the membrane, and any increase due to tissue development is negligible. The rates r_f , k_b and k_d were assumed to be equal to the values determined for calf cartilage explants [46, 49, 50].

Solution of a Compartmental Model

The solution of the compartmental model can be obtained if the initial conditions of the compartments and the parameters are known. The compartmental model was solved using the ODE solver, based on explicit Runge-Kutta methods, in Matlab (version 6.5, Mathworks, Natick, MA). Simulations were run with the parameters in Table 4.2 and varying values of the reflection coefficient, σ , to simulate effects of different membrane pore size on the matrix content and distribution within the system.

Table 4.2: Parameter values used in compartmental model simulation.

Parameters	Description	Value	Units
x_0	Initial conditions, amount of proteoglycan in x_{2b} at $t=0$	63	μg
k_{12}	rate of transfer of proteoglycan from compartment 1 to 2	0.8	day^{-1}
k_{21}	rate of transfer of proteoglycan from compartment 2 to 1	0.5	day^{-1}
k_{23}	rate of transfer of proteoglycan from compartment 2 and 2d to 3	3.7	day^{-1}
r_f	rate of formation of proteoglycan	35	$\mu\text{g}/\text{day}$
k_b	rate of binding of proteoglycan	1.0	day^{-1}
k_d	rate of degradation of proteoglycan	0.32	day^{-1}
σ	reflection coefficient of proteoglycan	0-1	none

Steady State Solution

A steady state solution was obtained by setting the rates of change with time to 0 for the following compartments, x_1 , x_2 and x_{2b} , and solving for the expressions:

$$\frac{dx_1}{dt} = \frac{dx_2}{dt} = \frac{dx_{2b}}{dt} = \frac{dx_{2d}}{dt} = 0 \quad (8)$$

For x_3 , the compartment increases indefinitely but when the other compartments reach steady state, the rate of increase becomes constant. The values for the construct and bulk fluid compartments steady state (calculated using parameter values from Table 4.2) were calculated for different reflection coefficients σ .

Statistical Analysis

All data were analyzed using one-way ANOVA. Systat 10.2 (Systat Software, Inc., Point Richmond, CA) was used for statistical analysis. Membranes are described by their pore sizes. ANOVA with pore size as a fixed factor was used to analyze effects of pore size on DNA, sGAG and collagen contents in constructs and media components. ARC and FIC results from different experiments were pooled for a total of n=4-6 for each culture condition and donor animal was treated as a random factor to take into account any variability from cell source. ARC and FIC culture conditions were analyzed independently since the cells were derived from different animal donors. When significant effects ($p < 0.05$) were found, Tukey post-hoc testing was performed. Arcsine transformation was applied to data that were in terms of percentages for normalization before analysis. Data are expressed as mean $\pm$ SEM.

4.4 Results

sGAG content

sGAG content (Figure 4.3A) was affected by pore size in ARC condition but not in FIC condition. Pore size did not have an effect on the total sGAG in either ARC ($p=0.91$) or FIC ($p=0.26$) condition. Although total sGAG content was similar within each culture type condition, the mean total sGAG in the ARC condition ($274 \pm 14 \mu\text{g}$) was more than five-fold higher than that in the FIC condition ($52 \pm 4 \mu\text{g}$). For constructs derived from ARC, sGAG content decreased with increasing pore size ($p<0.01$), with significant decreases from 0.05 to 0.4 μm ($p<0.05$) and from 0.05 to 1.0 μm ($p<0.01$). For the ARC bulk fluid compartments, while pore size did not significantly affect sGAG content ($p=0.19$), there was a general trend of decreasing sGAG content with increasing pore size. In the ARC permeate compartments, sGAG content increased with increasing pore size ($p<0.001$), with significant increase from 0.05 to 0.4 μm ($p<0.005$), 0.05 to 1.0 μm ($p<0.001$), and 0.4 to 1.0 μm ($p<0.05$) membranes. In the FIC condition, there was no effect of pore size on construct ($p=0.69$), bulk fluid ($p=0.08$) or permeate ($p=0.59$) sGAG content.

Similarly, distribution of sGAG within the bioreactor system (Figure 4.3C) was also affected by pore size in the ARC condition but not in the FIC condition. sGAG distribution decreased with increasing pore size ($p<0.05$) in ARC constructs, with differences between construct compartments for 0.05 and 0.4 μm , and 0.05 and 1.0 μm (both $p<0.001$). sGAG distribution in the ARC bulk fluid compartments was not significantly affected by pore size ($p=0.23$). sGAG distribution in the ARC permeate compartments increased with increasing pore size ($p<0.001$), with differences between permeate compartments for 0.05 and 0.4 μm ($p<0.001$), 0.05 and

1.0 μm ($p < 0.001$), and 0.4 and 1.0 μm ($p < 0.01$). There was no effect of pore size on the percent distribution of FIC construct ($p = 0.84$), bulk fluid ($p = 0.66$) or permeate ($p = 0.91$) compartments.

Cell content

Pore size did not affect total cell content (Figure 4.3B) in either the ARC ($p = 0.24$) or FIC ($p = 0.79$) conditions. Although the total number of cells was similar for both conditions, the ARC constructs (0.80×10^6 cells) were twice as cellular as the FIC constructs (0.40×10^6 cells). Pore size did not affect cell content in the construct ($p = 0.45$), bulk fluid ($p = 0.35$) or permeate ($p = 0.23$) compartments for the ARC condition. Similarly, pore size did not affect cell content in the construct ($p = 0.64$) or bulk fluid ($p = 0.82$) or permeate ($p = 0.49$) compartments for the FIC condition.

Distributions of cells (Figure 4.3D) in ARC construct ($p = 0.08$), bulk fluid ($p = 0.09$) and permeate ($p = 0.72$) compartments and in FIC construct ($p = 0.23$), bulk fluid ($p = 0.99$) and permeate ($p = 0.46$) compartments were also not affected.

Construct collagen content

Collagen content (Figure 4.3E) varied with pore size in ARC constructs, but not FIC constructs. In ARC conditions, collagen content was affected by membrane pore size ($p < 0.05$), decreasing with increasing pore size, with differences between constructs grown on membranes of pore size 0.05 and 1.0 μm , and 0.05 and 1.0 μm (both $p < 0.05$). There was no effect of pore size on collagen content in FIC conditions ($p = 0.50$). Overall, ARC constructs had higher collagen content than FIC constructs.

Construct thickness

Construct thickness (Figure 4.3F) varied with pore size in the ARC condition, but not the FIC condition. In the ARC condition, construct thickness was affected by membrane pore size ($p < 0.05$), with decreasing thickness corresponding with increasing pore size, and differences between constructs grown on membranes of pore size 0.05 and 1.0 μm ($p < 0.05$). In the FIC condition, there was no effect of pore size on construct thickness ($p = 0.77$). Overall, FIC constructs were thinner than ARC constructs.

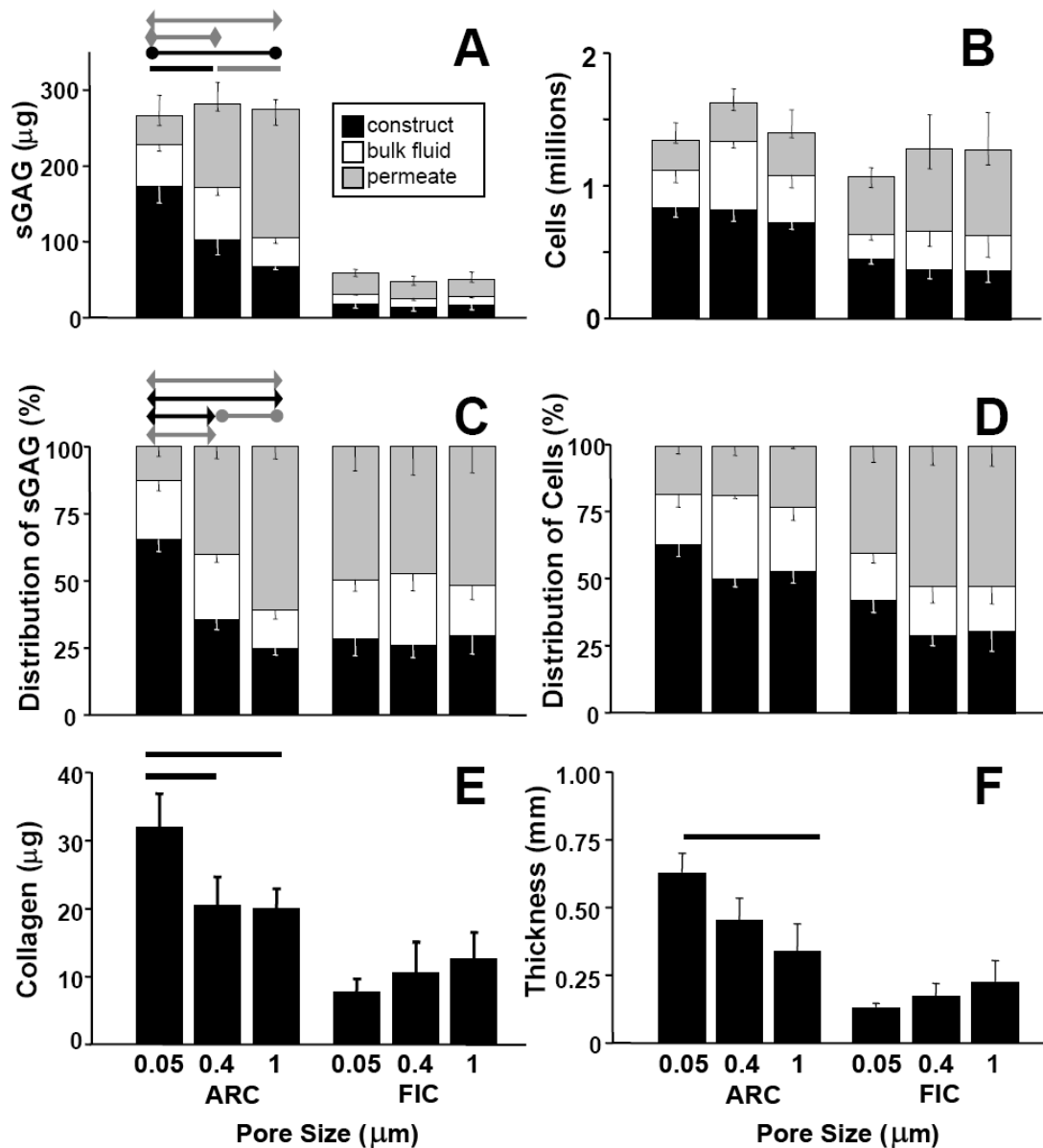


Figure 4.3: Effect of membrane pore size on construct properties. sGAG content (A) and percent distribution (B) and cell content (C) and percent distribution (D) and collagen content (E) and thickness (F) in ARC and FIC derived bioreactor construct ■, bulk fluid □, and permeate ■ compartments versus pore size. Significant effects are indicated as follows: — = $p < 0.05$, ● = $p < 0.01$, ◆ = $p < 0.005$, ◀ = $p < 0.001$. Data are mean $\pm$ SEM, $n=4-6$.

Size Analysis of Proteoglycans

Dissociative conditions

The size of the large ^{35}S labeled proteoglycan component varied slightly with construct, bulk fluid and permeate (Figure 4.4). ^{35}S labeled proteoglycans eluted in a single peak ($K_{av} \sim 0.226\text{-}0.298$) for all compartments and pore sizes. All three compartments also had a peak at total volume ($K_{av} \sim 1$) consistent with free ^{35}S radiolabel. The ^{35}S proteoglycan peak location (determined by parabolic fit of 4 to 5 points near peak) varied ($p < 0.05$) with pore size for construct samples with $K_{av} \sim 0.268 \pm 0.008$, 0.226 ± 0.014 , 0.258 ± 0.001 (mean $\pm$ SEM) for pore sizes 0.05, 0.4 and 1.0 μm respectively. For bulk fluid samples, ^{35}S proteoglycan monomer sizes were similar ($p = 0.78$) with peak locations of $K_{av} \sim 0.270 \pm 0.006$, 0.268 ± 0.009 , 0.274 ± 0.005 for pore sizes 0.05, 0.4 and 1.0 μm respectively, as well as permeate samples ($p = 0.19$) with peak locations of $K_{av} \sim 0.298 \pm 0.008$, 0.264 ± 0.018 , 0.287 ± 0.006 for pore sizes 0.05, 0.4 and 1.0 μm respectively). Overall, sizes of ^{35}S proteoglycans in the media compartments were smaller than in the construct.

There were differences in the peak sizes in bulk fluid samples, with the smaller pore sizes including larger peaks (more radiolabeled proteoglycans), and in permeate samples, the smaller pore sizes retaining smaller peaks (less radiolabeled proteoglycans). These differences are illustrated in Figure 4.5 which shows the distribution of the total amount of radiolabeled proteoglycans is similar to the trend shown for the total amounts of sGAG shown in Figure 4.3C.

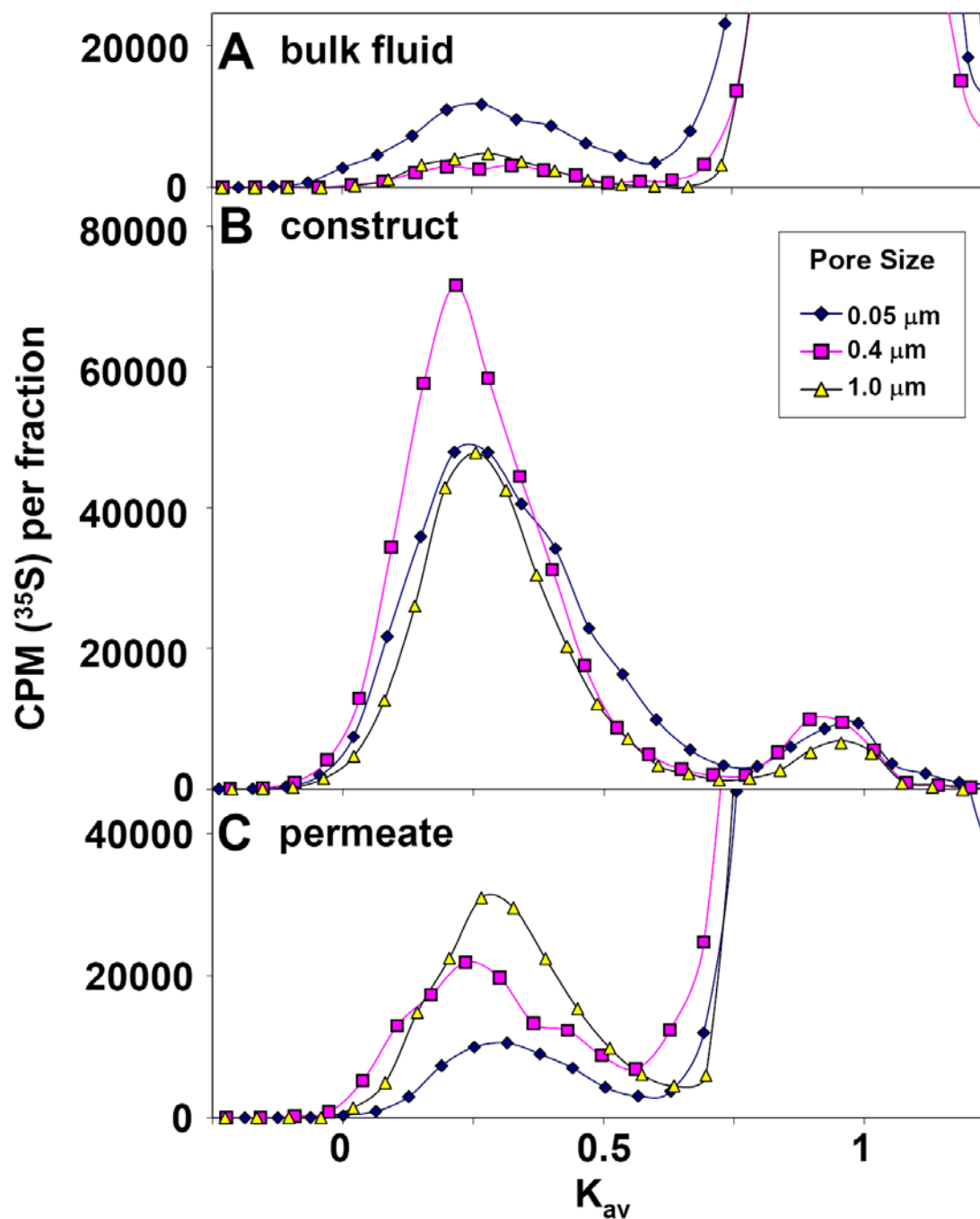


Figure 4.4: Size distribution of ^{35}S labeled proteoglycans under dissociative conditions within A) bulk fluid, B) construct and C) permeate samples. CPM values were scaled accordingly to total volume of sample, and are an average of 3 replicates for each compartment and pore size.

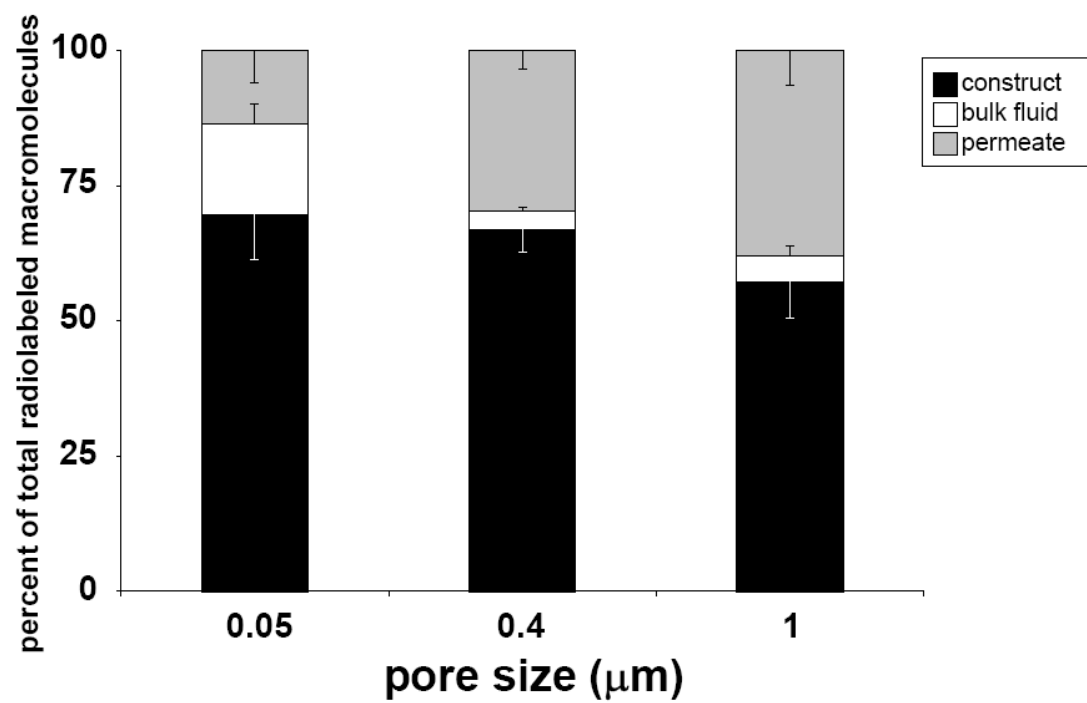


Figure 4.5: Distribution of ^{35}S labeled proteoglycans in compartments. Data are mean $\pm$ SEM.

Aggregation Ability Analysis of Proteoglycans

Associative conditions

^{35}S labeled proteoglycans eluted into two portions (Figure 4.6): the aggregated proteoglycans eluted in one steep peak located at the void volume ($K_{av} \sim 0$) and the proteoglycan monomers eluted in a second broad peak located between void volume and the total volume ($K_{av} \sim 0.5$). The aggregation ability of all the proteoglycans, including the ^{35}S labeled and the added aggrecan (Figure 4.7), was also demonstrated. The elution profiles of all proteoglycans were similar to that of the ^{35}S labeled proteoglycans, with two peaks located at $K_{av} \sim 0$ and $K_{av} \sim 0.5$. Percentages of the ^{35}S labeled and total proteoglycans in the aggregated versus monomer form are shown in Figure 4.8. For total proteoglycans, the percentage of aggregate was $\sim 70\%$ for all columns. For ^{35}S labeled (newly synthesized) proteoglycans, in constructs, the lowest percentage of aggregate was present for the smallest pore size: 60% aggregation for 0.05 μm versus 89 and 80% for 0.4 and 1.0 μm respectively. In contrast, the lowest percentage of aggregate was found in the largest pore size, 1.0 μm , in the permeate. These results indicated that there were more degraded monomers (unable to aggregate), which are smaller than intact aggrecan monomers, retained in the construct grown on the smallest pore size membrane.

The aggregability index (Table 4.3) was compared to the sizes of the ^{35}S proteoglycan monomers determined under dissociative conditions. Overall, the aggregability index was higher for larger ^{35}S proteoglycans (smaller K_{av}), which is consistent with the larger size of intact proteoglycan monomers which have the ability to aggregate, in comparison to the smaller degraded proteoglycan monomers which do not have the ability to aggregate.

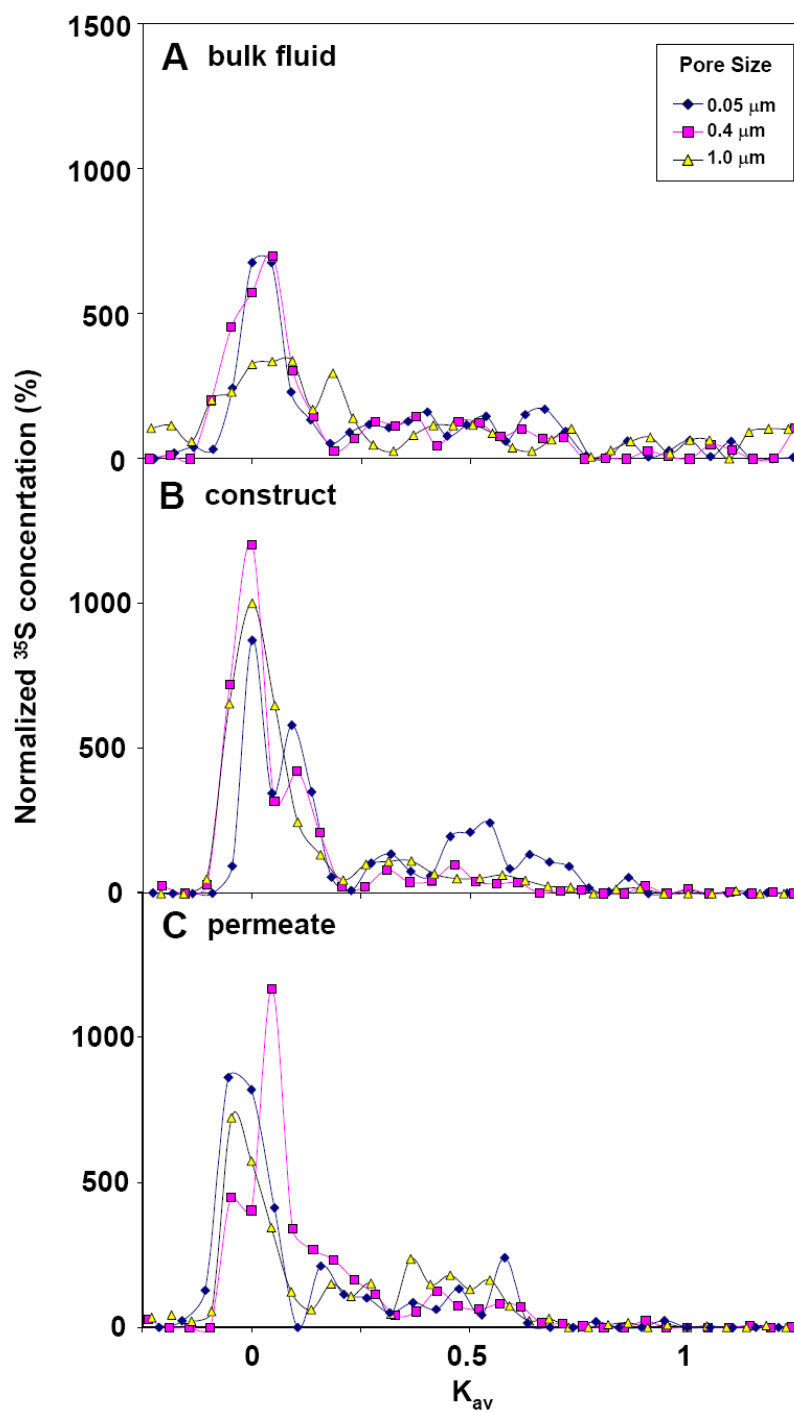


Figure 4.6: Aggregation ability of ^{35}S labeled proteoglycans under associative conditions within A) bulk fluid, B) construct and C) permeate samples. Samples were pooled from 3 replicates for each compartment and pore size.

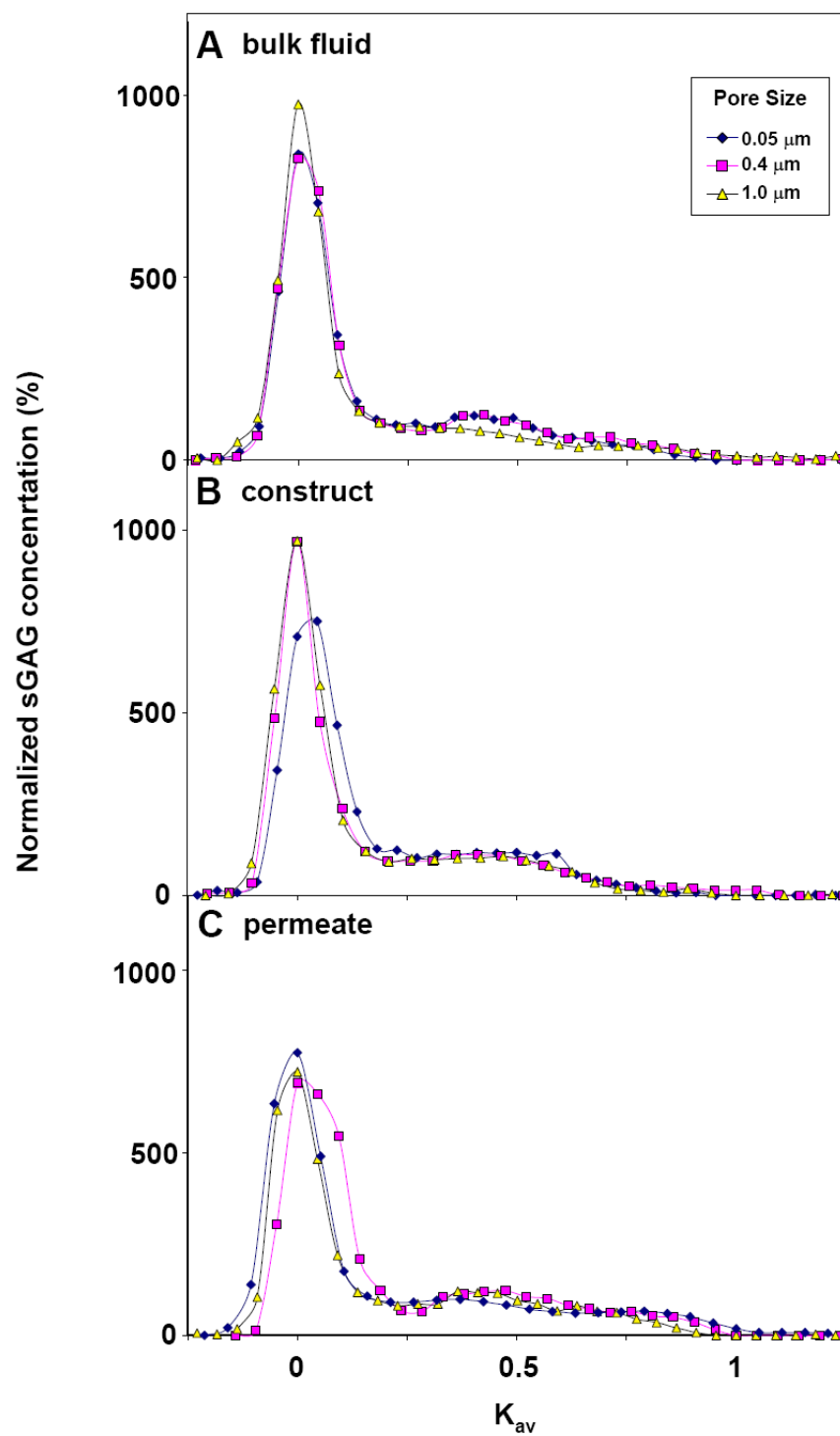


Figure 4.7: Aggregation ability of proteoglycans under associative conditions within A) bulk fluid, B) construct and C) permeate samples. Samples were pooled from 3 replicates for each compartment and pore size.

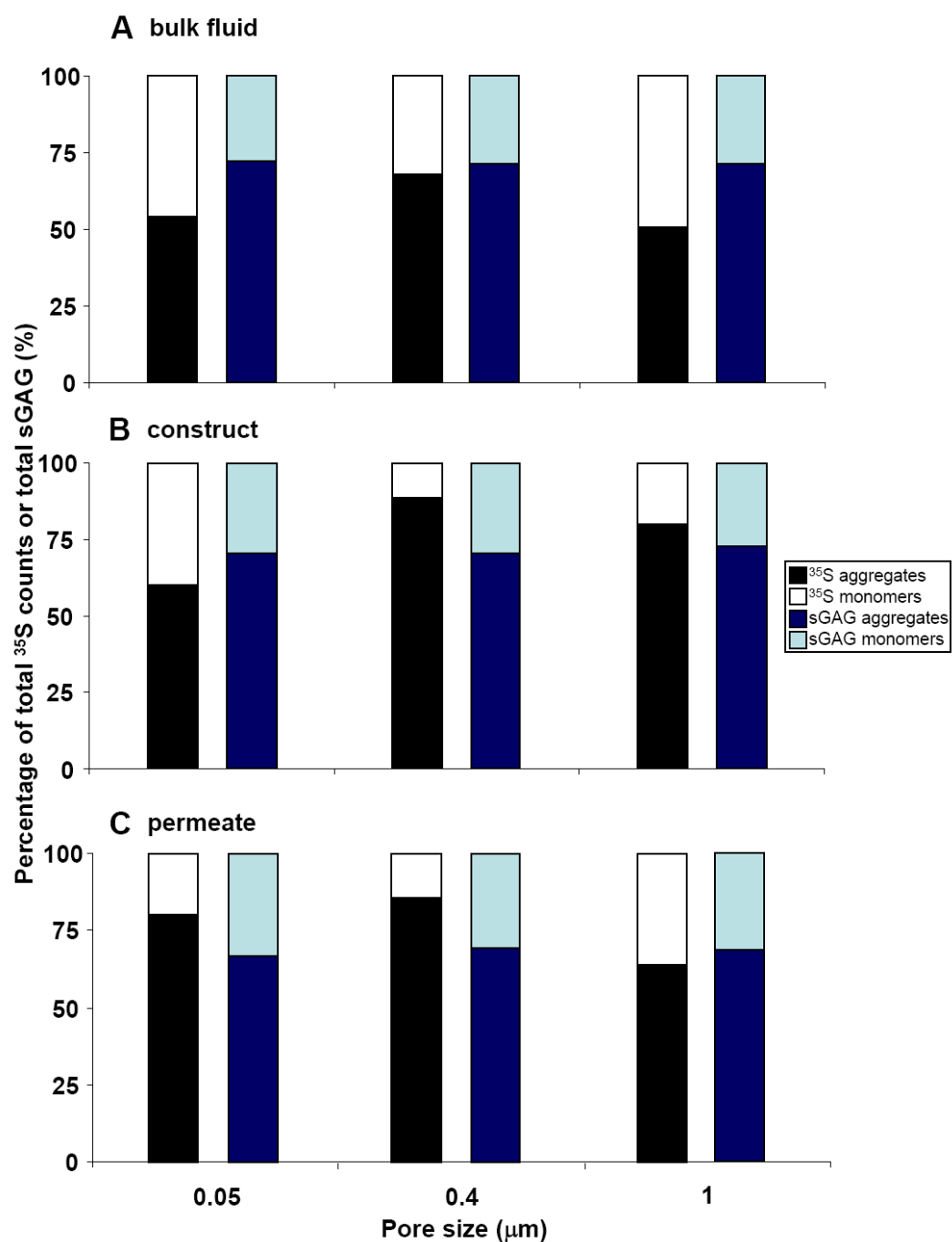


Figure 4.8: Percentage of ^{35}S labeled and total proteoglycan aggregates and monomers within compartments within A) bulk fluid, B) construct and C) permeate samples. Samples were pooled from 3 replicates for each compartment and pore size.

Table 4.3 : Aggregability index and relative size of ^{35}S labeled proteoglycans. The aggregability index is the ratio of the percentage of aggregation of ^{35}S labeled proteoglycans to the percentage of aggregation non-radiolabeled proteoglycans with hyaluronan and link protein under associative conditions. Relative sizes of ^{35}S labeled proteoglycans are represented by K_{av} as determined by gel filtration chromatography under dissociative conditions.

Membrane pore size (μm)	Compartment	Aggregability Index	K_{av}
0.05	bulk fluid	0.75	0.270
0.4		0.95	0.268
1		0.71	0.274
0.05	construct	0.85	0.268
0.4		1.26	0.226
1		1.10	0.258
0.05	permeate	1.20	0.298
0.4		1.23	0.264
1		0.93	0.287

Compartmental Model

Simulation results (Figure 4.9) described the change of proteoglycan content within each compartment during a 30 day culture. The results indicated that during the initial 3-day static culture period, there was a build up of proteoglycan within the construct and bulk fluid compartments. After perfusion started on day 3, there was an inflection point for both construct and bulk fluid compartments. In the construct compartment, for $\sigma \leq 0.5$, the proteoglycan decreased initially and then increased again at a slower rate; for $\sigma > 0.5$, the proteoglycan continued to increase after the perfusion start but at a slower rate. In the bulk fluid compartment, after start of perfusion, the proteoglycan decreased and then continued to decrease until it reached a plateau value; with increasing σ , the plateau was reached sooner. The permeate compartment had no proteoglycan until day 3 when perfusion started, and then it began to increase rapidly. As reflection coefficient σ increased (corresponding to decreasing pore size or porosity) the amount of proteoglycan in the construct and bulk fluid compartments increased, and the amount in the permeate compartment decreased. With σ of 1 (impermeable membrane), there was no proteoglycan in the permeate compartment.

Simulation results (Figure 4.10) for distribution of proteoglycan after a 7 day culture showed that with increasing σ , the bulk fluid compartment proteoglycan percentage and construct compartment proteoglycan percentage increased. The permeate compartment proteoglycan percentage decreased with increasing σ .

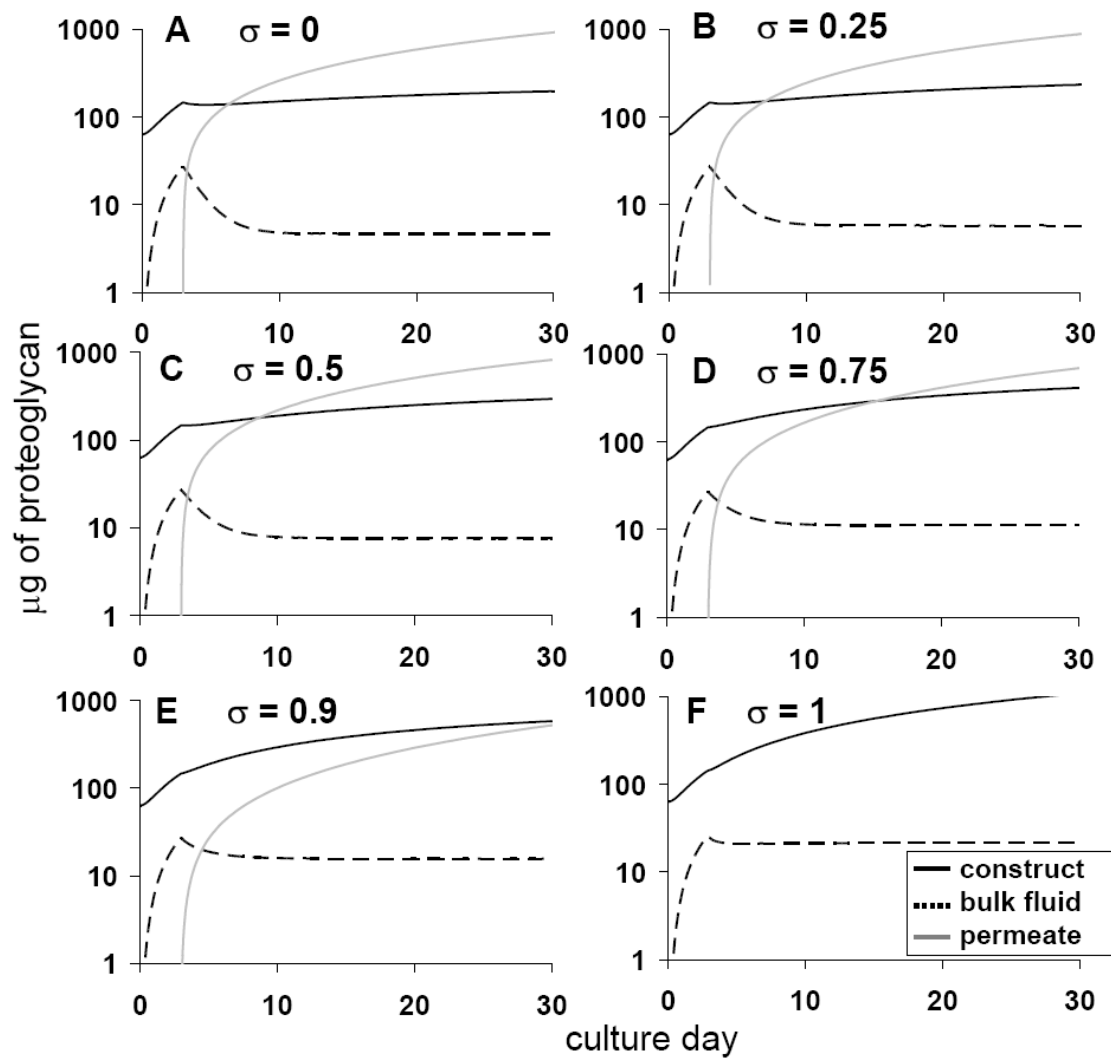


Figure 4.9: Model simulation results for proteoglycan content in a 30 day culture with different in reflection coefficient (σ): A) 0, B) 0.25, C) 0.5, D) 0.75, E) 0.9 F) 1.

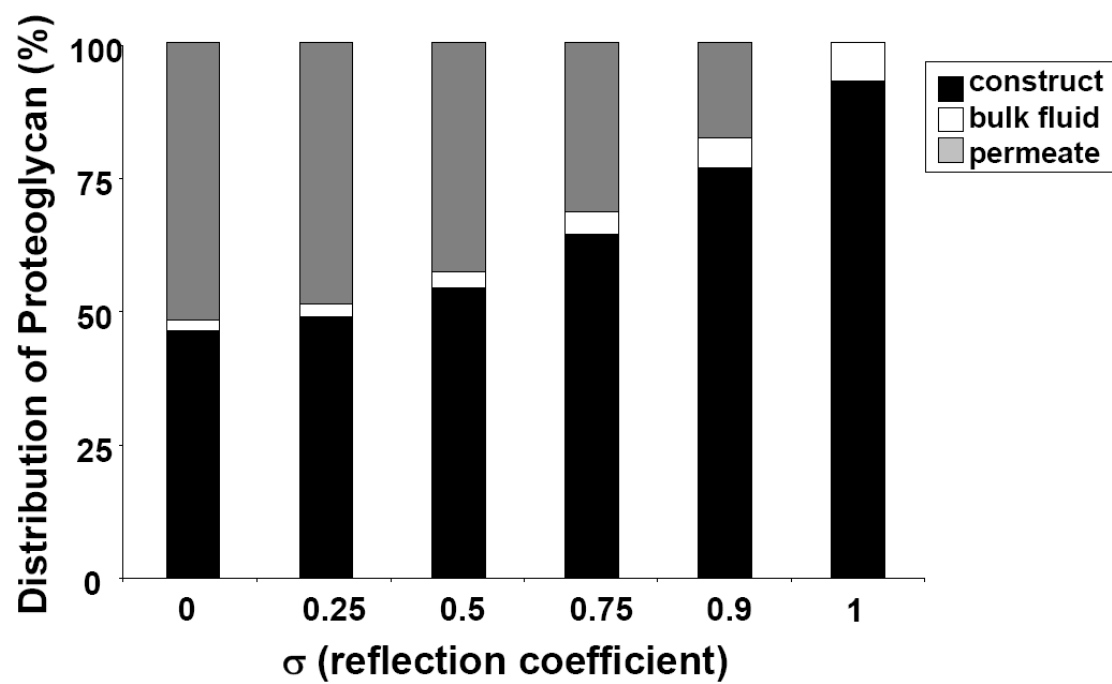


Figure 4.10: Model simulation results for percent distribution of proteoglycan in relation to reflection coefficient (σ) for a 7 day culture.

Steady state solution was obtained, and the expressions for the compartments are as follows:

$$x_1^{ss} = \frac{k_{21}}{k_{12}} \left(\frac{r_f}{(1-\sigma)k_{23} + k_b} \right) \quad (9)$$

$$x_2^{ss} = \frac{r_f}{(1-\sigma)k_{23} + k_b} \quad (10)$$

$$x_{2b}^{ss} = \frac{k_b}{k_d} \left(\frac{r_f}{(1-\sigma)k_{23} + k_b} \right) \quad (11)$$

$$x_{2d}^{ss} = \frac{k_b}{(1-\sigma)k_{23}} \left(\frac{r_f}{(1-\sigma)k_{23} + k_b} \right) \quad (12)$$

The construct compartment, which is made up of x_2 , x_{2b} , and x_{2d} can be represented as:

$$x_{construct}^{ss} = \left(1 + \frac{k_b}{k_d} + \frac{k_b}{(1-\sigma)k_{23}} \right) \left(\frac{r_f}{(1-\sigma)k_{23} + k_b} \right) \quad (13)$$

For the permeate (compartment 3) the rate of increase becomes constant at steady state and is equal to r_f , the rate of formation.

Table 4.4 shows the steady state values for different σ . (Note that there is no steady state value for $\sigma = 1$ since the construct can increase indefinitely in that case.) The values are consistent with the trends shown in the simulations (Figures 4.8, 4.9), with steady state values for construct and bulk fluid compartments increasing with increasing σ .

Table 4.4: Steady state values for construct and bulk fluid compartments.

σ	Construct ($\mu\text{g proteoglycan}$)	Bulk Fluid ($\mu\text{g proteoglycan}$)
0	251	5
0.25	312	6
0.5	415	8
0.75	620	11
0.9	907	16
0.99	2014	20

4.5 Discussion

The results described in this study indicate that a semi-permeable membrane in a perfusion bioreactor enhances the retention of proteoglycans and collagen. The extent of the retention by the membranes on cartilaginous constructs is dependent on the pore size and culture condition. In the ARC condition, smaller pore sizes retain larger amounts of proteoglycan and collagen in the construct, which also resulted in greater construct thickness, although the cellularity of the constructs was unaffected by pore size. Sizes of proteoglycan monomers formed from ARC were similar between pore sizes and compartments, although monomers were slightly smaller in media compartments. The aggregation ability of these monomers was also determined, with greater amounts of the smaller non-aggregating monomers retained with the smallest pore size membrane. Pore size had no significant effects on FIC conditions and overall values of construct proteoglycan, collagen, cellularity and thickness were considerably lower in these cultures.

The results that indicate pore size dependence of matrix retention are tempered by the fact that the porosities of the different pore size membranes were dissimilar. For the 0.05 μm pore size membrane, the porosity was only 1.2%, which was an order of magnitude less than the porosities of the 0.4 and 1.0 μm membranes, which had porosities of 12.7 and 15.7% respectively. Thus, the retention of matrix molecules was also dependent on the membrane porosities, with lower porosity increasing retention. The shape of the molecules may also influence their retention. As the smallest pore size is on the order of the diameter of the aggrecan monomer (30 nm) [5, 34], it would be expected that more of the newly synthesized molecules which are in monomer form were retained in the construct grown on top of the 0.05 μm pore size membrane. However, the aggrecan monomer is cylindrical, not spherical, in shape with a structure

consisting of a brush-like array of sGAG side chains attached to a central core protein [9, 29, 31], with an average length of 100-400 nm [5, 31, 33, 34]. Additionally, the orientation of the molecules as they approach the pores may also determine whether or not they pass through the membrane. If a molecule approaches the membrane with its length parallel to the pores it will be retained by a larger pore size, i.e. an aggrecan monomer with a length of 400 nm could potentially be retained by a 0.4 μm pore size membrane, whereas if it approaches the membrane perpendicular to the pores it may easily pass through. Most likely the molecules will approach the pores at some orientation between perpendicular and parallel, resulting in a distribution of molecules between compartments upstream and downstream of the membrane that is not dependent on pore size alone.

The size analysis portion of the study indicated that the proteoglycan retained within the ARC constructs were smallest on the membrane with the smallest pore size and lowest porosity, suggesting that more degraded monomers were retained. The shape and location of the elution peaks were similar to that for calf cartilage extract (data not shown), indicating that the newly synthesized monomers were mostly aggrecan as is expected for ARC. The aggregation ability of these monomers was also analyzed, and the results were consistent with the expectation that more of the smaller degraded (non-aggregating) proteoglycans would be retained in the construct with smaller pore size and lower porosity. As it may not be desirable to retain larger amounts of degraded proteoglycans within a construct, the pore size and porosity should be chosen to maximize retention of the intact monomers. It is also interesting to note that the proteoglycan monomers present in the bulk fluid and the permeate were slightly smaller in size than monomers within the construct and that sizes were similar for different membranes. There are variations in size for proteoglycan

monomers, so this difference between the size distribution was probably due to smaller proteoglycans (intact and non-intact) diffusing into the medium compartments faster than the larger proteoglycans (intact and non-intact) remaining in the construct compartment.

The amount of collagen retained by the membranes may also be related to the pore size but was most likely dependent on the porosity. Newly synthesized procollagen is 1.5 nm in diameter [20, 39] and significantly smaller than all of the membrane pore sizes used in this study. The largest amount of collagen was retained on the smallest pore size, which was also the lowest porosity. In contrast to the results for proteoglycan content, the amounts retained on the other two pore sizes, which had similar porosities, were not significantly different. As with aggrecan, the procollagen molecules are not spherical but instead have a more cylindrical shape with a length of ~300 nm [33, 39, 63]. Thus the retention of collagen may also depend on the orientation of the molecule as they approach the membrane pores as well as on the form of the molecule.

In contrast to the proteoglycan and collagen content, the cellularity of the constructs was not significantly affected by the pore size. Since the average diameter of a chondrocyte is around 10 μm [19, 60] which was much larger than any of the pore sizes studied, it was expected that the majority of cells would be retained on top of the membranes, regardless of pore size. However, the results indicated that some cells were present in the permeate portion of the media. This may be explained by considering that the cells in the permeate compartment were dead and were not intact and their debris passed through the membranes. When the permeate portion was examined under the microscope, there were no visible cells. Most likely, the DNA

content measured in the permeate compartment is derived from cell debris that has passed through the membranes.

The effect of membrane pore size on constructs derived from ARC but not FIC may be due to a number of reasons, including the phenotype of cells with ARC versus FIC constructs and the consequent production of aggrecan. ARC cells have been shown previously to form cartilaginous tissue *in vitro* [26]. Cells that are encapsulated in alginate maintain their phenotype and synthesize aggrecan molecules that will form aggregates, in contrast to those cells that have lost their phenotype which will synthesize smaller non-aggregating proteoglycans [17, 30]. Thus, the FIC cells may have been less phenotypically stable and the molecules produced by those cells passed through the membrane pores. Additionally, the ARC cells are seeded with their cell associated matrix, and this initial amount of matrix may also partly account for the higher total amounts of matrix retained in the ARC constructs. In this experiment, the seeded ARC cells contained 63 ± 5 μg of sGAG versus the FIC cells which contained no sGAG. The presence of this surrounding layer of cell associated matrix may also provide protective effects for the ARC cells under perfusion conditions, which may explain the higher proportion of dead FIC cells within the permeate compartment. Although it has been demonstrated that ARC constructs may be formed in this culture system, it may not be possible to create a robust construct from FIC without use of a scaffold, or without longer culture periods.

This study makes use of the mechanism of selective retention that has been traditionally used in industry for ultrafiltration applications, to concentrate suspensions of particles, colloids and macromolecules [27] and indicates an application for cartilage tissue engineering. The bioreactor setup of the membrane on top of the frit is analogous to ultrafiltration setups which consist of an anisotropic

membrane, in which a thin layer with small pore size supported by a thicker layer or larger pore size to decrease hydraulic resistance [27]. In addition to the expected effects of retention of based on pore size and porosity, there are also other effects that can occur with a membrane filtration process. Molecules which are smaller than or of a similar size to the pore size can also be retained by the membrane via effects such as pore plugging, pore adsorption, or hydrodynamic bridging [51]. Another effect that can occur is concentration polarization, in which the accumulation of retained molecules at the surface of the membrane can create a concentration gradient [27, 51]. This concentration polarization effect can develop into a phenomenon which is described as cake formation. During cake formation, a layer on top of the membrane forms, which differs in rheological and mechanical properties from the overlying solution, and becomes in essence another membrane through which the solutes and solvent must pass; this layer also has a maximum concentration of solutes at the surface of the membrane [3, 12, 27, 51]. The occurrence of this layer can decrease the permeability of the membrane.

The developing cartilage construct that forms on the surface of the membrane may exhibit aspects of the cake formation and concentration polarization phenomena. The cell-associated matrix initially present around the ARC cells may aid in the development of a cake layer and thus increase retention of matrix in comparison to the FIC cells. With the higher initial amount of matrix in the ARC culture and also the synthesis of larger molecules, there may also be a greater effect of concentration polarization in those cultures, where the molecules build up at the surface and act as a second membrane. With these effects, it is possible that there may be a depth-dependent matrix molecule concentration throughout the construct [21]. Native cartilage exhibits a lower concentration of proteoglycans at the articular surface and

within the superficial layer, with the concentration increasing with increasing depth into the middle and deep layers, and the deepest layers exhibiting a relatively constant concentration [8, 21]. The addition of a semi-permeable membrane to the perfusion bioreactor for cartilage tissue engineering also recapitulates the low-permeability behavior of the deep region of immature and mature articular cartilage. This system could potentially be used to recreate the depth-dependent properties of the tissue.

One of the overall goals of tissue engineering of cartilage is to create tissue with properties similar to native cartilage. In comparison to native bovine cartilage at various stages of maturation [58], sGAG content on a per cell basis of the tissue engineered constructs in the 0.05 and 0.4 μm pore size condition (0.20 and 0.13 mg sGAG per million cells, respectively) is comparable to sGAG content in the calf and fetal native tissue (0.25 and 0.15 mg sGAG per million cells, respectively). The collagen content for all constructs are considerably lower than the content found in native tissue at all stages of development, which is typical of cartilaginous constructs [14]. The construct properties that are required depend on the nature of the application; a construct with properties similar to immature cartilage may have more integration potential when implanted, whereas a construct with properties similar to mature cartilage may be useful for an application that requires mechanical load bearing [22]. In terms of proteoglycan content, these constructs may resemble immature native tissue, but there needs to be a significant increase in the collagen content if the goal is to mimic the properties of native cartilage.

The modeling portion of the study demonstrated that a compartmental model could be developed to describe the effects of a membrane in this bioreactor system. The model does not predict values that match experimental values exactly, but has potential utility in describing trends and approximating experimental behavior. The

simulation results showed patterns with changing pore size (σ) that were consistent with those expected and found in experimental data, with increasing proteoglycan content in construct and bulk fluid compartments and decreasing proteoglycan content in permeate compartments with decreasing pore size and porosity (increasing σ). However, in comparison to experimental data, the model appeared to underestimate the amount of proteoglycans distributed into the bulk fluid compartment and overestimate the amount of proteoglycans distributed into the construct compartment. Based on the results of the size analysis and aggregation ability portion of the experiment, there are degraded proteoglycans within the bulk fluid compartment, which this version of the model does not describe. For simplification, the degraded proteoglycans were assumed to only enter the permeate, since to account for them entering the bulk fluid would require the addition of another subcompartment within the bulk fluid to exchange with the construct subcompartment, since these proteoglycans cannot be bound and thus cannot be considered part of the soluble proteoglycan pool. Additionally the assumption of the same reflection coefficient for both intact and degraded proteoglycans does not account for the differences of the retention of the two types of proteoglycans, which was demonstrated experimentally. Modification of the model to include an additional reflection coefficient for the degraded proteoglycan and also to describe the inclusion of the degraded proteoglycans in the bulk fluid compartment may improve the estimation of the bulk fluid proteoglycan content.

More experimental data and experiments designed to directly measure values for model parameters within this system would help further refine the model and increase its utility. Measurements of parameter values for rate of formation, binding and degradation of proteoglycans (using radiolabel experiments) under both static and

perfused condition would be important, to determine whether or not the assumption that these values are constant is valid. Modification of the model to include a time-dependent term for the reflection coefficient term, to indicate the decreasing permeability of the membrane as the construct develops on its surface, might also increase the accuracy of the simulation. Reflection coefficients could be estimated by flowing solutions of proteoglycans through membranes of known pore size and measuring resulting permeate concentrations. With knowledge of parameters, this model could also be used to describe other molecules of interest and their distribution within the bioreactor system. Potentially, this model can be adapted for describing other similar systems with membranes, static or perfused.

This study has shown that implementation of a semi-permeable membrane in a perfusion bioreactor can enhance the retention of matrix molecules in a developing ARC cartilaginous construct. This membrane can be used to prevent the loss of molecules that are newly synthesized in a perfusion system that would otherwise be lost due to convection through the porous tissue. Through selection of the membrane pore size and porosity, the amount of matrix in the construct can be modulated, with larger amounts of matrix corresponding with smaller pore size and lower porosity. The retention of degraded monomers versus intact monomers should also be considered when selecting a membrane for construct modulation. A membrane with a pore size between 0.05 and 0.4 μm (and porosity between 1.2 and 15.7%) may maximize the retention of intact monomers. Thus, the perfusion system with a semi-permeable membrane may be a useful tool for accelerating the maturation of cartilaginous construct.

4.6 Acknowledgements

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CHAPTER 5

CONCLUSIONS

5.1 Summary of Findings

This dissertation examined two important aspects of cartilage tissue engineering: cell sourcing for large-scale clinical applications, and modulation of matrix content in cartilaginous constructs. Human chondrocytes from osteoarthritic (OA) knees were identified as a feasible source for creating large-scale human cartilaginous constructs (Chapter 2). The expansion of these cells was accelerated by the application of the growth factor cocktail containing TGF- β 1, FGF-2 and PDGF-bb (TFP), and redifferentiation post expansion was enhanced by the application of human serum (HS), which allowed the formation of chondron-like structures similar to those found in native cartilage. The ability of these expanded and redifferentiated chondrocytes derived from OA knees to form constructs was demonstrated, along with the effects of the growth factors GDF-5, IGF-I and TGF- β 1 and HS on their properties (Chapter 3). IGF-I and TGF- β 1 in combination were found to have increase proteoglycan and collagen content, whereas HS increased collagen content and compressive modulus. Semi-permeable membranes of pore sizes 0.05, 0.4 and 1.0 μ m with corresponding porosities of 1.2, 12.7 and 15.7%, were applied to modulate matrix content in cartilaginous constructs in a perfusion bioreactor system and a

compartmental model was formulated to describe the effects (Chapter 4). The amount of matrix retained within the construct was dependent on the pore size and porosity; smaller pore sizes and lower porosities resulting in larger amounts of matrix within the construct, and correspondingly, smaller amounts of matrix passing through the membrane into the medium. Additionally, the smallest pore size resulted in higher retention of degraded proteoglycans within the construct.

In summary, chondrocytes from OA cartilage can be used as a cell source for human cartilage tissue engineering, and a semi-permeable membrane can be used to modulate matrix content in cartilaginous constructs. These findings have potential application for clinical therapies, for advancing the use of cartilage tissue engineering for repair of large scale defects.

5.2 Discussion

Major contributions of the current work include the following: 1) development of methods for use of human chondrocytes derived from OA cartilage as a cell source for large scale human cartilage tissue engineering, 2) demonstration of ability of human chondrocytes derived from OA cartilage to form cartilaginous constructs, 3) demonstration of the ability to modulate construct matrix content by application of membranes of different pore sizes and porosities in a perfusion bioreactor system, 4) development of a compartmental model to describe membrane effects on construct matrix content in a perfusion bioreactor system. Clinical implications of this work include: 1) abundant and readily available cell source for resurfacing of OA joints with tissue engineered cartilage and 2) modulation of matrix content in tissue engineered cartilage using a semi-permeable membrane.

The methods in this work for expansion and redifferentiation of chondrocytes from OA cartilage were developed based on previous work with normal human chondrocytes [2, 3]. During the expansion phase, application of the growth factor cocktail TFP along with serum (bovine or human) increased the growth rate 5.5-fold from that under basal conditions. During the redifferentiation phase, application of HS increased the collagen type II content and also resulted in the formation of chondron-like structures (clusters of cells surrounded by pericellular matrix). This pericellular matrix may provide protective and signaling functions for the cells [7, 8, 15, 20] and encourage cartilaginous tissue formation. These methods are readily translatable to clinical processes, since they incorporate the use of HS and defined growth factors in medium. Chondrocytes derived from OA cartilage could also potentially be used in an autogenic or allogeneic manner as a cell source for large-scale human cartilage tissue engineering. In clinical practice, osteochondral allografts are commonly used for repair of defects, with minimal immunogenic response. In a similar manner, a tissue engineered cartilaginous construct from allogeneic sources; when chondrocytes are surrounded by matrix, they are considered to be immunoprivileged [14, 18, 23]. As an allogeneic cell source, with over half a million total joint replacement (knee and hip) surgeries, OA cartilage is readily available and abundant [10].

Constructs were formed from the expanded and redifferentiated chondrocytes derived from OA cartilage, indicating the utility of OA cartilage as a cell source. Based on the results of the expansion and redifferentiation study, the condition of TFP with 2% HS was used for expansion and 20% HS was used for redifferentiation. Additionally, the effects of HS and the growth factors GDF-5, IGF-I and TGF- β 1 (growth factors important during chondrogenesis and cartilage development) individually and in combination on construct properties were determined. The results

indicated that IGF-I and TGF- β 1 had synergistic effect on increasing the wet weight, proteoglycan and collagen content of the constructs. Application of HS increased compressive modulus, and increased collagen content per wet weight, but did not increase proteoglycan per wet weight correspondingly. This result indicates that HS may be useful for modulation of construct properties to have increased collagen to proteoglycan ratios, as tissue engineered cartilage typically have high proteoglycan to collagen ratios in comparison to native cartilage. These methods are also readily translatable to clinical application, with the usage of culture medium containing HS and defined growth factors.

A semi-permeable membrane was applied in a perfusion bioreactor system to enhance the retention of proteoglycans and collagen (that may otherwise be lost due to convection) within the cartilaginous constructs. Both alginate recovered chondrocytes (ARC) and freshly isolated chondrocytes were used for construct formation, but there were only membrane effects on matrix content in the constructs formed from ARC, with smaller pore sizes and lower porosities retaining larger amounts of proteoglycan and collagen in the construct. The size of the proteoglycan monomers was the smallest in constructs formed on the membrane of the smallest pore size (and lowest porosity), which was consistent with the result that those constructs also had the highest amount of degraded proteoglycan (which is smaller than intact proteoglycan). As larger amounts of degraded proteoglycan may not be desirable in a construct, the membrane pore size and porosity should be selected accordingly to maximize total retention of proteoglycan with reduced retention of degraded proteoglycan; based on the results of this study, this membrane would have a pore size larger than 0.05 μm and less than 0.4 μm (and porosity between 1.2 and 12.7%). Proteoglycan content was also measured in the medium compartments upstream (bulk fluid) and downstream (permeate) of the

membrane, and also varied accordingly with pore size and porosity, with smaller amounts of proteoglycan present in the bulk fluid and larger amounts of proteoglycan present in the permeate with increasing pore size and porosity. The resulting constructs are comparable to immature native bovine cartilage on a proteoglycan content per cell basis, although collagen content was much lower than that found in native cartilage, which is typical of tissue engineered cartilage. Potentially, the collagen content can be increased by the addition of growth factors, such as TGF- β 1 [6]. This study examined the effects of membranes of three pore sizes with three different porosities; other pore sizes may result in different matrix content and distribution within the construct and medium compartments and need to be tested. It is also of interest to test membranes of different pore sizes with the same porosities to separate effects of pore size and porosity on matrix retention in the construct. Based on repair needs, a semi-permeable membrane can be used to modulate the matrix properties in a cartilaginous construct by selection of the appropriate pore size and porosity to resemble immature cartilage (for integration), or mature cartilage (load bearing) [13].

The compartmental model was developed to describe the effect of the semi-permeable membrane on proteoglycan content and distribution with a developing cartilaginous construct in the perfusion bioreactor system. Model simulations approximated the behavior of membranes of different pore sizes and porosities as described by a reflection coefficient (σ). Consistent with experimental data, simulations indicated increases in proteoglycan content in construct and bulk fluid compartments and a decrease in permeate compartment with increasing pore size and increasing porosity. There were a number of assumptions in the formulation of the model for simplification of analysis, which can be re-examined to improve the utility

of the model. In particular, the reflection coefficient, σ , which is assumed to be constant, should be allowed to vary with time, indicating decreasing permeability of the membrane as the construct develops on the surface. For specific applications, direct measurement of model parameters will allow for more accurate simulation of experimental data. This model can be adapted to describe other systems containing membranes with or without perfusion, and also be used to describe other molecules of interest.

The findings of this work have implications for the development of clinical applications of tissue engineered cartilage for joint-scale repair. For example, chondrocytes from a patient's OA knee could be expanded and redifferentiated to the level needed for creating a cartilaginous construct for joint resurfacing. These chondrocytes could then be cultured with growth factors, HS and a semi-permeable membrane to modulate the matrix properties of the resulting construct. In the case of joint resurfacing, the properties of the tissue engineered cartilage would need to be similar to adult cartilage, to allow load-bearing function. Taken together, the results of this dissertation work have contributed to the development of methods for cartilage tissue engineering. The findings reported here are consistent with the overall hypothesis that chondrocytes from OA knees can be utilized as a cell source for large scale human cartilage tissue engineering and a semi-permeable membrane can be used to modulate the matrix properties of cartilaginous constructs.

5.3 Future Studies

The current work can be expanded in the future in a number of ways. The methods developed for expansion and redifferentiation of chondrocytes derived from

OA cartilage could be implemented with chondrocytes derived from intact normal cartilage. The chondrocytes from OA cartilage could be used to form constructs at increasingly larger scales, with the eventual goal of creating a construct on the order of the whole-joint scale. Constructs could also be tailored to have the zonal properties found in native cartilage by layering cells isolated from superficial, middle and deep zones. The semi-permeable membrane could potentially be used to create depth-dependent matrix properties, as the dynamics of membrane filtration include polarization phenomena that can create a concentration gradient. The compartmental model can be expanded upon to more fully describe the features of the membrane in the perfusion bioreactor system.

The methods used for expansion and redifferentiation of chondrocytes derived from OA cartilage in this study can be applied to normal human chondrocytes. In particular, it may be useful to examine if the effects of HS on redifferentiation (which has not previously been applied to articular chondrocytes) are similar or markedly different on chondrocytes with a normal phenotype, by designing an experiment where chondrocytes from OA and normal cartilage are compared directly. Additionally, in this study, only macroscopically normal cartilage from the OA knees were utilized, and it may be of interest to investigate the use of chondrocytes from portions of non-intact cartilage in OA knees. It has been shown that OA chondrocytes have a different gene expression profile than normal chondrocytes [1, 22, 24], and may also have decreased matrix synthesis [4, 22]. One study has shown that with expansion, OA and normal chondrocytes do not exhibit major differences in gene expression patterns [16]. It would be of interest to determine whether the expansion and redifferentiation methods in this work may similarly affect chondrocytes derived from OA and normal cartilage, with additional analysis of the expression patterns, in particular for the

aggrecan and collagen type II genes which are markers of the chondrogenic phenotype.

The ability of the expanded and redifferentiated chondrocytes from OA cartilage to form constructs on the scale of focal defects was demonstrated. Ultimately, the goal is to create constructs from these cells that can repair large scale defects and eventually resurface a whole joint. The constructs formed in this study were circular disks of 3.2 and 6.5 mm diameter, useful for purposes of determining the feasibility of forming constructs and screening various media formulations. Future studies should examine formation of constructs in the size range of 3-4 cm², for repair of typical size defects [11], eventually scaling up to ~120 cm² for the resurfacing of a entire knee joint [5]. For resurfacing of an OA joint, these constructs may have to be shaped during culture in order to fit the contours of the joint, which may be achieved with the usage of molds [9]. To obtain the desired mechanical properties, it may also be necessary to implement a whole joint bioreactor that provides mechanical stimulation via a continuous passive motion machine [19].

Native cartilage has a distinct zonal organization, where cells are separated in superficial, middle and deep zones, and matrix composition and content vary with depth. Recapitulating this zonal organization may be important to the functionality of a tissue engineered construct. The current study utilized cells from full thickness OA cartilage to ensure a high yield of isolated cells. However, there may be differences in the proliferation and redifferentiation of cells from the different zones, which can be determined experimentally using the same methods as for the full thickness cells. The cells from the various zones could be layered in a construct to mimic the organization in native cartilage. Previously, this technique has been used to achieve depth-dependent properties in tissue engineered constructs using bovine chondrocytes [13].

Of particular interest may be the ability to achieve the secretion of the lubricating molecule PRG4 (secreted by superficial zone chondrocytes) at the surface of the construct using this method [13], which is crucial to enabling the smooth movement in the knee joint.

In addition to using cells isolated from different cartilage zones to create a construct that more closely resembles native cartilage, the semi-permeable membrane may be useful for modulation of depth-dependent properties. Although the effect of membrane pore size and porosity was only examined for bulk matrix properties, there may be a potential application for depth-dependent matrix properties in constructs due to the concentration polarization effect. During filtration of molecules through a membrane, a concentration gradient results (with the highest concentration at the surface of the membrane) as the retained molecules diffuse back into the bulk fluid [17, 21]. Similarly, for constructs grown on top of a semi-permeable membrane, a depth-dependent matrix molecule concentration gradient can develop [12]. For proteoglycans, this depth-increasing gradient would be consistent with the depth-increasing content found in native cartilage.

The compartmental model describing the effects of a semi-permeable membrane on content and distribution of proteoglycans could be modified to more closely describe the system. Potential modifications include: 1) addition of a time-dependent reflection coefficient, to describe the effect of the development of the construct on the surface of the membrane on decreasing permeability, 2) an additional compartment to indicate degraded proteoglycans within the bulk fluid, 3) an additional reflection coefficient to describe the effect of the membrane on retention of degraded proteoglycans, which are smaller than intact proteoglycans and 4) perfusion-dependent rates of proteoglycan formation, binding and degradation. Time course

experiments should be designed to measure the amount of proteoglycan in the different compartments at different time points, to validate the model.

Overall, the results of this dissertation are a step toward the eventual goal of cartilage tissue engineering is the creation of a biological joint replacement in place of the currently available artificial joint replacement. With a readily available source of human chondrocytes, studies to scale up culture of cartilaginous cultures can be performed, with the target of designing a construct or constructs that can be used to create a layer of tissue engineered cartilage that can be attached to a bone substrate to form a complete joint. The semi-permeable membrane can be implemented in the culturing of this cartilage tissue to tailor the biochemical properties to resemble those found in native cartilage, to provide load-bearing capacity to the biological joint.

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