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BI-ZONAL CARTILAGINOUS TISSUES

ENGINEERED IN A ROTARY CELL CULTURE SYSTEM

A Marsano*, D Wendt*, TM Quinn**, TJ Sims°, M Heberer*, I Martin*

* Departments of Surgery and of Research, University Hospital Basel, CH

** Cartilage Biomechanics Group, Ecole Polytechnique Fédérale de Lausanne (EPFL), CH

° University of Bristol, Academic Rheumatology. Southmead Hospital, Bristol, UK.

Address correspondence to:

Ivan Martin

Institute for Surgical Research and Hospital Management

University Hospital Basel

Hebelstrasse 20,

4031 Basel, Switzerland

phone: + 41 61 265 2384; fax: + 41 61 265 3990

e-mail: imartin@uhbs.ch

Running head: Engineering of bi-zonal cartilage tissues

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Abstract

In this study, we aimed at validating a rotary cell culture system (RCCS) bioreactor with medium recirculation and external oxygenation, for cartilage tissue engineering. Primary bovine and human culture-expanded chondrocytes were seeded into non-woven meshes of esterified hyaluronan (Hyalograft®C), and the resulting constructs were cultured statically or in the RCCS, in the presence of insulin and TGFβ3, for up to 4 weeks. Culture in the RCCS did not induce significant differences in the contents of glycosaminoglycans (GAG) and collagen deposited, but markedly affected their distribution. In contrast to statically grown tissues, engineered cartilage cultured in the RCCS had a bi-zonal structure, consisting of an outgrowing fibrous capsule deficient in GAG and rich in collagen, and an inner region more positively stained for GAG. Structurally, trends were similar using primary bovine or expanded human chondrocytes, although the human cells deposited inferior amounts of matrix. The use of the presented RCCS, in conjunction with the described medium composition, has the potential to generate bi-zonal tissues with features qualitatively resembling the native meniscus.

1. Introduction

Hydrodynamic flow conditions generated using different culture systems have been demonstrated to modulate the development of engineered cartilage [5]. In particular, the use of Rotary Cell Culture Systems (RCCS), often referred to as rotating wall vessels, has been reported to promote the development of cartilaginous tissues with increased amounts of glycosaminoglycans (GAG) and collagen, and more uniform spatial distributions of extracellular matrix as compared to statically cultured constructs [13,14,15,16]. These findings were attributed to the dynamic laminar flow in the RCCS, which enhanced mass transport around the constructs, while generating minimal turbulent eddies and shear stresses.

The version of the RCCS typically used for scaffold-based tissue engineering approaches (i.e., the Slow Turning Lateral Vessel, STLV) has recently been extended beyond the basic *batch mode* models towards more integrated configurations in which culture medium is continuously recirculated through the culture vessel and an external flow loop (Fig 1). These perfused models allow for medium sampling and exchange without interrupting bioreactor rotation, facilitate inline monitoring of the culture media components [6,17], and provide a means for external gas exchange. Since medium is recirculated through an external oxygenator, the role of the inner co-axial cylinder as an oxygenator is obsolete in perfused systems. Removal of this cylinder would not only reduce the frequency of damaging collisions between constructs and vessel walls, but would allow for more flexible bioreactor designs. Larger constructs could be cultured without increasing the radial dimensions of the outer vessel, which would otherwise lead to a significant increase in the volume of required medium. This is particularly relevant in terms of operating costs if expensive medium supplements (e.g., growth factors) are to be used.

In this paper we aimed at validating a perfused STLV RCCS configuration without the concentric inner cylinder, designed with a relatively small (40 ml) culture chamber, for the engineering of cartilaginous tissues. The system was first tested with primary bovine chondrocytes, typically used in studies performed with other RCCS designs, and then with

human culture-expanded chondrocytes, representing a clinically relevant cell source. Cells were seeded in porous scaffolds made of esterified hyaluronan (Hyalograft®C) and the resulting constructs were cultured in the RCCS in the presence of soluble factors (i.e., insulin and TGFβ) known to enhance GAG and collagen deposition [2,10].

2 Materials and Methods

2.1 Bioreactor configuration

The RCCS used in this study, manufactured by Synthecon Inc. (Texas, USA), consisted of a culture vessel and a medium recirculation system (Fig 1). The cylindrical vessel was 3.5 cm in length and 3.8 cm in diameter, with a culturing volume of 40 ml. The medium recirculation system was composed of a peristaltic pump (314D pumphead, 4 roller; Watson Marlow), a valve manifold, a bubble trap, and an oxygenator.. The valve manifold was used for the initial filling of the bioreactor system and for the periodic changes of the culture medium. Recirculating medium entered the bubble trap (maintained approximately one-third full with medium) through a top port and exited through a port at the base to eliminate small bubbles entrapped in the culture medium supply lines The oxygenator consisted of a 2 m length coil of silicon tubing (Tygon 3350; 3/32" i.d., 5/32" o.d.). A total volume of 75 ml culture medium was used to fill the culture vessel and recirculation system. The RCCS was placed into a conventional humidified 5% CO_2 incubator.

2.2 Cell Isolation and Expansion

Bovine articular cartilage was collected from the femoropatellar grooves of two 6 months-old cows. Human articular cartilage was collected from the femoral condyle of two cadavers (20 and 32 years of age), with no known clinical history of joint disorders, within 24 hours after death, after informed consent of the relatives and approval by the local ethical committee. Cartilage tissues were finely minced and digested by incubation for 22 hours at 37°C in 0.15% type II collagenase (Worthington Biochemical Corporation, Lakewood, NJ) (1ml solution per 100mg

tissue). Isolated cells were resuspended in “complete medium”, composed of Dulbecco’s Modified Eagle Medium (DMEM; 4.5 g/L glucose with nonessential amino acids), 10% fetal bovine serum, 0.1mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.29 mg/mL L-glutamine. Human articular chondrocytes were expanded for two passages in complete medium further supplemented with 1 ng/mL Transforming growth factor-β1 (TGF-β1), 5 ng/mL Fibroblast growth factor-2 (FGF-2) and 10 ng/mL Platelet-derived growth factor-bb (PDGF-bb), previously shown to increase human chondrocyte proliferation rate and post-expansion chondrogenic capacity [1,9].

2.3 Cell seeding and culture in porous scaffolds

Expanded human or freshly isolated bovine articular chondrocytes were seeded into non-woven meshes (5.5 mm diameter, 2 mm thick disks) made of esterified hyaluronan (Hyalograft®C, Fidia Advanced Biopolymers, Abano Terme, Italy), at a density of 4E + 06 cells/scaffold (7E + 07 cells/cm^3). Briefly, 4E + 06 cells were resuspended in 28μL of complete medium and slowly dispersed over the top surface of the dry meshes with a micropipette. The seeded scaffolds were cultured for 2 days in 12 well-plates before being transferred either to the described RCCS or to 6 well-plates. Cell-scaffold constructs were then cultured for 4 weeks in complete medium further supplemented with 10 ng/ml of TGFβ3, 0.1 mM ascorbic acid 2-phosphate and 10 μg/ml of human insulin, in order to enhance chondrogenesis [2,10], with medium changes twice a week.

During culture in the RCCS, culture medium was continuously recirculated through the system at a flowrate of 0.6 ml/min. The angular velocity of the vessel was increased from 16 rpm at the beginning of the culture to 50 rpm after 4 weeks in order to maintain constructs in a continual freefall condition [11]. For each experiment, at least three specimens per experimental group were assessed histologically and biochemically as described below.

2.4 Histological assessment

Engineered tissues were rinsed in phosphate buffered saline, fixed in 4% buffered formalin for 24 h at 4°C, embedded in paraffin, and cross-sectioned (7 μm thick). Sections were stained with Safranin-O for GAG and with elastica-van gieson for collagen.

2.5 Biochemical assays

Engineered tissues were digested with 1 ml protease K solution (1mg/mL protease K in 50 mM Tris with 1 mM EDTA, 1 mM iodoacetamide, and 10 mg/mL pepstatin- A) for 15 h at 56°C [8]. DNA was quantified with the CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR), with calf thymus DNA as a standard. GAG was quantified with the dimethylmethylene blue colorimetric assay, with chondroitin sulfate as a standard [4]. Total collagen was quantified as follows: digested samples were hydrolyzed for 24 hours at 110°C in constant boiling hydrochloric acid and excess acid removed by lyophilization. Dried hydrolyzates were analyzed using a Biochrom 20 Plus amino acid analyzer equipped with post column Ninhydrin detection to measure the hydroxyproline amount. Collagen content was calculated using a hydroxyproline to collagen ratio of 1:10 [8].

2.6 Statistical analysis

Differences between the experimental groups were evaluated by non-parametric Mann Whitney U tests and considered to be statistically significant with $P<0.05$.

3. Results

3.1 Primary bovine articular chondrocytes

Cultivation of chondrocyte-scaffold constructs in the RCCS generated bi-zonal tissues, with distinct patterns of spatial distribution of GAG and collagen (Fig. 2). In particular, the central region was intensely stained for GAG, whereas the external region was negatively stained for GAG and contained a higher density of elongated cells. The external capsule was outgrowing from the original scaffold, as indicated by the lack of scaffold fibers, and was more intensely

stained for collagen than the central core. In statically grown constructs, the intensities of GAG and collagen stain were relatively uniform throughout the cross-sections and generally lower than those observed respectively in the central and outer regions of the RCCS-cultured tissues. The dry weight fractions of GAG and total collagen were similar in constructs cultured statically and in the RCCS for 2 or 4 weeks, with no significant increase with time (Fig. 3).

3.2 Expanded human articular chondrocytes

As compared to primary bovine chondrocytes, expanded human chondrocytes generated tissues which were less intensely stained for GAG, but with a similar pattern in the GAG and collagen distribution (Fig. 4). In particular, culture in the RCCS resulted in the formation of a fibro-cartilaginous tissue outgrowing from the original scaffold area, although more irregular in shape than using bovine chondrocytes. The outgrowing tissue was negatively stained for GAG but was more intensely stained for collagen than the construct internal region. The central construct region, corresponding to the original scaffold area, was more intensely stained for GAG following RCCS than static culture.

Similar to constructs generated from bovine cells, the dry weight fractions of GAG and total collagen were comparable in constructs cultured statically and in the RCCS (Fig. 5). However, the fractions of GAG and collagen increased between 2 and 4 weeks of culture, likely due to the progressive re-differentiation of the expanded cells.

4. Discussion

In this paper we describe the use of a perfused RCCS configuration for the engineering of cartilage tissues. While culture in the RCCS did not significantly affect the overall fractions of GAG and collagen in the engineered tissue, their distribution was strongly influenced, resulting in the formation of bi-zonal tissues, with an inner region stained predominantly for GAG and an outer capsule stained predominantly for collagen.

In previous studies which used a standard tissue culture RCCS vessel (i.e., concentric cylinder arrangement, without medium recirculation), the formation of relatively uniform cartilaginous tissues was reported [13,14,15,16]. Considering that hydrodynamics can have a dramatic impact on the development of engineered cartilage, we speculate that the fluid dynamics generated in the two configurations of the RCCS, with and without the central cylinder, may account for the apparent inconsistency. In the absence of 3D constructs, rotation of both vessel configurations would generate solid body rotation of the culture medium. However, with the introduction of 3D constructs into the flow (dynamically freefalling), it is likely that the mixing patterns and dissipation of turbulent eddies would be altered without the inner concentric cylinder. Interestingly, in these same studies, constructs engineered in mixed flask bioreactors were surrounded by fibrous external capsules, similar to those reported here. Perhaps the turbulent eddies and shear stresses in our RCCS are more similar to those in a mixed flask than in the standard RCCS design. Although the dynamic fluctuations of the freefalling construct represent a considerable challenge for a computational fluid dynamics model, flow visualization techniques could be used to better characterize the local velocity profiles and shear stresses around constructs in both vessel configurations.

Alternatively, the differing results could be explained by the medium supplementation with soluble factors not used in the previous studies. The presence of TGFb3, in conjunction with fluid flow, could have supported chondrocyte proliferation [12], which is known to be associated with a de-differentiated and fibroblastic phenotype [3,7]. The specific scaffold used is not likely to have determined the result, since uniform tissues were obtained when non-woven meshes of esterified hyaluronan were used in the standard RCCS design [14]. The outgrowth of a fibrous tissue in the RCCS occurred when either freshly isolated bovine or expanded de-differentiated human chondrocytes were used, and thus appeared to be generated by a mechanism common to various chondrocytic cells. Further experimental studies, in conjunction

with flow visualization techniques in the new configuration of the RCCS vessel, are necessary to elucidate the mechanisms underlying our findings.

The bi-zonal tissues generated in this study, with an outer fibrocartilage-like capsule and a central region containing larger amounts of GAG, in some respects resemble the structure of the meniscus. In this context, studies are ongoing to more precisely characterize the molecular composition and mechanical function of the engineered tissues and to compare them with those of native meniscus. Efforts in this direction could lead to the controlled use of hydrodynamic culture conditions and soluble factors for the engineering of meniscus substitutes.

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Figure captions

Figure 1. **Schematic diagram of the RCCS with medium re-circulating system**.

A: Bottle for waste medium; **B**: Valve manifold; **C**: Silicon tubing oxygenator; **D**: Bubble trap; **E**: Peristaltic pump; **F**: Cylindrical culture vessel, without inner core; **G**: Stepper motor.

Figure 2. **Histology of primary bovine chondrocyte-based engineered tissues.**

Representative sections of engineered tissues, generated after 4 weeks of culture statically (**A**, **C, E**) or in the RCCS (**B**, **D, F**). Sections were stained for GAG with Safranin O (**A, B, C, D**) or for collagen with elastica-van gieson (**E, F**). Scale bar = 200μm (**A**, **B**) or 100μm (**C, D, E, F**). The darker spots correspond to undegraded polymer fibers.

Figure 3. **Biochemistry of primary bovine chondrocyte-based engineered tissues.**

Biochemical quantification of GAG (**A**) and total collagen (**B**) in engineered tissues cultured statically or in the RCCS for 2 or 4 weeks.

Figure 4. **Histology of expanded human chondrocyte-based engineered tissues.**

Representative sections of engineered tissues, generated after 4 weeks of culture statically (**A**, **C, E**) or in the RCCS (**B**, **D, F**). Sections were stained for GAG with Safranin O (**A, B, C, D**) or for collagen with elastica-van gieson (**E, F**). Scale bar = 200μm (**A**, **B**) or 100μm (**C, D, E, F**). The darker spots correspond to undegraded polymer fibers.

Figure 5. **Biochemistry of expanded human chondrocyte-based engineered tissues.**

Biochemical quantification of GAG (**A**) and total collagen (**B**) in engineered tissues cultured statically or in the RCCS for 2 or 4 weeks.