Comparison of Chondrogensis in Static and Perfused Bioreactor Culture

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As a result of the low yield of cartilage from primary patient harvests and a high demand for autologous cartilage for reconstructive surgery and structural repair, primary explant cartilage must be augmented by tissue engineering techniques. In this study, chondrocytes seeded on PLLA/PGA scaffolds in static culture and a direct perfusion bioreactor were biochemically and histologically analyzed to determine the effects of fluid flow and media pH on matrix assembly. A gradual media pH change was maintained in the bioreactor within 7.4−6.96 over 2 weeks compared to a more rapid decrease from 7.4 to 6.58 in static culture over 3 days. Seeded scaffolds subjected to 1 μm/s flow demonstrated a 118% increase ($p < 0.05$) in DNA content, a 184% increase ($p < 0.05$) in GAG content, and a 155% ($p < 0.05$) increase in hydroxyproline content compared to static culture. Distinct differences were noted in tissue morphology, including more intense staining for proteoglycans by safranin-O and alignment of cells in the direction of media flow. Culture of chondrocyte seeded matrices thus offers the possibility of rapid in vitro expansion of donor cartilage for the repair of structural defects, tracheal injury, and vascularized tissue damage.

Introduction

Articular cartilage serves as the weight-bearing tissue of joint surfaces. Healthy articular cartilage is composed of immobilized chondrocytes in a secreted extracellular matrix (ECM). The ECM is composed of type II collagen (*1*) and proteoglycans rich in sulfated glycoaminoglycans (S-GAG). Joint loading from body movement induces fluid velocities in the tissue that maintain waste and nutrient flow, as well as ion movement. Since tissue pH is related to content of collagen and proteoglycan within the ECM, chondrocytes are able to mediate ECM biosynthesis through a pH feedback loop (*2*).

Demand for cartilage for reconstructive surgery to treat injury and disease such as osteoarthritis is high, leading to interest in cartilage tissue engineering techniques (*3*). The use of polyglycolic acid (PGA) and poly-L-lactic acid (PLLA) as scaffold materials to localize cells and promote focal matrix accumulation has shown much promise (*4*). Studies demonstrating that chondrocyte metabolism is dependent on pH (*2*) raise concerns about the use of these polymers that produce acidic degradation fragments. However, the effect of scaffold degradation on media pH in culture has not been determined.

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The growth of chondrocytes on PGA matrices has been augmented by the use of flow bioreactors to increase transport of nutrients to and waste away from cells (*3*). Various designs have been aimed at increasing mass transfer rates, including magnetic impellor stirring bioreactors and shaker flasks in which constructs are free-floating (*3*) and rotating bioreactors in which constructs are subjected to secondary flows (*5*). The limitations of such designs include unregulated and unknown fluid flows through the construct in culture. Fluid flows of ~1 μm/s have been shown to accelerate cartilage ECM assembly (*6*); this phenomenon has not been exploited in the process of bioreactor culture.

The objectives of this study were (1) to characterize ECM assembly by chondrocytes in PLLA/PGA scaffolds in static culture and in those perfused with a physiologically relevant fluid velocity and (2) to assess the relative roles of cell metabolism and polymer degradation in regulating environmental pH in both static and bioreactor culture.

Methods and Materials

Cell Isolation and Construct Seeding. Circular disk scaffolds of nonwoven PGA (Albany International Research, Mansfield, MA) mesh were cut to a 12.7 mm diameter × 1 mm thickness and coated with PLLA (Polysciences, Warrington, PA) by solvent evaporation (*7*). Chondrocytes isolated and purified from bovine calf glenohumeral joint surfaces (A. Arena Brothers, Hopkinton, MA) (*8*) were seeded on scaffolds for 24 h at a density of 2.5×10^6 cells/scaffold in 6-well plates with 5 mL of Ham's F-12 (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, 100

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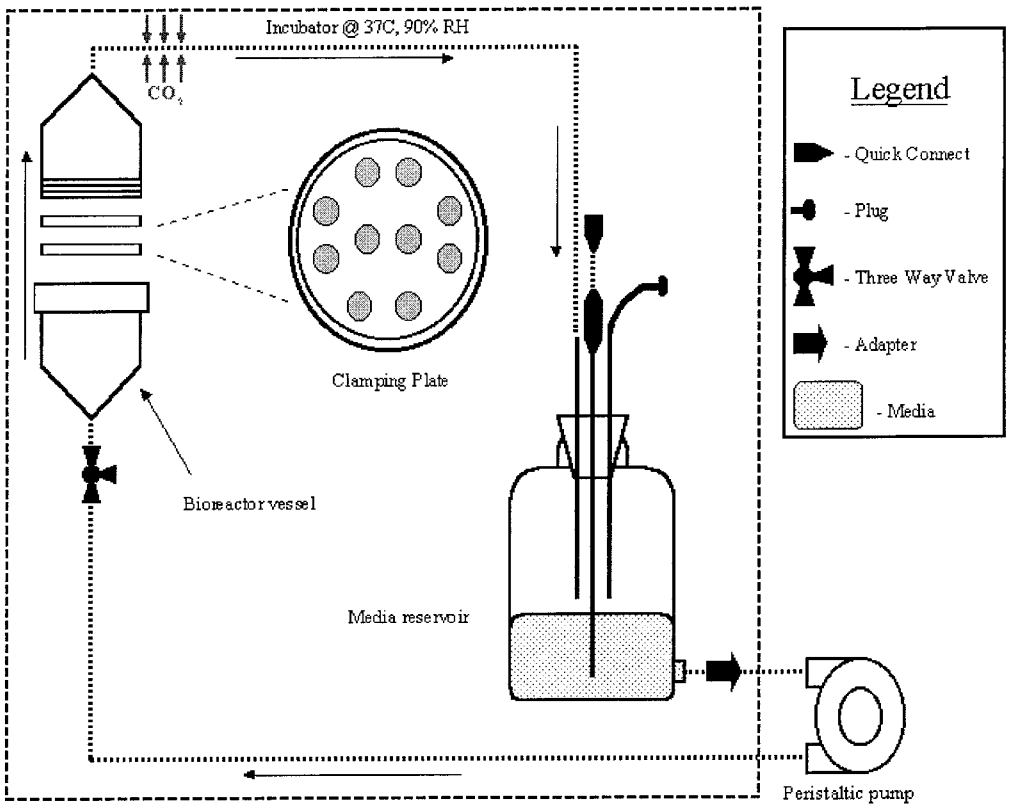

Figure 1. Schematic representation of the perfusion bioreactor system assembly.

μg/mL streptomycin, and 25 μg/mL L-ascorbic acid (Fisher Scientific, Pittsburgh, PA).

Bioreactor Design and Assembly. The perfusion bioreactor system was designed to direct constant fluid flow through developing tissue without stagnant and secondary flows. The bioreactor vessel forced influent media through cultured constructs as media flows from the bottom to the top of the bioreactor vessel (Figure 1). Gas exchange occurred across the silicone tubing that supplies and returns medium within the incubator. Ample gas exchange and equal fluid flow distribution were demonstrated during the operational and sterility testing phases of the bioreactor. Media flow was controlled by a peristaltic pump (Cole-Palmer, Vernon Hills, IL), which regulated flow rates from 0.1 to 2.0 mL/min, which translates to 0.3−10 μm/s in linear flow velocity, through the scaffolds. These studies were conducted at a fluid velocity of 1 μm/s, which is known to stimulate cartilage matrix biosynthesis (*6*).

Ten seeded PLLA/PGA disks were placed in the perfusion bioreactor, which imposed a 1 μm/s flow through the disks in the axial direction. A total volume of 500 mL of media perfused the entire system via a recirculating peristaltic pump. Two separate control groups were established to understand the effects of bioreactor culture. Static control disks were placed 6-well plates filled with 5 mL of media that was changed every 2−3 days, consistent with standard cartilage organ culture (*2*). In parallel, high volume control studies were established with seeded disks grown in static culture with media volumes equivalent to the 50 mL of media per disk of the bioreactor. This control group allows the assessment of the effect of media volume on the rate of chondrocyte matrix biosynthesis and proliferation.

Biochemical and Histological Analysis The pH of the media from both bioreactor and static culture were measured twice weekly. Media and disk samples were extracted from the bioreactor and static culture every 2 weeks. Disks were weighed and cut in halves used for histological and biochemical analysis, respectively. Portions used for biochemical analysis were digested with papain and assayed for DNA content, as a measure of chondrocyte number, by Hoechst 33258 dye binding (*9*), S-GAG content by 1,9-dimethylmethylene blue chloride (*10*), and hydroxyproline content by reaction with dimethylaminobenzaldehyde (DMAB) (*11*). Samples for histological examination were fixed in formalin, embedded in paraffin, sectioned via microtome, and stained with safranin-O/fast green (*12*).

Statistical Methods. Differences between DNA, S-GAG, and hydroxyproline content of static and bioreactor cultured constructs were determined by two-tailed Student's *t* test. Data presented here represent comparison of samples from a single run of the bioreactor. An additional two bioreactor runs yielded data within 20% of that presented here at each time point, with the same relative trends between static and bioreactor samples.

Results

Bioreactor media gradually decreased in pH from 7.4 to 7.0 over 2 weeks (Figure 2). In contrast, rapid, successive decreases in pH from 7.4 to 6.6 of seeded static cultures were observed over 3-day intervals. Unseeded matrices in static culture showed successive moderate decreases in pH from 7.4 to 7.1 in periods between media changes. Histological examination of safranin-O/fast green stained samples by light microscopy revealed slight staining for proteoglycans was observed in static cultures, with no orientation of cell growth and ECM biosythnesis (Figure 3a). Intense staining for proteoglycans and columns of chondrocytes and ECM aligned in the direction of media flow in the bioreactor (Figure 3b).

Biochemical analysis of static and bioreactor culture samples showed a 118% increase ($p < 0.05$) in DNA

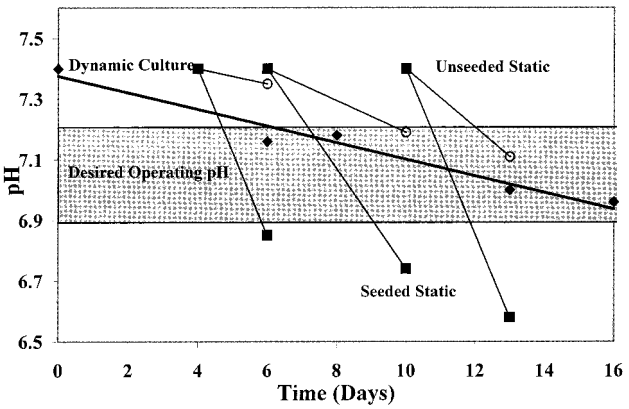

Figure 2. Comparison of environmental pH for seeded static, unseeded static, and bioreactor culture conditions.

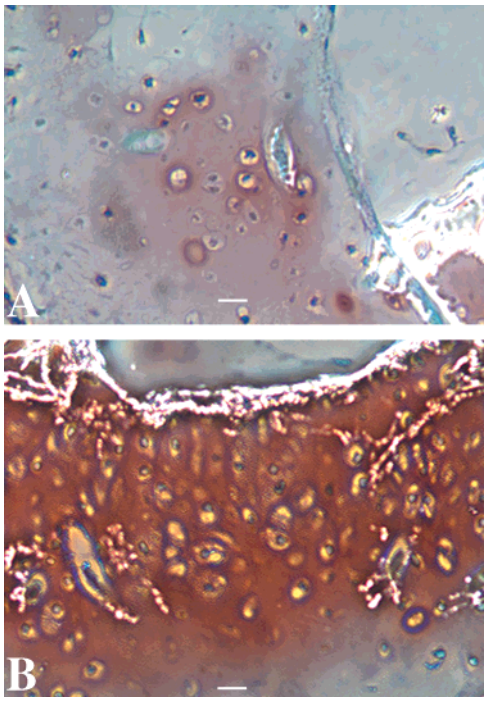

Figure 3. (A) Static sample at 2 weeks stained with safranin-O/fast green revealed light staining and no discernible orientation (400×, bar = 10 μm). (B) Bioreactor sample at 2 weeks stained with safranin-O/fast green (400×, bar = 10 μm). Intense staining was observed, as well as alignment of cells in the direction of media flow.

content in the bioreactor compared to static culture (Figure 4). At 4 weeks, GAG concentrations of bioreactor culture samples were 184% higher ($p < 0.05$) than in static culture (Figure 5) confirmed by the increased presence of proteoglycan in the safranin-O/fast green stained bioreactor samples. By 2 weeks in bioreactor culture, a 155% ($p < 0.05$) increase in hydroxyproline content was observed in bioreactor samples compared to static controls (Figure 6), and 4-week analysis revealed a 130% ($p < 0.05$) increase. Disks cultured in high media volume showed no difference in matrix assembly or chondrocyte proliferation compared to static controls, indicating that media volume had no effect on chondrogenesis in this system.

Discussion

The goal of this study was to compare cell proliferation and ECM biosynthesis in static and bioreactor culture systems and to assess the effects of these processes on

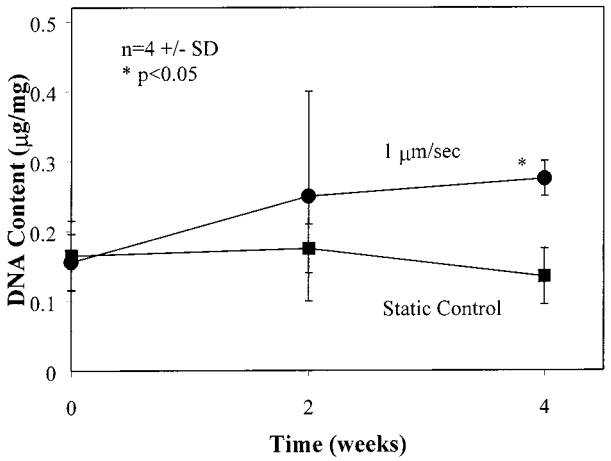

Figure 4. DNA analysis up to 4 weeks showed a 118% increase ($p < 0.05$) in DNA content of the bioreactor compared to static cultures. Values are mean ± SD ($n = 4$).

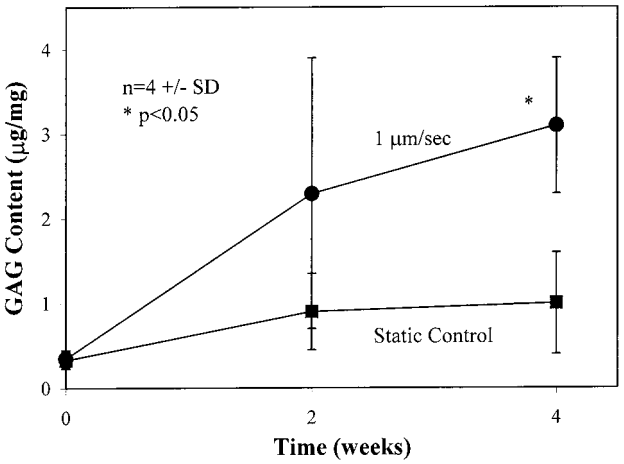

Figure 5. S-GAG analysis up to 4 weeks showed a 184% ($p < 0.05$) higher content of S-GAG in the bioreactor compared to static cultures. Values are mean ± SD ($n = 4$).

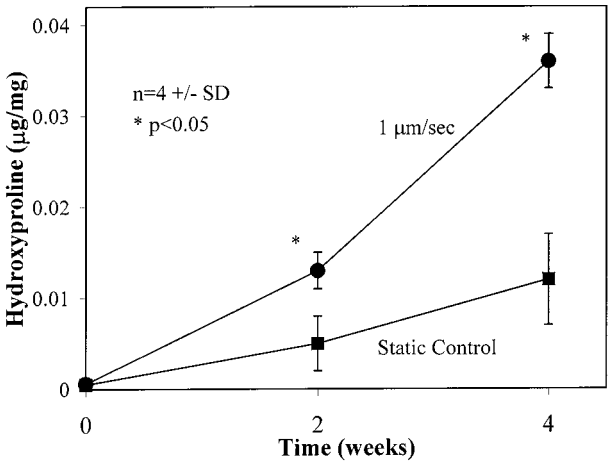

Figure 6. Collagen analysis at 2 and 4 weeks showed 155% and 130% increases ($p < 0.05$) in hydroxyproline content in bioreactor compared to static culture. Values are mean ± SD ($n = 4$).

media pH. Media pH from seeded constructs decreased sharply compared to unseeded static controls (Figure 2), suggesting that cell metabolism, not scaffold degradation, is the primary regulator of media pH in this system. This is of great importance given that acidic media is known to inhibit cartilage matrix assembly (*2*). The bioreactor system was more effective at maintaining stable pH,

possibly because of more efficient gas exchange through silicone tubing.

The axial flow through the sample induced in the bioreactor produced multiple focal areas of columnar cell orientation and matrix assembly. Given the known stimulatory effects of fluid velocities of this magnitude (~1 μm/s) (*6*), it is likely that fluid flow, as well as pH regulation, contributed to the higher rates of matrix assembly observed in samples cultured in the bioreactor. The ability to create aligned tissues may prove advantageous in the engineering of articular cartilage, which contains large regions of oriented cells and matrix (*1*).

Studies on the effects of fluid flow conducted offer the possibility of rapid in vitro expansion of donor cartilage for the repair of structural defects, tracheal injury, and vascularized tissue damage. Tissue-engineered cartilage on a 3-dimensional moldable framework offers the potential for ear as well as nose (*7*) and nipple (*13*) reconstructive surgeries. The technology described in this study may provide a more successful, cost-effective, and time-efficient method for the growth of tissue-engineered articular cartilage.

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