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Running head: Perfusion bioreactor to engineer uniform tissues

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Abstract

In this work, we assessed whether perfusion culture of cells uniformly seeded within porous scaffolds, at a flow rate maintaining a normoxic range of oxygen levels, can lead to the development of uniform engineered tissue grafts. An integrated bioreactor system was first developed to streamline the steps of perfusion cell seeding of porous scaffolds and perfusion culture of the cell-seeded scaffolds. Oxygen tensions in perfused constructs was monitored by in-line oxygen sensors incorporated at the construct inlet and outlet. Adult human articular chondrocytes were perfusion-seeded into foam scaffolds at a rate of 1mm/s. Cell-seeded foams were then either cultured statically in dishes or further cultured under perfusion at a rate of 100μm/s for 2 weeks. Following perfusion seeding, viable cells were uniformly distributed throughout the foams. Constructs subsequently cultured statically were highly heterogeneous, with cells and matrix concentrated at the construct periphery. In contrast, constructs cultured under perfusion were highly homogeneous, with uniform distributions of cells and matrix. Oxygen tensions of the perfused medium were always near normoxic levels (inlet≅20%, outlet>15%). In conclusion, we have demonstrated that culture of uniformly seeded chondrocytes under direct perfusion, maintaining cells within a normoxic range of oxygen levels, is necessary to generate uniform tissues.

1. Introduction

Tissues engineered *in vitro* by seeding and culturing cells into 3D porous scaffolds are frequently reported to have an inhomogeneous structure, consisting of a dense layer of cells and extracellular matrix (ECM) concentrated along the periphery, and a necrotic interior region. Such an inhomogeneous structure may limit the initial mechanical functionality and subsequent *in vivo* development of grafts of clinically relevant size.

In order to generate homogeneous tissue grafts, cells may have to be initially seeded into porous 3D scaffolds with a uniform distribution [7,8], thereby establishing a template for spatially uniform ECM deposition. The cells seeded within the interior regions must then be supplied with sufficient nutrients during prolonged 3D culture in order to maintain viability and support the production of ECM; possibly requiring the application of convective fluid flow. In addition, in order to have an indication of whether convective flow is sufficient, it may be necessary to monitor the range of oxygen concentrations to which cells within the scaffold are exposed; in fact, oxygen has a low solubility in culture medium and is likely to be one of the limiting nutrients within a 3D construct [4].

We previously described a bioreactor for the perfusion of cell suspensions through the pores of 3D scaffolds in alternate directions, resulting in high efficiency and high spatial uniformity of cell seeding [8]. In this work, we first developed an integrated bioreactor system to allow prolonged perfusion of culture medium through 3D constructs following cell seeding by perfusion, within a single device. To continuously monitor the range of oxygen levels in the perfused constructs, in-line oxygen sensors were incorporated within the culturing pathway near the inlet and outlet of the constructs.

Using the developed bioreactor system, we then tested the hypothesis that cells seeded uniformly throughout a 3D scaffold, when cultured under direct perfusion within a normoxic range of oxygen levels, will generate a homogeneous graft with a uniform distribution of cells and ECM. The hypothesis was tested using a *human* chondrocyte/foam scaffold model system.

2. Methods

2.1 Integrated perfusion bioreactor system

The bioreactor system is designed to perfuse first a cell suspension (Figure 1a), and subsequently culture media (Figure 1b), through the pores of a 3D scaffold within a single closed system. To avoid any risk of mechanically induced cell damage from a pumphead, we aimed to design a seeding flow path which did not recirculate the cell suspension through the scaffold and pump. Therefore, based on previous seeding bioreactor, the seeding pathway is designed to instead pump the headspace above the cell suspension back and forth from one Teflon column to the other, thereby generating an alternating flow of the cell suspension through the scaffold. Cell settling and cell attachment to bioreactor components are minimized by its vertical orientation, component material properties, and by minimizing the surface area of horizontal surfaces where cells tend to accumulate. Scaffolds are lightly press-fit and clamped within a scaffold chamber (Figure 1c), such that fluid flow cannot deviate around the scaffold but must flow through its pores. The chamber is manufactured from polycarbonate and polished until translucent, thus permitting the detection of possible air bubbles. Teflon FEP tubes (6mm i.d.; Cole Parmer) are connected to disposable three-way stopcocks (Hi-

Flow™; Medex GmbH) via polypropylene luer adaptors (EM-Technik GmbH), and stopcocks are then connected to the scaffold chamber via its luer connections.

Following the cell seeding phase, stopcocks can simply be rotated to divert flow through a separate perfusion loop for prolonged culture. Although the seeding path minimizes cell attachment to the bioreactor components, the separate pathway for prolonged culture eliminates the inadvertent culture of any attached cells which would nonetheless proliferate on the bioreactor components and consume vital nutrients. Gas exchange for medium oxygenation and pH buffering is achieved through platinum-cured silicon tubing (1/32" i.d., 1/16" o.d., 2m length; Cole Parmer). Media is exchanged through Interlink® injection sites (Becton Dickinson) connected to the reservoir bottle and can therefore be performed without removing the system from the incubator.

To ensure accurate, controlled, and reproducible perfusion through each construct (i.e., avoiding either preferential channeling or negligible flow through one particular construct), each construct can be seeded and cultured independently from the others using independent flow pathways and a multi-channel peristaltic pump. Up to eight integrated bioreactor units can be placed into a standard-sized incubator. Excluding the scaffold chamber, all components were selected for disposable use, eliminating risks of contamination and endotoxin build up associated with reuse. All bioreactor components were assessed for cytotoxicity in accordance with ISO10993-5.

2.2 Oxygen sensors

In order to eliminate artifacts (i.e., media re-oxygenation) associated with sampling and off-line analysis, *in-line* oxygen sensors were required. However, the perfusion bioreactor a tissue engineering application impose unique requirements on the sensors (e.g., small

size, accurate at low flow rates, long-term stability), necessitating non-traditional sensor technologies. Therefore, chemo-optic flow-through micro-oxygen sensors (FTC-PSt-3; PreSens GmbH, Germany), based on the quenching of luminescence by oxygen, which do not consume oxygen, are independent of flow rate, and maintain long-term stability, are incorporated into the culturing perfusion loop (Figure 1b). A fiber optic cable transmits the optical signals between the sensor and oxygen meter (Fibox-3; PreSens GmbH).

Sensors are connected directly to the stopcocks at the inlet and outlet of the scaffold chamber, in order to prevent significant oxygen ingress into the system through tubing and other components, as preliminarily determined when sensors were placed further upstream/downstream. Nitrogen-sparged water (i.e., 0% oxygen) was perfused through the system to demonstrate that negligible oxygen from the incubator was diffusing into the bioreactor between the inlet and outlet sensor.

2.3 Experimental model system

In two independent experiments, culture expanded adult human articular chondrocytes (AHAC) (1.3E+07 AHAC suspended in 8ml media) were perfusion seeded onto foam disks made of poly(ethylene glycol terephthalate)/poly(butylene terephthalate) (PolyActive, 300/55/45 composition, compression molded, 8mm diameter, 4.5mm thick; IsoTis OrthoBiologics, The Netherlands) (n=12 per experiment) at a superficial velocity of 1mm/s overnight using an oscillating bi-directional flow regime (0.008Hz). Following perfusion seeding, four constructs were examined by confocal microscopy to for the cell viability (staining with LIVE/DEAD® Viability/Cytotoxicity Kit; Molecular Probes) as previously described [8], and were assessed histologically for the uniformity of the cell

distribution (hematoxylin and eosin (H&E) stained cross-sections). Four seeded foams were transferred to agarose-coated dishes for static culture, and four remained within the bioreactor system for perfusion culture with continuous uni-directional flow at a superficial velocity of 100μm/s. Constructs were cultured in 25mL of DMEM (4.5g/L glucose with nonessential amino acids) supplemented with 10% FBS, 10mM HEPES, 2mM glutamine, 1mM sodium pyruvate, 100U/ml penicillin, 100μg/ml streptomycin, 0.1mM ascorbic acid 2-phosphate, 1U/ml insulin, and 10ng/ml TGFß-3 for two weeks in a 20% oxygen/5% CO_2 incubator, with media exchanged three times per week. Cultured constructs were examined with confocal microscopy for the distribution of viable cells and histologically for the uniformity of the cell and matrix distribution (H&E staining).

3. Results

3.1 Cell seeding into porous scaffolds

Following cell seeding, essentially all AHAC detected in the foams were viable, likely due to efficient nutrient transport and the flushing of dead cells out from the scaffold pores resulting from perfused flow. Furthermore, cells were seeded with a highly uniform distribution throughout the cross-sections, consistent with results obtained using a previously described perfusion bioreactor [8].

3.2 Static culture of cell-scaffold constructs

Perfusion seeded foams cultured statically for 2 weeks contained a dense layer of viable cells along the outer 0.5-1.0mm periphery of the construct which encapsulated an internal region with predominantly non-viable cells (Figure 2a). Histologically, constructs

cultured statically were highly heterogeneous, containing cells and ECM concentrated only along the outer 1mm periphery (Figure 3a).

3.3 Perfusion culture of cell-scaffold constructs

At all times throughout the culture period, oxygen tensions measured at the inlet remained near saturation levels (i.e., 20%), and those measured at the outlet remained above 15% (Figure 4). These data confirmed that at the flow rate used the bioreactor maintained an efficient and rather homogeneous oxygen supply to the cells within the perfused constructs, close to normoxic levels.

Perfusion seeded foams cultured under perfusion contained viable AHAC distributed throughout both the exterior and interior regions of the foams, with few non-viable cells detected (Figure 2b). In contrast to static cultures, constructs cultured under perfusion were remarkably homogeneous, containing a uniform distribution of both cells and ECM throughout the entire cross-section (Figure 3b).

4. Discussion

In this paper, we described the design of an integrated bioreactor system, which streamlines within a single device the phases of perfusion cell seeding and prolonged perfusion culture of the cell-seeded scaffolds. Using the developed bioreactor we then demonstrated that culture of uniformly seeded AHAC under direct perfusion, maintaining cells in a normoxic range of oxygen levels, is necessary to generate a homogeneous tissue with a uniform distribution of both cells and ECM.

Previous studies have used perfusion systems to culture chondrocytes into porous scaffolds, but either did not address the uniformity of the resulting tissues [1,5,6] or

reported the formation of heterogeneous tissues, despite using scaffolds that were rather thin [2]. The discrepancy between the latter study and our results could be explained by the fact that in our experiments perfusion culture was introduced following the uniform seeding of cells by perfusion, and/or by the higher flow rate (approximately 10-fold higher) used in our system, which may have provided a more efficient and homogeneous oxygen supply to the chondrocytes within the 3D constructs.

Despite initially having a uniform distribution of cells, statically cultured foams contained only a thin layer of cells and matrix concentrated at the construct periphery. Although oxygen tensions within statically cultured constructs were not assessed in this study, the heterogeneity could be explained by the steep oxygen gradients (from 20% at the surface to 2% at a depth of 1 mm from the surface) previously predicted [3] and measured [4] using the same Polyactive foams cultured in the absence of perfusion. The dramatically improved tissue uniformity generated under perfusion as compared to static culture may be attributed to the maintenance of a normoxic range of oxygen levels across the constructs, as well as to the increase of mass transport of other nutrients (e.g., glucose) and metabolic waste products. In this context, studies are ongoing, using the developed bioreactor system, to understand the specific influence of oxygen on chondrocyte metabolism and function within a 3D construct.

In conclusion, we have developed an integrated perfusion bioreactor system for the engineering of uniform 3D grafts based on AHAC. The described device could be used in conjunction with other cell types, not only for generating uniform tissue grafts, but also as a controlled model system to investigate fundamental mechanisms of cell function in a 3D environment.

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Figure Legends

Figure 1. Integrated perfusion bioreactor system. The bioreactor system streamlines the phases of cell seeding and prolonged culture of the cell-seeded scaffolds within a single device. (a) Cell seeding pathway. Alternating bi-directional flow of the cell suspension through the scaffold is generated by pumping the headspace above the cell suspension back and forth from one Teflon column to the other. In this way, cells are not recirculated through the pump, eliminating potential mechanically induced cell damage from the pumphead. (b) Prolonged culturing pathway. Following the cell seeding phase, the bioreactor system remains within the incubator, cell-seeded scaffolds are not handled or transferred to a separate bioreactor, but instead, stopcocks simply divert flow to a separate perfusion loop for prolonged culture. (c) Scaffold chamber. The scaffold is inserted into a removable holder, which simplifies sterile assembly and construct harvesting. The top of the chamber clamps the scaffold in place by its outer 1mm periphery. The flow path contains a gradual expansion and contraction to reduce flow separation, and has a straight cylindrical region to fully develop the flow before reaching the construct.

Figure 2. Cell viability after two weeks of culture. Presented confocal microscopy images show only the channel for viable cell staining. Statically cultured constructs contained (a) a high density of viable cells concentrated at the periphery, and (b) an essentially non-viable internal region. In contrast, perfusion cultured constructs contained high densities of cells both (c) at their periphery, and (d) throughout their internal region.

Figure 3. Representative H&E stained cross-sections following two weeks of culture. (a,b) Statically cultured constructs; (c,d) perfusion cultured constructs; (a,c) low magnification images show the tissue distribution throughout the entire cross-section (scale bar = 1mm); (b,d) higher magnification images identify the tissue "*t*" and scaffold "*s*" within the cross-sections. Statically cultured constructs contained cells and matrix only at the construct surface, reaching a depth of approximately 1mm into the scaffold. In contrast, perfusion cultured constructs were highly homogeneous, containing a uniform distribution of cells and matrix throughout the cross-section.

Figure 4. Profiles of the oxygen tensions measured at the inlet and outlet of the scaffold chamber. Inlet oxygen concentrations remained near saturation levels throughout the two week culture period. Oxygen levels measured at the outlet were less than 5% lower than the inlet, indicating that cells within the construct were exposed to relatively homogeneous oxygen tensions.