Effects of Medium Perfusion Rate on Cell-Seeded Three-Dimensional Bone Constructs in Vitro*

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ABSTRACT

Cellular activity at the center of tissue-engineered constructs in static culture is typically decreased relative to the construct periphery because of transport limitations. We have designed a tissue culture system that perfuses culture medium through three-dimensional (3D) porous cellular constructs to improve nutrient delivery and waste removal within the constructs. This study examined the effects of medium perfusion rate on cell viability, proliferation, and gene expression within cell-seeded 3D bone scaffolds. Human trabecular bone scaffolds were seeded with MC3T3-E1 osteoblast-like cells and perfused for 1 week at flow rates of 0.01, 0.1, 0.2, and 1.0 mL/min. Confocal microscopy after 1 week of culture indicated that a flow rate of 1.0 mL/min resulted in substantial cell death throughout the constructs whereas lowering the flow rate led to an increasing proportion of viable cells, particularly at the center of the constructs. DNA analysis showed increases in cell proliferation at a flow rate of 0.01 mL/min relative to 0.2 mL/min and static controls. Conversely, mRNA expressions of Runx2, osteocalcin, and alkaline phosphatase were upregulated at 0.2 mL/min compared with lower flow rates as quantified by real-time RT-PCR. These data suggest that medium perfusion may benefit the development of 3-D tissues in vitro by enhancing transport of nutrients and waste within the constructs and providing flow-mediated mechanical stimuli.

INTRODUCTION

IN THE ABSENCE of a vascular blood supply in vitro, nutrient delivery to cells throughout three-dimensional (3D) tissue-engineered constructs grown in static culture must occur by diffusion. As a result, thin tissues (e.g., skin) and tissues that are naturally avascular (e.g., cartilage) have been more readily grown in vitro than thicker, vascular tissues such as bone.1–3 When 3D cellular constructs are grown in static culture, cells on the outer surface of the constructs are typically viable and proliferate readily while cells within the construct may be less active or necrotic.4–6 For example, rat calvarial osteoblasts cultured statically within demineralized trabecular bone scaffolds (6.0 mm in diameter and 3.0 mm thick) for 56 days produce a thin (less than 500 μm) layer of mineralized matrix around the construct periphery (Fig. 1A). Tissue culture systems that provide dynamic medium flow conditions around or within tissue-engineered constructs are designed to enhance nutrient exchange and cell growth in vitro.7–9 Such tissue culture systems may be useful as bioreactors to engineer thicker, more uniform tissues for implantation or as test bed models that simulate aspects of the in vivo environment.

In addition to enhancing mass transport, bioreactor systems may be used to deliver controlled mechanical stimuli such as flow-mediated shear stresses, matrix strains, or hydrostatic pressures to tissue constructs. This general approach has been used to culture a variety of 3D constructs including bone, cartilage, muscle, liver, and blood vessels. Tissue culture systems that incorporate dynamic medium flow conditions for developing 3D bone and cartilage constructs include spinner flasks, rotary cell bioreactors, and perfusion systems. In general, improved cell viability, proliferation, and extracellular matrix production have been demonstrated in such systems relative to static controls.

The perfusion of culture medium through porous 3D cellular constructs for bone and cartilage tissue engineering purposes has only recently been analyzed. Glowacki et al. perfused culture medium through stromal cell seeded 90% porous type I collagen sponges at 1.3 mL/min. The perfused constructs, in comparison with the static controls, had increased cell viability and proliferation, especially in the center. Bujia et al. demonstrated successful long-term culture of chondrocytes seeded onto a polylactic acid fleece, using perfusion of medium. In addition, Goldstein et al. described an increase in alkaline phosphatase (ALP) production from osteoblasts seeded on 80% porous polyglycolic acid foams that were perfused with medium at 0.03 mL/s in comparison with static controls. Although medium perfusion has been shown previously to significantly influence cell-seeded constructs, no previous study has specifically addressed the effects of varying perfusion rate.

The goals of this study were to develop a novel 3D perfusion tissue culture system and investigate the influence of different continuous flow rates on bone constructs cultured for 1 week. Using MC3T3-E1 osteoblast-like cells seeded on cylindrical human trabecular bone scaffolds, we demonstrate the effects of varying the medium perfusion rate on cell viability, proliferation, and gene expression within the constructs.

MATERIALS AND METHODS

Freeze-dried, \( \gamma \) irradiation-sterilized human trabecular bone (Georgia Tissue Bank, Atlanta, GA) was rehydrated by submersion in sterile phosphate-buffered saline (PBS) for 1 h before sizing. A trephine drill bit was used under sterile conditions to produce cylindrical scaffolds measuring 0.25 in. in diameter and length from the trabecular bone of several femoral metaphyses. The cylinders were hydrated in sterile PBS at 4°C for 24 h before seeding with cells. Microcomputed tomography (micro-CT) imaging was used to evaluate trabecular bone scaffold architecture (Fig. 1B). Average porosity was 82% and average pore size was 645 \( \mu \)m as determined by 3D stereological analysis of micro-CT data.

Hydrated trabecular bone scaffolds were seeded in 24-well non-tissue culture-treated plates with 2 million MC3T3-E1 immature osteoblast-like cells suspended in 100 \( \mu \)L of culture medium (\( \alpha \)-minimum essential medium) (GIBCO, Grand Island, NY) containing 1% penicillin-streptomycin and 10% fetal bovine serum (FBS; HyClone, Logan, UT) per scaffold. MC3T3-E1 cells were selected for their ability to express proteins specific to the osteoblast phenotype and to produce mineralized nodules when cultured in vitro. After 20 min, 2 mL of culture medium was added to each sample. The cells were then allowed to adhere to the constructs over a period of 24 h before placement into the 3D tissue culture system.

A novel 3D tissue culture system was developed that facilitates controlled perfusion of a defined culture medium through cell-seeded constructs. For each perfusion rate experiment, eight cylindrical constructs were loaded into individual chambers of the 316L stainless steel bioreactor.
steel perfusion block shown in Fig. 2. Side holes in the perfusion block allowed the entrance and exit of defined culture medium to each of the independent chambers. The culture medium flow rate was controlled by a Masterflex console drive pump (Cole-Parmer, Vernon Hills, IL), and the flow loop was completed by a multichannel reservoir containing 15 mL of culture medium per chamber. Medium perfusion rates of 1.0, 0.2, 0.1, and 0.01 mL/min were applied continuously to the constructs for 7 days. Static controls consisted of cell-seeded constructs cultured unconfined in non-tissue culture-treated six-well plates with 8 mL of complete culture medium as well as cell-seeded constructs encased in stainless steel cubes with two lateral ports, simulating the interior of the perfusion system. Culture medium was changed on day 2 and day 4 and contained standard osteogenic supplements of 3 mM sodium β-glycerophosphate, ascorbic acid (50 μg/mL), and 10−8 M dexamethasone. Acellular controls were also employed for each analysis group. After 1 week, a live/dead fluorescent cell stain (L3224; Molecular Probes, Eugene, OR) that labels viable cells green (calcine) and dead cells red (ethidium homodimer) was used to assess cell viability within the perfused constructs and static controls. The cellular constructs were cut in half before staining to allow any cells in the center of the construct to be viewed. Cell viability within constructs taken down after 1 day in static culture was also assessed. The stained cellular constructs were viewed with a laser scanning confocal microscope (LSM 510UV; Carl Zeiss, Thornwood, NY).

At 1 week, constructs for each perfusion rate (n = 6) were retrieved for total DNA content analysis in order to assess cell proliferation. Each cellular construct was placed into 1 mL of PBE (phosphate-buffered ethylenediaminetetraacetic acid [EDTA]) and stored at −80°C. Two freeze-thaw cycles were completed before crushing each construct in a mortar and pestle. Each sample was then sonicated to rupture cell membranes and release the cellular DNA into solution. After centrifugation at 10,000 × g, 10 μL of each sample was added to 200 μL of working dye solution containing 10 ng of Hoechst dye (Sigma, St. Louis, MO) per milliliter of Tris-EDTA-sodium buffer. A standard of calf thymus DNA (Sigma) was also prepared at 10 μg/mL in PBE. The fluorescence of the samples was read on a 96-well black plate at an excitation wavelength of 365 nm and an emission wavelength of 458 nm.

One-week experiments were repeated at each flow rate to assess the effects of perfusion rate on gene expression. RNA from constructs was isolated with a RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s description. First-strand cDNA synthesis was performed according to a well-established procedure using SuperScript II as described by the manufacturer (GIBCO-BRL, Gaithersburg, MD). Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed on five of the constructs at each perfusion rate, using an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) sequence detector. This technique provides quantitative comparisons of gene expression among experimental groups by determining the real-time fluorescence emitted at each PCR amplification. Runx2, osteocalcin (OCN), and ALP were quantified by real-time RT-PCR and were normalized to 18S (Table 1). Data from sample groups were analyzed for statistical significance with a one-way analysis of variance (ANOVA) test and the Tukey least significance test for post-hoc comparisons, with a significance level of p < 0.05.

RESULTS

Cell viability

Confocal microscopy revealed a clear influence of perfusion rate on cell viability. At 1 day, constructs showed adherence of MC3T3-E1 cells on the surface of the tra-
beculae within the constructs. A high proportion of the cells was viable throughout the constructs on day 1. After 1 week in static culture, viable cells were nearly confluent on the periphery of the constructs; however, consistent with previous reports, only a few viable cells were found at the construct center. The highest flow rate, 1 mL/min, resulted in nearly complete cell death throughout the construct after 7 days. Lowering the flow rate increased cell viability at both the periphery and the center of the constructs after 1 week of continuous perfusion. Flow rates of 0.2 and 0.1 mL/min resulted in a mixture of viable and dead cells on the surface of the constructs, with limited cell viability in the center. However, a flow rate of 0.01 mL/min resulted in a high proportion of viable cells both on the outer surface of the constructs and within the construct interior.

Cell proliferation

The effects of medium perfusion rate on cell proliferation, as measured by total DNA content within the constructs, was consistent with the qualitative assessment of cell viability by confocal microscopy. High DNA levels were measured after 7 days of continuous perfusion at 0.1 mL/min in comparison with both the 0.2-mL/min and static control groups (Fig. 3, \( p < 0.05 \)). There was also significantly greater cell proliferation in the 0.1-mL/min group than in the 0.2-mL/min group; however, only the lowest perfusion rate of 0.01 mL/min enhanced cell proliferation relative to static controls. As expected, minimal DNA was recovered from acellular control samples.

Osteoblastic gene expression

Real-time RT-PCR showed that all three bone-related genes analyzed were detected in cellular constructs perfused at less than or equal to 0.2 mL/min. Expression of Runx2, ALP, and OCN was enhanced within cell-seeded constructs perfused at 0.2 mL/min relative to lower flow rates (Fig. 4, \( p < 0.05 \)). However, the 0.2-mL/min perfusion treatment increased only Runx2 expression relative to unconfined static controls. There was not enough RNA recovered from the control constructs encased in the stainless steel to produce reliable measurements. Likewise, there was no RNA recovered from acellular control samples.

<table>
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<th>Gene</th>
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<th>Reverse primer</th>
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<tr>
<td>OCN</td>
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<td>ALP</td>
<td>5’-GGG ACT GGT ACT CGG ATA ACG A</td>
<td>5’-CTG ATA TGC GAT GTC CTT GCT (copy complement)</td>
<td>71</td>
<td>AF285233</td>
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FIG. 3. DNA assay after 1 week of perfusion. 1 day: 1 day seeded scaffold before perfusion. static 7 day: static sample (not perfused). 0.01, 0.1, and 0.2 mL/min 7 day: flow rates, perfused for 1 week. Brackets: \( p < 0.05 \).
DISCUSSION

A critical barrier to the development of 3D tissues in vitro is the limited transport of nutrients and metabolites within the construct interior. As a result, reduced cell viability or activity is typically observed at the center of tissue constructs grown in static culture. In the present study, a perfusion tissue culture system has been developed and used to achieve enhanced cell viability and increased cell proliferation in 3D bone constructs at low continuous perfusion rates compared with static controls. Higher continuous perfusion rates activated expression of genes associated with osteoblast differentiation but also led to increased levels of cell death due to flow-induced shear stresses.

Various fluid flow conditions have been shown previously to regulate cell activity in 2D culture systems. Continuous fluid flow applied to osteoblasts in vitro has been shown to upregulate bone-related gene and phenotype expression. Pulsatile and oscillatory flow conditions applied to osteoblasts in vitro parallel-plate flow chambers have also been shown to increase gene expression, intracellular calcium concentration, and the production of nitric oxide and prostaglandin E2 in comparison with static controls. Furthermore, cell responsiveness has been reported to vary with fluid flow rate and frequency. Proposed mechanisms for the stimulation of cells by fluid flow include increased mass transport, generation of streaming potentials, and application of shear stresses to the cell membranes. Although these previous studies were performed in 2D cell culture systems, they suggest that variable flow conditions may also have differential effects in 3D tissue culture systems.

Total DNA results after 7 days of continuous perfusion were consistent with qualitative assessments of cell viability by confocal microscopy. Significantly more DNA was isolated from samples perfused at 0.01 mL/min in comparison with both static controls and samples perfused at 0.2 mL/min. Differences in the number of viable cells were most evident at the center of the constructs, suggesting that slow perfusion may have improved mass transport throughout the constructs. Perfusing medium at lower rates may also have allowed for autocrine effects, as soluble proteins released by cells may have been flushed away at higher flow rates before exerting an influence on neighboring cells. Continuous perfusion at low rates for 1 week did not significantly increase the expression of Runx2, ALP, or OCN bone marker genes compared with static controls.

A higher continuous perfusion rate, 0.2 mL/min, significantly upregulated expression of Runx2, ALP, and OCN relative to lower rates. Runx2 expression, but not ALP or OCN expression, was also upregulated compared with static controls. Runx2, ALP, and OCN are important markers of different stages of bone matrix production. Runx2 is a transcriptional activator essential for initial osteoblast differentiation and subsequent bone formation. ALP is expressed by preosteoblasts and osteoblasts before the expression of OCN. Finally, OCN is a late marker that binds hydroxyapatite and is produced by osteoblasts just before and during matrix mineralization.

Although this study measured responses at only one relatively early time point, the RT-PCR results in con-
cert with DNA content data indicate that lower continuous flow rates promoted cell proliferation whereas a higher perfusion rate of 0.2 mL/min may have accelerated the differentiation of cells within the construct toward an osteoblast phenotype. These results suggest that it may be possible to direct cellular behavior within 3D constructs via modulation of perfusion flow rate. Longer term studies that measure the effects of perfusion rate on matrix mineralization are ongoing to test this hypothesis.

Live/dead staining and confocal microscopy showed an increasing level of cell death within the constructs associated with increasing the perfusion rate from 0.1 to 1.0 mL/min. Increased shear stresses at the higher perfusion rates were likely responsible for the observed decrease in cell viability. The DNA present after 1 week of perfusion at 0.2 mL/min was lower than the 1-day seeded sample, indicating that the original cell number had actually decreased. The loss in cell number may be due to cells being sheared off the construct as a result of the higher flow rates as well as decreased cell proliferation. Although continuous perfusion at 0.1–1.0 mL/min was not beneficial to construct development, transient increases in flow rate superimposed on continuous slow perfusion may simultaneously enhance mass transport and accelerate osteoblast differentiation.

The flow rates used in this study (0.01–1.0 mL/min) are lower than those described in other 3D perfusion systems (1.3–1.8 mL/min). Even if the flow rates had been matched, however, differences in the architecture and porosity of the scaffolds used in the various perfusion experiments would result in different shear stresses applied to cells on the scaffold surfaces. The scaffolds used in this experiment were in general larger and less porous than those used in previous perfusion experiments, and local shear stresses at a given rate of perfusion would therefore be higher. Although modeling the flow of medium through 3D porous scaffolds with complex microarchitecture to estimate local shear stresses is a challenging computational problem, such analyses are necessary to compare results between different perfusion experiments. Computational fluid dynamics simulations that specifically model scaffold microarchitecture are currently under development to directly estimate local fluid velocities and shear stresses throughout perfused constructs.

A perfusion tissue culture system has been developed and used to demonstrate that altering the flow rate through 3D cell-seeded scaffolds affects cell viability, cell proliferation, and the expression of bone marker genes. Further characterization and optimization of perfusion systems may provide an effective strategy to overcome current diffusion limitations associated with culturing 3D tissues in vitro while simultaneously providing flow-mediated mechanical stimuli known to be potent regulators of cell function.

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REFERENCES


