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Bioreactor culture duration of engineered constructs influences bone formation by mesenchymal stem cells

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ABSTRACT

Perfusion culture of mesenchymal stem cells (MSCs) seeded in biomaterial scaffolds provides nutrients for cell survival, enhances extracellular matrix deposition, and increases osteogenic cell differentiation. However, there is no consensus on the appropriate perfusion duration of cellular constructs *in vitro* to boost their bone forming capacity *in vivo*. We investigated this phenomenon by culturing human MSCs in macroporous composite scaffolds in a direct perfusion bioreactor and compared their response to scaffolds in continuous dynamic culture conditions on an XYZ shaker. Cell seeding in continuous perfusion bioreactors resulted in more uniform MSC distribution than static seeding. We observed similar calcium deposition in all composite scaffolds over 21 days of bioreactor culture, regardless of pore size. Compared to scaffolds in dynamic culture, perfused scaffolds exhibited increased DNA content and expression of osteogenic markers up to 14 days in culture that plateaued thereafter. We then evaluated the effect of perfusion culture duration on bone formation when MSC-seeded scaffolds were implanted in a murine ectopic site. Human MSCs persisted in all scaffolds at 2 weeks *in vivo*, and we observed increased neovascularization in constructs cultured under perfusion for 7 days relative to those cultured for 1 day within each gender. At 8 weeks post-implantation, we observed greater bone volume fraction, bone mineral density, tissue ingrowth, collagen density, and osteoblastic markers in bioreactor constructs cultured for 14 days compared to those cultured for 1 or 7 days, and acellular constructs. Taken together, these data demonstrate that culturing MSCs under perfusion culture for at least 14 days *in vitro* improves the quantity and quality of bone formation *in vivo*. This study highlights the need for optimizing *in vitro* bioreactor culture duration of engineered constructs to achieve the desired level of bone formation.

1. Introduction

Large bone deficits due to trauma, surgery, or resulting from slow and non-healing defects represent a significant clinical problem. More than 1 million bone repair surgeries are performed annually, bringing the financial burden to over $5 billion in the Unites States alone [1,2]. Autograft bone is the current gold standard for repairing non-healing bone defects, yet its use has numerous drawbacks such as donor site morbidity, prolonged pain, limited availability, and increased risk of infection [3,4]. Tissue engineered bone constructs represent a promising alternative to autografts. To generate implantable osteogenic grafts, biomaterial scaffolds are commonly seeded with bone-forming cells and cultured *in vitro* under variable conditions and durations. Compared to static or continuous dynamic culture, scaffolds under continuous perfusion culture exhibit improved cell growth and survival, extracellular matrix (ECM) deposition, and enhanced osteogenic differentiation of mesenchymal stem cells (MSCs) due to increased nutrient availability and application of mechanical stimulation [5–8]. However, the effect of bioreactor culture duration *in vitro* on the bone forming capacity of MSCs upon transplantation *in vivo* is largely unknown.

An ideal bone scaffold for bioreactor culture of MSCs must be osteoconductive, providing a structure and network that supports cell attachment, growth, and osteogenic differentiation [9,10]. Composite scaffolds formed of hydroxyapatite (HA) and poly(lactide-*co*-glycolide) (PLG) offer tunable biodegradability, osteoconductivity, porosity, and mechanical properties [11–14]. Moreover, HA-PLG

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scaffolds can regulate osteogenesis and trophic factor secretion by MSCs *in vitro* and *in vivo* [11]. We and others reported that the pore size of three-dimensional macroporous scaffolds can regulate cell differentiation under static or dynamic conditions [15–19]. Pore size also directly influences the shear stress experienced by cells when scaffolds are maintained under continuous perfusion, thus regulating osteogenic differentiation of MSCs [10,17]. Therefore, selecting the appropriate pore size for constructs cultured in perfusion bioreactors is vital for bone tissue engineering applications.

While the exposure of osteoblasts and osteoprogenitor cells to fluid shear stress enhances their bone forming potential *in vivo* [6,20], the contribution of perfusion culture duration to bone formation has not been thoroughly investigated. Furthermore, the effect of culture duration on the bone-forming potential of human MSCs, a more clinically relevant cell source compared to osteoblasts, has not been reported in the literature. Bioreactor culture durations ranging from 5 days [21] to 5 weeks [22–24] have been used for creating implantable tissue engineered bone. Shorter culture durations may be sufficient to prime MSCs towards osteogenic differentiation and boost their proangiogenic potential [25]. Extended culture durations such as 2–5 weeks may result in a more mature construct with increased cellularity, differentiated cells and a dense ECM, which can act as a scaffold for host cell infiltration and differentiation [10,26,27]. Thus, there is a critical gap in our knowledge for the appropriate *in vitro* perfusion culture duration to maximize bone formation *in vivo* with osteogenic grafts.

We hypothesized that HA-PLG scaffolds would serve as effective osteoconductive biomaterials to promote MSC osteogenic differentiation and that longer culture durations would result in mature constructs suitable for implantation. We investigated the role of perfusion on bone formation by culturing human MSCs in porous HA-PLG scaffolds of varying pore sizes and durations in a continuous perfusion bioreactor. Thus, the goals of this study were to 1) determine the effect of pore size of HA-PLG scaffolds on MSC osteogenic differentiation *in vitro*; 2) characterize the osteogenic response of MSCs in HA-PLG scaffolds when cultured in perfusion bioreactors for up to 21 days; and 3) investigate the effect of *in vitro* perfusion culture duration on *in vivo* bone forming capacity of tissue engineered constructs in an ectopic site.

2. Materials and methods

2.1. Scaffold preparation

Scaffolds were fabricated using a gas foaming/particulate leaching method as described [11,16]. Briefly, microspheres composed of PLG (85:15, DLG 7E; Lakeshore Biomaterials, Birmingham, AL) were prepared using a double-emulsion process and lyophilized to form a free-flowing powder. 9.2 mg of lyophilized microspheres were combined with 23.1 mg of HA crystals (particle size 100 nm, Berkeley Advanced Biomaterials, Berkeley, CA) and 175.6 mg of NaCl to yield a 2.5:1:19 mass ratio of ceramic:polymer:salt. Porogen diameter ranges were achieved by passing salt crystals through sieves of distinct size ranges (small: 125–300 μm, medium: 300–500 μm, and large: 500–850 μm). Control PLG scaffolds were fabricated without HA using 250–425 μm salt crystals, as this is the range currently employed by our group and others to support cellular ingrowth [11,28–31]. The powdered mixture was then compressed for 1 min into solid disks (final dimensions: 8 mm in diameter and 2 mm in height) using a Carver Press (Carver, Inc., Wabash, IN). Compressed disks were exposed to high pressure CO_2 gas (5.5 MPa) for at least 16 h followed by rapid pressure release to cause polymer fusion. NaCl particles from the scaffolds were then leached in distilled H_2O for 24 h. Scaffolds were sterilized by 70% ethanol under gentle vacuum followed by two rinses in sterile PBS and dried in a sterile biosafety cabinet. Prior to cell seeding, scaffolds were soaked in cell culture medium for at least 30 min to facilitate cell adhesion.

2.2. Scanning electron microscopy

To visualize gross morphology and pore architecture, scaffolds were gold coated using a sputter coater (Desk II; Denton Vacuum, Moorestown, NJ) and imaged using scanning electron microscopy (Hitachi 3500-N, Hitachi Science Systems Ltd, Tokyo, Japan) at 5 kV. Pore diameter was measured in ImageJ (National Institutes of Health, Bethesda MD). Briefly, pores were measured edge to edge along the maximum and minimum axis and averaged to generate a representative diameter. Eight pores from four separate images per group were measured to determine pore diameter.

2.3. Measurement of fluid permeation velocity through scaffolds

Permeation velocity of fluid flow through the scaffolds was measured as an indicator of pore connectivity and indirect measure of fluid shear stress (Fig. 1D, insert). Prior to analysis, scaffold height was measured using digital calipers. Pristine, acellular scaffolds were held in place between two bioreactor adaptors housed between two rubber syringe stoppers in a syringe barrel to create a seal preventing fluid flow around the scaffold. The syringe was filled with media, and the volume that passed through the scaffold was recorded. Permeation velocity was calculated using a derivative of Darcy's Law [16].

2.4. Cell culture

Human bone marrow-derived MSCs (Lonza, Walkersville, MD) were expanded without further characterization in growth medium (GM) consisting of minimum essential alpha medium (α-MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin/streptomycin (Gemini Bio-Products, Sacramento, CA). Cells were cultured under standard conditions (37 °C, 21% O_2, 5% CO_2) and utilized at passages 4–5. For all experiments, cells were initially seeded in GM and then exposed to osteogenic medium (OM: GM supplemented with 10 mM β-glycerophosphate, 50 μg/mL ascorbate-2-phosphate, and 100 nM dexamethasone). Media was changed every 2–3 or 3–4 days for dynamic and bioreactor cultures, respectively.

2.5. Cell seeding and maintenance

For characterizing the effect of pore size on cell seeding efficiency, cells were seeded statically or in perfusion bioreactors (Fig. 2A). Scaffolds were soaked in medium for 30 min before seeding. For static seeding, 1.2×10^6 MSCs were suspended in 75 μL GM and applied dropwise to scaffolds. MSCs were allowed to attach for 3 h before transferring scaffolds into well plates with GM. For bioreactor seeding, scaffolds were installed in U-CUP flow perfusion bioreactors (Cellec Biotek, Basel, Switzerland) and 10 mL of GM was injected through the bottom port into the bioreactor (Fig. 1A). 1.2×10^6 MSCs suspended in 2 mL GM were injected *via* the top port into the bioreactor, bringing the final media volume in each bioreactor to a total of 12 mL. Up to 10 individual bioreactors were then connected to a syringe pump (Harvard Apparatus, Holliston, MA) to maintain media at a superficial velocity of 3 mL/min for 15–18 h.

For all *in vitro* studies, scaffolds were seeded using bioreactors to ensure homogenous cell distribution for all experimental groups. After the seeding phase (15–18 h), GM was replaced with OM, and constructs were maintained under continuous perfusion culture for up to 21 days. For culture under dynamic conditions, scaffolds were