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Leading opinion

Measuring stem cell dimensionality in tissue scaffolds

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ABSTRACT

Many scaffold systems have evolved for tissue engineering and in vitro tissue models to provide a 3D (three-dimensional) microenvironment that enables cells to behave more physiologically. We hypothesized that cells would adopt morphologies with more 3D character during culture in scaffolds as compared to planar substrates. Cell shape and function are tightly linked and effects of scaffold niche properties on cell shape and dimensionality are important for directing cell function. Herein, primary human bone marrow stromal cells (hBMSCs) were cultured in 6 different scaffolds and on a planar control substrate. hBMSCs were imaged using 3D confocal microscopy, and 3D image analyses were used to assess hBMSC shape and dimensionality. A characteristic gyration tensor ellipsoid was calculated for hBMSCs in the different scaffolds which enabled hBMSC dimensionality to be classified based on shape. A "Dimensionality Matrix" was developed that showed that hBMSC shape and dimensionality were influenced by scaffold properties, and that scaffolds could drive hBMSCs into 1D, 2D or 3D shapes. In addition, the hBMSC Z-Depth was measured to determine if hBMSCs became less flat during culture in scaffolds. Z-Depth results showed that all 6 scaffolds caused an increase in cell Z-Depth compared to the 2D planar substrate. These results demonstrate that hBMSCs take on morphologies with greater 3D character in scaffolds than on a planar substrate and that scaffold properties can be adjusted to modify cell dimensionality. In addition, biomaterialists can use this measurement approach to assess and compare scaffold design modifications as they strive to create optimal cell niches that provide a 3D microenvironment.

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1. Introduction

Many scaffolds have been advanced as 3D systems for tissue engineering, drug screening and in vitro tissue models [1,2]. These include, among others, collagen gels [3,4], matrigel [5,6], fibrin gels [7], porogen-leached scaffolds [8,9], gas-foamed scaffolds [10,11], nanofiber scaffolds [12–14], freeform-fabricated scaffolds [15,16], poly(ethylene glycol) hydrogels [17–19], self-assembled peptide gels [20,21], decellularized extracellular matrix (ECM) [22,23] and spheroid culture [24,25]. Scaffolds are used to provide a 3D microenvironment for cells that is a more natural milieu for cells than planar tissue culture polystyrene (TCPS) plates. It is suggested that the 3D microenvironment enables cells to behave more physiologically, or to behave more closely to how they behave in vivo. The terms "3D culture" and "3D scaffold" suggest that cell shape may have more 3D character during culture in a scaffold that provides a 3D microenvironment.

Cells exist within and interact with their microenvironment, the cell niche, and the structural, chemical and mechanical properties of their niche can direct cell function [26]. Cells are highly responsive to the structural properties of their surroundings such as topographical surface features and 3D structure [13,16,27,28]. Substrate properties influence the energetics of cell–substrate interactions causing changes in cell shape [29,30]. Structural queues in the cell environment can guide cells into morphologies that direct their function and there is a strong link between cell shape and cell function [31–35]. The dimensionality of the cell niche will also influence cell shape and function [1,13,36–38]. For instance, cells on planar surfaces may adopt a planar morphology or become polarized with all matrix adhesions on their basolateral surface. In contrast, cells in a 3D niche may take on a 3D morphology and develop matrix contacts in multiple planes, enabling them to form extensions in 3D space. Further, cell dimensionality can influence signal transduction, where flatter cell morphologies cause inhomogeneities in the distribution of membrane-bound signaling

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molecules [39]. Thus, scaffold properties will dictate cell niche dimensionality and 3D cell shape, which will ultimately influence cell behavior.

Despite the interest in scaffolds, the physical effects of scaffolds on cell shape and dimensionality have not been described. There is not a clear approach to compare scaffold niche dimensionalities which makes it challenging to improve scaffold designs so that they provide the proper microenvironment for cells. For these reasons, we have developed an approach for measuring cell dimensionality during culture in scaffolds. Primary human bone marrow stromal cells (hBMSCs) were cultured in a variety of scaffolds and imaged using 3D confocal fluorescence microscopy. Since hBMSCs adopt complex morphologies that are difficult to assess, a characteristic ellipsoid was fit to each hBMSC using a gyration tensor calculation. A "Dimensionality Matrix" was developed to assess the gyration tensor ellipsoids and classify them as 1D, 2D or 3D. In addition, other cell shape metrics, such as the cell Z-Depth, which assesses how deeply hBMSCs penetrated into the *Z*-direction, have been measured to determine the effect of different scaffolds on hBMSC dimensionality.

2. Methods and materials

2.1. Cell culture

Primary human bone marrow stromal cells (hBMSCs) (also known as mesenchymal stem cells) were obtained from Tulane Center for Gene Therapy (Dr. Darwin Prockop, isolated from iliac crest of a healthy 29 year old female, Donor 7038) [40]. hBMSCs were cultured in α-minimum essential media (Invitrogen) containing 16.5% by volume fetal bovine serum (Atlanta Biologicals), 4 mmol/L L-glutamine (Invitrogen), and 1% by volume of Penicillin/Streptomycin (Cellgro). hBMSCs were trypsinized (0.25% by mass containing 1 mmol/L ethylenediaminetetraacetate (EDTA), Invitrogen) at 70% confluency and seeded onto the various scaffolds studied. Passage 5 hBMSCs were used for all experiments. hBMSCs were cultured at 37 °C under 5% CO_2 (by volume) before fixation and staining for image analysis.

2.2. Collagen Gel

Collagen gels were fabricated from Type I collagen (PureCol, 3 mg/mL, bovine, Advanced BioMatrix) under sterile conditions using 8 parts collagen and 1 part 10× Dulbecco's Modified Eagle's Medium (Sigma) neutralized to pH 7.2 with 0.1 mol/L of NaOH. hBMSCs were mixed with the collagen solutions (1 mL) and plated into TCPS wells (12-well plate). Collagen was allowed to gel for 2 h at 37 °C (2.6 mm thickness and 22 mm dia.) which encapsulated the hBMSCs at a density of 2×10^4 cells/well (2×10^4 cells/cm^3). hBMSCs in collagen gels were cultured 24 h in 1.5 mL of medium. Previous work showed that these collagen gel preparations can make fibrils of diameter of 435 nm [41].

2.3. PCL-SC (PCL spun-coat films)

PCL (poly(ε-caprolactone), relative molecular mass 80 000 g/mol, Sigma) solutions (10% by mass in glacial acetic acid) were spun-coated (1000 rpm, 30 s) onto TCPS dishes (100 mm dia.), air-dried, annealed at 60°C for 30 s and hot-punched into PCL-SC film disks (20 mm dia.) [13]. PCL-SC disks were affixed to the bottom of 12-well TCPS plates with silicon grease, sterilized with ethylene oxide (Anderson Products), degassed 1 d (house vacuum), incubated 2 d in complete medium (with serum), seeded with hBMSCs (2×10^4 cells/well) and cultured 24 h in medium (1.5 mL).

2.4. PCL-SNF ("Small" PCL nanofiber scaffolds)

PCL was dissolved at 10% by mass in 3:1 volume ratio chloroform: methanol for "Small" nanofiber scaffold fabrication (PCL-SNF). PCL-SNF were electrospun (18 gauge needle, 2 mL/h, 16.5 kV) onto 20 mm dia. TCPS disks [13], affixed to the bottom of 12-well TCPS plates with silicon grease, sterilized with ethylene oxide, degassed 1 d (house vacuum), incubated 2 d in complete medium (with serum), seeded with hBMSCs (2×10^4 cells/well) and cultured 24 h in medium (1.5 mL). All nanofiber scaffold mats completely covered the TCPS so that hBMSCs seeded onto the scaffolds came into contact with the nanofibers and did not touch the TCPS substrates below.

2.5. PCL-BNF ("Big" PCL nanofiber scaffolds)

PCL was dissolved at 30% by mass in 9:1 volume ratio chloroform: methanol for "Big" nanofiber scaffold fabrication (PCL-BNF). PCL-BNF were electrospun (18 gauge needle, 0.5 mL/h, 15 kV) onto 9 mm dia. TCPS disks [13], affixed to the bottom of 48-well TCPS plates with silicon grease, sterilized with ethylene oxide, degassed 1 d (house vacuum), incubated 2 d in complete medium (with serum), seeded with hBMSCs (1×10^4 cells/well) and cultured 24 h in medium (0.5 mL).

2.6. PDLLA-NF (PDLLA nanofiber scaffolds)

PDLLA (poly(D, L-lactic acid), relative molecular mass 100 000 g/mol, SurModics) was dissolved at 15% by mass in hexafluoroisopropanol (1,1,1,3,3,3-hexasfluoro-2-propanol) for nanofiber scaffold fabrication (PDLLA-NF). PDLLA-NF were electrospun (18 gauge needle, 1.5 mL/h, 15 kV) onto 9 mm dia. TCPS disks [13], affixed to the bottom of 48-well TCPS plates with silicon grease, sterilized with ethylene oxide, degassed 2 d (house vacuum), pre-wetted 1 min under house vacuum in complete medium (with serum), seeded with hBMSCs (1×10^4 cells/well) and cultured 24 h in medium (0.5 mL).

2.7. PCL-FFF (PCL freeform-fabricated scaffolds)

Freeform fabricated scaffolds (5 mm dia., 2 mm height, 300 μm strut diameter, 500 μm strut spacing, 0°/90° lay-down pattern) made by precision extrusion deposition using PCL (relative molecular mass 43,000–50,000 g/mol, Sigma) were purchased from 3D Biotek (PCL-FFF) (13; 15; 16). PCL-FFF were put into 96-well non-tissue culture treated polystyrene plates, sterilized with ethylene oxide, degassed 2 d (house vacuum), pre-wetted 1 min under house vacuum in complete medium (with serum), seeded with hBMSCs (5×10^3 cells/well) and cultured 24 h in medium (0.2 mL).

2.8. PEGTM (poly(ethylene glycol) tetramethacrylate gels)

Four-arm PEG (total relative molecular mass 20,000 g/mol, each arm 5000 g/mol, JenKem Technology) was mixed with methacrylic anhydride (20-fold molar excess, Sigma) and microwaved (consumer model) for 10 min to prepare poly(-ethylene glycol) tetramethacrylate (PEGTM) [19]. Under sterile conditions, PEGTM solution [50 μL of 10% by mass PEGTM and 0.05% by mass Irgacure 2959 photo-initiator (Ciba Chemicals) in 0.1 mol/L phosphate buffered saline (PBS)] containing hBMSCs (10^6 cells/mL) was placed in cylindrical Teflon molds (5 mm dia., 3 mm height) and photopolymerized (365 nm light, 2 mW/cm^2, 15 min). PEGTM gels with cells were cultured 4 h in 96-well TCPS plates with medium (0.2 mL) before fixation and staining.

2.9. Scaffold characterization

Scanning electron microscopy (SEM) was used to determine the fiber diameter for PCL-SNF, PCL-BNF and PDLLA-NF and to determine strut diameter and strut spacing for PCL-FFF. Scaffolds were sputter-coated with gold for 90 s and imaged (SEM, 15 kV, Hitachi s-4700eII FE-SEM). Fiber diameters were determined by electron micrograph image analysis using ImageJ software (NIH). PEGTM gel modulus was measured by uniaxial static compressive load (strain rate of 0.03 mm/s, Dynamic Mechanical Analyzer, TA Instruments, calculated from slope of the linear fit for 5%–10% strain) [19]. Gravimetry was used to measure total porosity of PCL-FFF scaffolds using an equation, "Total porosity $= 1 - [(m/d)/v]$", where "m" is mass of the scaffold (g), "d" is PCL density (1.1 g/mL), and "v" is volume of scaffold (mL) (caliper measurements).

2.10. Fluorescent staining of hBMSCs and imaging

After indicated cell culture times, scaffolds with cells were washed with PBS and fixed with 3.7% by volume formaldehyde (in PBS) for 15 min, permeabilized with 0.5% by volume Triton X-100 for 10 min, and then stained with Alexa Fluor 546-phalloidin (20 nmol/L) and 4′,6-diamindo-2-phenylindole, dihydrochloride (DAPI, 5 mg/L) in 2% by mass bovine serum albumin and 20 mmol/L Trizma-HCl buffer to stain for F-actin and nuclei, respectively (Invitrogen). For PEGTM, nuclei were stained with TOPRO-3 (carbocyanine monomer nucleic acid stain, 1 μmol/L) instead of DAPI. hBMSCs were imaged by confocal microscopy using a 40× water immersion objective (0.8 N.A.) on a Ziess LSM 510, or a 63× water immersion objective (0.9 N.A.) or a 20× dry objective (0.7 N.A.) on a Leica SP5 II confocal microscope. High resolution z-stacks of images (0.2–0.5 μm z-step size, except for cells in PEGTM, which was at 2 μm z-step size) of hBMSCs were captured for each scaffold type. Care was taken to image single hBMSCs that were not near the scaffold boundaries and that were not touching other cells (one nucleus per object). Images were 8-bit with a pixel resolution of 512 × 512 or greater (line averaging was $n = 2$–4). Fifteen cells were imaged for each scaffold type ($n = 15$ cells).

2.11. Data sources

Confocal image stacks for hBMSCs in PCL-SC, Collagen Gel, PCL-SNF and PCL-BNF were collected anew for the current work. Image stacks for hBMSCs in PDLLA-NF [13], PCL-FFF [16] and PEGTM [19] were collected previously and analyzed for dimensionality herein. Note that hBMSCs from the same donor were cultured under identical conditions in all cases.

2.12. hBMSC image analysis: Cell Area and Z-depth

For calculating Cell Area and Z-Depth, z-stacks of tif images were compiled into 3D renderings with *x*, *y*, and *z* dimensions using ImageJ (*x*–*y*–*z* coordinates). Since