Biomaterials 32 (2011) 2256–2264

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Modulus-driven differentiation of marrow stromal cells in 3D scaffolds that is independent of myosin-based cytoskeletal tension

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ARTICLE INFO

Article history:
Received 4 November 2010
Accepted 26 November 2010
Available online 21 December 2010

Keywords:
Three-dimensional culture
Bone marrow stromal cell
Cytoskeleton
Hydrogels
Matrix modulus

ABSTRACT

Proliferation and differentiation of cells are known to be influenced by the physical properties of the extracellular environment. Previous studies examining biophysics underlying cell response to matrix stiffness utilized a two-dimensional (2D) culture format, which is not representative of the three-dimensional (3D) tissue environment *in vivo*. We report on the effect of 3D matrix modulus on human bone marrow stromal cell (hBMSC) differentiation. hBMSCs underwent osteogenic differentiation in poly (ethylene glycol) hydrogels of all modulus (300-fold modulus range, from 0.2 kPa to 59 kPa) in the absence of osteogenic differentiation supplements. This osteogenic differentiation was modulus-dependent and was enhanced in stiffer gels. Osteogenesis in these matrices required integrin-protein ligation since osteogenesis was inhibited by soluble Arginine-Glycine-Aspartate-Serine peptide, which blocks integrin receptors. Immunostained images revealed lack of well-defined actin filaments and microtubules in the encapsulated cells. Disruption of mechanosensing elements downstream of integrin binding that have been identified from 2D culture such as actin filaments, myosin II contraction, and RhoA kinase did not abrogate hBMSC material-driven osteogenic differentiation in 3D. These data show that increased hydrogel modulus enhanced osteogenic differentiation of hBMSCs in 3D scaffolds but that hBMSCs did not use the same mechanosensing pathways that have been identified in 2D culture.

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1. Introduction

Stem cell-based therapeutics have generated great interest as potential treatments for organ replacement and regenerative medicine [1]. Understanding how cells respond to the physical properties of their surroundings is critical to further development of these therapies. Cell function is strongly influenced by the chemical and physical properties of the local extracellular matrix (ECM). Numerous studies have shown that matrix modulus affects cell behaviors such as migration, organization, proliferation and differentiation [2–4], which ultimately play important roles in physiological processes such as organismal development [5] and cancer progression [6,7].

Human bone marrow stromal cells (hBMSCs) are being widely studied as a source of multipotential cells for regenerative medicine [8]. Optimizing the biomaterial modulus is critical for determining hBMSC fates in a tissue scaffold to maximize generation of the desired tissue. Studies have examined the role of modulus in directing cell response using a two-dimensional (2D) culture format [2–4,6,7,9]. It was reported that hBMSCs cultured on low (<1 kPa), intermediate (8 kPa–15 kPa), and high (25 kPa–40 kPa) modulus gels exhibited increased expression of neuronal, myogenic and osteogenic markers, respectively [2]. This study concluded that lineage commitment of hBMSCs could be directed by the substrate modulus alone through a modulus-sensing mechanism mediated by integrin engagement and myosin-based contraction of the actin cytoskeleton.

Subsequently, hBMSC differentiation was shown to be profoundly influenced not only by substrate modulus but also by the ECM protein that was crosslinked onto the substrate [4]. Indeed, mRNA expression of myogenic (MyoD) and osteogenic (Runx2) markers changed significantly when different ECM proteins were crosslinked to gels of the same modulus [4]. In addition to directing cell differentiation, gel modulus was also shown to regulate hBMSC proliferation. Cells on soft gels (250 Pa), similar to the modulus of bone marrow, assumed a quiescent, non-proliferative state while

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0142-9612/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.biomaterials.2010.11.065

cell proliferation resumed on stiffer gels (7.5 kPa) and was most robust on rigid glass surfaces [3]. Whereas considerable progress has been demonstrated in understanding cellular response to substrate stiffness in 2D formats, the role of matrix modulus in 3D tissue scaffolds is less established. Cellular responses *in vivo* are often different than that observed in planar 2D culture formats, and studies indicate that 3D scaffolds better recapitulate the native tissue environment [10–14]. A recent study has described biophysical mechanisms underlying murine BMSC differentiation in peptide-functionalized hydrogels of different moduli cultured in the presence of both osteogenic and adipogenic supplements. These authors showed that differentiation was regulated by a complex interplay between mechanical and biochemical cues [15].

The objective of the current study was to test the effect of hydrogel modulus on stem cell differentiation in 3D scaffolds and establish the biophysical mechanisms of modulus-driven hBMSC differentiation due to physical cues alone. We describe the response of hBMSCs encapsulated in photopolymerized poly(ethylene glycol) (PEG)-based hydrogels of varying stiffness. PEG hydrogels provide a hydrated environment and are widely used as tissue engineering scaffolds [13,16]. hBMSCs were encapsulated in PEG hydrogels with compressive moduli varying over a 300-fold range and cultured in growth medium without addition of exogenous biomolecules such as medium supplements (dexamethasone, ascorbic acid, β-glycerophosphate), growth factors, or vectors. Cell differentiation was assayed to investigate the effect of hydrogel modulus on hBMSC fate. Further experiments were performed to identify the mechanostransduction pathway through which hBMSCs interacted with the 3D scaffolds. We used an Arginine-Glycine-Aspartate-Serine (RGDS) peptide that binds to integrin receptors and a series of small molecule inhibitors for actin filaments, microtubules, myosin-mediated contraction, and RhoA kinase (ROCK) to determine if these mechanosensing elements mediated the response of encapsulated hBMSCs to scaffold modulus.

2. Materials and methods

2.1. Preparation and characterization of scaffolds

4-arm PEG (total relative molecular mass 20000 g/mol, each arm 5000 g/mol, Jemkem Technology) was reacted with 20-fold molar excess of methacrylic anhydride (Sigma–Aldrich) in a consumer microwave for 10 min to prepare PEG- tetramethacrylate (PEGTM) [16]. Hydrogels were prepared by dissolving a known mass fraction of PEGTM (2%, 3%, 5% or 10%) in 0.1 mol/L phosphate-buffer saline (PBS) containing 0.05 mass % of the photoinitiator Irgacure 2959 (Ciba Chemicals) with 10^6 cells suspended per 1 mL. 50 μL of solution was transferred to Teflon molds (5 mm diameter × 3 mm height) and cured with 365 nm light at 2 mW/cm^2 for 15 min. For mechanical characterization, hydrogels were subjected to a uniaxial static compressive load at a strain rate of 0.03 mm/s by a Dynamic Mechanical Analyzer (TA Instruments).The slope of the linear fit for 5%–10% strain was taken as the measure of the compressive modulus. Shear moduli for the softer gels (2% and 3% PEGTM) were measured between parallel plates using a dynamic strain frequency sweep (ARES-LS, TA Instruments). The loss modulus at 10 Hz was used as measure of the shear modulus of the hydrogel.

2.2. Cell culture

Passage 1 human bone marrow stromal cells (hBMSCs) from a 29 year old female donor were obtained from TulaneUniversity Center for Gene Therapy. hBMSCs were characterized by established guidelines for multipotency by the supplier [17]. Medium was prepared from α-modification of Eagle's minimum essential medium containing L-glutamine (α-MEM, Lonza) supplemented with 16% (volume fraction) fetal bovine serum (Atlanta Biologicals), 2 mmol/L L-glutamine (Sigma–Aldrich), 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma–Aldrich) [18]. Cells from passages 5 and 6 were used for all experiments reported in this study. Control experiments in well plates using osteogenic and adipogenic medium were used to confirm that hBMSCs maintained their multipotency (not shown). For L929 fibroblast (ATCC, CCL-1) control experiments, passage 3 and 4 cells were encapsulated in PEGTM gels and cultured with MEM (Gibco), 10% fetal bovine serum (Gibco), 2 mmol/L L-glutamine (Sigma–Aldrich), and 1 mmol/L sodium pyruvate (Sigma). In all 3D scaffold experiments, cells were encapsulated at 10^6 cells/mL of pre-polymer solution.

For inhibitor studies (all from Sigma), latrunculin A (350 ng/mL), blebbistatin (50 μmol/L), and colchicine (1 μg/mL) were added to the growth medium at each medium change whereas ML-7 (10 μmol/L) and Y-27632 (10 μmol/L) were added to the cultures daily. RGDS peptide (GGGRGDS) was synthesized with an automated peptide synthesizer (APEX 396, Aapptec) using standard solid-phase F-moc chemistry. For RGDS treatments, cells were trypsinized and incubated with RGDS (3.3 mmol/L) for 2 h and RGDS was included in culture medium and refreshed in all medium changes.

2.3. Measurement of cell response

Cell viability was determined semi-quantitatively by staining with calcein AM (Live, 2 μmol/L) and ethidium homodimer-1 (Dead, 2 μmol/L) (Invitrogen) [16]. Resulting live and dead images (3 images per gel) were counted for fractional

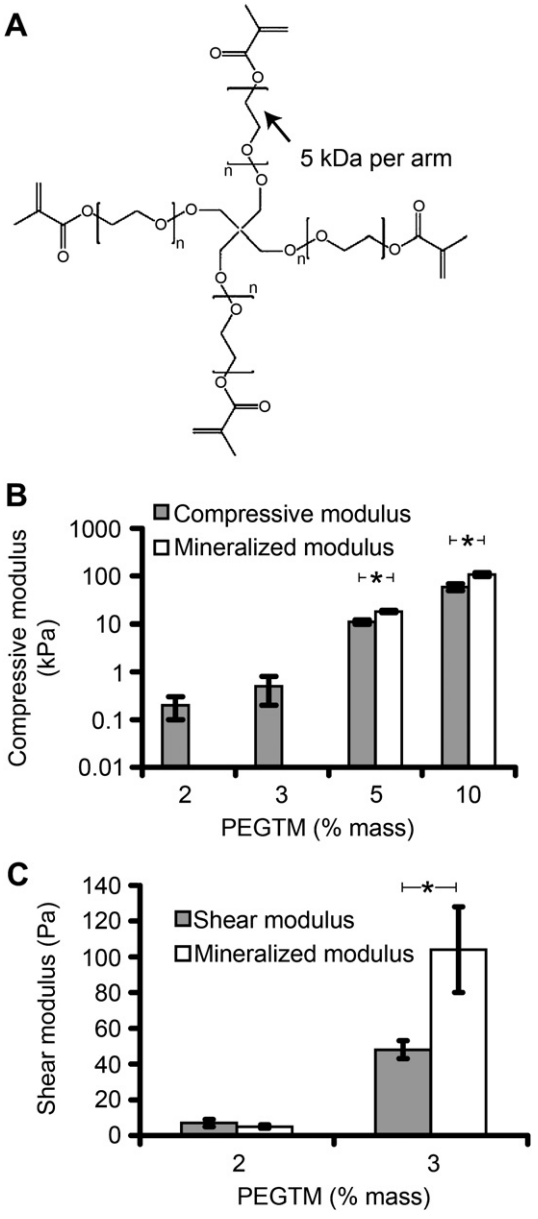

Fig. 1. (A) Chemical structure of the PEGTM monomer with four methacrylate end-groups that facilitate formation of the hydrogel by chemical crosslinking. (B) Plot of compressive modulus of hydrogels for different mass fractions of PEGTM. (C) Softer gels (2% and 3% PEGTM) were characterized in shear modulus as well due to their lower compressive modulus. Graphs also show compressive (B) and shear (C) moduli of mineralized scaffolds at 42 d culture with hBMSCs. Statistical differences ($p < 0.05$, t-test, $n = 4$) are indicated by asterisk.