Biomaterials 31 (2010) 1–8

Contents lists available at ScienceDirect

Biomaterials

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

In situ elasticity modulation with dynamic substrates to direct cell phenotype

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ARTICLE INFO

Article history:
Received 11 August 2009
Accepted 7 September 2009
Available online 27 September 2009

Keywords:
Hydrogel
Elasticity
Photolithography
Fibroblast
Heart valve
Cell activation

ABSTRACT

Microenvironment elasticity influences critical cell functions such as differentiation, cytoskeletal organization, and process extension. Unfortunately, few materials allow elasticity modulation in real time to probe its direct effect on these dynamic cellular processes. Here, a new approach is presented for the photochemical modulation of elasticity within the cell's microenvironment at any point in time. A photodegradable hydrogel was irradiated and degraded under cytocompatible conditions to generate a wide range of elastic moduli similar to soft tissues and characterized using rheometry and atomic force microscopy (AFM). The effect of the elastic modulus on valvular interstitial cell (VIC) activation into myofibroblasts was explored. In these studies, gradient samples were used to identify moduli that either promote or suppress VIC myofibroblastic activation. With this knowledge, VICs were cultured on a high modulus, activating hydrogel substrate, and uniquely, results show that decreasing the substrate modulus with irradiation reverses this activation, demonstrating that myofibroblasts can be de-activated solely by changing the modulus of the underlying substrate. This finding is important for the rational design of biomaterials for tissue regeneration and offers insight into fibrotic disease progression. These photodegradable hydrogels demonstrate the capability to both probe and direct cell function through dynamic changes in substrate elasticity.

1. Introduction

Fabrication of cell culture substrates with different elastic moduli has been explored for the past decade, as researchers identified that cell contractile forces and related cell functions, such as motility [1], cytoskeletal organization [2], and differentiation [3,4], are influenced by the elasticity of the underlying substrate [5,6]. For example, in response to an injury, fibroblasts are known to differentiate into myofibroblasts, a wound healing phenotype responsible for repairing and replacing damaged extracellular matrix (ECM) in injured tissues and organs [7]. As ECM is secreted, the elastic modulus of the cell microenvironment increases. Once the desired matrix modulus is achieved, the myofibroblasts deactivate and undergo a number of cellular processes, including senescence [8] and apoptosis [7], the exact pathways of which are not well understood. If this de-activation is misregulated and the myofibroblast phenotype persists, ECM secretion continues, increasing the matrix modulus and effecting fibrosis [9]. Understanding the role of the matrix modulus in dynamic cellular signaling processes such as these is important for treatment of fibrotic diseases, as well as in the design of tissue regeneration strategies [3]. Many researchers have thus sought to explore the influence of substrate elasticity on cell function by the development of materials with highly regulated properties.

Researchers have used discrete poly(acrylamide)-, poly(ethylene glycol) (PEG)-, or poly(dimethyl siloxane)-based gels with different moduli to explore cellular processes such as myotube differentiation [10], embryonic cardiomyocyte beating [11], and neuronal cell process extension [12]; smooth muscle cell (SMC) proliferation and focal adhesion formation [13]; and myofibroblastic activation [14], respectively. These materials are useful but require the preparation of a unique formulation for each gel (e.g., different crosslinker concentration) to vary the modulus, and more importantly, the material properties are fixed upon formation. To address some of these limitations, advanced processing techniques have been developed to create modulus gradients within hydrogels [15,16]. For example, gradient hydrogels formed from mixtures of acrylamide and bis-acrylamide have been used to study fibroblast migration [17]. Improved gradient fabrication techniques, such as using

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doi:10.1016/j.biomaterials.2009.09.025

microfluidics with poly(acrylamide)-hydrogels [18] and advanced patterning with poly(dimethylsiloxane) (PDMS) [19] gels, have been used to examine vascular SMC spreading and cytoskeletal organization [18] and fibroblast and endothelial cell migration [19], respectively. While these approaches have been proven versatile, the material properties are static. Recently, Frey and Wang developed a polyacrylamide-based photodegradable hydrogel whose modulus can be decreased 20–30% of its initial value with irradiation in the presence of NIH 3T3 fibroblasts [20]. This dynamic modulation of substrate rigidity was used to study the influence of modulus on 3T3 cell morphology and migration. In parallel to this work, we were interested in probing the influence of dynamic changes in substrate modulus on cell function (e.g., differentiation), which often requires a large variation in modulus. Specifically, we have developed a photodegradable monomer for synthesizing PEG-based hydrogels that degrade in response to light, and the chemistry is compatible with cell encapsulation and 3D cell culture [21]. Here, we exploit this chemistry to create a 2D cell culture platform whose elasticity can be tuned over a wide range of moduli and subsequently varied in real time by exposure to light. Experiments were designed to answer how *in situ* changes in substrate modulus might influence the fibroblast–myofibroblast differentiation process, especially how myofibroblast de-differentiation might be directed by a step change in the elasticity of its microenvironment. In general, a cell culture substrate that allows on-demand creation of materials with variable elasticity, either in a discrete or gradient fashion, would allow the design of unique experiments to further explore the influence of elasticity on cellular functions. Moreover, such materials would improve the understanding of how cells respond to dynamic microenvironmental changes (e.g., the role of matrix elasticity in promoting or suppressing fibrosis).

2. Materials and methods

2.1. Hydrogel preparation

The photodegradable crosslinker (PEGdiPDA) was synthesized as previously described [21]. PEGdiPDA ($M_n \sim 4070$ g/mol, 8.2 wt%) was copolymerized with PEGA ($M_n \sim 400$ g/mol, 6.8 wt%, Monomer–Polymer and Dajac Laboratories, Inc) in PBS via redox-initiated free radical polymerization using 0.2 M ammonium persulfate (AP) and subsequently adding 0.1 M tetraethylmethylenediamine (TEMED) while vortexing. The polymerization is complete within 5 min, based on modulus evolution followed by rheometry. The hydrogels were formed *in situ* for rheometry experiments. For all other experiments, hydrogels were polymerized between glass coverslips (22 mm × 22 mm) separated by a 0.25-mm thick spacer (9 mm × 9 mm). For cell experiments, fibronectin (100 nM, BD Bioscience) was mixed in the monomer solution and entrapped within the hydrogel. Uniform presentation of fibronectin with degradation was verified by fluorescent labeling of the protein and subsequent imaging *in situ* (Supplemental Figure S1). The hydrogel was covalently linked to one of the coverslips during polymerization by methacrylation of the coverslip directly prior to use. The methacrylated coverslip was prepared by cleaning of the slip with Piranha (30 min, 3:1 by volume sulfuric acid:hydrogen peroxide), rinsing with copious amounts of deionized (DI) water, rinsing with acetone, and chemical vapor deposition of methacrylopropyltrimethoxysilane (Gelest) at 60 °C for 3 h under argon. After preparation, hydrogels were transferred to PBS for AFM characterization or to cell culture media (M199, Invitrogen) with 1 μg/mL amphotericin B, 50 U/mL penicillin, and 50 μg/mL streptomycin. Media was refreshed after 12 h to remove any remaining monomer or initiator.

2.2. Hydrogel characterization with rheometry

Hydrogel degradation was characterized with a photorheometer ($\gamma = 10\%$, $\omega = 10$ rad/s for linear viscoelastic regime, ARES, TA). A thin hydrogel film (0.05 mm) was polymerized *in situ* between an 8 mm diameter flat quartz plate and a temperature-controlled Peltier flat plate (25 °C). Upon complete polymerization, the hydrogel was surrounded with a small amount of water to prevent dehydration, and the hydrogel storage modulus (G') was monitored, which dominates for these elastic polymer networks ($G \approx G'$ when $G' > G''$) [22], while irradiating the sample with UV light (365 nm at 10 mW/cm^2, EXFO, Novacure, high-pressure mercury arc lamp with light guide and collimating lens). To follow degradation, G' was normalized to its initial value G_0'. The storage modulus was converted to Young's modulus (E) using rubber elasticity theory, where $G = E/2(1+\nu)$, assuming a Poisson's ratio (ν) of 0.5 for bulk measurements of elastic hydrogel polymer networks [22,23].

2.3. Hydrogel film degradation and characterization with AFM

A surface elasticity gradient was created by irradiating the surface of a photodegradable hydrogel with a light gradient (365 nm at 10 mW/cm^2 for 0–5 min across the surface, where light penetration and gel degradation are limited to the top ~50 μm of the gel). This light gradient was achieved by irradiating the hydrogel while continuously covering the hydrogel surface with an opaque plate from one side (0 mm) to another (9 mm) with a cover plate attached to a linear motion stage [24]. The covering rate is set so that the entire sample was covered prior to complete degradation of the sample surface (<10 min of 365 nm at 10 mW/cm^2 as determined by rheometry). This degradation gradient creates a gradient in surface elasticity from the initial hydrogel modulus on one side of the gel to a modulus that is ~20% of the initial value after 5 min of degradation on the other side of the gel. The resulting surface elasticity gradient was measured with atomic force microscopy [25] (AFM, PicoPlusTM scanning probe microscope, Molecular Imaging, Inc., pyramidal silicon nitride tip with force constant 0.12 N/m, radius = 10 nm, height = 2.5–3.5 nm, and angle of pyramid = 35°), where E at various linear positions across a gel surface was obtained using the Hertz model and $\nu \approx 0.2$ for the surface of a PEG hydrogel [26]. Discrete samples with high and low moduli were similarly prepared except with either no irradiation or flood irradiation of the entire sample for 5 min (365 nm at 10 mW/cm^2). In addition, elasticity was modulated in the presence of cells on discrete, high-modulus samples with flood irradiation for 5 min (365 nm at 10 mW/cm^2).

2.4. Cell culture

VICs were isolated from porcine aortic valve leaflets by sequential collagenase digestion as previously described within 24 h after sacrifice [27]. Isolated cells were cultured in growth media (Medium 199, 15% fetal bovine serum (FBS), 2% penicillin/streptomycin (100 U/mL), 0.4% fungizone (0.5 μg/mL)) and successively passaged (passage 2 and 3 used for experiments). All experiments were performed in low serum (1% FBS supplemented) media to minimize cell proliferation and plated at 40,000 cells/cm^2.

2.5. Real-time cell tracking

Time lapse images of cell movement were captured every 15 min over a 3 day period using a Nikon TE 2000 PFS fluorescent microscope equipped with a motorized stage and an environmental sample chamber. Images were collected and analyzed using MetaMorph software (Molecular Devices). Velocities were calculated by taking the change in cell position divided by the time interval (i.e., 15 min) over 3 days, which were subsequently averaged for each cell. The velocities presented are average velocities for several cells over 3 days on the gradient substrates.

2.6. Immunostaining

Samples were fixed with 10% buffered formalin, permeabilized in 0.05 wt% Tween 20, blocked with 3 wt% bovine serum albumin (BSA), and incubated with mouse anti-α-smooth muscle actin (αSMA) (Abcam) antibody. Following primary antibody coupling, samples were washed and incubated with goat-anti-mouse Alexa 488 (Invitrogen) and phalloidin-tetramethylrhodamine B isothiocyanate (Sigma–Aldrich). Samples were subsequently counter-stained with DAPI and imaged on a Nikon TE 2000 epi-fluorescence microscope with 20× magnification objective. Images from each fluorescent channel were merged, and the background was flattened using MetaMorph. The number of cells was counted using the DAPI channel and ImageJ (Analyze Particles function, NIH). The number of myofibroblasts was counted manually by identifying cells with αSMA organized into fibrils, and the percentage of myofibroblasts was calculated by dividing the number of myofibroblasts by the total number of cells and multiplying by 100%.

2.7. Gene expression

Cells were transfected with a αSMA-luciferase reporter construct (5 μg plasmid), developed by N.A. Rice [28], using an Amaxa Nucleofector (program U-23 as recommended by the manufacturer). Cells were then seeded on hydrogels, and samples were analyzed for luciferase activity on Day 1 and 3 (Promega Bright-Glo Luciferase Assay System per manufacturers instructions).

2.8. Statistics

All data collected are presented as mean ± standard error of three or more samples. A Student's t-test was used to compare data sets, and the resulting p values that were used to determine statistical significance are noted.