Acta Biomaterialia 9 (2013) 7640–7650

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

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

Stiffness and adhesivity control aortic valve interstitial cell behavior within hyaluronic acid based hydrogels

Bin Duan [a], Laura A. Hockaday [a], Edi Kapetanovic [b], Kevin H. Kang [a], Jonathan T. Butcher [a,*]

[a] Department of Biomedical Engineering, Cornell University, Ithaca, NY, USA
[b] College of Human Ecology, Cornell University, Ithaca, NY, USA

ARTICLE INFO

Article history:
Received 11 March 2013
Received in revised form 24 April 2013
Accepted 26 April 2013
Available online 3 May 2013

Keywords:
Stiffness
Cell spreading
Microenvironment
Tissue engineering
Phenotype

ABSTRACT

Bioactive and biodegradable hydrogels that mimic the extracellular matrix and regulate valve interstitial cells (VIC) behavior are of great interest as three-dimensional (3-D) model systems for understanding mechanisms of valvular heart disease pathogenesis in vitro and the basis for regenerative templates for tissue engineering. However, the role of stiffness and adhesivity of hydrogels in VIC behavior remains poorly understood. This study reports the synthesis of methacrylated hyaluronic acid (Me-HA) and oxidized and methacrylated hyaluronic acid, and the subsequent development of hybrid hydrogels based on modified HA and methacrylated gelatin (Me-Gel) for VIC encapsulation. The mechanical stiffness and swelling ratio of the hydrogels were tunable with the molecular weight of the HA and the concentration/composition of the precursor solution. The encapsulated VIC in pure HA hydrogels with lower mechanical stiffness showed a more spreading morphology compared to their stiffer counterparts and dramatically up-regulated alpha smooth muscle actin expression, indicating more activated myofibroblast properties. The addition of Me-Gel in Me-HA facilitated cell spreading, proliferation and VIC migration from encapsulated spheroids and better maintained the VIC fibroblastic phenotype. The VIC phenotype transition during migration from encapsulated spheroids in both Me-HA and Me-HA/Me-Gel hydrogel matrixes was also observed. These findings are important for the rational design of hydrogels for controlling the VIC morphology, and for regulating the VIC phenotype and function. The Me-HA/Me-Gel hybrid hydrogels accommodated with VIC are promising as valve tissue engineering scaffolds and 3-D models for understanding valvular pathobiology.

1. Introduction

The physiochemical similarity to the native extracellular matrix (ECM) and the permeability to nutrients make hydrophilic hydrogels advantageous for tissue engineering applications [1,2]. By carefully selecting a hydrogel material, crosslinking method and conjugatable biomolecules, hydrogels can support both two-dimensional (2-D) surface-seeded cell culture and three-dimensional (3-D) cell encapsulation [3,4]. Numerous efforts have been made to investigate the role of 3-D microenvironments on cell behavior, especially the effects of stiffness, matrix components and chemical stimuli [5,6].

Among various biodegradable synthetic and natural polymers, hyaluronic acid (HA), a glycosaminoglycan (GAG) component richly present in connective tissues, have been reported to play an important role in cell signaling [7], cell migration [8], wound repair [9] and morphogenesis [10]. HA-based hydrogels are thus widely used as wound healing dressing, tissue engineering scaffolds, and cell/molecule delivery carriers [11,12]. The mechanical properties of hyaluronic acid based hydrogels are tunable by varying crosslinking degree [13–16], photoinitiator concentration [17], or crosslinker concentration [18,19]. The stiffness of HA-based hydrogels can regulate phenotypic changes and differentiation of human hepatic stem cells [18], mesenchymal stem cells (MSC) [15] and neural progenitor cells [13]. With combination of matrix metalloproteinase (MMP) degradable crosslinkers and arginine–glycine–aspartic acid (RGD) peptide, HA-based hydrogels can modulate the spreading and migration of mouse MSC [20].

Valve interstitial cells (VIC) are the major cell type found in heart valve leaflets and play a critical role in maintaining homeostasis and driving tissue remodeling within the dynamic environment of the valve [21]. VIC are a heterogeneous population, with up to five distinct phenotypes [22]. The predominant VIC phenotype in healthy adult valves is quiescent fibroblasts, with only 2–5% expressing myofibroblast markers [23]. VIC are activated and show a myofibroblastic phenotype with more alpha smooth muscle actin (αSMA) expression during remodeling and disease states [24,25]. Prolonged activation of this myofibroblast phenotype may result in pathological heart valve matrix remodeling, formation of

* Corresponding author. Tel.: +1 607 225 3575.
E-mail address: jtb47@cornell.edu (J.T. Butcher).

1742-7061/$ - see front matter © 2013 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.actbio.2013.04.050

calcific nodules on the valvular leaflets and further stenosis of heart valve [23,26,27]. The characterization of VIC in response to substrate stiffness in 2-D culture has been widely investigated [28–32]. For 3-D culture, VIC are usually encapsulated in collagen hydrogels [33,34] and poly(ethylene glycol) (PEG) based hydrogels [35,36], which are model biomaterials rather than serving a tissue engineering purpose. Collagen hydrogels can support the spreading and proliferation of VIC well, but they can be caused to contract quite easily and are mechanically inadequate [37]. PEG hydrogels are considered as blank slates and can control the fate of cells through co-polymerization with other macromolecules and multiple functional moieties [38]; however, PEG cannot be totally degraded or remodeled. VIC in 3-D methacrylated hyaluronic acid (Me-HA) based hydrogels had very limited spreading morphology without incorporation of any cell adhesion motif [39], and the encapsulated VIC may have a different phenotype from the native tissue. Although VIC have been shown to be able to achieve their native morphology within methacrylated gelatin (Me-Gel) hydrogels [40], the role of hydrogel stiffness on VIC behavior in 3-D environment remains unclear. In addition, hybrid hydrogels such as collagen and GAG are better ECM analogs with which to investigate the role of the 3-D microenvironment on VIC physiological and pathological phenotypes [41]. It is thus important to develop appropriate 3-D hydrogels with biochemical and biophysical tunability for mimicking ECM to better regulate VIC activity and for living valve replacements as well.

In this study, we developed photocrosslinked hydrogels formed from oxidized and methacrylated HA with different molecular weights and tunable stiffness. Combining the modified HAs with Me-Gel, we assessed the roles of stiffness and adhesion on VIC proliferation, spreading and phenotype. Furthermore, we studied the migration rate and phenotype of VIC from encapsulated VIC spheroids in HA-based hydrogels as well as in hybrid hydrogels.

2. Materials and methods

2.1. Modification of HA and gelatin

Oxidized HA (OHA) with different molecular weights was first prepared by reacting HA (Novozymes) with sodium periodate (Sigma). Briefly, 10 ml of aqueous sodium periodate solution (0.05 or 0.1 mol ml^{-1}) was added to HA solution (0.5 g in 100 ml of distilled water) with continuous stirring in the dark at room temperature. The reaction was stopped after 10 min by the addition of ethylene glycol (Sigma; molar ratio of ethylene glycol:sodium periodate = 1:1). The OHA was purified by dialysis (MWCO 1000; Spectra/por, Spectrum) against distilled water for 3 days. Then methacrylated HA and OHA (Me-HA and MOHA) were synthesized by the addition of a methacrylate functional group to the HA as described by Smeds et al. [42] and Brigham et al. [43]. Briefly, 10 ml of methacrylic anhydride (MA; Sigma) was reacted with HA (or OHA) solution at 40 °C for 6 h and the pH of mixture during reaction was maintained at 8.5 by adding 5 N NaOH. The Me-HA and MOHA solution was dialyzed for 3 days and lyophilized.

The molecular weight and polydispersity of HA and various modifications of HA were determined by a Waters gel-permeation chromatography (GPC) system equipped with three ultrahydrogel columns (i.e. 2000, 500 and 250 Å in series, Waters) in series, a 1515 isocratic high-performance liquid chromatography pump (Waters) and a 2414 refractive index detector (Waters). The mobile phase employed was 0.1 M phosphate buffer saline (PBS, pH 7.4) with 0.125% sodium azide at a rate of 0.8 ml min^{-1} calibrated with eight individual pullulan standards.

Degree of methacrylation was determined using ^1H nuclear magnetic resonance (^1H NMR) spectroscopy. Lyophilized modified HA was dissolved in D_2O at a concentration of 10 mg ml^{-1} at 40 °C and spectra were recorded on a Bruker ARX-300 spectrometer using tetramethylsilane as the internal standard. The degree of methacrylation was calculated as the ratio of the relative peak integration of the double bond protons in the methacrylate groups (peaks at ~5.6 and ~6.1 ppm) to the methyl protons of HA (peak at ~1.9 ppm) normalized according to the number of protons per group.

Me-Gel was synthesized as previously described [44]. Briefly, type A porcine skin gelatin (Sigma) was dissolved at 10% (w/v) into distilled water at 40 °C and then MA (1:5 v/v to gelatin solution) was added dropwise under continual stirring at 40 °C for 1 h. The solution was dialyzed at 40 °C to remove methacrylic acid and lyophilized for 3 days.

2.2. Preparation and characterization of HA-based hydrogels

Photocrosslinkable HA-based polymers (Me-HA or MOHA, 0.75% w/v) with or without Me-Gel (1% w/v) were dissolved in cell culture medium with 0.03% w/v 2-hydroxy-1(4-(hydroxyethox)pheny)-2-methyl-1-propanone (Irgacure 2959; BASF The Chemical Company). The gel precursor solution was transferred into molds and subsequently exposed to 365 nm UV light (EN-280L, Spectroline, 2.0 mW cm^{-2}) for 5 min. Six types of hydrogels were fabricated using methacrylated HA of various molecular weights ($MO_{0.1}$HA, $MO_{0.05}$HA and Me-HA) with the addition of Me-Gel. These were denoted as $MO_{0.1}$HA, $MO_{0.05}$HA, Me-HA, $MO_{0.1}$-HA/Me-Gel, $MO_{0.05}$HA/Me-Gel and Me-HA/Me-Gel.

Uniaxial compressive testing of different hydrogels (8 mm in diameter, 2 mm in thickness, $n = 4–6$) was performed using an ELF 3200 (Bose EnduraTEC) mechanical test-frame. A 250 g load cell (Sensotec) was attached to the bottom plate and a displacement sensor to the top plate with the cross-head speed of 0.01 mm s^{-1}. The bulk compressive moduli were calculated from the slope of the initial linear region (5–10% strain for compressive test) of the respective stress–strain curves.

For mass swelling ratio measurement, different hydrogel samples were dried and weighed (weight W_d) and reimmersed in DMEM at 37 °C for 48 h. The weight of the samples ($n = 5$) in the swollen state (W_s) was then measured. The mass swelling ratio (Q) was calculated by $Q = (W_s - W_d)/W_d$.

2.3. Cell culture and cell encapsulation in 3-D hydrogels

Porcine aortic VIC were isolated from porcine aortic valves obtained at a slaughterhouse (Shirk Meats, Himrod NY) using collagenase digestion of the leaflets, as previously described [37]. Cells were used at passages 5–8.

VIC were encapsulated in the 3-D hydrogels using two different strategies: homogeneous encapsulation with individual cells throughout the hydrogel and spheroid encapsulation with a single cluster of cells inside the hydrogel. The hydrogels containing homogeneously encapsulated cells were used to study VIC spreading and phenotype while the encapsulated VIC spheroids were used to study cell migration. For the homogeneous encapsulation, VIC were resuspended in different precursor solutions (polymer plus culture medium with Irgacure at concentration described previously) at a density of 5×10^6 cells ml^{-1}, then 50 μl of cell encapsulated precursor solution was transferred into molds and subject to photopolymerization with UV light for 5 min. VIC spheroids were prepared by the hanging drop method. Briefly, 20 μl of the cell resuspension at a density of 5×10^6 cells ml^{-1} was deposited onto the lid of a 48-well plate and cultured at 37 °C in a cell culture incubator overnight. For VIC spheroid encapsulation, a poly(ethylene glycol) diacrylate (PEGDA, MW 3350) layer was first prepared from 30 μl precursor solution (with 0.03% w/v Irgacure) to ensure that cells would not migrate out of the gel and grow onto the dish.