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Mitral valvular interstitial cell responses to substrate stiffness depend on age and anatomic region

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ABSTRACT

The material properties of heart valves depend on the subject's age, the state of the disease and the complex valvular microarchitecture. Furthermore, valvular interstitial cells (VICs) are mechanosensitive, and their synthesis of extracellular matrix not only determines the valve's material properties but also provides an adhesive substrate for VICs. However, the interrelationship between substrate stiffness and VIC phenotype and synthetic properties is poorly understood. Given that the local mechanical environment (substrate stiffness) surrounding VICs differs among different age groups and different anatomic regions of the valve, it was hypothesized that there may be an age- and valve-region-specific response of VICs to substrate stiffness. Therefore, 6-week-, 6-month- and 6-year-old porcine VICs from the center of the mitral valve anterior leaflet (MVAC) and posterior leaflet (PML) were seeded onto poly(ethylene) glycol hydrogels of different stiffnesses and stained for markers of VIC activation (smooth muscle alpha-actin (SMaA)) and collagen synthesis (heat shock protein-47 (HSP47), prolyl 4-hydroxylase (P4H)). Six-week-old MVAC demonstrated decreased SMaA, P4H and HSP47 on stiffer gels, while 6-week-old PML only demonstrated decreased HSP47. Six-month-old MVAC demonstrated no difference between substrates, while 6-month-old PML demonstrated decreased SMaA, P4H and HSP47. Six-year-old MVAC demonstrated decreased P4H and HSP47, while 6-year-old PML demonstrated decreased P4H and increased HSP47. In conclusion, the age-specific and valve-region-specific responses of VICs to substrate stiffness link VIC phenotype to the leaflet regional matrix in which the VICs reside. These data provide further rationale for investigating the role of substrate stiffness in VIC remodeling within diseased and tissue engineered valves.

1. Introduction

Valve disease afflicts a substantial portion of the population: 1–2% of 26- to 84-year-olds are afflicted by mitral valve disorders [1]. Valve disease incurs significant morbidity and mortality, requiring over 100,000 surgeries in the USA each year [2]. In many of these disease states the mechanical properties of these valves are altered, often contributing to the poor valve function requiring surgical intervention. Valvular interstitial cells (VICs) are the dynamic, living component of heart valves responsible for synthesizing and maintaining the valve matrix composition, which in turn determines the valve's material behavior. VICs and valves are known to be responsive to changes in their mechanical environment [3–5]. However, the interplay between matrix-driven material properties such as stiffness and the phenotype and synthetic behavior of VICs, particularly in the mitral valve (MV), has largely been overlooked. Recent work has demonstrated age-related significant changes in valve composition [6–8] and material properties [9]; other studies have shown substantial heterogeneity in material behavior among the different anatomic regions of the MV [10]. Given that different aged VICs and VICs from different regions of the MV reside in valve tissues with distinct stiffnesses [9], it was hypothesized that there may be an age- and valve-region-specific response of VICs to substrate stiffness.

In order to test this hypothesis, separate groups of VICs from three distinct age groups and from two different regions of the MV were cultured on poly(ethylene) glycol (PEG) hydrogels of two different stiffnesses. After 48 h, the resulting VIC expression of cell phenotype and collagen synthesis markers was assessed using immunocytochemistry (ICC).

PEG hydrogels were chosen for this experiment based on their promise as a potential platform for the design of scaffolds for tissue-engineered heart valves. PEG hydrogels are extremely hydrophilic, providing prevention against protein adsorption, a critical step in the immunogenicity and degradation of bioprosthetics [11]. They are also highly permeable, allowing the exchange of nutrients and waste materials [12]. Their stiffness can be regulated by changing the molecular weight and concentration of PEG [13]. However, one of the factors that makes these gels particularly

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attractive is the ability to customize them by conjugating to the PEG backbone various peptides, including cell ligands and growth factors, as well as incorporating enzyme-degradable sequences allowing tunability of the degradation rate of the hydrogel. This designer biofunctionality makes PEG hydrogels advantageous for the tissue engineering of heart valves. In the present study PEG hydrogels were conjugated with an Arg–Gly–Asp–Ser (RGDS) peptide, enabling VIC attachment to the hydrogel, and methacrylated heparin, which is necessary for normal VIC morphology [14,15]. These functionalized PEG hydrogels of two different stiffnesses were formulated to keep the concentration of biological cues constant, thus isolating the effect of stiffness on VIC phenotype.

2. Materials and methods

2.1. Synthesis of PEG hydrogel components

PEG-diacrylate (PEG-DA) of 3400 Da MW was synthesized from PEG (Sigma–Aldrich, St. Louis, MO) as previously described [13]. ^{1}H nuclear magnetic resonance (NMR) analysis revealed >95% acrylation. Methacrylated heparin was synthesized as described previously [14]. Briefly, a 10 mg ml^{-1} solution of heparin (Sigma–Aldrich) dissolved in ultrapure water was reacted with 40 molar excess methacrylic anhydride (Sigma–Aldrich). The pH of the solution was adjusted to 7.5 using 4 M NaOH and stirred for 24 h. Methacrylated heparin was then precipitated using cold 95% ethanol. The precipitate was then filtered, dried and dialyzed against ultrapure water using a 1000 Da MWCO membrane (Spectrum Laboratories, Rancho Dominguez, CA). The product was then lyophilized. ^{1}H NMR analysis revealed 5% methacrylation per disaccharide.

The RGDS peptide (Bachem, Bubendorf, Switzerland) was attached to hetero-bifunctional PEG (PEG-SCM; Laysan Bio, Arab, AL) by reacting the peptide with hetero-bifunctional PEG and catalyst diisopropylamine (Sigma–Aldrich) in dimethyl sulfoxide (DMSO; 1.2 M RGDS:1.0 M PEG-SCM:2.0 M disopropylamine) for 24 h. The product was then dialyzed against ultrapure water using a 1000 Da MWCO membrane (Spectrum Laboratories) and lyophilized. The Trp–Arg–Gly–Asp–Ser (WRGDS) peptide (GenScript, Piscataway, NJ) was similarly attached to hetero-bifunctional PEG. Evaluation of PEG-RGDS by gel permeation chromatography revealed 81% conjugation.

2.2. Polymerization of functionalized PEG hydrogels

PEG hydrogels were synthesized by dissolving the appropriate amounts of PEG-RGDS, methacrylated heparin and PEG-DA in phosphate-buffered saline. A volume of 10 μl ml^{-1} 2,2-dimethoxy-2-phenyl-acetophenone (300 mg ml^{-1} in 1-vinyl-2-pyrrolidone) was added and the solution was poured between two sterile glass slides separated by a 0.4 mm spacer and exposed to ultraviolet light for 2 min (365 nm, 10 mW cm^{-2}). Hydrogels were then removed from the mold and soaked in phosphate-buffered saline with 2% antibiotic (Mediatech, Herndon, VA) for at least 24 h allowing the hydrogels to swell to equilibrium. Hydrogel thickness was optically measured using a Leica DFC 320 CCD camera (Wetzlar, Germany) and ImagePro acquisition software (Media Cybernetics, Bethesda, MD). Hydrogel thicknesses were determined from the acquired images using ImageJ software (NIH, Bethesda, MD).

2.3. Optimization of functionalized PEG hydrogels

Optimization studies were performed to determine the concentrations of PEG-RGDS and methacrylated heparin in the pre-polymer solution necessary to yield equivalent amounts of these bioactive ligands in the swollen, polymerized gels of the two different weight–volume fractions of PEG-DA. The concentration of methacrylated heparin in polymerized, swollen gels was determined using an uronic acid assay, as described by Blumenkrantz and Asboe-Hansen [16] after gels were hydrolyzed by reacting them with 0.1 N NaOH for 34 h at 37 °C. These studies determined that 9.0 mg ml^{-1} of methacrylated heparin in the pre-polymer hydrogel solution for the 5% weight–volume PEG-DA hydrogel and 10.6 mg ml^{-1} of methacrylated heparin in the solution for the 15% weight–volume PEG-DA hydrogel yielded equivalent concentrations of methacrylated heparin in the polymerized, swollen gels of the different PEG-DA weight–volume fractions (Fig. 1). Optimization studies utilizing tryptophan (which was detected by its absorbance at 280 nm using a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyavale, CA)) in the RGDS peptide (WRGDS, conjugated to PEG using the same reaction as for RGDS; gel permeation chromatography revealed 83% conjugation), determined that 7.49 mg ml^{-1} of PEG-RGDS in the pre-polymer solutions for both weight–volume PEG-DA hydrogels yielded equivalent final concentrations of PEG-RGDS in the swollen, polymerized gels (Fig. 2).

2.4. Determination of elastic modulus of functionalized PEG hydrogels

Strips of the two weight–volume fraction PEG hydrogels (5 mm in width) were uniaxially tensile tested using an EnduraTec ELF 3200 (Bose, Eden Prairie, MN). The strain rate was 10 mm s^{-1} and load–elongation data were recorded until failure occurred. Displacement was converted to strain based on the initial hydrogel length between grips. The elastic modulus was determined as the slope of the least-squares linear fit to the stress–strain curve.

2.5. Cell culture and cell seeding onto PEG

Mitral valves were dissected from hearts from 6-week-, 6-month- and 6-year-old pigs obtained from an abattoir (the 6-week- and 6-month-olds from Fisher Ham and Meat, Spring, TX; the 6-year old from Animal Technologies, Tyler, TX). Based on previous studies demonstrating that the mitral valve anterior center (MVAC) is stiffer than the posterior leaflet [10], VICs were isolated from the MVAC (representing a stiffer valve region) and the PML (representing a less stiff valve region) from the same mitral valves according to previously published protocols [17]; cells were cultured in medium containing 10% bovine growth serum (HyClone, Logan, UT) and 2% antibiotic/antimycotic (Mediatech). The medium was changed every 2–3 days and cells were passaged after reaching

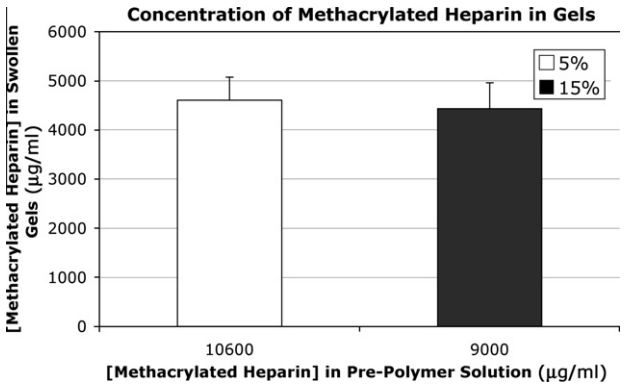

Fig. 1. Concentration of methacrylated heparin in the two different weight–volume fraction PEG swollen gels as determined by uronic acid assay. Four PEG hydrogel samples of each weight–volume fraction were tested. Error bars on all graphs indicate standard error of the mean.

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