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Correlation between valvular interstitial cell morphology and phenotypes: A novel way to detect activation

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ABSTRACT

Valvular interstitial cells (VICs) constitute the major cell population in heart valves. Quiescent fibroblastic VICs are seen in adult healthy valves. They become activated myofibroblastic VICs during development, in diseased valves and *in vitro*. 2D substrate stiffness within a 5–15 kPa range along with high passage numbers promote VIC activation *in vitro*. In this study, we characterize VIC quiescence and activation across a 1–21 kPa range of substrate stiffness and passages. We define a cell morphology characterization system for VICs as they transform. We hypothesize that VICs show distinct morphological characteristics in different activation states and the morphology distribution varies with substrate stiffness and passage number. Four VIC morphologies - tailed, spindle, rhomboid and triangle - account for the majority of VIC in this study. Using α-smooth muscle actin (α-SMA), non-muscle myosin heavy chain B (SMemb) and transforming growth factor β (TGF-β) as activation markers for validation, we developed a system where we categorize morphology distribution of VIC cultures, to be potentially used as a non-destructive detection method of activation state. We also show that this system can be used to force stiffness-induced deactivation. The reversibility in VIC activation has important implications in *in vitro* research and tissue engineering.

1. Introduction

Heart valve diseases are a major source of morbidity and mortality. Approximately 5 million people are burdened with heart valve disease in USA (Nkomo et al., 2006). Heart valves are passive tissues that control direction of blood flow in the heart. They have three major components: valvular interstitial cells (VICs), valvular endothelial cells (VECs) and extracellular matrix (ECM) (Donnelly, 2008). VICs constitute the major cell population in heart valves. Functions of VIC include maintenance of structural integrity, synthesis of ECM components, homeostasis, repair and remodeling, *etc.* (Mulholland and Gotlieb, 1996; Taylor et al., 2003). Specific VIC functions are performed by specific phenotypes of VIC (Liu et al., 2007; Rabkin-Aikawa et al., 2004). VICs can have five phenotypes described by Liu et al. (2007) as follows: embryonic progenitor endothelial/mesenchymal cells, quiescent VIC (qVIC), activated VIC (aVIC), progenitor VIC (pVIC) and osteoblastic VIC (obVIC). qVICs reside in healthy adult heart valves and are responsible for physiological maintenance (Rabkin-Aikawa et al., 2004). qVICs have fibroblastic properties (Liu et al., 2007). aVICs, also known as the myofibroblastic phenotype, are activated forms of qVICs. aVICs remodel the ECM and take part in proliferation and other cellular activities (Liu et al., 2007; Rabkin et al., 2001; Walker et al., 2004). Increased population of aVIC results in abnormal valve shapes and degenerative diseases (Rabkin et al., 2001). aVICs are more abundant in developing (Hinton et al., 2006) and degenerative heart valves (Rabkin-Aikawa et al., 2004). Myofibroblastic aVICs show high expression of α-smooth muscle actin (α-SMA), non-muscle myosin heavy chain B (SMemb) and transforming growth factor β (TGF-β), typically absent from qVICs (Liu et al., 2007; Rabkin-Aikawa et al., 2004; Rabkin et al., 2001).

VIC activation *in vivo* and *in vitro* occurs due to abnormal mechanical and chemical environments (Donnelly, 2008; David Merryman, 2010; Sacks and Yoganathan, 2007). Substrate stiffness has an important effect on VIC activation or phenotype transformation (Kloxin et al., 2010; Quinlan and Billiar, 2012; Yip et al., 2009). It has been shown that VICs, in 2D *in vitro* conditions, become activated on substrate stiffness close to single digit values in kPa. Kloxin et al. (2010) showed that VICs in 2D culture become activated and deactivated above and below 15 kPa substrate stiffness. Later publications from the same research group showed VIC activation above 7 kPa substrate

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stiffness (Wang et al., 2012; Gould et al., 2014; Kirschner et al., 2014; Wang et al., 2013). Quinlan and Billiar (2012) observed the stiffness range for VIC activation and deactivation *in vitro* to be 4.8–9.6 kPa. Chen et al. (2011) showed that TGF-β, which induces activation in VICs, fails to activate VICs *in vitro* on 2D substrates with stiffness lower than 11 kPa. Hydrogels were used as substrates in these *in vitro* experiments as their stiffness can be readily tuned by changing monomer or crosslinking density. Another activation variable often overlooked in *in vitro* VIC research is passage number. Primary VICs collected from healthy adult valves are largely quiescent (α-SMA negative) (David Merryman, 2010). As VICs spend more time and cell division cycles *in vitro* without physiological fluid flow, the overall VIC population tends to shift from quiescent to activated (Liu et al., 2007).

VIC phenotypes show specific protein markers such as aVIC marker α-SMA and obVIC marker RUNX2. However, VIC phenotype detection using these markers requires immunoassay-based techniques which render the VICs unusable for future experimentation. A much simpler and non-destructive way of VIC phenotype detection would involve the use of well-characterized cell morphologies. A complete understanding of the distribution of VIC morphologies may help us identify the state of the population of VICs. Liu and Gotlieb (2007) described six morphologies of VICs *in vitro*, which we adapted during these studies. Liu described round, rhomboid, tailed, spindle, multi-extension and half-moon. Liu's observations regarding these six morphologies are as follows (Liu and Gotlieb, 2007). Round and rhomboid morphologies are less motile, express α-SMA as stress fiber cytoskeletal components and synthesize more ECM. More elongated tailed and spindle morphologies are more motile, express α-SMA in lamellipodia and cell extensions, and synthesize less ECM. Multi-extension and half-moon morphologies are much less common *in vitro* compared to the other four types.

So far VIC morphologies and their distribution have been studied on stiff polystyrene culture plates and with high passage number VICs only (Liu and Gotlieb, 2007). These conditions and number of cell divisions may have had significant effect on VIC phenotype transformation and as a result on morphology and phenotype distribution. So, VIC morphology distribution studies on substrates of physiological stiffness with early passages of VICs are of absolute importance. A thorough molecular characterization of these morphologies and their intrinsic potential for transformation would be beneficial for tissue engineering of novel valve repair products.

The first goal of this study was to identify *in vitro* VIC morphology distribution across a range of substrate stiffness and passage numbers and to correlate VIC morphologies with VIC phenotypes. The second goal of this study was to investigate reversal of VIC activation by lowering substrate stiffness. Porcine aortic VICs were cultured on polyacrylamide hydrogels of stiffness 1, 7 and 21 kPa. As determined by others Kloxin et al. (2010), Quinlan and Billiar (2012), Wang et al. (2012), Gould et al. (2014), Kirschner et al. (2014) and Wang et al. (2013), VICs have a narrow threshold of 5–7 kPa for activation. So, substrate stiffness used in this study fall below, in between and above the activation range, respectively. Six VIC morphologies were detected and their distributions according to substrate stiffness and passage number were quantified. The morphologies detected are spindle, tailed, rhomboid, triangle, round and multi-extension.

2. Methods

2.1. Polyacrylamide gel preparation and rheology measurements

Aqueous solutions of 6, 10 and 16% acrylamide were prepared with a 120:1 acrylamide to bisacrylamide mass ratio (both from Bio-Rad Laboratories, Hercules, CA). 15 μL of 10% ammonium persulfate and 3 μL of tetramethyl ethylenediamine (both from Fisher Scientific, Waltham, MA) were added to 1 mL of acrylamide-bisacrylamide solutions and gels of 0.75 mm thickness were cast. At least 6 h were allowed for complete gel polymerization. Gels were washed in water overnight, then trimmed to fit 6-well culture plates. Prior to cell seeding, gels were treated with 0.25 mM sulfosuccinimidyl 6-(4′-azido-2′-nitrophenylamino) hexanoate crosslinker (Fisher Scientific) and exposed to ultraviolet light (302 nm wavelength) at an irradiance of 1.9 mW/cm^2 for 15 min. Gels were then incubated with 25 μg/mL type I bovine collagen (Advanced Biomatrix, San Diego, CA) at 37 °C for at least 1 h. A phosphate buffer saline (137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate dibasic and 1.46 mM potassium phosphate monobasic (all from Fisher Scientific)) sterile wash was performed before cell seeding.

Rheometry (ARES, TA Instruments, New Castle, DE) was used for substrate stiffness measurements following the protocol by Calvet et al. (2004). Parallel plate geometry of 20 mm diameter was used. Gels were cast directly on rheometer lower plate and upper plate was lowered to a gap of 500 μm. A soak time of 20 min without any strain or angular frequency was performed to allow the gel to polymerize. After the soak time, a frequency sweep was performed at constant strain of $\gamma^0 = 0.01$ over a frequency range of 0.1–100 rad/s. Storage and loss moduli were obtained. Temperature was maintained at 20 °C during rheological measurements. Gels before and after the collagen treatment were also tested. For this purpose, premade gels with or without collagen treatment underwent the same rheometry protocol.

2.2. Cell culture and morphology detection

VICs were extracted from porcine aortic heart valves. Aortic valve leaflets were digested using 1200 U/mL collagenase (Sigma-Aldrich, St. Louis, MO) in culture medium for 10 min and scrubbed for VEC removal. VECs were removed by centrifugation and scrubbed leaflets were digested overnight with collagenase. VICs were cultured from P1 to P6 passages on polyacrylamide gels. Cell seeding density of 2.5×10^4 cells/cm^2 was used. Dulbecco's Modified Eagle Medium:Ham's F-12 1:1 medium (Mediatech, Corning, Manassas, VA) supplemented with 10% bovine growth serum (Sigma-Aldrich) and 1% antibiotics/antimycotics (10,000 U/mL penicillin G, 10 mg/mL streptomycin sulfate and 25 μg/mL amphotericin B (Quality Biologicals, Gaithersburg, MD)) was used as cell culture medium. At 80% confluence VICs were incubated with 200 U/mL collagenase in culture medium at 37 °C for 30 min to aid cell detachment from collagen coated surfaces. During the transfer of VICs from one passage to next, the substrates, soft or stiff, were kept constant. Two exceptions to this were made to P3 and P5 passage VICs when they were transferred from stiffest to softest substrate to test VIC deactivation. All experiments were performed with three biological replicates, *i.e.*, cells harvested from three individual porcine hearts.

Cell morphologies were detected using phase contrast images. VIC morphology was determined based on similarity to sample images in Fig. 1. The total counts for each morphology in populations of different substrate stiffness and passage number were reported. VIC morphology was determined based on definitions of spindle, tailed, rhomboid and triangle. Rhomboid and triangle morphologies follow their intrinsic definitions and are of low aspect ratio. Spindle and tailed morphologies have high aspect ratio, with respectively two or one long extension from the nuclear bulge. The total counts for each morphology in populations of different substrate stiffness and passage number were reported. For each particular substrate stiffness and passage number, images containing at least 200 cells were analyzed to generate morphology distributions.

2.3. Cell morphology measurement and analysis

Image analysis was performed using FracLac V.2.5 plugin for ImageJ software (Image processing and analysis in Java by NIH Image, National Institute of Health, Bethesda, MD). Images were converted to binary form. A convex hull was drawn by connecting the outermost foreground pixels and joining them with straight lines. A bounding