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Myofibroblastic activation of valvular interstitial cells is modulated by spatial variations in matrix elasticity and its organization

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ABSTRACT

Valvular interstitial cells (VICs) are key regulators of the heart valve's extracellular matrix (ECM), and upon tissue damage, quiescent VIC fibroblasts become activated to myofibroblasts. As the behavior of VICs during disease progression and wound healing is different compared to healthy tissue, we hypothesized that the organization of the matrix mechanics, which results from depositing of collagen fibers, would affect VIC phenotypic transition. Specifically, we investigated how the subcellular organization of ECM mechanical properties affects subcellular localization of Yes-associated protein (YAP), an early marker of mechanotransduction, and α-smooth muscle actin (α-SMA), a myofibroblast marker, in VICs. Photo-tunable hydrogels were used to generate substrates with different moduli and to create organized and disorganized patterns of varying elastic moduli. When porcine VICs were cultured on these matrices, YAP and α-SMA activation were significantly increased on substrates with higher elastic modulus or a higher percentage of stiff regions. Moreover, VICs cultured on substrates with a spatially disorganized elasticity had smaller focal adhesions, less nuclear localized YAP, less α-SMA organization into stress fibers and higher proliferation compared to those cultured on substrates with a regular mechanical organization. Collectively, these results suggest that disorganized spatial variations in mechanics that appear during wound healing and fibrotic disease progression may influence the maintenance of the VIC fibroblast phenotype, causing more proliferation, ECM remodeling and matrix deposition.

1. Introduction

Valvular interstitial cells (VICs), the most abundant cell type in heart valve tissue, actively regulate the structure and composition of the extracellular matrix (ECM) during wound healing and disease progression [1,2]. VICs fulfill their functional role in ECM remodeling by transitioning from a quiescent fibroblast phenotype to an activated myofibroblast phenotype with more contractility, higher matrix metalloproteinase (MMP) activity and more collagen production [2–5].

Under normal physiological conditions, the ECM that VICs synthesize and reside in has a highly organized distribution of collagen fibers [6], but during disease progression and wound healing, the organization of the collagen fibers that are newly synthesized by VICs is disturbed and alignment is lost [7]. Deposition of more collagen fibers results in a higher stiffness of the ECM, and the disorganization of new deposited collagen fibers leads to spatial variation of matrix mechanics [8,9]. *In vivo* studies showed that disorganized collagen deposition is a common phenomenon in different types of tissue. As one specific example, the group of Scully demonstrated that during valve fibrosis and heart disease

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progression with age, collagen fibers in human aortic heart valves become disorganized and lose alignment [7]. Collagen organization has also been studied during wound healing processes. Doillon and co-workers observed that the collagen distribution was poorly organized during wound healing in a full thickness defect in rat skin [10]. Furthermore, the group of Bodi showed that in swine, and more importantly in human, the collagen organization at the outer region of the myocardial infarcted area was more disorganized and random compared to the core region [11]. Other mechanical properties that changed during valvular ECM remodeling have been shown to affect VIC phenotypic change and functions, for example stress relaxation [12] and transvalvular pressure [13]. For this manuscript, we are specifically interested in the effect of randomized stiffness organization, which results from new deposited collagen fibers on VIC phenotypic change.

The observations of disorganized collagen distributions *in vivo* for fibroblast-rich regions also corresponded with findings from *in vitro* studies with VICs using a scratch wound model. At the edge of the wound, VICs were found to be more proliferative and to form more α-smooth muscle actin (α-SMA) fibers compared to VICs distal from the wound's edge [14]. Based on the different behavior of VICs as a function of their matrix environment and organization, the question arose if it was the organization of the ECM or the change in overall stiffness of the local microenvironment that affected VIC proliferation and phenotypic transition during disease progression and wound healing. We hypothesized that local variations in matrix mechanics (e.g., from organized to disorganized) would influence the phenotypic properties of VICs through a mechanotransduction process [15].

To test this hypothesis, we used a synthetic hydrogel platform with photo-tunable mechanical properties that allows us to spatially vary the matrix mechanics, as the nitrobenzyl groups in our hydrogel platform enable spatial control over the crosslinking density [15–17]. Photomasks with different lithographic patterns were used to fabricate hydrogels with patterned mechanical properties, both organized and disorganized, through photodegradation. Subsequently, porcine VICs, which similarly to other sources of fibroblasts, can undergo a fibroblast-to-myofibroblast transition, were cultured on these hydrogel substrates and VIC activation was characterized by the appearance of organized α-SMA stress fibers, a key myofibroblast marker [2,18–21]. During the transition into myofibroblasts, VICs form α-SMA stress fibers in the cytoplasm (α-SMA activated cells or activated VICs), while a diffuse α-SMA expression in the cytoplasm (α-SMA deactivated cells or deactivated VICs) has been observed for quiescent fibroblasts [2,20,21].

Yes-associated protein (YAP), a transcriptional co-activator that shuttles from the cytoplasm to the nucleus (YAP activation), is an indicator for cell mechanotransduction [22]. The foundation and majority of studies about YAP as a mechanotransduction indicator have been completed using mesenchymal stem cells as a model [23,24], but some recent studies with myofibroblasts have also shown that YAP activation is involved during the transition from fibroblasts into myofibroblasts [25–27]. However, our knowledge about how YAP is involved in the mechanotransduction process for VICs is still limited, especially the relationship and temporal order between the intracellular localization of YAP and α-SMA activation. Therefore, studies were conducted to investigate the relationship between YAP and α-SMA activation as a function of time and how mechanotransduction affects the VICs phenotypic transition. Ultimately, these studies provide insight into the impact of the organization of matrix mechanics on a wide range of VIC functions, as well as the role of mechanotransduction through YAP signaling on the VIC phenotypic transition.

2. Materials and methods

2.1. Synthesis of photo-degradable hydrogel components

Poly(ethylene glycol) di-photodegradable acrylate (PEGdiPDA) was synthesized and characterized as previously described [13,14]. Briefly, 4-[4-(1-acrylethyl)-2-methoxy-5-nitrophenoxy]butanoic acid (8 eq.) and poly(ethylene glycol) bis-amine (M_n ~ 3400 Da; Laysan Bio Inc.) (1 eq.) were dissolved in *N*-Methyl-2-pyrrolidone (NMP) (156 mmol). (1-[bis (dimethylamino)methylene]-1H-1,2,3-triazolo [4,5-b]pyridinium 3-oxid hexafluorophosphate) (HBTU) (9 eq.) and 1-hydroxybenzotriazole (HOBt) (9 eq.) were added and allowed to react over night. The product was precipitated in ice-cold diethyl ether and dialyzed against water (SpectraPor 7, MWCO 2000 Da; Spectrum Labs). Purity and conjugation was confirmed by ^{1}H NMR (~90% conjugation). The adhesive peptide, OOGRGDSG (diethylene glycol-diethylene glycol-glycine-arginine-glycine-aspartic acid-serine-glycine), was synthesized on a Tribute peptide synthesizer (Protein Technologies) using standard Fmoc solid phase peptide synthesis protocols [15]. The N-terminal amine was coupled with acrylic acid using HATU conditions on resin to yield the Acryl-OOGRGDSG monomer (acrylated RGD) [15]. The peptide was cleaved from the resin in 1 h (95% TFA, 2.5% triisopropylsilane (TIPS) and 2.5% DI H_2O). This mixture was precipitated in ether and centrifuged. The peptide was washed with ether and centrifuged two additional times and then dried under vacuum. Peptide purification was conducted using semi-preparative reversed-phase high performance liquid chromatography (RP-HPLC; Waters 2767, 2489, 2545) with a gradient of 5:95 acetonitrile:water with 0.1% (vol/vol) TFA over 70 min at 20 mL/min on a C18 5 μm preparation column. The purity of peptides was confirmed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry using α-cyano-4-hydroxycinnamic acid as a matrix. (MALDI-TOF MS, Applied Biosystems DE Voyage) as previously described in Ref. [15].

2.2. Photo-degradable hydrogel formulations

Photo-degradable hydrogels were polymerized as described previously [15]. Briefly, PEGdiPDA was copolymerized with PEG monoacrylate, M_n ~400 Da (PEG400A) (Monomer-Polymer and Dajac Laboratories, Inc.) and acrylated RGD through a redox-initiated free radical polymerization in PBS. Gel solutions were prepared with 5.25 wt% PEGdiPDA, 7.25 wt% PEG400A, 2.5 mM acrylated RGD and 200 mM ammonium persulfate (APS). A final concentration of 100 mM tetramethylethylenediamine (TEMED) was added to initiate the polymerization. Gels were formed in 6 min on acrylated cover glass with a 12 mm diameter.

2.3. Rheological characterization of bulk hydrogel properties

The dynamic moduli of hydrogels both before and during photodegradation were measured on a Discovery Hybrid Rheometer (TA Instruments) at room temperature. Optically thin hydrogels with a thickness of 100 μm were formed *in situ* between a Quartz bottom plate and an 8 mm diameter stainless steel upper plate. The gel network evolution was monitored using a dynamic time sweep at 1% strain and an angular frequency of 3 rad/s. After the storage modulus (G') reached its plateau at 3.37 ± 0.02 kPa, degradation was started by collimated 365 nm light ($I_0 = 10$ mW/cm^2; Omnicure 1000, Lumen Dynamics), and the change in G' was monitored using the same dynamic time sweep parameters until the G' reached 1.93 ± 0.17 kPa, 1.31 ± 0.02 kPa and 0.69 ± 0.03 kPa, respectively. Young's modulus was calculated with $E = 2 * (1 + \nu) * G'$, where a Poisson's ratio (ν) of 0.5 for the PEG hydrogels was assumed [28].