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Characterization of valvular interstitial cell function in three dimensional matrix metalloproteinase degradable PEG hydrogels

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ABSTRACT

Valvular interstitial cells (VICs) maintain functional heart valve structure and display transient fibroblast and myofibroblast properties. Most cell characterization studies have been performed on plastic dishes; while insightful, these systems are limited. Thus, a matrix metalloproteinase (MMP) degradable poly (ethylene glycol) (PEG) hydrogel system is proposed in this communication as a useful tool for characterizing VIC function in 3D. When encapsulated, VICs attained spread morphology, and proliferated and migrated as shown through real-time cell microscopy. Additionally, fibronectin derived pendant RGD was incorporated into the system to promote integrin binding. As RGD concentration increased from 0 to 2000 μM, VIC process extension and integrin $\alpha_v\beta_3$ binding increased within two days. By day 10, integrin binding was equalized between conditions. VIC morphology and rate of process extension were also increased through decreasing the hydrogel matrix density presented to the cells. VIC differentiation in response to exogenously delivered transforming growth factor-beta1 (TGF-β1) was also examined within the hydrogel networks. TGF-β1 increased expression of alpha smooth muscle actin (αSMA) and collagen-1 at both the mRNA and protein level by day 2 of culture, indicating myofibroblast differentiation, and was sustained over the course of the study (2 weeks). These studies demonstrate the utility, flexibility, and biological activity of this MMP-degradable system for the characterization of VICs, an important cell population for tissue engineering viable valve replacements and understanding valvular pathobiology.

1. Introduction

Valvular interstitial cells (VICs) are the main cell population of the cardiac leaflets. When these cells are isolated from fresh tissue and plated on traditional two-dimensional plastic dishes, they undergo a wound-healing response and begin to differentiate from quiescent fibroblasts to activated myofibroblasts, giving rise to a heterogeneous population [1,2]. Furthermore, VIC monolayer contractility leads to the formation of multicellular aggregates and calcified nodule structures on traditional culture surfaces [3–5]. Hence, a culture system that would allow VIC characterization under conditions more reminiscent of native valve properties is highly desirable. Polymeric 3D hydrogel materials based on a variety of chemistries have been introduced for this purpose [6–8]. Development of appropriate 3D culture scaffolds for VIC characterization is exceedingly important for tissue engineers to generate viable valve replacements, in addition providing tools for studying VIC biology and pathology in efforts to better treat valvular disease.

In past studies, VICs have been cultured primarily in 3D matrices derived from natural materials. The most widely studied being collagen followed by fibrin-based materials [7,9,10]. Although these materials provide 3D scaffolds that support cell viability, their material properties are difficult to control and cannot be loaded under physiologically relevant conditions. Furthermore, due to the contractile and remodeling activity of VICs, collagen matrices are quickly compacted by the cells to at least half their original size [4,10,11]. Beyond material properties, protein matrices interact with cells in a complex manner by coupling membrane receptors and initiating signaling cascades that direct cell differentiation, proliferation, and migration [7,12,13]. Natural proteinaceous materials also serve as a reservoir for bioactive molecules by sequestering growth factors and cytokines from the media [14–16]. These intricate biological interactions make it exceedingly difficult to deconvolute the effects of specific material interactions on VIC functions. To address these challenges, we investigated a newly developed bioactive synthetic hydrogel [17] for 3D VIC culture.

This hydrogel system consists of four-arm poly(ethylene glycol) (PEG) chains connected with enzymatically degradable peptides.

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PEG is a versatile synthetic polymer utilized extensively for 3D encapsulation due to its bioinert nature. Moreover, PEG can be easily functionalized with numerous reactive groups to allow cytocompatible encapsulation with light irradiation [18–20]. Here, PEG was functionalized with norbornene reactive groups to allow radical photopolymerization with thiol functional groups on cysteine containing peptides. In this mechanism, gels form by radical mediated, one-to-one addition, leading to a regular polymer network structure [17]. In these gels, a matrix metalloproteinase (MMP) degradable peptide (GPQGIWGQ) was chosen to crosslink PEG chains to allow cell-dictated gel remodeling, as VICs are known to secrete a wide range of these enzymes [21–23]. This peptide sequence is derived from collagen motifs [24,25] and has been utilized previously in fabrication of degradable networks [8,26]. Finally, this system allows incorporation of additional peptides, such as those based on adhesion proteins (e.g., RGD) to tune integrin binding [27]. Here, we explore these novel MMP-degradable PEG hydrogels functionalized with RGD as a potential platform to study 3D VIC morphology, differentiation, proliferation, and migration.

2. Materials and methods

2.1. Hydrogel and peptide preparation

Four-arm, poly(ethylene glycol) (PEG) (20,000 M_n) (JenKemUSA) was functionalized with norbornene functional groups by addition of norbornene acid by symmetric anhydride *N,N′*-dicyclohexylcarbodiimide (DCC) coupling as previously described [17]. Briefly, 4-arm PEG was dissolved in dichloromethane (DCM) with pyridine and 4-(dimethylamino)pyridine (DMAP) (Sigma). In a separate vessel, DCC was reacted at room temperature with 5-norbornene-2-carboxylic acid to form the anhydride. After stirring for 30 min, the PEG solution was added and stirred overnight. The mixture was then filtered, washed with sodium bicarbonate, and precipitated in ice-cold diethyl ether. Substitution and purity of the PEG–norbornene product was determined to be >95% as characterized by ^{1}H NMR with particular attention paid to the alkene proton peaks occurring between 6.3 and 5.9 ppm.

The MMP-degradable peptide and adhesive RGD peptide were synthesized on solid Rink-amide resin using Fmoc chemistry on a model 433A peptide synthesizer from Applied Biosystems or a Tribute peptide synthesizer from Protein Technologies. Lysines were added to the peptides to enhance solubility. All peptides were analyzed by reverse phase high-pressure liquid chromatography (HPLC) and MALDI mass spectroscopy. When purity was less than 95%, peptides were purified with HPLC. All peptides were lyophilized from water or water/acetonitrile solution. As peptides prepared by this method often contain substantial amounts of bound water, true peptide content was determined by using the absorbance of the peptide solution at 280 nm with a molar extinction coefficient for tryptophan of 5500 M^{-1} cm^{-1}. To verify the absorbance results and to verify the presence and content of reduced thiols, Ellman's assay (Pierce) was performed using a cysteine standard. The resulting PEG–norbornene and peptide sequences are given in Fig. 1.

2.2. Cell culture and encapsulation

VICs were isolated from surgically removed aortic cardiac leaflets from porcine hearts (Hormel) received within 24 h after sacrifice by sequential collagenase digestion as previously described [28]. 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 with trypsin digestion. All experiments utilized VICs at the second or third passage and were performed in low serum (1% FBS supplemented) media to minimize cell proliferation except where noted. Hydrogel VIC cultures were supplemented with TGF-β1 at 5 ng/mL where indicated.

VICs were suspended in stoichiometrically balanced monomer solutions (i.e., equal thiol to ene ratio) comprised of either 5 or 10 wt% PEG–norbornene and MMP-degradable peptide, 0.05 wt% Igracure 2959 (photoinitiator), and 1000 μM CRGD adhesive peptide unless otherwise specified, in phosphate buffered saline (PBS) at a density of 15 million VICs/mL monomer solution. The cell–gel solution was injected between glass slides separated by a 1 mm spacer in circular molds (7 mm diameter), and exposed to UV light centered at 352 nm at 5 mW/cm^2 for 10 min to allow complete polymerization. The resulting cell–gel polymer discs were then transferred to cell media with indicated supplements and cultured for up to 21 days in a humid incubator at 37 °C and 5% CO_2.

2.3. Live/dead staining and morphology analysis

VIC viability and morphology within the MMP-degradable PEG hydrogels were determined by live/dead staining (Invitrogen). Briefly, hydrogels were rinsed with PBS, and placed in phenol red-free media containing the live/dead stain for 30 min. VIC containing MMP-degradable PEG hydrogels were then rinsed and imaged utilizing confocal microscopy (LSM 5 Pascal, Achroplan 10× NA 0.3 W, Carl Zeiss, Inc). For each gel, three image z-stacks 200 μm (10 μm slices) in height were taken and projected for image analysis. Cell area and circularity (circularity = 4π(area/perimeter^2)) were determined from projections of the live/dead image stacks using NIH ImageJ software analyze particles feature.

2.4. Immunostaining

Hydrogel constructs were fixed with 10% buffered formalin overnight at 4 °C, then transferred to a 30 wt% sucrose solution in PBS for an additional 24 h at 4 °C. Samples were then mounted in cryostat mounting medium, frozen, sectioned into 30 μm slices, and mounted on glass slides for immunostaining. Slides were rinsed and permeabilized in 0.05 wt% Tween 20-supplemented PBS. Non-specific antibody staining was blocked with 3 wt% bovine serum albumin (BSA) containing PBS. Slides were then incubated with the specified primary antibodies (mouse anti-αSMA (Abcam), mouse anti-collagen-1 (Abcam), or mouse anti-integrin $\alpha_v\beta_3$ (Abcam)), at previously determined dilutions in 1 wt% BSA containing PBS. Following primary antibody coupling, samples were washed and incubated with mouse anti-goat alexa 488 (Invitrogen) and phalloidin–tetramethylrhodamine B isothiocyanate (Sigma–Aldrich). Slides were subsequently mounted with DAPI and imaged on a Nikon TE 2000 epi-fluorescence microscope. Images from each fluorescent channel were merged, and background flattened using MetaMorph (Molecular Devices). The number of cells was counted using the DAPI channel and ImageJ (NIH). Myofibroblasts and integrin positive cells were counted manually by identifying cells with positive staining, normalized to cell number from DAPI counts and expressed as a percentage.

2.5. mRNA isolation and quantifiable real-time polymerase chain reaction (qRT-PCR)

Messenger RNA (mRNA) was isolated from liquid nitrogen snap frozen VIC laden MMP-degradable PEG hydrogels using Trizol (Sigma–Aldrich). mRNA was then column purified with the Ambion RiboPure kit per manufacturer's instructions. Purity and amount of mRNA were confirmed with a NanoDrop spectrophotometer (Fisher). Reverse transcription was performed using the iScript cDNA Synthesis kit (Bio-Rad). Polymerase chain reaction (PCR) was then conducted using an iCycler qRT-PCR machine (Bio-Rad). Primers for GAPDH, α-SMA, and collagen-1 (Integrated DNA technologies) were previously reported [29]. Threshold cycle (C_T) and primer efficiency were analyzed according to the Pfaffl method and normalized to GAPDH [30].

2.6. Real-time cell tracking

Time lapse images of cell morphology and movement were captured in 400 μm z-stacks (15 μm slices) every 30 min over a 5 day period using a Nikon TE 2000 PFS fluorescent microscope equipped with a motorized stage and environmental sample chamber. Images were collected and analyzed using MetaMorph (Molecular Devices). Cell velocity was calculated from the average x and y displacements divided by the interval time between images (30 min).

2.7. dsDNA assay

Double stranded DNA (dsDNA) was isolated from similarly sized hydrogel constructs by papainase (Worthington) digestion at 60 °C overnight. For each sample, two hydrogels were combined per digest sample. The resulting digestion solution was analyzed for dsDNA content using the Quant-It PicoGreen dsDNA assay (Invitrogen) per manufacturer's instructions.

2.8. Statistics

Data are presented as mean ± standard error of three or more samples. ANOVA analysis was used to compare data sets, and the resulting p values that were used to determine statistical significance are indicated in figure captions.

3. Results

3.1. VIC proliferation in MMP-degradable PEG hydrogels

MMP-degradable PEG hydrogels form versatile ideal networks with tailorable bioactivity through peptide incorporation (Fig. 1). Within these constructs we investigated VIC proliferation, migration, and differentiation. VIC proliferative ability in response to serum induced growth conditions within MMP-degradable PEG hydrogels was studied. To visualize cell proliferation, VIC laden MMP-degradable PEG hydrogels were placed on a real-time cell tracking microscope in high serum (15% FBS) media. A filmstrip of recorded VIC division is shown in Fig. 2A. In these images classic