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Discrete microstructural cues for the attenuation of fibrosis following myocardial infarction

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ABSTRACT

Chronic fibrosis caused by acute myocardial infarction (MI) leads to increased morbidity and mortality due to cardiac dysfunction. We have developed a therapeutic materials strategy that aims to mitigate myocardial fibrosis by utilizing injectable polymeric microstructures to mechanically alter the microenvironment. Polymeric microstructures were fabricated using photolithographic techniques and studied in a three-dimensional culture model of the fibrotic environment and by direct injection into the infarct zone of adult rats. Here, we show dose-dependent down-regulation of expression of genes associated with the mechanical fibrotic response in the presence of microstructures. Injection of this microstructured material into the infarct zone decreased levels of collagen and TGF-β, increased elastin deposition and vascularization in the infarcted region, and improved functional outcomes after six weeks. Our results demonstrate the efficacy of these discrete anti-fibrotic microstructures and suggest a potential therapeutic materials approach for combatting pathologic fibrosis.

1. Introduction

Coronary heart disease is a daunting challenge in the United States with nearly one million new or recurrent MIs each year [1]. Although strategies for the acute management of an MI have proven to be effective at improving mortality and morbidity outcomes, the development of fibrous scar tissue in the infarct zone often leads to challenging chronic complications and functional insufficiencies [2]. These subsequent morbidities highlight the importance of the cardiac microenvironment in the development of pathology following MI as stiffening and thinning of the infarcted wall leads to diminished pumping efficiency with further progression to heart failure [3–8].

Recent work has focused on developing therapies to reintegrate healthy muscle tissue or stem cell sources into the infarcted heart to induce regeneration of the damaged muscle [9–12]. Others have demonstrated the advantage of mechanically supportive bulk injectable gels composed of processed biological materials to improve functional outcomes after MI [13–17]. Despite the early successes of some of these strategies, the dense fibrotic tissue that begins to arise within days of the MI presents a barrier to both the biologic and mechanical mechanisms of these potential therapies [18]. Cell survival or invasion into the scar tissue is met with resistance from the pathologically altered microenvironment that is hostile to cellular integration [19]. Meanwhile, stiffening of the muscle tissue with subsequent decrease in contractile function limits the positive mechanical influence of bulk material injections [15]. Therapies that directly target fibrosis at the local level after an infarct could alleviate the maladaptive mechanical reaction responsible for the chronic decline in heart function.

In addition to laying down the extracellular matrix (ECM) that creates the physiologic mechanical environment in the heart, cardiac fibroblasts have been shown to play a critical role in the injury response and limited regenerative potential of heart muscle [3–8,20,21]. After MI, a population of highly contractile

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myofibroblasts develops in the wake of inflammatory matrix metalloproteinases, which degrade the injured muscle. Transformation initiated by increased local tensile stresses on resident fibroblasts that are no longer able to offload mechanical stress by attachment to a robust network of ECM proteins may contribute to this pathologic response, resulting in accelerated ECM production and increased release of signaling factors [7,8,20,22,23].

We have previously utilized a system of polymeric microstructures, termed "microrods" and "microcubes" that act as anchors for cell traction and demonstrated their effects on fibroblast growth in 2D and 3D culture [24–28]. Using basic techniques in photolithography, this system allows us to tune the shape and mechanical stiffness of these microstructures to enhance interactivity and efficacy in mitigating myofibroblastic transformation. This platform is also highly versatile and can be studied both *in vitro* and *in vivo* to assess the mechanistic interactions and therapeutic potential of polymeric microstructures. Here we demonstrate the effects that micromechanical environmental cues have on cells and tissues by elucidating changes in the regulation of fibrotic activation at the transcriptional level and correlating this to demonstrated therapeutic efficacy of microstructure injections into infarcted myocardium.

2. Materials and methods

2.1. Microstructure fabrication

Microrods (100 μm × 15 μm × 15 μm) and microcubes (15 μm × 15 μm × 15 μm) were fabricated from PEG-DMA as previously described using commercially available materials [24] and re-suspended in complete media for *in vitro* studies or sterile saline solution for intra-cardiac injections (Fig. 1). Briefly, polyethylene glycol dimethacrylate (PEG-DMA) (MN = 750, Sigma Aldrich, St. Louis, MO) was diluted with Calcium and Magnesium-free 1x Phosphate Buffered Saline (PBS). The photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DMPA) (Sigma Aldrich, St. Louis, MO) solubilized at 100 mg/mL in 1-vinyl-2-pyrrolidinone (Sigma Aldrich, St. Louis, MO) was then added to this mixture in equal volume to the PBS added and vortexed thoroughly. The solution was spun to a 15 μm thick layer on a piranha-solution-cleaned silicon wafer (Addison Engineering, San Jose, CA) and exposed through a photomask to a 405 nm UV light source using a Karl Suss MJB3 mask aligner (Suss Microtec, Garching, Germany) to crosslink the desired regions in the shape of microrods (100 μm × 15 μm × 15 μm) or microcubes (15 μm × 15 μm × 15 μm). Microstructures were rinsed, scraped from the surface gently using a cell scraper, and sterilized in 70% ethanol. Before use, microstructures were centrifuged to allow aspiration of the ethanol and re-suspension at the desired number-density in saline solution.

2.2. Cell culture and qPCR

Murine 3T3 fibroblasts (ATCC, Manassas, VA) were harvested between passages 18 and 20 and mixed with either microrods at a high ratio (1:1) or a low ratio (1:5) of structures to cells, or microcubes at a high ratio (20:3) or low ratio (4:3) and added to liquid state Growth Factor Reduced, High Concentration Matrigel (Lot 42155) (BD Biosciences, San Jose, CA) doped with 10% (v/v%) of a 0.2% gelatin solution (Sigma Aldrich, St. Louis, MO) to a final protein concentration of 4 mg/mL. The mixture was then seeded into ~2 mm thick cultures in a 96-well plate before gelling at 37 °C. After gelation, fresh media was added on top and replaced every other day for four days. Genetic material was harvested by standard TRIzol (Life Technologies, Carlsbad, CA) extraction protocols.

A Viia7 qPCR machine (Life Technologies, Carlsbad, CA) was used to measure relative expression levels of gene targets as compared to housekeeping gene 60s ribosomal protein L19 (rpL19). Expression levels of genes for mmp2, SRF, YAP, and TAZ were evaluated using Fast SYBR Green Mastermix (Life Technologies, Grand Island, NY) and custom made DNA primers (Integrated DNA Technologies, Coralville, IA) in triplicate for three biological replicates (See Supplementary Table 1).

2.3. Infarct model and microstructure delivery

The animal protocol for induction of MI was approved by the Committee for Animal Research of the University of California San Francisco and was performed in accordance with the recommendations of the American Association for Accreditation of Laboratory Animal Care. The ischemia-reperfusion model used in this study has been extensively tested in our lab. All injections were performed successfully and there were no complications resulting from surgery or injection in any animal. Protocols were approved by the IACUC of UCSF. All studies were performed in two rounds of experiments (Experimental Group 1: Saline – $n = 10$, Microrod – $n = 20$, Microcubes – $n = 9$, Experimental Group 2: Saline – $n = 8$, Microrod – $n = 11$, Microcube – $n = 11$).

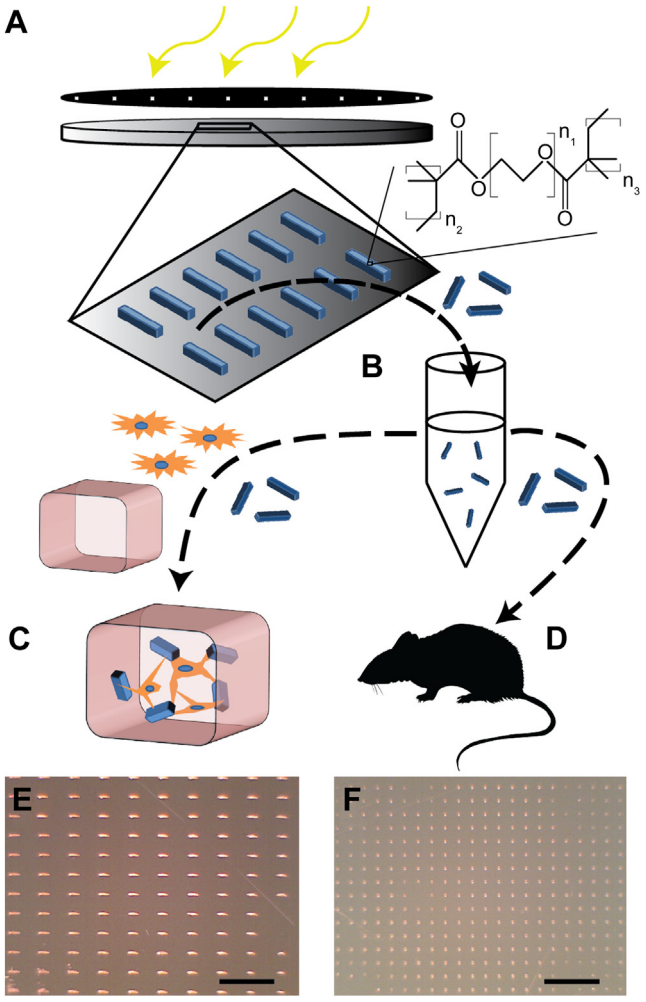

Fig. 1. Microstructure fabrication and experiment design. Microstructures are fabricated photolithographically by exposure of a thin film of hydrogel polymer to UV energy through a patterned photomask (A). Microrods and microcubes are released from the surface in sterile saline solution (B). From here, the microstructures are either added to a solution of fibroblasts and liquid phase Matrigel to create *in vitro* 3D tissue constructs for study of cell–microstructure interactions (C), or are injected in saline solution into the infarct zone of Sprague–Dawley rats under ultrasound guidance 48 h after temporary LAD ligation and reperfusion (D). Light micrographs show high throughput arrays of microrods (E) and microcubes (F) on a silicon wafer. Scale bar = 500 μm.

To produce the MI model, female Sprague–Dawley rats (180–220 g) underwent occlusion of the left anterior descending coronary artery for 30 min followed by reperfusion while under general anesthesia achieved by inhalation of 2% L/min isoflurane. The chest was then closed and the animal was allowed to recover. The rats were randomized two days after MI to saline-injected, microrod-injected, or microcube-injected treatment groups, and were given one intramuscular injection into the heart wall under blinded conditions via ultrasound guided transthoracic injection using a 29-gauge syringe. Each injection consisted of 50 μL of sterile 0.9% sodium chloride solution (APP Pharmaceuticals, LLC, Schaumburg, IL) containing no microstructures ($n = 18$), 2.5×10^5 microrods ($n = 31$), or 1.65×10^6 microcubes ($n = 20$) and was delivered to the center of the infarct region as visualized by hyperechoic signal on ultrasound. Successful injection was confirmed by local increase in ultrasound signal in the vicinity of the syringe.

2.4. Echocardiography

Transthoracic echocardiography was performed with a 15-MHz linear array transducer system (Sequoia c256, Acuson, Erlangen, Germany) on all animals under 2% L/min isoflurane. Echocardiography was done prior to injection on day two post-MI and six weeks post-injection using standard methods that have been performed reproducibly in our lab [29,30]. To determine the ejection fraction at 48 h and six weeks, the ventricular shadow was outlined in both systole and diastole and the single plane area length algorithmic method was applied. Two-dimensional images