Acta Biomaterialia 50 (2017) 234–248

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Engineered extracellular microenvironment with a tunable mechanical property for controlling cell behavior and cardiomyogenic fate of cardiac stem cells

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ARTICLE INFO

Article history:
Received 19 September 2016
Received in revised form 26 December 2016
Accepted 1 January 2017
Available online 4 January 2017

Keywords:
Cardiac stem cells
Microenvironment
Elastic modulus
Cell behavior
Cardiomyogenic fate

ABSTRACT

Endogenous cardiac stem cells (CSCs) are known to play a certain role in the myocardial homeostasis of the adult heart. The extracellular matrix (ECM) surrounding CSCs provides mechanical signals to regulate a variety of cell behaviors, yet the impact in the adult heart of these mechanical properties of ECM on CSC renewal and fate decisions is mostly unknown. To elucidate CSC mechanoresponses at the individual cell and myocardial level, we used the sol-to-gel transitional gelatin-poly(ethylene glycol)-tyramine (GPT) hydrogel with a tunable mechanical property to construct a three-dimensional (3D) matrix for culturing native myocardium and CSCs. The elastic modulus of the GPT hydrogel was controlled by adjusting cross-linking density using hydrogen peroxide. The GPT hydrogel showed an ability to transduce integrin-mediated signals into the myocardium and to permit myocardial homeostatic processes *in vitro*, including CSC migration and proliferation into the hydrogel from the myocardium. Decreasing the elastic modulus of the hydrogel resulted in upregulation of phosphorylated integrin-mediated signaling molecules in CSCs, which were associated with significant increases in cell spreading, migration, and proliferation of CSCs in a modulus-dependent manner. However, increasing the elastic modulus of hydrogel induced the arrest of cell growth but led to upregulation of cardiomyocyte-associated mRNAs in CSCs. This work demonstrates that tunable 3D-engineered microenvironments created by GPT hydrogel are able to control CSC behavior and to direct cardiomyogenic fate. Our system may also be appropriate for studying the mechanoresponse of CSCs in a 3D context as well as for developing therapeutic strategies for *in situ* myocardial regeneration.

Statement of Significance

The extracellular matrix (ECM) provides a physical framework of myocardial niches in which endogenous cardiac stem cells (CSCs) reside, renew, differentiate, and replace cardiac cells. Interactions between ECM and CSCs might be critical for the maintenance of myocardial homeostasis in the adult heart. Yet most studies done so far have used irrelevant cell types and have been performed at the individual cell level, none able to reflect the *in vivo* situation. By the use of a chemically defined hydrogel to create a tunable 3D microenvironment, we succeeded in controlling CSC behavior at the myocardial and individual cell level and directing the cardiomyogenic fate. Our work may provide insight into the design of biomaterials for *in situ* myocardial regeneration as well as for tissue engineering.

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http://dx.doi.org/10.1016/j.actbio.2017.01.002
1742-7061/

1. Introduction

For decades, the adult mammalian heart was considered a post-mitotic organ, yet recent studies have demonstrated that cardiomyocytes (CMCs) are regenerated at a low but detectable rate, indicating the presence of an intrinsic homeostasis mechanism [1,2]. The search for the origin of regenerating CMCs led to the discovery of endogenous myocardium-resident cardiac stem cells (CSCs) in the adult heart; these play important roles in myocardial homeostasis [1,3,4]. CSCs appear to be multipotent in their capacity to differentiate into all myocardial cell types including CMCs, endothelial cells (ECs), and vascular smooth muscle cells [1–5]. Subsequent clinical studies demonstrated a good safety profile and efficacy for improving myocardial regeneration [5,6], indicating that CSCs might have potential uses in the development of regenerative therapeutics for cardiac disease.

The accumulated evidence indicates that mechanical signals transmitted by the myocardial extracellular matrix (ECM) play important roles in cardiac organogenesis and morphogenesis [7–9]. After ligation with cellular integrin receptors, these ECM-mediated signals consequently mediate a variety of intracellular signaling pathways [8,9]. Since CSCs are surrounded by myocardial ECM in myocardial niches [1,4], it is possible that interplays between CSCs and ECMs contribute to CSC renewal, migration, proliferation, and cardiomyogenic fate. Yet the literature remains unclear about the role of ECMs in regulating CSC behavior and fate. This is due in part to a lack of suitable *in vitro* tools for probing interactions between ECMs and CSCs residing in the myocardium. We and others described unique organ culture systems enabled to recapitulate endogenous tissue-specific homeostatic processes through preservation of structural and functional integrity of stem cell niches [10–15]. All of these cultures used ECM-mimicking hydrogels to construct a 3-dimensional (3D) artificial extracellular microenvironment. This artificial microenvironment allows embedded organ fragments to be supported 3-dimensionally, preserving structural and functional integrity, and permitting a tissue-specific homeostatic mechanism, including cell renewal, migration, proliferation, and differentiation into tissue-specific committed cells. Therefore, a hydrogel-supported organ culture method might be a useful tool for investigating the role of ECMs in regulating CSC behavior at the myocardium-level.

A wide variety of hydrogels fabricated from synthetic and natural polymers have been applied to construct 3D platforms for cell culture and tissue engineering [16], yet only natural polymer-derived hydrogels, such as Matrigel [15], collagen [10–12], and fibrin [13,14], have been used in organ culture. Growing evidence indicates that the physical properties of hydrogels, including elastic modulus, topographic features, and ligand type and density, affect cell-to-ECM interactions [17–19]. Although natural polymer-derived hydrogels provide a highly permissive substratum supporting a wide range of cell behaviors, adjusting their physical properties is difficult [19,20]. Due to rapid biodegradation, maintaining their structural stability as an artificial ECM during extended culture periods is also problematic [20]. Therefore, hydrogels used for probing CSC-to-ECM interaction are required to have physical properties that can adjust to tissue-specific extracellular microenvironments.

Finding the mechanisms and factors that govern CSC renewal, migration, and fate in the adult heart remains a formidable challenge [21]. Regarding control of stem cell behavior and fate, most of the studies done so far have focused on the identification and modification of soluble biochemical and molecular factors [22]. Concurrent with these factors, convincing evidence indicates that mechanical signals also play important roles in cell behavior and fate decision [23]. Recently, biomaterials-guided approaches have been used in attempts to integrate mechanical signals for regulating stem cell behavior and directing cell fate [17,23,24]. Most of these attempts have exclusively used mesenchymal stem cells (MSCs), which have been shown to have an ambiguous cardiomyogenic potential, fetal-, neonatal-, or pluripotent stem cell-derived CMCs [23–28]. Since neither of these types of cells are adult cardiac cells, their responses to mechanical signals cannot not reflect intrinsic myocardial homeostasis in the adult heart. Moreover, the striking differences in the mechanoresponses of cells under two-dimensional (2D) and 3D culture conditions have been widely acknowledged [29]. Yet most studies have been conducted in a 2D setting to determine the cellular mechanoresponse, which may not reflect the *in vivo* situation. It is worth noting that little is known about which physical properties of an extracellular microenvironment are important for regulating CSC renewal, migration, and cardiomyogenic fate.

We recently developed a chemically defined hydrogel made from gelatin–poly(ethylene glycol)–tyramine (GPT) copolymer [30]. This synthetic hydrogel has the ability to finely control the elastic modulus and protease-mediated degradability by adjusting cross-linking reactions. Here, we investigated the feasibility of chemically defined GPT hydrogel to construct a myocardial-mimetic microenvironment for potential application in 3D culture of the native myocardium and CSCs. We further explored the impact of the elastic modulus of GPT hydrogel on integrin-mediated signaling cascades, cell outgrowth, and cardiomyogenic fate of CSCs in a 3D setting.

2. Materials and methods

2.1. Reagents

Gelatin (Type A, porcine skin, >300 bloom), polyethylene glycol (PEG; molecular weight = 4.0 K), horseradish peroxidase (HRP), hydrogen peroxide (H_2O_2), 4-dimethylamino pyridine (DMAP), and *p*-nitrophenylchloroformate (PNC) were purchased from Sigma-Aldrich (St. Louis, MO). Tyramine (TA) was obtained from Acros Organics (Geel, Belgium). Triethylamine (TEA) was supplied by Kanto Chemical Corporation (Tokyo, Japan) and aluminum oxide was purchased from Strem Chemicals (Newburyport, MA). All media and reagents for myocardium and cell cultures were obtained from Gibco (Grand Island, NY) unless otherwise specified.

2.2. Synthesis of the gelatin–PEG–TA copolymer

As described in our previous study, the gelatin-PEG-tyramine (GPT) copolymer was synthesized by conjugating the PEG–TA on the gelatin backbone [30]. The terminal hydroxyl groups of the PEG were activated with PNC to obtain amine reactive PEG (PNC-PEG-PNC) conjugate. The PNC-PEG-PNC conjugate was then reacted with TA, followed by the gelatin, to synthesize the GPT copolymer. The chemical structure of the GPT copolymer was characterized by ^{1}H NMR spectroscopy; δ 4.8 (m, the proton of anomeric carbon of gelatin), δ 0.8–4.6 (m, alkyl proton of gelatin), δ 3.5–3.8 (m, $-CH_2-CH_2-$ of PEG ethylene), and δ 6.8 and 7.1 (m, aromatic protons of TA).

2.3. Fabrication of the in situ forming GPT hydrogel with varying elastic moduli

The GPT polymer was dissolved in 10 mM phosphate-buffered saline (PBS; pH 7.4) to make 3% *w*/*v* GPT working solution. To fabricate the hydrogel, the GPT working solution containing either HRP or H_2O_2 was mixed 1:1 and incubated at room temperature (RT) for 5 min. The elastic modulus of the GPT hydrogel was