Biomaterials 35 (2014) 92–104

Contents lists available at ScienceDirect

Biomaterials

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

The effect of bioartificial constructs that mimic myocardial structure and biomechanical properties on stem cell commitment towards cardiac lineage

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ARTICLE INFO

Article history:
Received 15 July 2013
Accepted 17 September 2013
Available online 4 October 2013

Keywords:
Biocompatibility
Cardiac tissue engineering
Cell morphology
ECM (extracellular matrix)
Scaffold
Stem cell

ABSTRACT

Despite the enormous progress in the treatment of coronary artery diseases, they remain the most common cause of heart failure in the Western countries. New translational therapeutic approaches explore cardiomyogenic differentiation of various types of stem cells in combination with tissue-engineered scaffolds. In this study we fabricated PHBHV/gelatin constructs mimicking myocardial structural properties. Chemical structure and molecular interaction between material components induced specific properties to the substrate in terms of hydrophilicity degree, porosity and mechanical characteristics. Viability and proliferation assays demonstrated that these constructs allow adhesion and growth of mesenchymal stem cells (MSCs) and cardiac resident non myocytic cells (NMCs). Immunofluorescence analysis demonstrated that stem cells cultured on these constructs adopt a distribution mimicking the three-dimensional cell alignment of myocardium. qPCR and immunofluorescence analyses showed the ability of this construct to direct initial MSC and NMC lineage specification towards cardiomyogenesis: both MSCs and NMCs showed the expression of the cardiac transcription factor GATA-4, fundamental for early cardiac commitment. Moreover NMCs also acquired the expression of the cardiac transcription factors Nkx2.5 and TBX5 and produced sarcomeric proteins. This work may represent a new approach to induce both resident and non-resident stem cells to cardiac commitment in a 3-D structure, without using additional stimuli.

1. Introduction

Myocardial infarction causes heart muscle loss and decrease of function. Although surgical and pharmacological therapies have improved the survival of patients, these therapies cannot effectively compensate for the loss of cardiomyocytes [1]. Recently, stem cell therapy has emerged as a promising approach for both heart regeneration and function restoration [2,3].

Various types of stem cells have cardiac commitment potential, including mesenchymal stem cells (MSCs) [4–6] and cardiac resident progenitor cells [7]. Some of the initial efforts at promoting cardiomyogenesis using MSCs involved the use of the demethylating agent 5-azacytidine [8], which, however, induces apoptosis *in vivo* [9]. Thereafter, a variety of different approaches have been attempted, including electrical stimulation, chemical induction, use of biological agents and cell co-culturing. However, evidence of MSC differentiation to a cardiomyogenic phenotype *in vivo* has been controversial [10], leading to the concept that functional benefits of MSCs may be largely due to paracrine mechanisms [11,12]. New hope for cardiac tissue regeneration has been raised by

Abbreviations: 3-D, three-dimensional; ECM, extracellular matrix; MSCs, mesenchymal stem cells; NMCs, non myocytic cells; PHBHV, poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid).

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http://dx.doi.org/10.1016/j.biomaterials.2013.09.058

the discovery of resident cardiac stem cells in the adult murine heart [7]. Several recent reports demonstrated that clonally expanded human cardiac progenitor cells [13–15] can generate new viable myocardium and support ventricular function when injected into infarcted hearts [16]. However the delivery of stem cells and the timing of their implantation are particularly problematic, leading to the loss of up to 90% of transplanted cells soon after delivery due to hypoxia, myocardial inflammation, or the physical stresses associated with the implantation procedure [17,18]. Since the cell niche provides crucial support [19–21], tissue-engineered scaffolds, resembling the characteristics of the native tissue, may be a feasible therapeutic approach. In fact biomaterials have been increasingly used in targeting heart repair [22,23], as they can provide a proper platform for stem cell survival, proliferation and differentiation, as well as a guide for three-dimensional (3-D) tissue reconstruction [24]. Proper myocardium regeneration and function may be largely dependent on the properties of the scaffolds. For instance, biomimetic scaffolds mimicking features of the myocardial extracellular matrix (ECM) may facilitate tissue development, as they provide a native-like template that allows cells to organize into the tissue specific structure [25,26]. Yet, the effectiveness of stem cell therapy depends on proper cell differentiation, thus the ideal tissue constructs should stimulate correct stem cell commitment towards cardiac lineage.

Over the past years, polyhydroxyalkanoates (PHAs) were used as biomaterials for applications in medical devices and tissue engineering scaffolds [27]. Poly 3-hydroxybutyrate (PHB) was also investigated for cardiovascular patches [28]. 3-D scaffolds consisting of pure gelatin have already been applied for the regeneration of cardiac tissue both *in vitro* and *in vivo*. In particular, *in vitro* tests have shown spontaneous rhythmic contractility and cell migration of cells seeded on these structures [29]. Yet *in vivo* tests showed a dissolution process of gelatin patches, which starts at four weeks and is over at twelve with inflammatory response [30]. However there is no information in literature about the use of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBHV) as pure polymer or in combination with biological polymers for cardiac reconstruction.

We hypothesized that a construct for cardiac regeneration with optimal characteristics could be obtained by combining gelatin, providing good biocompatibility and adequate degradation kinetics with PHBHV having easy processability and a minimal inflammatory response. Moreover, the non-crosslinked gelatin introduced in the bioartificial system can favor water adsorption into the scaffold enhancing hydrolytic degradation of synthetic component. Therefore, we investigated the role of chemical composition and surface properties of the substrates in controlling stem cell response. Chemical structure and molecular interaction between material components give different properties to the substrate including hydrophilicity/hydrophobicity, roughness, porosity, chemical compatibility, mechanical characteristics and degradation kinetics associated to the material in analogy to those of the native tissue [31,32]. One important aspect is to select material microstructure geometry depending on the specific cell type of which is to promote growth. Recently, cell adhesion of cardiomyocytes was increased by honeycomb structures with pores slightly higher than cells [33]. Increased elongation efficiency of human MSCs (hMSCs) was observed on polycaprolactone (PCL) substrates having anisotropic geometry obtained by uniaxial stretch [34]. Aligned fibrous scaffolds of biodegradable polyurethane, obtained by electrospinning, improved the differentiation of murine embryonic stem cell-derived cardiomyocytes in co-culture with fibroblasts [35].

Hence, in this study we investigated whether PHBHV/gelatin tissue constructs, which have specific physico-chemical properties and a micro-topography that mimics structural and mechanical properties of the myocardium, would induce MSCs and cardiac resident cells (non myocitic cells, NMCs) to differentiate into a cardiac lineage without the need for additional stimuli.

2. Materials and methods

2.1. Preparation and physico-chemical/mechanical characterization of PHBHV/gelatin scaffolds

2.1.1. Reagents

Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBHV, Sigma–Aldrich, USA, PHV content 12% mol) and gelatin from porcine skin [gelatin A (GelA), Sigma–Aldrich] were used without further purification for scaffold preparation. As solvent, dichloromethane (DCM, Carlo Erba Reagenti, Italy, ACS purity degree) and milliQ water were used for the preparation of PHBHV and GelA solutions respectively.

2.1.2. Preparation of bioartificial blends

Bioartificial blends were obtained starting from different solutions of PHBHV/DCM and a GelA/water solution with concentration 10% w/v obtained at 50 °C. Polymer solution was maintained under high stirring at room temperature and the gelatin solution was added dropwise to avoid the segregation of the protein in DCM. Four different PHBHV/gelatin blends were prepared with following composition (w/w): 95/05, 90/10, 85/15 and 80/20 [36].

2.1.3. Preparation of ECM-like porous bioartificial membranes

Using prepared blends, microstructured scaffolds were obtained by soft lithography. The geometry of the ECM-like scaffold and the procedure to obtain the geometry, based on the morphological analysis of a decellularized suine cardiac tissue, were already described in a previous work [34]. A productive drawings obtained by CAD systems has been realized in accordance with the predefined geometry. CAD model was applied to soft lithography technique to obtain a silica master and the corresponding soft moulds were prepared by polymerization of a vinyl-terminated polydimethylsiloxane (PDMS) oligomer (Sylgard® 184 Silicone Elastomer Kit, Dow Corning Corporation, USA) in an under-vacuum oven at 40 °C for 24 h. At the end of the polymerization, the soft mould was cut around the silica master and carefully removed. A predefined volume of bioartificial blend obtained as previously described was deposited by using a Gilson syringe on the PDMS mould then lyophilized for 18 h to obtain a controlled solvent casting and homogeneous microstructured materials. Bioartificial blends were laid on glass flat supports and the same procedure previously described was followed to obtain non-microstructured materials as control. Thickness of obtained biomatrices varied on the basis of the chemical composition of the bioartificial blend from 70 μm to 300 μm.

2.1.4. Scaffold characterization

Morphological analysis was carried out onto Au sputtered samples by scanning electron microscopy (SEM, Jeol JSM 5600, Japan). Chemical analysis was carried out by FT-IR Chemical Imaging (Perkin Elmer Spotlight 300, USA) and high-performance liquid chromatography (HPLC, Perkin Elmer Series 200, USA); for FT-IR analysis, a) attenuated total reflectance (ATR) sampling technique on small fragments of scaffolds was performed, the use of the Chemical Imaging allowed evaluating the distribution of components in the manufacts, obtaining chemical and correlation maps to visualize their distribution, b) near infrared spectroscopy was used to evaluate anisotropic hydrophilicity (FT-NIR Spectrum 400, Perkin Elmer, USA). Spectral images were acquired in transmission and μATR mode (spectral resolution was 4 cm^{-1}, spatial resolution was 100 × 100 μm) using the infrared imaging system Spotlight 300 (Perkin Elmer). Spectra were collected by touching the ATR objective on the sample and collecting the spectrum generated from the surface layer of the sample. The Spotlight software used for acquisition was also used to pre-process the spectra. Near infrared spectra were measured in the region 7800–4000 cm^{-1} in reflection mode at spatial resolution of 6.25 μm; Dynamic mechanical analysis was performed by DMA (DMA8000, Perkin Elmer, USA) to evaluate the viscoelastic behavior of the materials at different temperatures, samples (length 1 cm, width 0.5 cm, thickness dependent on the composition) underwent a strain scan analysis, imposing a cyclic deformation of 5% in respect to sample length, tests were carried out in dry and wet conditions, at room temperature and at 37 °C. In addition, in order to evaluate the mechanical anisotropy of obtained scaffolds, tests were carried out applying the strain in two directions, parallel and perpendicular to the grating direction. Mechanical parameters of the microstructured materials were evaluated taking the cross section geometry into account. In particular, the actual section of microfabricated specimens was evaluated as:

$$A_{act} = l \cdot s - n_c \cdot l_2 \cdot s \quad (1)$$

where A_{act} is the actual cross section, l is the width of the matrix, s is the thickness, n_c is the number of unit cells that were repeated along the width of the sample, l_2 is the sum of the widths of cell voids along the sample width. The value of A_{act} was divided by the sample width to obtain an actual width l_{act}. With this parameter the geometry constant K was evaluated as: