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The behavior of cardiac progenitor cells on macroporous pericardium-derived scaffolds

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ABSTRACT

Cardiovascular diseases hold the highest mortality rate among other illnesses which reveals the significance of current limitations in common therapies. Three-dimensional (3D) scaffolds have been utilized as potential therapies for treating heart failure following myocardial infarction (MI). In particular, native tissues have numerous properties that make them potentially useful scaffolding materials for recreating the native cardiac extracellular matrix (ECM). Here, we have developed a pericardium-derived scaffold that mimics the natural myocardial extracellular environment and investigated its properties for cardiac tissue engineering. Human pericardium membranes (PMs) were decellularized to yield 3D macroporous pericardium scaffolds (PSs) with well-defined architecture and interconnected pores. PSs enabled human Sca-1^+ cardiac progenitor cells (CPCs) to migrate, survive, proliferate and differentiate at higher rates compared with decellularized pericardium membranes (DPMs) and collagen scaffolds (COLs). Interestingly, histological examination of subcutaneous transplanted scaffolds after one month revealed low immunological response, enhanced angiogenesis and cardiomyocyte differentiation in PSs compared to DPMs and COLs. This research demonstrates the feasibility of fabricating 3D porous scaffolds from native ECMs and suggests the therapeutic potential of CPC-seeded PSs in the treatment of ischemic heart diseases.

1. Introduction

Cardiovascular diseases are the leading cause of death in industrialized nations and the developing world, account for approximately 40% of all human mortality [1]. A massive cardiomyocyte loss occurs following myocardial infarction (MI) which consequently leads to the development of non-contractible scar tissue [2]. Due to the shortage of organ donors and limited success of cell therapy, stem cell scientists, engineers and physicians continually seek new strategies for the regeneration of structural and functional features of an injured heart [3].

Myocardial tissue engineering is a promising approach that relies on combining appropriate cells with biomaterials to develop and create biological substitutes that resemble myocardium [4]. Although various natural and synthetic biomaterials have been employed within myocardial tissue engineering strategies, challenges remain in their ability to mimic the native extracellular matrix (ECM) microenvironment and effectively stimulate essential cellular responses [5–7]. Decellularized biological scaffolds, by transferring the appropriate signals to implanted cells, have the capability to support their retention, migration, proliferation and differentiation [8,9]. Upon decellularization, a significant amount of

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immunogenic material would be removed, allowing the preserved ECM to orchestrate cell communications [10]. As a result, readily available natural platforms can be widely used as ideal candidates for regenerative medicine [11,12].

Due to the demonstrated benefits of decellularized organs for engineering other organs, the use of this approach may be advantageous for fabrication of myocardial patches [13,14]. Numerous decellularized scaffolds such as small intestine submucosa (SIS), urinary bladder and pericardium ECMs have been evaluated for use in ischemic myocardium treatment [15–17]. In this regard, pericardium membrane (PM) is an appropriate candidate for myocardial tissue engineering applications due to its biochemical and biomechanical properties that mimic the myocardial ECM [18]. Previous efforts at using PM as functional scaffolds have generated permeable constructs that enable cell migration with some challenges [19,20]. Recently, an injectable form of PM was proposed as a myocardial-engineered scaffold [21]. Despite many merits, a potential limitation of this approach was that the migration of cells within the gel was not improved compared to the collagen scaffolds (COLs). Additionally, the three-dimensional (3D) structure of the native tissue was lost during the fabrication process [21]. Several studies have shown that 3D macroporous scaffolds provide good physical, mechanical and structural support for proliferation and differentiation of seeded cells. It has been demonstrated that the interconnectivity of pores within such 3D structures enhances oxygen diffusion, nutrition transfer, cell migration and vascularization [22,23]. However, so far the use of 3D macroporous decellularized ECM scaffolds have not been broadly explored.

In this study, we aimed to fabricate a 3D macroporous cardiac patch that can mimic the myocardium ECM. It was anticipated that the 3D PM structure and mechanical properties would enhance desired cell–matrix interactions. In particular, to evaluate the synergistic effect of biological cues and the interconnected microstructure, we fabricated 3D macroporous PSs from human decellularized pericardium membranes (DPMs) by the freeze-drying method. We further evaluated the *in vitro* viability, migration, proliferation and differentiation of cardiac progenitor cells (CPCs) within these scaffolds. CPCs were used due to their ability to self-renew and differentiate into cardiomyocytes, fibroblasts, endothelial and smooth muscle cells. Furthermore, CPCs do not form teratomas [24,25] and improve heart function *in vivo* after being injected into the damaged myocardium following MI [26,27]. The placement of these cells in proper natural matrix may bring more promising outcomes in myocardial regeneration. Finally, we studied the potential of PSs to induce angiogenesis and direct the cardiac differentiation of CPCs compared to COLs and DPM *in vitro* and *in vivo* to test the suitability of this platform for myocardial treatment.

2. Materials and methods

2.1. Decellularization of PM

Human PM was provided by the Royan Tissue Department from cadavers and approved by the Ethical Committee for research applications. PM was maintained on ice while transported to the laboratory to prevent cellular lysis and damage to the matrix, then decellularized as previously described [21]. In brief, the sample was washed with Dulbecco's phosphate-buffered saline (PBS, Invitrogen, 21600-051) for 2 h and sodium dodecyl sulfate (SDS, Sigma L4390, 0.1% w/v) for 24 h, both in the presence of protease inhibitors. The tissue was finally treated with a nuclease solution (RNase/DNase), then washed in sterile PBS for 24 h by agitation, frozen overnight at −80 °C and lyophilized (Christ, Alpha 1-2 LD) for 24 h.

2.2. Scaffold fabrication

To fabricate PSs, decellularized pericardium was solubilized by enzymatic digestion using previously published protocols [21], after which the pericardium gel was fabricated. Briefly, fine milled pericardium powder (1.5% wt) was allowed to digest in pepsin (Merck, 107185) at 1 mg/ml that was dissolved in 0.1 M HCl for about 60 h under constant stirring. Afterwards, the solution was brought to pH 7.4 by the addition of 1 M NaOH and 10X PBS, which formed a viscous gel solution. Thereafter, solubilized ECM from the pericardium was poured into 24-well plates (TPP, Switzerland), frozen at −80 °C and lyophilized overnight.

DPM and COL were prepared for use as control materials. To fabricate DPMs, the decellularized pericardium was treated with 0.2 M acetic acid, followed by a wash with PBS, after which it was freeze-dried for 24 h in order to increase porosity within the structure. To fabricate COLs, collagen type I, extracted from rat tail by treatment with acetic acid, was dissolved at a 1% (w/v) concentration as previously described [28]. COLs were prepared according to the procedure mentioned for the PSs.

PS, DPM and COL were then chemically crosslinked for 24 h at 25 °C with a sterile solution of 50 mM 2-(N-morpholino) ethanesulfonic (MES, Sigma, M3671) in 70% ethanol (pH 5.4), 30 mM 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC, Sigma, E7750) and N-hydroxysulfosuccinimide (NHS, Sigma Aldrich, 130672) at a molar ratio of 1:1. Following crosslinking, the scaffolds were thoroughly rinsed several times with double distilled water (d.d.H_2O) followed by three washes for 24 h with PBS, after which the samples were frozen at −80 °C and lyophilized until further use. The non-crosslinked samples of PS, DPM and COL were mentioned as N-PS, N-DPM and N-COL, respectively.

2.3. Evaluation of the decellularization process

2.3.1. Histological characterization

Native pericardium and DPM were fixed for 24 h in a 10% PBS/neutral buffered formalin solution (pH 7.4) at 25 °C. Subsequently, samples were washed in d.d.H_2O, dehydrated in a graded alcohol series, embedded in paraffin, and sectioned into 5 μm sections. The tissue slides were stained with hematoxylin and eosin (H&E, Sigma–Aldrich), Masson's trichrome (MT, Sigma–Aldrich), and alcian blue (AB, Sigma–Aldrich), after which they were assessed under a light microscope (BX51, Olympus). In addition, the tissue slides were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich, D8417) for confirmation of the absence of cell nuclei in the decellularized pericardium. Collagen and GAG contents were calculated for DPM relative to PM as fold change in intensity of green and blue hues in MT and AB histological sections, respectively. At least 100 fields were counted by three blinded investigators for each sample.

2.3.2. DNA quantification

Total DNA content was quantified in samples of native pericardium and DPM. Samples were completely homogenized and solubilized in 1 ml lysis buffer (50 mM tris-HCl, 50 mM EDTA, 1% SDS, 10 mM NaCl, pH 8.0) and subsequently digested in the presence of proteinase K, overnight in a water bath at 65 °C, followed by a phenol/chloroform extraction. DNA was precipitated from the aqueous phase with 100% ethanol, after which the extracts were subsequently washed with 70% ethanol. After dissolving the resultant pellet in RNase-free water, spectrophotometer was used to ascertain the concentration of DNA at 280 nm. The amount of DNA was averaged from a set of three independent runs and expressed as μg/mg dry weight of samples. For further examination, extracted DNA samples from both native and decellularized tissue were electrophoresed on 0.7% agarose gel in the presence of 1 μg/ml ethidium bromide (EthBr).

2.4. Physico-chemical characterization of scaffolds

2.4.1. Scanning electron microscope (SEM) analysis

Sample structure and morphology was analyzed with a scanning electron microscope (SEM, VEGA\TESCAN, Czech Republic) at an operating voltage of 15 kV. Samples were fixed in 2% glutaraldehyde in 0.1 M PBS and left for 24 h at 4 °C. After washing with 0.1 M PBS, the material was fixed in 1% OsO_4 in 0.1 M PBS (pH 7.3) for 2 h at 25 °C. Specimens were dehydrated in a graded ethanol-water series to 100% ethanol, then allowed to completely dry. Finally samples were mounted on aluminum stubs and coated with a thin layer of gold. We determined the average pore size by SEM after measuring the dimensions of at least 500 pores that were randomly chosen from the samples by Image Analyzer software (Image J 1.44p). The porosity of the scaffolds was calculated by determining the volume (V) and mass (m) of the scaffolds. Porosity of scaffolds was defined as:

$$\text{Porosity}(\%) = \left[1 - \frac{d_s}{d_p}\right] \times 100$$

where d_s and d_p are the density of the scaffold and average protein density (1.32 gr/cm^3), respectively [29,30].

For the cell-seeded samples, the scaffolds were fixed in 2% glutaraldehyde and subsequently processed for SEM examinations.

2.4.2. SDS-Page

Proteins were boiled with denaturing PAGE loading buffer (200 mM tris-HCl, 50% glycerol, 8% SDS, 400 mM DTT, 0.04% bromophenol blue, pH 6.8). One-dimensional gel electrophoresis was performed on pericardium solubilized ECM and compared with DPM and COL. The three solutions were run on 0.5 mM tris-HCl (pH 6.8), 8% polyacrylamide gel in tris/glycine/SDS buffer; a recombinant molecular weight marker (Sigma, M0671) was used as a standard protein. Samples were run