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A naturally derived cardiac extracellular matrix enhances cardiac progenitor cell behavior in vitro

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ABSTRACT

Myocardial infarction (MI) produces a collagen scar, altering the local microenvironment and impeding cardiac function. Cell therapy is a promising therapeutic option to replace the billions of myocytes lost following MI. Despite early successes, chronic function remains impaired and is likely a result of poor cellular retention, proliferation, and differentiation/maturation. While some efforts to deliver cells with scaffolds have attempted to address these shortcomings, they lack the natural cues required for optimal cell function. The goal of this study was to determine whether a naturally derived cardiac extracellular matrix (cECM) could enhance cardiac progenitor cell (CPC) function in vitro. CPCs were isolated via magnetic sorting of c-kit$^+$ cells and were grown on plates coated with either cECM or collagen I (Col). Our results show an increase in early cardiomyocyte markers on cECM compared with Col, as well as corresponding protein expression at a later time. CPCs show stronger serum-induced proliferation on cECM compared with Col, as well as increased resistance to apoptosis following serum starvation. Finally, a microfluidic adhesion assay demonstrated stronger adhesion of CPCs to cECM compared with Col. These data suggest that cECM may be optimal for CPC therapeutic delivery, as well as providing potential mechanisms to overcome the shortcomings of naked cell therapy.

1. Introduction

Cardiovascular disease is the leading cause of death in the USA. There were an estimated 1.5 million cases of myocardial infarction (MI) in 2011 [1]. Following MI in animal models, there is a 40–60% reduction in myocyte number in the myocardium with billions of myocytes being lost within the first several days [2,3]. These myocytes are not replaced and this results in extensive inflammation and fibrosis, leading to a loss of contractility. Fibroblasts within the damaged tissue proliferate and secrete high levels of collagen to prevent the heart from rupturing, ultimately leading to heart failure. The only comprehensive cure for heart failure is cardiac transplant, which is greatly limited by the number of available donor hearts. This has forced clinicians to find new ways to improve chronic cardiac function, such as the use of beta-blockers, angiotensin receptor blockers, and other pharmacological interventions [4]. While these therapies may sustain cardiac function, they do little to regenerate functional tissue.

Cellular therapy has shown early success as a potential treatment for improving acute cardiac function post MI [5–8]. Mesenchymal stem cell injection into the infarcted myocardium shows decreased fibrosis and an improvement in certain heart function parameters [5,9]. While exciting, this finding was not due to reconstitution of the myocardium, but attributed to increased angiogenesis [8]. In 2003 the heart was found to have a population of stem/progenitor cells capable of cardiac differentiation, termed cardiac progenitor cells (CPCs) [10]. These cells are clonogenic, self-renewing, and capable of differentiation into the four major cardiac cell types (cardiomyocytes, endothelial cells, smooth muscle cells, and fibroblasts) [11,12]. For these reasons, and because CPCs do not form teratomas during cell therapy, they are a good candidate to repair the myocardium. Intramyocardial injections of CPCs have shown improvements in cardiac function after injury, potentially through myocardial regeneration [10–14]. Phase 1 clinical trials are underway with injection of autologous CPCs, and are promising [15]. However, while many cell therapy trials have shown show acute success, improvements in chronic function remain a

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challenge. This is most likely due to the fact that the local delivery of cells faces several shortcomings, such as poor retention of the cells in the myocardium, reduced survival, and poor differentiation and maturation of implanted cells [16]. Due to these issues the mechanisms by which positive effects are produced are controversial (i.e. paracrine factors vs. regeneration) [9,16].

Cellular phenotypes are influenced by their microenvironment. Matrix stiffness [15,17,18], organization [19], and biochemistry [19–21] have been shown to influence cell fate. These signals are transduced intracellularly through receptor–ligand interactions, mainly integrins [22]. It is important to consider that these trends are likely to be matrix and cell type specific. By providing cells with an ideal microenvironment it is plausible that the cells will have a more favorable outcome (i.e. improved survival, proliferation, and differentiation). This is achieved either in vitro by culturing cells on a matrix or in vivo by administering cells within a matrix that can assemble into a three-dimensional (3-D) scaffold. Injectable biomaterials are potential attractive cell delivery vehicles as they can provide a suitable microenvironment and can potentially be delivered via minimally invasive catheters [23]. Cellular therapies have been combined with various matrices to treat MI [24–30]. The major disadvantage of the currently used biomaterials for myocardial regeneration is that they lack the complexity and specificity of the native myocardial extracellular matrix (ECM) [31].

In this study a naturally derived porcine cardiac ECM (cECM) was examined for the ability to improve CPC function. Our hypothesis was centered on the fact that this would mimic the biochemical cues of a healthy myocardium, while collagen would represent both the diseased area and a commonly used cell delivery vehicle [30]. Our results demonstrate that CPCs prefer naturally derived cECM over collagen (Col) as measured by cardiomyogenic gene expression, cell survival, proliferation, and adhesion.

2. Materials and methods

2.1. CPC isolation

CPCs were isolated from adult male Sprague–Dawley rats (about 250 g) by removing the heart and homogenizing the tissue, as approved by Emory University's Institute Animal Care and Use Committee. The tissue homogenate was further digested with type-2 collagenase (1 mg ml^{-1} in Hank's balanced salt solution (HBSS), Worthington Biochemical) and passed through a 70 μm filter. Cells were incubated with Dynabeads (Dynal) conjugated to a c-kit antibody (Santa Cruz H-300) prior to magnetic sorting. Sorted cells were plated in a T-75 tissue culture flask and expanded to confluence. Following isolation, CPCs were characterized by flow cytometric analysis of c-kit (Santa Cruz H-300), multi-drug resistance protein (MDR) (Santa Cruz H-241), Gata-4 (Santa Cruz H-112) and Nkx2.5 (Santa Cruz H-114). Only clones with >90% c-kit expression were used for subsequent studies. Supplementary Fig. 1 shows representative flow cytometry histograms for c-kit, MDR, Gata-4, and Nxk2.5.

2.2. Decellularized cardiac extracellular matrix (cECM) generation

Decellularized porcine ventricular ECM was obtained and processed into a cell culture coating as previously described [32,33]. Briefly, porcine ventricular tissue was isolated and cut into small rectangular pieces, rinsed in phosphate-buffered saline (PBS) (Fisher), and decellularized using 1% sodium dodecyl sulfate (SDS) (Fisher) for 4–5 days. The decellularized cECM was then rinsed with Triton X-100 (Integra Chemical Co.) for 30 min and with deionized water overnight, frozen at −80 °C overnight, lyophilized (Labconco) overnight, and milled into a fine powder. The powder was digested using pepsin at 1 mg ml^{-1} in 0.1 M HCl (Fisher) for at least 54 h prior to use, as modified from a previously published protocol, at a ratio of 10:1 ECM matrix to pepsin [34]. The material was then raised to a basic pH by adding 1 M NaOH (Fisher), and brought to a salt concentration of 1× PBS through the addition of 10× PBS. Then the material was brought to the physiological pH 7.4 using HCl and NaOH, and diluted to 6 mg ml^{-1} using 1× PBS. The cECM was then frozen at −80 °C overnight, lyophilized for 24 h (Labconco) and stored at −80 °C prior to use.

2.3. Cell culture

Matrix solutions were made by reconstituting cECM in sterile water and then diluting to 1 mg ml^{-1} in 100 mM acetic acid. Collagen I (Col) (rat tail, Invitrogen) was diluted to 1 mg ml^{-1} in 100 mM acetic acid. Plastic tissue culture plates were coated with cECM or Col and incubated for 1 h at 37 °C to allow adsorption. Coated plates were then washed twice with 1× PBS to remove acetic acid. CPCs were seeded on the coated plates and incubated in the appropriate medium for the desired time periods (see subsequent methods sections for details specific to each experiment).

2.4. RNA and protein isolation

Cell culture was performed as described above in 6-well plastic tissue culture plates coated with 500 μl of the appropriate matrix. Two wells were prepared for each condition with 5×10^5 cells per well. Cells were cultured in treatment medium (Ham's F-12 (Mediatech) with 0.1 μg ml^{-1} basic fibroblast growth factor (bFGF) (Sigma), 1× insulin–transferrin–selenium (ITS) (Cellgro), and 1× penicillin–streptomycin–glutamine (P/S/G) (Cellgro)) with the medium exchanged every 48 h. Cells were harvested 2 and 7 days following plating, using Trizol (Invitrogen) to isolate RNA and protein. The Trizol solution was frozen at −80 °C until RNA isolation was performed. RNA and protein extraction were performed according to the manufacturer's protocols. Samples were stored at −20 °C.

2.5. Reverse transcription and quantitative real time PCR

RNA quantification and purity was determined by absorbance readings at 260 and 280 nm using a BioTek Synergy2 Spectrophotometer. Reverse transcription was performed with M-MLV (Invitrogen) as follows. Samples were prepared with 2 μg RNA and 0.1 μg hexamers (Thermo Scientific), 0.1 μg oligo(dT) (Fermentas), 25 nmol dNTP (Fermentas) and Rnase-free water in a final volume of 12 μl. No-template controls were performed by replacing RNA with Rnase-free water. Samples were heated at 65 °C for 5 min to denature the RNA, followed by 25 °C for 10 min to allow the hexamers and oligonucleotides to anneal. First strand buffer (1× final concentration, Invitrogen), 0.2 μmol dithiothreitol (Invitrogen), 40 U RNaseOUT Inhibitor (Invitrogen) and 200 U M-MLV (Invitrogen) were added to each sample. Samples were heated at 37 °C for 60 min for reverse transcription, followed by 70 °C for 15 min to inactivate the enzyme. cDNA products were stored at −20 °C.

Gene expression was measured by quantitative real time PCR in an Applied Biosystems StepOne Plus real time PCR system. Reaction mixtures contained 7.5 μl Power SYBR Green (Invitrogen), 5.1 μl Rnase-free water (Hyclone), 1.4 μl of the appropriate primer at 1 μM (IDT) and 1 μl 1:5 cDNA (total volume 15 μl). The running protocol was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s for all SYBR Green primers. A melt curve was calculated at 2 °C intervals for the same cycling conditions. Each sample was run in duplicate. A Taqman gene expression assay was performed to quantify GAPDH expression (Applied Biosystems) (50 °C for 2 min, 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min). The results are