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Young developmental age cardiac extracellular matrix promotes the expansion of neonatal cardiomyocytes in vitro

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ABSTRACT

A major limitation to cardiac tissue engineering and regenerative medicine strategies is the lack of proliferation of postnatal cardiomyocytes. The extracellular matrix (ECM) is altered during heart development, and studies suggest that it plays an important role in regulating myocyte proliferation. Here, the effects of fetal, neonatal and adult cardiac ECM on the expansion of neonatal rat ventricular cells in vitro *are* studied. At 24 h, overall cell attachment was lowest on fetal ECM; however, ~80% of the cells were cardiomyocytes, while many non-myocytes attached to older ECM and poly-L-lysine controls. After 5 days, the cardiomyocyte population remained highest on fetal ECM, with a 4-fold increase in number. Significantly more cardiomyocytes stained positively for the mitotic marker phospho-histone H3 on fetal ECM compared with other substrates at 5 days, suggesting that proliferation may be a major mechanism of cardiomyocyte expansion on young ECM. Further study of the beneficial properties of early developmental aged cardiac ECM could advance the design of novel biomaterials aimed at promoting cardiac regeneration.

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1. Introduction

Congenital heart defects (CHD) are the leading cause of mortality in live-born infants and young children [1]. Current surgical procedures to treat severe CHD, such as hypoplastic left heart syndrome, are palliative and do not replicate native heart anatomy and function [2], and these patients often suffer from secondary complications throughout life [2,3]. The ability to develop engineered cardiac tissue in vitro or stimulate cardiac regeneration in vivo has the potential to greatly improve treatments and outcomes in pediatric patients suffering from CHD. Although recent results with engineered vascular grafts in children are promising [4,5], no engineered heart tissues have yet been used in the clinic [6]. A major challenge to moving cardiac tissue engineering towards clinical relevance is the limited proliferative capacity of postnatal cardiomyocytes [7].

While the timing of the process is species-dependent [8–10], mammalian cardiomyocytes undergo a switch from hyperplastic to hypertrophic growth after birth [8]. In contrast to their postnatal counterparts, embryonic and fetal cardiomyocytes are highly proliferative and have been shown to restore function to damaged or diseased hearts in animal models [11–16]. Although a number of factors, such as cell–cell interactions [17,18], growth factor signaling [18] and mechanical forces [19,20], can regulate myocyte proliferation in the developing heart, it is likely that the extracellular matrix (ECM) also plays an important role. Collagen synthesis [21] and fibronectin expression [22] change with development, and integrin isoforms change concurrently with the transition from proliferation to terminal differentiation [23]. Other studies have demonstrated a significant effect of ECM signaling on cardiomyocyte function. For example, fibronectin and collagen III, up-regulated by mouse embryonic fibroblasts, enhanced embryonic cardiomyocyte proliferation in response to growth factors [18,24]. Periostin, an ECM protein expressed during fetal cardiac development [25,26], was found to promote myocyte proliferation in vitro and improved heart function after myocardial infarction in adult rats [27]. Collagen V resulted in better expansion of cardiac-like cells derived from mesenchymal stem cells compared with collagen I [28], which is highly expressed in the adult heart [25]. While these findings point to a critical role for the developing ECM in promoting or mediating cardiomyocyte proliferation, none of the aforementioned studies investigated the cardiac ECM as a whole.

Decellularized organs can provide complex, tissue-specific cues and are thus attractive for tissue engineering and regenerative medicine approaches [29]. Indeed, adult cardiac tissues have been studied extensively and have shown promise for certain applications [30–35], such as providing mechanical support [35] or

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promoting neovascularization [30] in the adult heart. However, adult ECM may lack the necessary cues for myocyte proliferation, as the role of most signaling in the adult organ is to maintain homeostasis. The only known study to date that specifically investigated the developmental age of the ECM showed that cells were better able to repopulate decellularized kidney sections from young rhesus monkey compared with those from adults, further supporting this concept [36,37]. Since cardiomyocyte proliferation is highest during prenatal development, mimicking fetal ECM may be more appropriate for promoting cardiac regeneration, but has not yet been explored.

The purpose of this study was to determine the effect of fetal cardiac ECM on the expansion of cardiomyocytes in vitro. Given the clinical need for novel tissue engineering and cell therapy-based treatments for the myocardium, ventricular cell response to fetal, neonatal and adult cardiac ECM was studied. It was found that cardiomyocytes had better adhesion and expansion on fetal ECM compared with older ECM, with myocytes remaining a large percentage of the cell population and increasing ~4-fold over 5 days in culture. Furthermore, it was found that significantly more myocytes were positive for the mitosis marker phospho-histone H3 on fetal ECM compared with other substrates, suggesting that myocyte expansion is due to proliferation, at least partially. The long-term goal for this work is to develop biomaterials that mimic developmental cues to stimulate or maintain cardiomyocyte proliferation for tissue engineering or regenerative medicine approaches for treating CHD.

2. Materials and methods

2.1. Heart harvest from fetal, neonatal and adult rats

All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee at Tufts University and the NIH Guide for the Care and Use of Laboratory Animals. Pregnant Sprague–Dawley rats (~3 months old) were deeply anesthetized with a mixture of ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) and then euthanized by harvest of the heart. Fetal pups (embryonic day 18–19) were isolated from the uterus, euthanized by decapitation and then the hearts were isolated. Neonatal pups (postnatal day 2–4) were euthanized by conscious decapitation prior to heart harvest. Freshly isolated hearts from all three ages were fixed for assessment of native cardiac cell proliferation, processed for analysis of ECM composition or decellularized for cell culture experiments, as described below.

2.2. Proliferation of cardiac cells in native hearts

To determine proliferation in native fetal, neonatal and adult hearts, samples were immediately immersed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) after harvest and fixed at 4 °C overnight. The samples were cryoprotected in a 30% sucrose solution and then embedded and frozen in Tissue-Tek OCT compound (VWR). The hearts were sectioned into 7-μm-thick slices in the circumferential direction on a Cryostat (Leica CM 1950). The tissue slices were stored at −20 °C until immunohistochemical staining. Samples were equilibrated to room temperature (RT), washed in phosphate-buffered saline (PBS) to remove OCT and then blocked with 5% donkey serum and 0.1% bovine serum albumin (BSA) (Sigma) in PBS for 1 h at RT. Cells undergoing mitosis were labeled with phospho-histone H3 (PHH3) antibody (Ser 10-R, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at RT followed by incubation with Alexa Fluor 488 donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 h at RT. Cells of the cardiomyocyte lineage were then labeled with cardiac α-actin antibody (5C5; Santa Cruz) and Alexa Fluor 555 donkey anti-mouse secondary antibody (Invitrogen). Samples were mounted with Vectashield medium containing DAPI nuclear stain and imaged on an Olympus IX8I microscope, using Metamorph Basic software (version 7.7.4.0, Molecular Devices).

2.3. Composition of ECM in fetal, neonatal and adult hearts

To determine the composition of the ECM, hearts were first decellularized in sodium dodecyl sulfate (SDS) with concentrations of 0.05%, 0.5% and 1% (wt./vol.) in distilled water (diH_2O) for fetal, neonatal and adult hearts, respectively. Fetal and neonatal hearts were immersed in SDS with gentle agitation; adult hearts underwent perfusion decellularization [33] or were minced and soaked in SDS. Once the tissue had turned white, samples were transferred to a wash in TritonX-100 (Amresco, Solon, OH) at the same concentration as the respective SDS solution for several hours. Next, the samples were washed with large volumes of diH_2O at least three times to remove residual detergent. Samples were frozen at −20 °C and then lyophilized overnight (Labconco, Kansas City, MO). Dry weights were determined, and the samples were digested at a concentration of 5 mg ml^{-1} in a solution containing 5 M urea, 2 M thiourea, 50 mM DTT and 0.1% SDS in PBS [38] with constant stirring at 4 °C for ~48 h. Afterwards, the samples were sonicated on ice (Branson Digital Sonifier, 20 s pulses, 30% amplitude), and protein was precipitated with acetone and analyzed via liquid chromatography tandem mass spectrometry (LC-MS/MS) at the Beth Israel Deaconess Medical Center Mass Spectrometry Core Facility. The 15 most abundant ECM components for each developmental age were identified from spectrum count data ($N = 2$ for each developmental age).

2.4. Second harmonic generation microscopy

Images of decellularized heart tissues were acquired on a Leica TCS SP2 confocal microscope equipped with a Ti:sapphire laser (Spectra Physics, Mountain View, CA). With the laser tuned to 800 nm, second harmonic generation (SHG) images were collected in the forward direction using a photomultiplier tube (PMT) with a 400(±10) nm bandpass filter. Two-photon excited fluorescence emission was also simultaneously collected between 500 and 550 nm from a non-descanned PMT in the epi-direction. Using a 63× water immersion objective (1.2 NA), a series of image slices (512 × 512 pixels, 238 × 238 μm^2 field of view) were acquired within the first 180–200 μm from the outer surface of the tissue in 2.5–5 μm increments. Image intensities were normalized for PMT gain and laser power as previously described [39], and the mean SHG signal was computed from each image volume. Average intensity projections in the z-direction are displayed in false color with the same normalized intensity scale.

2.5. Optimization of fetal heart decellularization

The use of 1% SDS for adult hearts is well established [30,33,34], but few studies have reported decellularization of younger organs [36]. Therefore, SDS and TritonX-100 were explored at various concentrations ranging from 0.1 to 1% for fetal heart decellularization to determine the optimal conditions. DNA was measured using a DNA Quantitation Kit (Sigma) per the manufacturer's instructions to assess cellular content in the fetal hearts after decellularization. Equal dry weights of native tissue controls and decellularized samples were tested. In addition, a sub-set of ECM proteins was analyzed using a modified ELISA [40] to determine ECM preservation after decellularization. Approximately 20 μg of ECM per well, with each sample run in triplicate, was adsorbed in a 96-well plate.

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