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A simplified protocol for culture of murine neonatal cardiomyocytes on nanoscale keratin coated surfaces

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ABSTRACT

Objective: We aim to develop a simple, efficient and cost-effective protocol for culturing the neonatal cardiomyocytes using keratin derived from human hair, which can be used for studying cardiac hypertrophy *in vitro*.
Methods: Keratin was extracted from human hair and applied as nanoscale coating onto the culture dishes. Physical parameters such as surface morphology and roughness of the coating were studied by SEM and AFM. Cardiomyocyte specific markers were assessed by immunofluorescence. Signaling pathways activated in hypertrophy were analyzed by western blotting and changes in the expression of fetal genes were analyzed by qPCR. The changes in the calcium fluxes were observed microscopically using Fluo-4.
Results: Keratin coated surfaces displayed a uniform coating and comparable roughness across dishes. Our optimized protocol for isolating cardiomyocytes yielded up to ~10^6 cells per heart. Characterization of cardiomyocytes with specific markers revealed that they can attach, grow and show spontaneous contractions on keratin-coated substrates similar to fibronectin-coated surfaces. Phenylephrine (PE) treated cardiomyocytes grown on keratin-coated substrates exhibited increased cell size, sarcomere organization and perinuclear ANP expression indicating the development of cardiac hypertrophy. In addition, we observed increased activation of Akt and ERK pathways, induction of the fetal genes and increased protein synthesis upon PE treatment, which are characteristics of cardiomyocyte hypertrophy. The protocol was extended to mouse cardiomyocytes and found to show similar results upon examination.
Conclusion: We demonstrate that keratin can act as an efficient yet cost effective alternative substrate for the attachment, growth and differentiation of neonatal murine cardiomyocytes.

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1. Introduction

Neonatal murine cardiomyocyte cultures have been widely used as a model for studying the spontaneous contractions, arrhythmia, hypoxia and ischemia at the cellular level. It not only permits the use of biochemical, morphological and electrophysiological approaches to understand the cardiovascular function but also offers great potential to study toxicological and pharmacological effects of drugs [1]. Besides, the primary cardiomyocyte culture has been extensively used to gain insights into the molecular mechanisms governing defects in cardiac regeneration and wound healing [2]. It is a validated and well accepted model to study the pathophysiology of the heart *in vitro*.

The isolation of primary cardiomyocytes was first reported in 1963 by Harary and Farley and since then many modifications of the original protocol have been reported [3–6]. However, it is still a challenge to obtain superior quality cardiomyocytes in terms of viability, spontaneous contractions and increased yield of cardiomyocytes *in vitro* [6]. From previous studies, it is well known that substrates on which cardiomyocytes are grown play a crucial role in enhancing cell attachment, growth and differentiation [7]. For this purpose, a variety of extracellular matrix (ECM) proteins such as collagen, fibronectin, laminin and gelatin have been shown to be useful, most of which are sourced from slaughtered animals while fibronectin is purified from human plasma [7–10]. While the skin, tendons, ligaments, cartilages or bones are treated chemically to obtain gelatin, their tails and other tissues are used for obtaining collagen or laminin [10,11]. Besides the sacrifice involved, products prepared from animal sources carry a significant risk of contamination and heterogeneity.

Recent research has focused on the alternatives for these conventionally used substrate coatings. Over the past decade, a family of intermediate filament proteins, namely keratins has been gaining substantial interest as a substrate for surface coating [12,13]. Even though they are

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structural proteins, they possess great potential as biomaterials due to their abundance, biodegradability, bioactivity and the fact that they can be easily harvested from discarded human hair [14]. Even though it has previously been shown to be a useful coating material for various cell lines including human mesenchymal stem cells (hMSCs), keratins have never been studied for the cell attachment and growth pattern of primary neonatal cardiomyocytes obtained from murine model systems [12,15]. Thus, we tested whether human hair derived keratin could be a cost effective alternative to previously reported coating materials such as fibronectin, gelatin, collagen and laminin. In this study, the use of human hair derived keratins has been shown to be an equally efficient substrate coating as compared to fibronectin and gelatin for culturing primary cardiomyocytes from neonatal murine. Further, we have verified the use of keratin coated surfaces for studying cardiac failure (CF) by assessing the development of agonist mediated cardiac hypertrophy in these cultured primary cardiomyocytes [16].

2. Materials and methods

2.1. Extraction of keratin from human hair

The keratins were extracted from human hair according to a previously reported procedure known as the 'Shindai method' [17]. Discarded human hair was obtained from a local hair salon in Bengaluru, India. They were washed thoroughly in detergent and then with distilled water. The cleaned hair were further immersed in ethanol for 2 h and dried at room temperature. The dried hair was then de-lipidized (removal of external lipids) by soaking in a mixture of chloroform and methanol (2:1 *v*/*v* ratio) for 24 h and dried again. Approximately 20–25 mg of de-lipidized hair was added to 5 ml of extraction buffer containing 25 mM Tris–HCl pH 8.5, 2.6 M thiourea, 5 M urea and 5% β-mercaptoethanol (β-ME) and the mixture was kept at 50 °C for 24–48 h under gentle stirring. The mixture was filtered through Whatman filter paper. After cooling, the filtered mixture was dialyzed using a dialysis membrane (MW cut off ~10 kDa) in ultrapure water to allow complete removal of all salts and β-ME. In order to obtain powdered keratin, the freeze-dry method was followed. The dialyzed mixture was frozen at −20 °C overnight and then lyophilized for 12 h. The extracted fraction of keratin was tested for solubility in various solvents and found to be soluble in 0.5 M NaOH.

2.2. Substrate coating

The glass coverslips or tissue culture polystyrene (TCPS) plates were coated with a thin film of keratin solution (500 μg/ml, extracted from human hair), fibronectin (25 μg/ml, Sigma, #F2006), gelatin (0.2% *w*/*v*, Sigma Aldrich) or poly-L-lysine (0.1% *v*/*v*, Sigma Aldrich) followed by air drying and UV sterilization. The keratin coated surface was washed with PBS prior to cell culture to remove excess NaOH.

2.3. Characterization of the coated surfaces

Surface morphology of the keratin, fibronectin, gelatin and poly-L-lysine coated surfaces were examined under a scanning electron microscope (SEM, Zeiss Gemini with MonoCL). The samples were dried in a desiccator and then mounted on an aluminum stub and sputter coated with gold before imaging. Images were acquired at 10,000× magnification using a secondary electron detector (SE2).

Surface topography was measured using a NX-10 atomic force microscope (Park Systems). The coverslips were mounted on a magnetic stub using a double-sided carbon tape and transferred to the AFM stage for imaging. A silicon AFM cantilever probe tip (Acta, Park Systems) in non-contact mode was used with a resonating frequency of 300 kHz. The data sets were subjected to first-order flattening using the XEI software (Park Systems Corporation). Mean surface roughness values (Ra) of four different fields per sample were calculated.

2.4. Biological studies

2.4.1. Harvest of the neonatal hearts

All experimental protocols involving the use of animals were reviewed and approved by the Institutional Animal Ethics Committee (IAEC), Indian Institute of Science, Bangalore constituted as per the article number 13 of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)-rules, laid down by Government of India (http://cpcsea.nic.in/Auth/index.aspx). Neonatal Sprague Dawley or Wistar rats and BALB/c mice (aged between 12 and 48 h) were procured from the Central Animal Facility (http://caf.iisc.ernet.in/), Indian Institute of Science (IISc), Bangalore. Pups were deeply anaesthetized with 1.0% isoflurane and euthanized by decapitation. Central thoracotomy was performed in the midline to expose the heart. A fresh pair of sterile scissors was used to carefully extract out the heart avoiding any damage to the myocardial tissue. The excised heart was placed in ice cold PBS containing 0.01 M of D-glucose. The heart was gently squeezed with the help of sterile forceps to drain out all the residual blood from the extracted heart. Heart tissue was minced into small pieces of about 1 mm^3 volume using a fresh pair of sterile scissors and were collected in a 1.5 ml microcentrifuge tube.

2.4.2. Enzymatic dissociation of cells

In order to get a high number of isolated cells, the heart tissue was digested using a mixture of 0.2% trypsin, 0.4 mg/ml Collagenase Type II (Gibco, Invitrogen) and 0.01 M of D-glucose in PBS. 65–75 μl of the digestion mixture per heart was used for each digestion. Each digestion was performed for 5 min at 37 °C with gentle shaking at 250 rpm. Supernatant from the first digestion which contains unwanted erythrocytes and debris was discarded. The digestion steps were repeated for a total of ten times and the supernatant containing a single cell suspension was collected in a 15 ml Falcon tube containing 100% horse serum (Thermo Scientific) and kept at 37 °C throughout the digestion process.

2.4.3. Pre-plating and cardiomyocyte culture

The horse serum enriched with a single cell suspension was then plated onto tissue culture polystyrene (TCPS) plates and incubated at 5% CO_2 and 37 °C for 1 h for differential attachment of non-myocardial cells. The non-adherent cells were then transferred to a sterile 15 ml tube and centrifuged at 1000 rpm for 10 min. The supernatant was discarded and the cell pellet was re-suspended in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco). The cells were counted using a haemocytometer and were plated at a cell density of one million cells per well of a 6 well plate (Costar) or 0.5 million cells per well of a 12 well plate (Costar) coated with fibronectin (25 μg/ml, Sigma Aldrich #F6005), or keratin (500 μg/ml), or bovine gelatin (0.2% *w*/*v*, Sigma Aldrich) or poly-L-lysine (0.1% *w*/*v*, Sigma Aldrich). The cells were incubated in a humid 5% CO_2 incubator at 37 °C. The medium was replenished after every 24 h. In order to evaluate the efficiency of seeding, cells were seeded at a density of 1.5 million cells per well of a 6 well plate and incubated for 24 h. After washing with PBS and detaching by 0.25% trypsin–EDTA, the cells were counted using a haemocytometer. The seeding efficiency was calculated by dividing the number of cells attached by the number of cells that were seeded. All experiments were performed on keratin and fibronectin coated plates in triplicates. To understand the influence of the growth substrate on trypsinization time, the cardiomyocytes were seeded in a 12-well plate coated with keratin or fibronectin. After 24 h post seeding, the cells were incubated with 200 μl of trypsin-EDTA. At different time intervals, detached cells were separated and the cell suspension was collected and counted. The cumulative total number of trypsinized cells was plotted against time.

2.4.4. Immunofluorescence

Cardiomyocyte population was characterized by immunofluorescence with specific markers as described previously [18]. The cells were fixed with 3.7% formaldehyde for 15 min exposure and permeabilized with 0.2% Triton-X for 5 min at room temperature. Cells were washed with PBS prior to each step. Blocking with 5% BSA dissolved in PBS for 1 h at room temperature was carried out to prevent nonspecific binding of the antibodies. The cells were incubated overnight at 4 °C with primary antibody: α-sarcomeric actinin (monoclonal, EA-53; 1:100, Sigma), desmin (monoclonal, DE-U-10, 1:20, Sigma), ANP (rabbit polyclonal, ab14348, 1:300, Abcam), Troponin I (Thermo), Troponin T (Abcam) or Nkx 2.5 (Abcam). The next day, cells were incubated with fluorophore conjugated secondary antibody for 1 h: fluorescent labeled Alexa Fluor 488 (green) and 546 (red) antibodies (Life technologies). Cells were washed and mounted on glass slides using a ProLong Gold antifade reagent with DAPI (Life Technologies) or Hoechst (1:2000). Samples were imaged using a confocal microscope (LSM 550, LSM 710 or LSM 880, Zeiss, USA).

2.4.5. Measurement of calcium transients

Cytosolic Ca^{2+} transients were measured in cardiomyocytes grown on live cell imaging culture dishes coated with keratin or fibronectin. The cells were loaded with Fluo-4 for 30 min at 37 °C, washed and imaged in serum free culture medium. Cardiomyocytes grown on keratin or fibronectin coated glass bottom culture dishes were placed at 37 °C in a 5% CO_2 incubator in a special chamber mounted on a Zeiss LSM 880 Airyscan microscope. The fluorescence emission from control and PE-treated cells were measured with 488 nm excitation for Fluo-4 with high speed fluorescence imaging.

2.4.6. Live cell imaging of organelles

In order to visualize the lysosomes, the cardiomyocytes were stained with 100 nM of Lyso-tracker Red DND 99 for 1 h at 37 °C, and thereafter washed and imaged in serum free DMEM using a Zeiss LSM 880 microscope. The fluorescence emission from beating cells was measured with 546 nm excitation for Lyso-tracker Red with high speed fluorescence imaging.

Similarly, to visualize the mitochondria, the cardiomyocytes were stained with 200 nM of Mito-tracker Deep Red for 1 h at 37 °C, and imaged in serum free culture medium free of the dye. The fluorescence emission from live cells was measured with 546 nm excitation for Mito-tracker Red with high speed fluorescence imaging using a Zeiss LSM 880 microscope.

2.4.7. Western blotting

Cardiomyocytes were treated with vehicle or 100 μM phenylephrine (PE) for 1 h and western blotting was performed as described in our previous reports [18,19]. Briefly, total cellular protein was isolated using a standard lysis buffer containing protease inhibitors. Protein concentration of the samples were estimated using a Bradford reagent, and an equal amount of proteins was loaded onto a 10% SDS-PAGE gel and resolved by electrophoresis at constant voltage. The proteins were then transferred onto a 0.22 μm PVDF membrane (BioRad Laboratories, USA) by overnight wet transfer. Primary antibodies for α-sarcomeric actinin (1:500; Sigma), MEF2C (1:1000, Pierce), Troponin T (1:1000, Abcam) and β-actin (1:1000; Sigma-Aldrich) were used along with goat anti-rabbit or goat anti-mouse horseradish peroxidase conjugated secondary antibodies (1:1000; Sigma-Aldrich). Antibody-reactive proteins were detected by chemiluminescence.

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