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Exogenous treatment with eicosapentaenoic acid supports maturation of cardiomyocytes derived from embryonic stem cells

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ABSTRACT

Embryonic stem cells offer multiple advantages over adult stem cells in terms of achieving acceptable number of functional cardiomyocytes to be exploited in cell therapy. However, differentiation efficacy is still a major issue to be solved before moving to regenerative medicine. Although a vast number of chemical compounds have been tested on efficiency of cardiac differentiation, the effect of fish oil components, such as eicosapentaenoic acid (EPA) on developmental bioenergetics, and hence cardiac differentiation, remained unstudied. EPA has been reported to have several cardioprotective effects, but there is no study addressing its role in cardiac differentiation. After mesoderm induction of embryoid bodies (EBs) derived from mouse embryonic stem cells (mESCs) in hanging drops initiated by ascorbic acid, they were treated with various concentrations of EPA. Gene and protein expression and functional properties of cardiomyocytes derived from ESCs were evaluated following treatment with various concentrations of EPA. Exposure to low concentrations of EPA (10 μM) increased percentage of beating colonies and beating area. This treatment also resulted in up to 3 fold increase in expression of *NKX2-5*, *MEF2C*, *MYH6*, *TNNT2* and *CX43*. FACS analysis confirmed gene expression analysis with increased percentage of MYH6 positive cells in EPA-treated group compared to the control group. In contrast, the expression of genes coding for cardiac differentiation, remained constant or even declined with higher concentrations of EPA. In conclusion, we have demonstrated that treatment of mESCs undergoing cardiac differentiation with low concentration, but not high concentration of EPA up-regulate transcription of genes associated with cardiac development.

1. Introduction

Ischemic heart disease is the major cause of death worldwide [1]. Embryonic stem cells (ESCs) are promising therapeutic agents that can potentially generate an unlimited source for cell therapy. Several lines of evidence have shown that cardiomyocytes derived from ESCs can replenish infarcted heart and rescue heart function [2,3]. However, preclinical studies in animal models of myocardial infarction (MI), showed low cell survival and inefficient electrical coupling following transplantation [4]. Therefore, providing conditions that lead to better survival, higher differentiation and better expression of electrical coupling-related channels in cardiomyocytes developed from pluripotent stem cells, could help improve methods for producing efficient cells in terms of regenerative medicine.

Recently, a vast number of growth factors and compounds such as transforming growth factor-β (TGF-β) [5], 5-azacytidine [6], hepatocyte growth factor [7] and angiotensin II [8] have been reported to induce cardiac differentiation. Although ample number of studies has been conducted on cardiogenic effect of growth factors and chemical compounds, effect of providing metabolic and energetic requirements of beating cardiomyocytes during transition from stem cells requiring little energy to high-demand cardiomyocytes are only partly understood. Moreover, in addition to

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being an important energy source in cardiomyocytes [9], fatty acids can modulate signaling pathways during heart development [10] and are also the major components of membranes and active ligands for receptors and transcription factors [11]. More recently a metabolomics study revealed the robust significance of metabolites such as fatty acids in directing fate of embryonic stem cell toward cardiomyocyte. They reported that treatment of ESCs with metabolites involving in oxidative metabolism such as saturated fatty acids enhanced cardiac differentiation [12]. However, effect of treatment with unsaturated fatty acids during cardiac differentiation has not been well defined yet.

n-3 polyunsaturated fatty acids (n-3 PUFA) such as eicosapentaenoic acid (EPA) have been reported to decrease risk of cardiovascular diseases and arrhythmia through various mechanisms [13–15]. Some studies showed that n-3 PUFA consumption leads to its incorporation into cellular membrane and also mitochondrial membranes, which could modify the activity of receptors and ion channels [16–20]. Moreover, increasing evidence indicated potential role of n-3 PUFAs in modulating bioenergetics of cardiac cells through increasing mitochondria number and improved mitochondrial function efficacy [21,22]. Besides, fatty acid treatment can modulate gene expression profile through various pathways, peroxisome proliferator-activated receptors (PPARs) being the most widely studied target [23]. Taking into account importance of coordination of genetic and bioenergetic transformations during differentiation into cardiomyocytes [24], it is of interest to study the possible effect of n-3 PUFA on cardiac differentiation and characteristics of cardiomyocytes derived from ESCs.

This study aims to explore the possible effect of a range of EPA concentrations on cardiomyocytes differentiation from mouse embryonic stem cells (mESCs). Moreover, we addressed their potential effect on electrophysiological characteristics of cardiomyocytes derived from mESCs by multi-electrode array study of beating cardiomyocytes.

2. Methods

2.1. Cell culture and differentiation

mESCs were cultured and maintained on gelatin in knock out Dulbecco's minimum essential medium (Knockout™ DMEM, Gibco, 10829–018) growth media supplemented with 1000 U/mL leukemia inhibitory factor (LIF, Royan Institute), glutamine (Gibco, 25030–024), penicillin/streptomycin (Gibco, 15070–063), nonessential amino acids (Gibco, 11140–035), β-mercaptoethanol (Sigma Aldrich, M7522), and 15% fetal bovine serum (Gibco, 12483–020).

mESCs were differentiated using hanging drop method, a previously described protocol [25]. Briefly, mESCs were trypsinized and hanging drops of 800 cells in 20 μL of cultivation medium without LIF were generated. On day 3 of differentiation, the generated EBs were transferred to petri dishes and re-suspended for more 2 days. On day 5, the EBs were plated on gelatin-coated plates and observed for the appearance of beating EBs. From day 3 onwards, cells were treated with ascorbic acid (100 μM) and different concentrations of EPA (10, 50, 100 μM).

2.2. Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, 15596-018). Subsequently RNA was reverse transcribed into cDNA using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, K1632). In this reaction, the samples were incubated in a thermocycler at 37 °C for 15 min and then at 85 °C for 5 s. Quantitive real-time PCR was performed using SYBR Premix Ex TaqTM II (Takara Bio, Inc., SYBR® Premix Ex Taq™ II RR081Q) in a final volume of 20 μL on a Rotor Gene real-time thermocycler (Qiagen, Hilden, Germany). The thermal profile for SYBR Green PCR was 95 °C for 15 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 30 s. The sequence of primers for amplification was as shown in Supplementary Table 1. The specificity of amplification was monitored by DNA melting curve during gradual temperature increments. β-tubulin was used as the endogenous control. The comparative Ct method (ΔΔCt) was utilized to calculate the fold change in genes expression in EPA-treated groups compared to non-treated group.

2.3. Immunofluorescence staining

Contracting EBs were dissociated using collagenase type II for 40 min in a 37 °C incubator. After dissociation, single cells were plated on gelatin-coated 4-well plates and allowed to grow for two days. Cells were fixed with phosphate buffered saline (PBS, Gibco, 21600–051) containing 4% paraformaldehyde (Sigma–Aldrich, P6148) at 4 °C for 20 min, washed with PBS containing 0.05% (v/v) Tween, permeabilized and blocked in PBS containing 0.2% (v/v) Triton X-100 and 10% serum for 40 min at room temperature and then incubated with first antibodies against MYH6, TNNT2, NKX2-5, CX43 or respective isotype controls in PBS containing 1% BSA overnight at 4 °C. Cells were washed with PBS containing 0.05% (v/v) Tween for three times and stained with secondary antibodies in PBS containing 1% BSA for 2 h at room temperature. Cells were washed again and Nuclei were stained with 4,6-diamidino-2-phenylin-dole (DAPI, Sigma-Alrich, D8417). Immunocytochemistry-stained images were obtained using fluorescent microscope (Olympus, IX71). All primary and secondary antibodies, dilutions and suppliers are listed in Supplementary Table 2.

2.4. Flow cytometry

Dissociated cells were transferred to flow cytometry tubes and fixed with PBS containing 4% paraformaldehyde at 4 °C for 20 min, fixed cells were washed with PBS containing 0.05% (v/v) Tween, permeabilized and blocked in PBS containing 0.2% (v/v) Triton X-100 and 10% serum for 40 min at room temperature and then stained with first antibody against MYH6 overnight at 4 °C. Cells were washed as above and stained with secondary antibodythen washed again and Fluorescence was detected with a BD-FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Data were analyzed using Flowing software version 2.5.1.

2.5. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) release assay (LDH Cytotoxicity Detection Kit; Biovision; USA) was performed to evaluate the possible cytotoxic effect of EPA according to manufacturer's protocol. Briefly, 50 μL of cell medium and 50 μL of cell medium collected from the control group and the groups treated with different concentrations of EPA and 50 μL of reaction mixture were added to each well of a 96-well plate and incubated at room temperature for 30 min. The release of LDH was assayed at absorbance of 490 nm.

2.6. Microelectrode array

Extracellular recordings were performed with a microelectrode array (MEA) data acquisition system (Multi Channel Systems, Reutlingen, Germany). The MEA plates contained a matrix of 60 titanium nitride electrodes (30 μm) with an inter-electrode distance of 200 μm. The MEA plates were sterilized and coated with fibronectin for 2 h. The beating colonies were micro-dissected and