International Journal of Cardiology 203 (2016) 964–971

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Efficient attenuation of Friedreich's ataxia (FRDA) cardiomyopathy by modulation of iron homeostasis-human induced pluripotent stem cell (hiPSC) as a drug screening platform for FRDA☆

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ARTICLE INFO

Article history:
Received 21 September 2015
Accepted 16 November 2015
Available online 17 November 2015

Keywords:
Friedreich's ataxia
Induced pluripotent stem cells
Cardiomyopathy
Idebenone
Deferiprone

ABSTRACT

Background: Friedreich's ataxia (FRDA), a recessive neurodegenerative disorder commonly associated with hypertrophic cardiomyopathy, is caused by silencing of the frataxin (FXN) gene encoding the mitochondrial protein involved in iron–sulfur cluster biosynthesis.
Methods: Application of our previously established FRDA human induced pluripotent stem cell (hiPSC) derived cardiomyocytes model as a platform to assess the efficacy of treatment with either the antioxidant coenzyme Q10 analog, idebenone (IDE) or the iron chelator, deferiprone (DFP), which are both under clinical trial.
Results: DFP was able to more significantly suppress synthesis of reactive oxygen species (ROS) than IDE at the dosages of 25 μM and 10 nM respectively which agreed with the reduced rate of intracellular accumulation of iron by DFP treatment from 25 to 50 μM. With regard to cardiac electrical-contraction (EC) coupling function, decay velocity of calcium handling kinetics in FRDA-hiPSC-cardiomyocytes was significantly improved by DFP treatment but not by IDE. Further mechanistic studies revealed that DFP also modulated iron induced mitochondrial stress as reflected by mitochondria network disorganization and decline level of respiratory chain protein, succinate dehydrogenase (CxII) and cytochrome c oxidase (COXIV). In addition, iron-response protein (IRP-1) regulatory loop was overridden by DFP as reflected by resumed level of ferritin (FTH) back to basal level and the attenuated transferrin receptor (TSFR) mRNA level suppression thereby reducing further iron uptake.
Conclusions: DFP modulated iron homeostasis in FRDA-hiPSC-cardiomyocytes and effectively relieved stress-stimulation related to cardiomyopathy. The resuming of redox condition led to the significantly improved cardiac prime events, cardiac electrical-coupling during contraction.

1. Introduction

Friedreich's ataxia is the most common hereditary ataxia encountered in clinical practice. While the disease is well known for its neurological involvements such as progressive gait and limb ataxia [1,2], most affected individuals died prematurely due to Friedreich's ataxia related cardiomyopathy [3]. To date, no therapeutic intervention has been shown to effectively modify or alleviate the pathophysiological process. The disease is caused by GAA triplet codon expansion within the first intron of the frataxin (*FXN*) gene encoding the mitochondrial protein, frataxin, leading to heterochromatin-mediated silencing of *FXN* in affected individuals [4,5]. While controversial, it is commonly accepted that frataxin plays an important role in mitochondrial iron metabolism particularly in the biosynthesis of iron–sulfur cluster, an essential component of Complex I–III enzymes in respiratory electronic transport chain. As such therapeutic strategies have been developed primarily targeting the increased reactive oxygen species (ROS) [6–9] to possibly resume the impaired energy generation related to these pathways. For instance, idebenone (2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone), a structural analog of co-enzyme Q10, shuttling electrons from Complex I and II and other flavoprotein dehydrogenase to Complex III of the mitochondrial respiratory chain [10,11] as well as acting an ROS scavengers, [12] have been demonstrated protective

☆ These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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http://dx.doi.org/10.1016/j.ijcard.2015.11.101

effects in cellular and animal models of Friedreich's ataxia. However, subsequent clinical trials have failed to demonstrate the efficacy of idebenone in Friedreich's ataxia patients with any meaningful clinical benefit of the therapy [13], thus raising the possibility whether the increased oxidative stress and the impaired ATP production are the key pathological mechanisms to the clinical phenotypes or just an epiphenomenon. In fact, this could also be related to the lack of an appropriate-yet-reliable disease model that could be recapitulating the pathophysiological process and allowing functional validation of candidate drugs. Using human induced pluripotent stem cells (hiPSCs) derived cardiomyocytes generated from patients with Friedreich's ataxia, we have previously reported that the enhanced iron uptake via upregulated transferrin receptor 1 and the resultant iron accumulation has a key pathophysiological role to induce downstream cardiomyopathic changes such as the reduced ATP synthesis, the increased reactive oxidative species (ROS) production, and the impaired calcium handling properties [14]. Based on our previous findings, we hypothesize that iron chelating could be one of the alternatives for treatment of FRDA by modulation of iron homeostasis. The orally active, iron chelating drug, deferiprone (DFP, 1, 2 dimethyl-3-hydroxy-pyrid-4-one) and its derivatives were primarily designed and screened for the treatment of iron overload and toxicity conditions [15]. They were identified as powerful free radical damage inhibitor since 30 years ago [16] and many *in vitro*, *in vivo* and clinical studies have confirmed these results [17,18]. Nevertheless, the studies of DFP and its derivatives were limited in neuroprotective effects [19].

In the present study, we exploited our previously established hiPSC-cardiomyocyte based Friedreich's ataxia model as a drug-testing platform to compare relative efficacy of the antioxidant, idebenone (IDE), Co-enzyme Q10 analog and an iron chelator, deferiprone (DFP), on mitochondrial function, reactive oxidative species production and calcium handling properties. Our results not only provide pathophysiological insights to therapeutic strategy for treatment of Friedreich's ataxia related cardiomyopathy, but also more importantly our approach illustrates that hiPSC-based drug testing strategy can be an integral part of future pre-clinical drug development.

2. Methods

2.1. Generation of human induced pluripotent stem cells and their cardiac derivatives

Skin biopsies were obtained under standard aseptic technique from a patient with Friedreich ataxia and documented GAA triplet repeat expansions within the first intron of the *FXN* gene and one healthy age- and sex-matched control subject. Detailed methods on hiPSC generation and characterization, and in vitro cardiac differentiation have been previously reported [14,20–22].

2.2. Cardiac differentiation

To induce cardiac differentiation, undifferentiated hiPSCs were maintained in mTeSR™1 medium (STEMCELL Technologies Inc., Vancouver, BC, Canada) as previously described [23–25]. Four days prior to induction, hiPSCs were dissociated into single cells with accutase (Invitrogen, CA, USA) and then seeded onto 12-well matrigel-coated plate (Thermos Scientific Inc., Walham, MA, USA) supplemented with Y27632 (5 μM) (Stemgent, Cambridge, MA, USA). On the first induction day, the culture medium was switched to RPMI medium (Life Technologies, Maryland, USA) without insulin and supplemented with B27 (Life Technologies, Maryland, USA) and a GSK-β inhibitor, CHIR99021 (12 μM) (Selleckchem, Houston, TX, USA), which was refreshed 24 h later. On Day 4, a Wnt signaling inhibitor, IWP2 (5 μM) (Selleckchem, Houston, TX, USA), was added to the culture medium. Typically, spontaneously beating cardiomyocytes could be observed around 9 days after induction. The cells were maintained in the culture and were dissociated approximately 30 days after induction using 0.25% Trypsin-EDTA for further experiments.

2.3. Friedreich's ataxia related cardiomyopathy model

To recapitulate the cellular features of Friedreich ataxia cardiomyocytes, hiPSC-derived cardiomyocytes were cultured for 48 h in a medium supplemented with 200 μM iron (II) sulfate as previously described [14]. Iron content of hiPSC-derived cardiomyocytes was assayed with rate fluorescence quenching of calcein by iron. The dissociated cardiomyocytes from iPSCs were plated onto a 96-well white microtiter plate. Calcein-AM (Life Technolgies, Maryland, USA) was dissolved in DMSO with a stock concentration of 10 mM. The dissociated cardiomyocytes were incubated in 10 μM calcein-AM in 5% FBS cardiomyocytes maintenance medium for 15 min, and washed twice with fresh medium before recording by M200 plate-reader (Tecan, Männedorf, Switzerland) for every 5 min. The intracellular iron (II) ion levels were expressed as reciprocal of the initial rate of fluorescence intensity rise.

2.4. Drug testing

To test the potential therapeutic effects, idebenone (Sigma-Aldrich, St. Louis, MO) and deferiprone (Selleckchem, Houston, TX, USA) were dissolved in DMSO at stock concentration 50 mM, stored at −20 °C, and were freshly diluted to the test concentration according to previous viability testing [6].

2.5. Reactive oxygen species (ROS) assay

hiPSC-derived cardiomyocytes treated with iron (II) sulfate for 48 h were seeded into a 96-well black microtiter plate at a density of 2×10^4/well. The ROS contents in hiPSC-derived cardiomyocytes were determined using DCFDA Cellular ROS Detection Assay Kit (Abcam, Cambridge, UK) according to manufacturer instructions. Briefly, hiPSC-derived cardiomyocytes were washed with DPBS and incubated with 25 μM DCFDA mix for 45 min at 37 °C in dark. Cells were then washed once with buffer solution before drug treatment. The signal was then detected every 5 min by M200 plate-reader (Tecan, Männedorf, Switzerland) with excitation wavelength at 485 nm and emission at 535 nm. Columns of non-stained cells were reserved as blank control.

2.6. Measurement of intracellular calcium homeostasis

Cytosolic calcium transients were measured in isolated hiPSC-derived cardiomyocytes using a confocal imaging system (Olympus Fluoview System version 4.2 FV300 TIEMPO) mounted on an upright Olympus microscope (IX71) as previously described as previously described [24,26–28]. Briefly, cells were loaded with 1:1 (v/v) amount of 20% Pluronic®-F127 (Invitrogen, life technologies) and 5 μM Fluo-3 AM (Sigma-Aldrich, St. Louis, MO) dissolved in DMSO with stock concentration of 5 mM for 45 min at 37 °C in Tyrode solution containing 140 mM NaCl, 5 mMKCl, 1 mMMgCl2, 1.8 mM CaCl2, 10 mM glucose and 10 mM HEPES at pH 7.4. Calcium transients of single cardiomyocytes were recorded with a temporal resolution of the line scan at 274 frames per second. All confocal calcium imaging experiments were performed within 48 h after isolation in order to minimize contamination of time-dependent changes in calcium handling property in culture. Fluorescence intensity was recorded by area against time mode (XYT) as a line plot, the calibration curve showed that there is linear relationship between fluo-3 intensity recorded with calcium concentration up to 630 nM [Ca^{2+}]. The data were then quantified as the background subtracted fluorescence intensity changes normalized to the background subtracted baseline fluorescence using Image J (National Institutes of Health. Amplitudes, maximal upstroke and decay velocity of calcium transient were analyzed by Clampfit version 9.2.0.09. (Axon Instruments, Inc., Foster City, CA).