Regenerative Therapy 3 (2016) 90–96

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original article

Heterogeneous filament network formation by myosin light chain isoforms effects on contractile energy output of single cardiomyocytes derived from human induced pluripotent stem cells

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ARTICLE INFO

Article history:
Received 7 December 2015
Received in revised form
19 February 2016
Accepted 22 February 2016

Keywords:
Human induced pluripotent stem cell
Cardiomyocyte
Traction force
Strain energy
Myosin II regulatory light chain
Collagen gel

ABSTRACT

Cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CMs) are expected to play an important role in heart therapies, in which hiPSC-CMs should generate sufficient contractile force to pump blood. However, recent studies have shown that the contractility of myocardial mimics composed of hiPSC-CMs is lower than that of adult human myocardium. To examine the mechanism by which contractile force output of hiPSC-CMs is weakened, we measured the contractile force of single hiPSC-CMs and observed the fibrous distribution of myosin II regulatory light chain (MRLC) of cardiac (contributes to beating) and non-cardiac (does not contribute to beating) isoforms. Single hiPSC-CMs were cultured on an extracellular matrix gel, and the contractile force and strain energy exerted on the gel were measured. Strain energy was not uniform between cells and ranged from 0.2 to 5.8 pJ. The combination of contractile force measurement and immunofluorescent microscopy for MRLC isoforms showed that cells with higher strain energy expressed the weakened non-cardiac myosin II fibers compared to those of cells with lower strain energy. Observation of cardiac and non-cardiac MRLC showed that the MRLC isoforms formed heterogeneous filament networks. These results suggest that strain energy output from single hiPSC-CMs depends both cardiac and non-cardiac myosin fibers, which prevent deformation of the cell body.

1. Introduction

Application of cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CMs) for *in vivo* repair and regenerative medicine has been examined (see review articles [1,2] and references therein). The physiological function of cardiomyocytes is to output contractile force to the extracellular matrix (ECM), where the force is sufficient to pump blood throughout the body. Schwan et al. stated that the contractile force of myocardial mimics composed of hiPSC-CMs was 10-fold lower than that of the adult human myocardium [3]. Moreover, Lu et al. repopulated hiPSC-CMs to a decellularized mouse heart, observed its beating, but found that the heart tissues was insufficient for pumping blood [4]. Thus, it is important to produce hiPSC-CMs with greater contractility. Recently, the group of Dr. Yamashita developed an efficient method for purifying hiPSC-CMs from human induced pluripotent cell derivatives [5] and demonstrated its application in infarcted hearts [6]. To expand the applications of hiPSC-CMs, contractile force and strain energy output should be examined.

Some methods for measuring the contractile force of single cardiomyocytes have been developed, including polyacrylamide hydrogel substrates with embedded beads [7,8], polydimethylsiloxane micro pillars [9], and carbon fiber tip [10]. Although hydrogel with embedded beads is advantageous for avoiding the effect of the constrained cellular adhesion area to the substrate, it is unclear whether the embedded microbeads reflect the gel surface. Thus, visualization of the polymer network itself should be conducted. Recently, covalently bonded collagen to a fluorescent dye and observed the cellular response to the collagen [11]. Since the elastic modulus of the gelled collagen was constant

Abbreviations: hiPSC-CMs, cardiomyocytes derived from human induced pluripotent stem cells; ECM, extracellular matrix; GFP, green fluorescent protein; MRLC, myosin II regulatory light chain.

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Peer review under responsibility of the Japanese Society for Regenerative Medicine.

http://dx.doi.org/10.1016/j.reth.2016.02.009

for the small strain and the viscosity was negligible over the time scale of cell migration [12], fluorescent collagen gel is useful for measuring cellular contractile force.

The mechanical properties of cells are closely related to the function of myosin II. Beating contractility of cardiomyocyte depends on the cardiac isoform of myosin II [2]. Based on Hooke's law, force generated by cardiac myosin II activity balances the sum of the restoring force to the deformation of the ECM and the cell itself. Assuming that cardiac myosin II activity is constant, if the cell has higher elasticity, contractile force output to the ECM will be lower. In contrast, if the cell has lower elasticity, the contractile force output will be higher. In non-cardiac cells, cellular elasticity is highly dependent on the non-cardiac isoform of myosin II [13,14]. Taking previous studies into consideration, we hypothesized that the contractile force and strain energy output of hiPSC-CMs depend not only on cardiac myosin II but also on non-cardiac myosin II.

In this study, we measured the contractile force and strain energy output of the purified single hiPSC-CMs. The difference in the strain energy output between beating hiPSC-CMs was evaluated in terms of expression of non-cardiac myosin II. The expression of non-cardiac myosin II in hiPSC-CMs is also discussed.

2. Materials and methods

2.1. Cells culture and plasmid construction

Purified hiPSC-CMs (Cellartis Cardiomyocytes; catalogue #Y10071; source #P11012) were purchased from Takara Inc. (Shiga, Japan). Procedure of the cell culture was conducted following the manufacturer's instructions. Two weeks after cultivation, hiPSC-CMs were trypsinized and used in subsequent experiments. The human fibroblast cell line (MRC-5 SV1 TG1) was purchased from RIKEN Cell Bank (Tsukuba, Japan) and the culture protocols were used according to those described previously [15]. Transfection of a plasmid into hiPSC-CMs and MRC-5 SV1 TG1was performed using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

To construct the green fluorescent protein (GFP)-tagged cardiac isoform of myosin regulatory light chain (MRLC), cDNA encoding full-length MYL2 (accession number: BC031006) was amplified by PCR, using the cDNA pool of the hiPSC-CMs, and inserted into the *Xho*I-*Kpn*I sites of pAcGFP-N3 (Takara). Primers and plasmid DNA sequences are shown in Supplementary data. GFP-MRLC expressing cells were observed by confocal laser scanning microscopy using a 63× objective lens (TCS-SP5; Leica Microsystems, Wetzlar, Germany). Beating hiPSC-CMs were observed at 512 × 512 pixels and 4 frames/s.

2.2. Preparation and mechanical properties of collagen gel

We purchased porcine-derived type I collagen solution (3 mg/mL; Cellmatrix-IP; Nitta Gelatin Inc., Osaka, Japan). The collagen was labeled with Alexa Fluor 546 according to the manufacturer's instructions (Alexa Fluor protein labeling kit; Thermo Fisher Scientific). A related method has been reported previously [16]. According to previous reports, collagen gel can be considered an elastic material under a small strain range (approximately around 0.2, depending on the experimental system) and the strain range can be widened with increasing collagen concentration [17,18]. To concentrate the collagen solution, the fluorescent-labeled collagen solution was centrifuged (Centrisart I; 20,000 MW cut off; Satorius AG, Goettingen, Germany). Collagen gelation was performed in a glass-bottom dish (8-well Nunc™ Lab-Tek™ II Chamber Slide™ System; Thermo Fisher Scientific) according to the Nitta Gelatin instructions and the final concentration of the collagen gel was 6 mg/mL. The thickness of the collagen gel was approximately 200 μm.

Prior to contractile force measurement of the gel, we evaluated Poisson's ratio and elasticity (Young's modulus) of the collagen gel. We used non-labeled collagen gel (final concentration was 6 mg/mL) in the following Poisson's ratio and the elasticity measurements assuming that the mechanical properties of the fluorescent-tagged collagen gel and non-labeled collagen gel were the same.

For Poisson's ratio measurement, we prepared a columnar collagen gel (diameter = 16 mm, height = 3 mm). The gel was transferred onto a transparent and flat-bottom vat supplied with HEPES buffered low-glucose Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA). We measured changes in the diameter and the height of the gel using a reading microscope with increasing load to the gel. Changes in the diameter and height were converted to longitudinal and lateral strains. Poisson's ratio was analyzed by dividing lateral strain by longitudinal strain. The averaged Poisson's ratio was 0.36 (standard deviation was 0.17, calculated from seven observations).

Measurement of collagen gel elasticity using atomic force microscopy has been previously described [19]. Briefly, a spherical bead 100 μm in diameter was indented into the collagen gel, and the force acting on the bead was recorded as a function of indentation distance. The relationship between force and distance was analyzed using the Hertzian contact theory. Averaged elasticity was 3.0 kPa (standard deviation was 0.8 kPa based on at least 1000 points).

2.3. Contractile force and strain energy analysis

Time lapse images of the fluorescent-labeled collagen gel surface with beating hiPSC-CMs was observed using a fluorescent microscope with a 20× objective lens (Ti-E and CFI-Plan Apo λseries; NIKON, Tokyo, Japan) and sCMOS camera (Zyla 4.2; Andor Technology Ltd., UK) at 1500 × 1500 pixels and 10 frames/s. An example of collagen deformation is shown in Supplemental movie 1. Contractile force (traction force) of hiPSC-CMs was analyzed by particle image velocimetry and Fourier transform traction cytometry method using ImageJ software plugins [20] (National Institutes of Health, Bethesda, MD, USA). Briefly, the displacement field of the collagen gel surface between the force-free image and another image was estimated by particle image velocimetry. The traction force field was obtained by deconvolution of Green's function and the displacement field from Fourier space. The regularization parameter was set at 1×10^{-8} for traction force reconstructions. Total strain energy U of the collagen gel by the hiPSC-CMs beating was evaluated using the following equation [21]:

$$U = \frac{1}{2} \int \boldsymbol{T}(x,y) \cdot \boldsymbol{u}(x,y) \mathrm{d}x\mathrm{d}y$$

where $\boldsymbol{T}$ denotes the traction force vector and $\boldsymbol{u}$ denotes the displacement vector in a laboratory frame (x, y). Analyzed displacement, traction force and local strain energy were visualized using MATLAB software. Representative total strain energy for each cell was determined as the average of the five peak values (denoted as “Max” in the Fig. 1(C)).

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.reth.2016.02.009.

2.4. Immunofluorescent microscopy

The hiPSC-CMs were fixed with 4% formaldehyde/PBS and permeabilized with 0.5% Triton X-100/PBS. The cells were stained with specific primary and Alexa-labeled secondary antibodies (Thermo