Toxicology in Vitro 54 (2019) 130–136

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Establishment of an in vitro cytotoxicity assay platform using primary monkey cardiomyocytes

Takuma Iguchi[a,*], Kazunori Fujimoto[a], Shinichiro Nakamura[b], Hiroyuki Kishino[a], Noriyo Niino[a], Kazuhiko Mori[a]

[a] Medicinal Safety Research Laboratories, Daiichi Sankyo Co., Ltd., 1-16-13 Kita-Kasai, Edogawa-ku, Tokyo 134-8630, Japan
[b] Department of Stem Cells and Human Disease Models, Research Center for Animal Life Science, Shiga University of Medical Science, Seta, Tsukinowa-cho, Otsu, Shiga 520-2192, Japan

ARTICLE INFO

Keywords:
Cardiomyocyte
Cynomolgus monkey
In vitro cytotoxicity
Microarray
Doxorubicin
Endoplasmic reticulum stress

ABSTRACT

To establish an in vitro cytotoxicity assay platform using monkey cardiomyocytes, we isolated primary cardiomyocytes from fetal cynomolgus monkeys at different gestation days (from day 39 to 90) using the trypsin and collagenase digestion method, which was identical to the standard procedure for rat cardiomyocytes. Under these conditions, the primary cells obtained from monkeys at gestation day 63 or earlier showed spontaneous beating, with > 80% cells being viable from all fetuses. Transcriptome analysis of the monkey cardiomyocytes indicated that the cells have essential components of cardiac functions, such as myosins, α-actin, cardiac troponins, and calcium-related molecules. The susceptibility to doxorubicin-induced cytotoxicity in monkey cardiomyocytes was comparable to that in rat cardiomyocytes, as evaluated based on intracellular ATP levels. Microarray analysis with Ingenuity Pathway Analysis revealed that doxorubicin predominantly increased the expression of several key genes involved in the endoplasmic reticulum stress pathway in monkey cardiomyocytes than in rat cardiomyocytes. In conclusion, we isolated primary monkey cardiomyocytes that showed similar sensitivity to doxorubicin as compared with rat cardiomyocytes. This in vitro monkey cardiomyocyte assay platform would serve as a powerful tool for the investigation of the interspecies differences in drug-induced cardiotoxicity and its underlying mechanism.

1. Introduction

Drug-induced cardiotoxicity is the leading cause of drug attrition during the preclinical and clinical development, due to serious adverse events such as arrhythmia, myocardial infarction, and myocardium necrosis (Kola and Landis, 2004; Laverty et al., 2011). In vitro toxicity evaluation during the early drug screening phase using cardiac myocytes is one of the gold standard practices that provides inherent advantages over in vivo studies, such as the quick evaluation with small amounts of test compounds, precise control of experimental conditions with less confounding variables, and reduction in the animal use (Allen et al., 2005).

Several cardiac myocyte cell lines such as H9c2 (Kimes and Brandt, 1976) and HL-1 (Claycomb et al., 1998) are used as in vitro models for toxicity screening in a cost-effective manner. Indeed, H9c2 cells can mimic the hypertrophic responses against angiotensin II and endothelin-1 observed in primary cardiomyocytes from neonatal rats (Watkins et al., 2011). However, the characteristics of these cell lines are different from those of naïve cardiac myocytes in that their morphologies and gene expression patterns are more similar to skeletal muscle myoblasts than to primary cardiomyocytes (Branco et al., 2015; Menard et al., 1999; Peter et al., 2016). Unlike primary neonatal cardiac myocytes, H9c2 cells do not spontaneously beat in culture (Peter et al., 2016). In addition, H9c2 cells exhibit excessive susceptibility to doxorubicin owing to its cytotoxic potential as compared to primary rat cardiomyocytes (Konorev et al., 2008). Inoue et al. (2008) demonstrated that the IC_{50} values for the cardiotoxicity of anthracyclines in rat neonatal cardiomyocytes showed a close correlation with the in vivo dose causing cardiotoxicity to rats. Consequently, primary cardiomyocytes are widely used to assess the effects of various compounds on

Abbreviations: IPA, Ingenuity Pathway Analysis; GO, Gene Ontology; hiPSC-CM, human pluripotent stem cell-derived cardiomyocyte; ER, endoplasmic reticulum

* Corresponding author.
E-mail addresses: iguchi.takuma.kz@daiichisankyo.co.jp (T. Iguchi), fujimoto.kazunori.wr@daiichisankyo.co.jp (K. Fujimoto), snakamur@belle.shiga-med.ac.jp (S. Nakamura), kishino.hiroyuki.jt@daiichisankyo.co.jp (H. Kishino), niino.noriyo.ve@daiichisankyo.co.jp (N. Niino), mori.kazuhiko.md@daiichisankyo.co.jp (K. Mori).

https://doi.org/10.1016/j.tiv.2018.09.012
Received 22 June 2018; Received in revised form 16 September 2018; Accepted 18 September 2018
Available online 24 September 2018
0887-2333/

functional (electrophysiology) and biological (cytotoxicity) activities.

Cynomolgus monkey (*Macaca fascicularis*) is one of the most commonly used animals for preclinical toxicity evaluation as a non-human primate, as it is considered to be anatomically, physiologically, and genetically closer to humans than are any other laboratory animals, including rats and mice (Register, 2009; Uchida et al., 2012). Despite the importance of the cytotoxicity assay using monkey cardiomyocytes, neither standardized procedures for isolation nor characterization of cells have yet been established. As species differences are observed in the pre-clinical phase, there is a need for the development of a model that may account for the molecular events that occur in response to drug exposure.

In this study, we established an in vitro cytotoxicity assay platform using monkey fetal cardiomyocytes at different gestation days. A few modifications in the fundamental procedures for the rat cardiomyocytes were required to isolate the monkey counterparts. Next, we characterized the monkey cardiomyocytes in comparison with other cell sources at the transcriptome level and evaluated their ability to show a cytotoxic response to doxorubicin as a representative cardiotoxicant.

2. Materials and methods

2.1. Animals

For monkeys, all the experiments were approved in advance by the Animal Care and Use Committee of Shiga University of Medical Science (Shiga, Japan). Oocytes were collected from sexually matured female cynomolgus monkeys (age, 4.3 to 11.3 years), and other females (age, 4.2 to 10.9 years) were the recipient of the embryo transfer. Fresh sperm was collected from male cynomolgus monkeys (age, 12.5 to 15.3 years). Cynomolgus monkeys were housed in a stainless-steel cage (W 500 mm × H 800 mm × D 800 mm). The animal room was maintained at 23 °C to 27 °C, with a relative humidity of 45% to 55% and 12 h light-dark cycles. Each animal was fed a commercial pellet monkey chow (20 g·kg^{-1}·day^{-1}; CMK1, CLEA Japan, Tokyo) in the morning, supplemented with 20 to 50 g/day of sweet potato in the afternoon. Water was given ad libitum by an automatic supplier.

For rats, all the experiments were approved in advance by the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Pregnant Sprague-Dawley rats at gestational days 15 to 19 were purchased from Charles River Laboratories (Tokyo, Japan). The animals were housed in a cage under controlled conditions (room temperature: 20 °C to 26 °C; humidity: 30% to 70%; and 12 h light-dark cycles) with ad libitum access to tap water and a certified pellet diet (CRF-1; Oriental Yeast, Tokyo, Japan).

2.2. Monkey fetal cardiomyocytes

Hearts were freshly collected from fetuses between 39 and 90 days of gestation in pregnant cynomolgus monkeys, which were conceived by an intracytoplasmic sperm injection and embryo transfer as previously described (Yamasaki et al., 2011). These fetal hearts were obtained from the animals that were sacrificed for other purposes than in vitro cultures. The fundamental procedures for the isolation of monkey cardiomyocytes were nearly identical to those of rats, with a few modifications; incubation time for collagenase digestion was extended from 30 to 45 min, and the centrifugation condition was changed to 100 ×*g* for 10 min.

2.3. Rat fetal cardiomyocytes

Pregnant rats of gestational day 20 were sacrificed by exsanguination under deep isoflurane-induced anesthesia. After all the fetuses were freshly removed, fetal hearts were collected. Fetal rat cardiomyocytes were isolated using the neonatal cardiomyocyte isolation system (Worthington biochemical, Lakewood, NJ) according to the manufacturer's instructions and previous reports (Louch et al., 2011; Vandergriff et al., 2015). In brief, fetal hearts were excised and chopped in Worthington Trypsin at a final concentration of 50 μg/mL. The tissues were minced and placed in a refrigerator overnight (16 to 20 h) at 2 °C to 8 °C. After the addition of 1 mL of a trypsin inhibitor, the tissues were digested in a collagenase solution (provided with the neonatal cardiomyocyte isolation system) with shaking instrument (20 cpm) at 37 °C for 30 min. The cells were gently triturated 10 times using a pipette and filtered through a 70-μm cell strainer into a fresh 50-mL centrifuge tube. The cells were centrifuged at 50 ×*g* for 5 min and resuspended in a culture medium comprising Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 5% heat-inactivated fetal bovine serum (FBS), 0.1 mM ascorbate, 1 mM sodium pyruvate, 100 mM nonessential amino acids, 1% insulin, transferrin, and selenium (ITS), and 1% penicillin/streptomycin. Cardiomyocytes were plated at a density of 9 ± 3 × 10^4 cells/cm^2 in collagen-coated tissue culture 24- or 96-well plates and incubated at 37 °C in the presence of 5% CO_2.

2.4. Cell viability

Cell viability was determined by the trypan blue exclusion assay; viable (unstained) and non-viable (stained) cells were counted using an inverted microscope.

2.5. Doxorubicin treatment

Doxorubicin hydrochloride (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich Japan, Tokyo, Japan) at various concentrations (10, 30, 100, 300, and 1000 nM; the final concentration of DMSO was < 0.5%). These concentrations were based on a preliminary experiment, wherein 1000 nM doxorubicin showed severe cytotoxicity, as evident from > 200% release of lactate dehydrogenase (LDH) from rat cardiomyocytes. After 72 h of preculture, the supernatant was removed and the cells were incubated with a medium supplemented with doxorubicin at 37 °C for 72 h.

2.6. Cytotoxicity assay

After the doxorubicin treatment, cellular ATP level was determined using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI). The kit was used according to the manufacturer's instructions. Cytotoxicity value of each sample was calculated as the percentage of corresponding control samples that was determined as 100%.

2.7. RNA extraction and cDNA microarray

Total RNA was isolated with Trizol (Thermo Fisher Scientific, Waltham, MA) from cardiomyocytes of rats and monkeys after doxorubicin treatment. Microarray analysis was performed using GeneChip 3'IVT PLUS Reagent Kit (Thermo Fisher Scientific) for cDNA synthesis and purification as well as for biotin-labeled cRNA synthesis according to the manufacturer's instructions. Every biotin-labeled cRNA target sample (10 μg) was individually hybridized to GeneChip Rat Genome 230 2.0 Array for rats or GeneChip Human Genome U133A 2.0 Array (Thermo Fisher Scientific) for monkeys at 45 °C for 16 h, followed by washing and staining with streptavidin-phycoerythrin using Fluidics Station 450 and scanning on GeneChip Scanner 3000. The scanned image was analyzed with a MAS5 algorithm using GeneChip Operating Software. All the MAS5-analyzed data were scaled by global normalization. Differentially expressed genes were selected according to the following filtering criteria: First, absent or marginally expressed genes as well as "no change," "marginal increase," and "marginal decrease" probe set comparisons were excluded from the subsequent analysis.