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Original Article

Preparation of iPS cell-derived CD31$^+$ endothelial cells using three-dimensional suspension culture

Shinako Masuda [a], Katsuhisa Matsuura [a, b, *], Tatsuya Shimizu [a]

[a] Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku, Tokyo 162-8666, Japan
[b] Department of Cardiology, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku, Tokyo 162-8666, Japan

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ABSTRACT

A well-organised vascular network is essential for metabolic exchange to maintain homoeostasis in the body. Therefore, for progress in regenerative medicine, it is particularly important to establish methods of vascularization in bioengineered three-dimensional (3D) functional tissues. In addition, it is necessary to develop methods to supply a large number of iPS cell-derived endothelial cells for fabricating the vascular network structure. There are already many reports on the method of inducing the differentiation of endothelial cells from iPS cells using 2D culture. However, there are few reports on methods for preparing a large number of iPS cell-derived endothelial cells. Therefore, we developed methods for inducing vascular endothelial cells from human inducible pluripotent stem (hiPS) cells using 3D suspension culture. hiPS cell-derived CD31$^+$ cells expressed several endothelial marker genes and formed endothelial cell network structures, similar to human umbilical vein endothelial cells. These results indicate that hiPS cell-derived CD31$^+$ cells may be a useful cell source for pre-vascularised network structures in 3D functional tissues, and it is important to develop 3D mass culture system for preparing a large number of cells to fabricate bioengineered tissues.

1. Introduction

It is anticipated that the application of three-dimensional (3D) bioengineered tissues in the fields of regenerative medicine and drug screening will become possible. The limitation of perfusion from the medium into 3D tissue is about 100–200 μm, although this depends on the type of tissue [1]. In addition to oxygen exchange, vascular network structures play essential roles in the supply of nutrition and the removal of metabolic waste products. Therefore, it is essential to establish methods for fabricating vascular structures within 3D tissues. We previously developed cell sheet engineering to create functional 3D tissues by layering two-dimensional confluent cell sheets harvested from temperature-responsive culture surfaces [2–5]. In the transplantation of layered neonatal rat cardiac cell sheets, CD31$^+$ cells within the graft formed a pre-vascular network, resulting in the connection between transplanted graft and host tissue and therefore to better engraftment [6,7]. We also established novel 3D tissue models with a perfusable vascular structure using *ex vivo* or *in vitro* vascular beds [8,9]. Because of the incomplete vascular structures within the abovementioned 3D tissue models, the establishment of fully vascularised host-connectable tissue is considered to be one of the major challenges for future work. An important factor in this context is human umbilical vein endothelial cells (HUVECs), which are currently used as vascular cells when reconstructing various tissues. However, to reconstruct the tissues more accurately, it is considered necessary to perform tissue-specific optimisation of the type of blood vessels, such as arterial or venous, and the vessel diameter.

Pluripotent stem cells are a promising cell source for fabricating bioengineered 3D tissues because of their potential to differentiate into various types of cells and their ability to supply a large number of cells. We previously reported on large-scale bioreactor systems for cardiovascular differentiation from mouse embryonic stem (ES) cells and human inducible pluripotent stem (hiPS) cells, as well as the fabrication of cardiac cell sheets from these pluripotent stem cell-derived cardiovascular cells [10–12]. It has been reported that pluripotent stem cell-derived cardiac tissues prepared by co-

* Corresponding author. Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku, Tokyo 162-8666, Japan. Fax: +81 3 3359 6046.
E-mail address: matsuura.katsuhisa@twmu.ac.jp (K. Matsuura).
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culture of vascular cells enhance the performance of transplanted grafts [13,14]. Building on previous work with the aim of providing a large number of endothelial cells for fabricating 3D-functional vascularised tissues, we here developed methods for inducing $CD31^+$ cells from hiPS cells using a bioreactor system, demonstrated pre-vascular network formation of hiPS cell-derived $CD31^+$ cells by co-culture with normal human dermal fibroblasts (NHDFs) and compared their characteristic features with those of tissue-derived endothelial cells.

2. Methods

2.1. Antibodies

Monoclonal antibodies for human kinase-insert domain receptor (KDR) conjugated with phycoerythrin (R&D Systems, Minneapolis, MN, USA) and monoclonal antibodies for human CD31 conjugated with phycoerythrin (R&D Systems) were used for magnetic-activated cell sorting (MACS) separation. Phycoerythrin-conjugated monoclonal antibodies for human vascular endothelial (VE)-cadherin (R&D Systems) and monoclonal antibodies for human CD31 conjugated with phycoerythrin were used for immunocytochemistry. Fluorescein-conjugated monoclonal antibody for murine human CD31 (R&D Systems) was used as the primary antibody for immunocytochemistry.

2.2. Cell culture

NHDFs and HUVECs were purchased from Lonza (Walkersville, MD) and maintained in accordance with the manufacturer's instructions. Human iPS (hiPS) cells (253G1) were purchased from RIKEN (Tsukuba, Japan) and maintained in Primate ES Cell Medium (ReproCELL Inc., Tokyo, Japan), supplemented with 5 ng/mL basic fibroblast growth factor (ReproCELL) on mitomycin C-treated mouse embryonic fibroblasts. Cells were passaged as small clumps every 3 days using CTK solution (ReproCELL).

2.3. Preparation of $CD31^+$ cells

$CD31^+$ cells were prepared from differentiated hiPS cells (253G1). A single-use bioreactor and a magnetic stirrer were purchased from ABLE Corporation & Biott Corporation (Tokyo, Japan). To induce differentiation, small colonies of hiPS cells were seeded into culture vessels (approximately 2×10^5 cells/mL mTeSR1 containing Y27632 [10 μM]) and cultured until day 2. From day 2 to day 7, embryoid bodies (EBs) were cultured in StemPro34 containing 50 μg/mL ascorbic acid (Sigma–Aldrich, St. Louis, MO), 2 mM L-glutamine (Life Technologies, Carlsbad, CA) and 400 μM 1-thioglycerol (Sigma–Aldrich). On day 2, medium was supplemented with 12 ng/mL BMP4, 5 ng/mL bFGF and 6 ng/mL Activin A (R&D Systems) and removed them at day 5. On day 5, medium was supplemented with 10 ng/mL vascular endothelial growth factor (VEGF) (R&D Systems) and 10 ng/mL bFGF and removed them at day 7. On day 7, EBs were enzymatically dissociated and subjected to MACS (Miltenyi Biotec GmbH, Germany) to separate KDR^+ cells. KDR^+ cells were re-cultured with 10 ng/mL VEGF and 10 ng/mL bFGF onto ColIV-coated tissue culture dishes. Three days after the re-culture, induced $CD31^+$ cells were isolated from re-cultured KDR^+ cells by MACS.

2.4. Immunocytochemistry

Cells were fixed with 5% dimethyl sulfoxide in methanol and blocked with 1% skimmed milk. The fixed cells were then stained with primary antibody overnight at 4 °C, followed by incubation with secondary antibody for 3 h at 4 °C. Nuclei were visualised with Hoechst 33342.

2.5. Image acquisition and data analysis

Images of $CD31^+$ cells were collected using an ImageXpress Ultra confocal high-content screening system (Molecular Devices, LLC, Sunnyvale, CA, USA). The number of $CD31^+$ cells, tube length and branch point of the $CD31^+$ cell network structure were assessed using MetaXpress software (Molecular Devices, LLC) [15].

2.6. Quantitative real-time polymerase chain reaction

First-strand cDNA was synthesised using a High Capacity cDNA Reverse Transcription Kit (ABI) from purified total RNA isolated using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). First-strand synthesis was performed on a T3000 ThermoCycler (Biometra). Quantitative real-time PCR was carried out using a StepOnePlus system (ABI), in accordance with the manufacturer's instructions. The expression levels of genes for cadherin 5 (CDH5), KDR, platelet/endothelial adhesion molecule 1 (Pecam1) and von Willebrand factor (vWF) were analysed by TaqMan gene expression assay (ABI) and gene expression was normalised to endogenous β-actin.

2.7. RNA extraction and microarray analysis

Total RNA of human umbilical artery endothelial cells (HUAECs) was purchased from Toyobo (Osaka, Japan). Total RNA of HUVECs and hiPS cell-derived $CD31^+$ cells was extracted from cells using an RNeasy Plus Mini Kit (Qiagen), in accordance with the manufacturer's instructions. RNA quantity and quality were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.) and an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA), as recommended. Total RNA was amplified and labelled with Cyanine 3 (Cy3) using Agilent Low Input Quick Amp Labeling Kit, one-colour (Agilent Technologies), following the manufacturer's instructions. For each hybridisation, 0.60 μg of Cy3-labelled cRNA was fragmented and hybridised at 65 °C for 17 h to an Agilent SurePrint G3 Human GE v3 8 × 60K Microarray (Design ID: 072363). Intensity values of each scanned feature were quantified using Agilent feature extraction software version 11.5.1.1, which performs background subtractions. We only used features that were flagged as no errors (detected flags) and excluded features that were not positive, not significant, not uniform, not above background, that were saturated and population outliers (not detected and compromised flags). Normalisation was performed using Agilent GeneSpring software version 14.8 (per chip: normalisation to 75th percentile shift). There are a total of 50,599 probes on the Agilent SurePrint G3 Human GE v3 8 × 60K Microarray (Design ID: 072363) without control probes.

2.8. Integrated bioinformatic analysis of differentially-expressed genes

Gene Ontology annotation of the differentially-expressed genes within the main category of biological process was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID 6.8; https://david.ncifcrf.gov). A three-way Venn diagram was also constructed using VENNY 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/).

2.9. Statistical analysis

Data are presented as means ± standard deviations. Student's *t*-tests or paired *t*-tests were used to analyse differences between two