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Original article

Formation of vascular network structures within cardiac cell sheets from mouse embryonic stem cells

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ABSTRACT

Bioengineered cardiac tissues represent a promising strategy for regenerative medicine. However, methods of vascularization and suitable cell sources for tissue engineering and regenerative medicine have not yet been established. In this study, we developed methods for the induction of vascular endothelial cells from mouse embryonic stem (ES) cells using three-dimensional (3D) suspension culture, and fabricated cardiac cell sheets with a pre-vascularized structure by co-culture of mouse ES cell-derived endothelial cells. After induction, isolated CD31+ cells expressed several endothelial cell marker genes and exhibited the ability to form vascular network structures similar to CD31+ cells from neonatal mouse heart. Co-culture of ES cell-derived CD31+ cells with ES cell-derived cardiomyocytes and dermal fibroblasts resulted in the formation of cardiac cell sheets with microvascular network formation. In contrast, microvascular network formation was reduced in co-cultures without cardiomyocytes, suggesting that cardiomyocytes within the cell sheet might enhance vascular endothelial cell sprouting. Polymerase chain reaction array analysis revealed that the expression levels of several angiogenesis-related genes, including fibroblast growth factor 1 (FGF1), were up-regulated in co-culture with cardiomyocytes compared with cultures without cardiomyocytes. The microvascular network in the cardiac sheets was attenuated by treatment with anti-FGF1 antibody. These results indicate that 3D suspension culture methods may be used to prepare functional vascular endothelial cells from mouse ES cells, and that cardiomyocyte-mediated paracrine effects might be important for fabricating pre-vascularized cardiac cell sheets.

1. Introduction

Well-organized, functional tissues are composed of various cell types. Tissue engineering represents a new therapeutic approach for the treatment of congenital defects and functional disorders, and methods for constructing 3D functional tissues are therefore required. We previously developed cell-sheet engineering that allowed the creation of functional 3D tissues by layering two-dimensional confluent cell sheets harvested from temperature-responsive culture surfaces [1–4]. A well-organized vascular network is essential for metabolic exchange throughout the engineered 3D tissues. In the case of neonatal rat cardiac cell sheets, CD31+ cells formed a network structure connecting host and graft vessels upon *in vivo* transplantation [5,6]. Furthermore, large numbers of cells are needed to fabricate functional 3D tissues. Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are considered as likely sources of cells for regenerative medicine.

Abbreviations: ES cell, embryonic stem cell; FGF1, fibroblast growth factor 1; 3D, three-dimensional; iPS cell, inducible pluripotent stem cell; Flk1, fetal liver kinase 1; VEGF, vascular endothelial growth factor; MACS, magnetic-activated cell sorting; VE-cadherin, vascular endothelial cadherin; eNOS, endothelial nitric oxide synthase; SMA, smooth muscle actin; Col4a3, collagen type IV alpha 3; EYFP, enhanced yellow fluorescent protein; FBS, fetal bovine serum; NEAA, non-essential amino acids; LIF, leukemia inhibitory factor; PCR, polymerase chain reaction; Gusb, glucuronidase, beta; qRT-PCR, quantitative real-time PCR; Acvrl1, activin receptor like 1; CXCR4, chemokine receptor type 4; Dll4, delta-like ligand 4; Efnb2, ephrin-B2; Ephb4, ephrin type-B receptor 4; Flt1, fms-related tyrosine kinase 1; KDR, kinase insert domain receptor; Notch1, Notch homolog 1; Nr2f2, nuclear receptor subfamily 2, group f, member 2; Pecam1, platelet/endothelial adhesion molecule 1; Pou5f1, POU class 5 homeobox 1; Cxcl5, chemokine (C-X-C motif) ligand 5; Egf, epidermal growth factor; Mdk, midkine; Tgfa, transforming growth factor, alpha; Tymp, thymidine phosphorylase; TIMP2, tissue inhibitor of metalloproteinase 2.

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We developed methods for inducing the differentiation of mouse ES cells in large-scale suspension cultures, and recently reported the creation of cardiac cell sheets by co-culture with cardiomyocytes derived from mouse ES cells and cardiac fibroblasts [7,8]. Large-scale differentiation systems were also applicable for the collection of cardiomyocytes and formation of cardiac cell sheets from human iPS cells [9].

Several studies have reported the induction of cardiovascular cells from pluripotent stem cells [10–12]. Yamashita et al. reported that cardiovascular cells could be differentiated from mouse ES and iPS cells through mesodermal progenitor fatal liver kinase 1 (Flk1)+ cells [13–15], and administration of vascular endothelial growth factor (VEGF) and cAMP resulted in the effective induction of arterial endothelial cells by activation of Notch signaling [16]. Prevascularization of cardiac cell sheets is important for prompt microvascular communication between transplanted grafts and host tissue, leading to better engraftment upon transplantation. Although cardiac cell sheets have been prepared from cardiomyocytes, endothelial cells, and mural cells derived from mouse ES or iPS cells [17,18], microvascular network formation *in vitro* has not been clear. Several issues regarding the use of pluripotent stem cell-derived endothelial cells for the fabrication of bioengineered cardiac tissue remain to be resolved, including: (1) genetic and functional differences in endothelial cells derived from heart tissue and pluripotent stem cells; (2) the contribution of pluripotent stem cell-derived endothelial cells to the microvascular network *in vitro*; and (3) the molecular mechanism underlying endothelial cell microvascular network formation in cardiac cell sheets.

With the aim of reconstructing ES cell-derived well-vascularized 3D cardiac tissues, we induced CD31+ cells from mouse ES cells using spinner flask cultures and generated cardiac cell sheets with a microvascular network structure by co-culturing CD31+ cells with mouse ES cell-derived cardiomyocytes and dermal fibroblasts.

2. Methods

2.1. Animals

Wild-type C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). Animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Women's Medical University.

2.2. Antibodies

Biotin-conjugated monoclonal antibodies for murine Flk1 (eBioscience, San Diego, CA, USA) and murine CD31 (BD Biosciences, San Jose, CA, USA) were used as primary antibodies for magnetic-activated cell sorting (MACS) separation. Phycoerythrin-conjugated monoclonal antibody for murine vascular endothelial (VE)-cadherin (BioLegend, San Diego, CA, USA) and monoclonal antibodies for cardiac troponin T (Thermo Fisher Scientific, Waltham, MA, USA), murine CD31, endothelial nitric oxide synthase (eNOS; BD Biosciences), murine α-smooth muscle actin (SMA; Sigma–Aldrich, St. Louis, MO) and collagen type IV alpha 3 (Col4a3; kindly provided by Dr. Sado, Shigei Medical Research Institute, Japan) were used as primary antibodies for immunocytochemistry. Antibodies for SM22α (Abcam, Cambridge, MA, USA) and fibroblast growth factor (FGF) 1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were also used as primary antibodies for immunocytochemistry. Anti-mouse, anti-rat, or anti-rabbit IgG antibodies conjugated with Alexa488, Alexa568 or Cy5 (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies for immunocytochemistry.

2.3. ES cell culture

R1 ES cells expressing the neomycin phosphotransferase gene under the control of the α-myosin heavy chain promoter (R1-neo) [19] with the enhanced yellow fluorescent protein (EYFP) gene (R1-neo EYFP) [20] were maintained on 1% gelatin-coated tissue culture dishes in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids (NEAA), 1 mM sodium pyruvate, 1% penicillin/streptomycin and 1000 U/ml leukemia inhibitory factor (LIF).

EMG7 ES cells expressing the enhanced green fluorescent protein gene under the control of the α-myosin heavy chain promoter [15] were maintained in Glasgow's minimum essential medium supplemented with 10% fetal bovine serum, 0.1 mM 2-mercaptoethanol, 0.1 mM NEAA, 1 mM sodium pyruvate, 1% penicillin/streptomycin and 1000 U/ml LIF.

2.4. Preparation of cardiac cells

ES cell-derived cardiomyocytes were prepared by neomycin selection of differentiated R1 ES cells, as described previously [7] with a few modifications. To induce differentiation, R1 ES cells were cultured in the presence of noggin (150 ng/ml) (R&D Systems, Minneapolis, MN, USA) from day −3 to day 1 of induction of differentiation. The differentiation medium for R1 ES cells was Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 0.1 mM NEAA, 1 mM sodium pyruvate and 1% penicillin/streptomycin. Trypsinized R1 ES cells were seeded at 5×10^4 cells/ml (total, 200 ml/flask) into spinner flasks and cultured in the absence of LIF until day 18. On day 5, the volume of medium was increased to 400 ml/flask. Granulocyte colony-stimulating factor (1 ng/ml) was added to the differentiation medium from day 5 to day 10, and fetal bovine serum in the differentiation medium was replaced with bovine serum from day 7 to day 18. For selection of ES cell-derived cardiomyocytes, differentiated cells were cultured in the presence of G418 from days 10 to 18. On day 18, undifferentiated cells included in the suspension culture were depleted by MACS using anti SSEA-1 microbeads.

2.5. Preparation of CD31+ cells

CD31+ cells were prepared from differentiated EMG7 or R1-neo EYFP ES cells. To induce differentiation, trypsinized ES cells were seeded at 1×10^5 cells/ml into spinner flasks and cultured in the absence of LIF until day 5. Differentiation medium for EMG7 ES cells consisted of MEM-α supplemented with 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol and 1% penicillin/streptomycin. Differentiation medium for R1-neo EYFP ES cells consisted of Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 0.1 mM NEAA, 1 mM sodium pyruvate and 1% penicillin/streptomycin. On day 5, embryoid bodies were enzymatically dissociated and subjected to MACS to separate Flk1+ cells. Flk1+ cells were re-cultured with both VEGF (50 ng/ml) and 8-bromo-cAMP (0.5 mmol/l) onto collagen IV-coated tissue culture dishes. Three days after re-culture, induced CD31+ cells were isolated from re-cultured Flk1+ cells by MACS.

2.6. Mouse primary cell culture

Dermal fibroblasts were obtained from young adult mice. Dermal tissue specimens were cut into pieces 1 cm^2 and incubated at 4 °C for 24 h with 500 U/ml dispase. The skin was separated into