Biomaterials 34 (2013) 9441–9450

Contents lists available at ScienceDirect

Biomaterials

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

Hypoxia-induced therapeutic neovascularization in a mouse model of an ischemic limb using cell aggregates composed of HUVECs and cbMSCs

Chieh-Cheng Huang [a, 1], Ding-Yuan Chen [a, 1], Hao-Ji Wei [b, c, 1], Kun-Ju Lin [d, e, 1], Cheng-Tse Wu [a], Ting-Yin Lee [a], Hsin-Yi Hu [a], Shiaw-Min Hwang [f], Yen Chang [b, c, **], Hsing-Wen Sung [a, *]

[a] Department of Chemical Engineering and Institute of Biomedical Engineering, National Tsing Hua University, Hsinchu, Taiwan, ROC
[b] Division of Cardiovascular Surgery, Veterans General Hospital, Taichung, Taiwan, ROC
[c] College of Medicine, National Yang-Ming University, Taipei, Taiwan, ROC
[d] Department of Medical Imaging and Radiological Sciences, Chang Gung University, Taoyuan, Taiwan, ROC
[e] Department of Nuclear Medicine and Molecular Imaging Center, Chang Gung Memorial Hospital, Linkou, Taiwan, ROC
[f] Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC

ARTICLE INFO

Article history:
Received 17 July 2013
Accepted 4 September 2013
Available online 17 September 2013

Keywords:
Tissue ischemia
Cell-based therapy
Cell transplantation
Hypoxia-inducible factors
Tissue engineering

ABSTRACT

Cell transplantation for therapeutic neovascularization holds great promise for treating ischemic diseases. This work prepared three-dimensional aggregates of human umbilical vein endothelial cells (HUVECs) and cord-blood mesenchymal stem cells (cbMSCs) with different levels of internal hypoxia by a methylcellulose hydrogel system. We found that few apoptosis occurred in these cell aggregates, despite developing a hypoxic microenvironment in their inner cores. *Via* effectively switching on the hypoxia-inducible factor-1α-dependent angiogenic mechanisms, culturing the internally hypoxic HUVEC/cbMSC aggregates on Matrigel resulted in formation of extensive and persistent tubular networks and significant upregulation of pro-angiogenic genes. As the level of internal hypoxia created in cell aggregates increased, the robustness of the tubular structures developed on Matrigel increased, and expression levels of the pro-angiogenic genes also elevated. Transplantation of hypoxic HUVEC/cbMSC aggregates into a mouse model of an ischemic limb significantly promoted formation of functional vessels, improved regional blood perfusion, and attenuated muscle atrophy and bone losses, thereby rescuing tissue degeneration. Notably, their therapeutic efficacy was clearly dependent upon the level of internal hypoxia established in cell aggregates. These analytical results demonstrate that by establishing a hypoxic environment in HUVEC/cbMSC aggregates, their potential for therapeutic neovascularization can be markedly enhanced.

1. Introduction

Peripheral artery disease (PAD) is a consequence of atherosclerotic occlusion, which can lead to critical limb ischemia, tissue necrosis, and ultimately amputation of the lower extremities [1,2]. Despite advances in pharmacological and surgical approaches for therapeutic neovascularization, prognosis for PAD patients remains poor [3]. Neovascularization *via* cell transplantation has emerged as a promising therapeutic modality for treating ischemic diseases [1,3–5]. Prior to cell transplantation, the desired cell types must be expanded into a large scale *in vitro* and then dissociated from their cultured tissue culture polystyrene (TCPS) dishes using proteolytic enzymes. However, dissociated cells that are grafted into ischemic tissues typically have low therapeutic efficacy due to their poor cell retention, which is problematic in clinical settings [5–7]. To improve applications of cell-based approaches for patients with tissue ischemia, optimization strategies are needed to improve their therapeutic potential.

A cell-based therapeutic strategy, using three-dimensional (3D) aggregates of human umbilical vein endothelial cells (HUVECs) and cord-blood mesenchymal stem cells (cbMSCs), for modulating

* Corresponding author. Department of Chemical Engineering, National Tsing Hua University, Hsinchu 30013, Taiwan, ROC. Tel.: +886 3 574 2504.
** Corresponding author. Division of Cardiovascular Surgery, Veterans General Hospital, Taichung, Taiwan, ROC.
E-mail addresses: ychang@vghtc.gov.tw (Y. Chang), hwsung@che.nthu.edu.tw (H.-W. Sung).
[1] The first four authors (C.C. Huang, D.Y. Chen, H.J. Wei, and K.J. Lin) contributed equally to this work.

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.09.010

neovascularization to rescue tissues from critical limb ischemia has been recently reported by our group [8]. According to those results, transplantation of 3D HUVEC/cbMSC aggregates restored blood perfusion in a mouse model of hindlimb ischemia more effectively than their dissociated counterparts. The transplanted HUVEC/cbMSC aggregates enhanced functional vessel formation within the ischemic limb and protected it from degeneration.

Cell aggregates develop hypoxia in their inner core at distances that exceed the diffusion capacity of oxygen [5,9,10]. When in hypoxic environments, cells have a variety of biological responses that activate neovascularization and regulate cell survival [11,12]. Induction of angiogenesis by hypoxia is an important component to maintain oxygen homeostasis for metabolic demand [13]. By switching on a series of signal transduction mechanisms, hypoxia-inducible factors (HIFs) can induce transcriptional activation of several pro-angiogenic genes, causing formation of functional neovasculatures [14]. Additionally, exposure of ECs and MSCs to low oxygen tension significantly promotes the secretion of numerous angiogenic growth factors, such as vascular endothelial growth factor (VEGF), adrenomedullin (ADM), and angiopoietin-1 (Ang-1), stromal cell–derived factor-1 (SDF-1), and insulin-like growth factor (IGF-1) [11,14,15].

In this study, we hypothesize that by establishing their internal oxygen-deprived environment, the capacity of 3D HUVEC/cbMSC aggregates to secrete angiogenic growth factors and induce neovascularization can be enhanced significantly, thereby improving therapeutic efficacy. To verify this hypothesis, HUVEC/cbMSC aggregates with different levels of internal hypoxia were fabricated, by controlling their sizes and cell densities. Their expression of hypoxia-responsive genes and formation of tubular structures *in vitro* on Matrigel was measured. The therapeutic potential of hypoxic HUVEC/cbMSC aggregates for reperfusion of tissues in a mouse model of an ischemic limb was also evaluated.

2. Materials and methods

2.1. Cell culture

Human cbMSCs and HUVECs were kindly provided by Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan. The cbMSCs, transfected non-virally with red fluorescent protein (RFP) and human telomerase reverse transcriptase, were cultured in Minimum Essential Medium (MEM) Alpha Medium (Life Technologies, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS, HyClone, Logan, UT, USA), 30 μg/mL hygromycin B, and 200 μg/mL geneticin (Life Technologies). The HUVECs were cultivated in Medium 199 (Life Technologies) containing 10% FBS and 1% penicillin-streptomycin (Life Technologies). Cells were grown in an incubator maintained at 37 °C in 5% humidified CO_2.

2.2. Fabrication and characterization of hypoxic HUVEC/cbMSC aggregates

The 3D cell aggregates were fabricated in MEM Alpha Medium supplemented with 20% FBS and 1% penicillin–streptomycin, by using a thermo-responsive methylcellulose (MC) hydrogel system created in 96-well plates as reported previously by our group [8,16,17]. Briefly, equal amounts of HUVECs and cbMSCs were suspended in culture medium, added into each well of the MC hydrogel system, and then cultivated for 24 h on a rotating orbital shaker. The obtained cell aggregates were observed by using an inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss, Jena, Germany), and their diameters were determined by using the Image-Pro Plus software (Version 4.5, Media Cybernetics, Bethesda, MD, USA).

To analyze their gene expression profiles, total RNA of the as-prepared cell aggregates was extracted using TRIzol (Life Technologies) and then reverse-transcribed into complementary DNA (cDNA) by using the High Capacity Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. The quantitative real-time polymerase chain reaction (qPCR) was performed by the Applied Biosystems 7500 Real-Time PCR System using Power SYBR Green PCR Master Mix (Life Technologies) in triplicate for each sample and each gene. The primer sequences used are listed in Table 1. The cDNA that was prepared from monolayers of co-cultured cbMSCs and HUVECs was used as a reference.

Cell viability was investigated by the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Life Technologies) and examined using a Zeiss LSM 780 confocal microscope (Carl Zeiss). To count the viable cells, test cell aggregates were dissociated enzymatically and subjected to trypan blue dye exclusions.

Table 1
Primer list used for quantitative real-time polymerase chain reaction analyses.

Gene	Forward	Reverse
GAPDH	5′-GGAGCGAGATCCCTCCAAA-3′	5′-GGCTCCCCCCTGCAAA-3′
HIF-1α	5′-CGTTCCTTCGATCAGTTGTC-3′	5′-TCAGTGGTGGCAGTGGTAGT-3′
Bax	5′-ATGGACGGGTCCGGGGAG-3′	5′-TCAGAAAACATGTCAGCTGCC-3′
Bcl-2	5′-ATGTGTGTGGAGAGCGT CAA-3′	5′-CAAAGGCATCCCAGCCTC-3′
VEGF	5′-ATGACATGCTTTCCCTCCT-3′	5′-GAAGGAGGAGGGCAGA ATCAT-3
ADM	5′-TCGGAGTTTCGAAAGA AGTG-3′	5′-GGAAGTTGTTCATGCTCTGG-3′
Ang-1	5′-TTCCTTTCCTTTGCTTTCCTC-3′	5′-CTGCAGAGCGTTTGTGTTGT-3′
SDF-1	5′-TCGTGCTGACCGCGCTCTG CCTCA-3′	5′-TCTGAAGGGCACAGTTTGG AGTGT-3′
CXCR4	5′-TCAGTGGCTGACCTCCTCTT-3′	5′-TTTCAGCCAGCAGTTTCCTT-3′
IGF-1	5′-GTGTGGAGACAGGGGCTT TTAT-3′	5′-GGACAGAGCGAGCTGACTTG-3′
SMA	5′-CTGGGGTATTGGGGGCATC-3′	5′-CTGTTCCAGCCATCCTTCAT-3′
SM22	5′-TTTTGCTTGACGAACAGCAC-3′	5′-AACAGCCTGTACCCTGATGG-3′
PDGFRβ	5′-ACACCTCCTCAACCATCTCC-3′	5′-GAAGCTATCCTCTGCTTCCG-3′
NG2	5′-CTGCAGGTCTATGTCGGTCA-3′	5′-TTGGCTTTGACCCTGACTATG-3′

For immunofluorescence staining, the harvested cell aggregates were fixed in 4% paraformaldehyde and then stained with antibodies against HIF-1α or fibronectin (Abcam, Cambridge, MA, USA), followed by incubation with Alexa Fluor 488 Phalloidin and proper secondary antibodies (Life Technologies). The samples were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich, St. Louis, MO, USA) and observed by confocal microscopy.

2.3. Tube formation assay

The fabricated cell aggregates were harvested and seeded in the μ-Dish (ibidi, Munich, Germany) coated with growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA). On days 1, 4, 7, and 14, images of tube formation were taken on a fluorescence microscope (Carl Zeiss); the number of branching points and the spreading length of tubular structures were then measured ($n = 10$). Immunofluorescence staining of the grown tubular structures was performed with anti-RFP (Life Technologies), anti-von Willebrand factor (vWF), anti-smooth muscle actin (SMA), or anti-SM22 (Abcam) antibodies, visualized by fluorophore-conjugated secondary antibodies (Life Technologies), counterstained with DAPI, and assessed by confocal microscopy. To determine the levels of gene expression, the formed tubular structures were analyzed by qPCR; detailed methods used were described in Section 2.2.

2.4. Animal study

The use and care of animals in this study was consistent with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council and published by the National Academy Press in 1996. All animal experiments were conducted with institutional approval from the IRB of Veterans General Hospital (Taichung, Taiwan). Balb/C mice (8 weeks old) were subjected to left femoral artery ligation to induce unilateral hindlimb ischemia. Both the proximal and distal portions of the femoral artery were ligated, and the segment between the ligatures was excised. For cell transplantation, test HUVEC/cbMSC aggregates (2×10^6 cells in total) with different levels of internal hypoxia were injected into the quadriceps muscle adjacent to the proximal ligation site. Immunosuppression was achieved with cyclosporine A administered intramuscularly at the dose of 10 mg/kg/day until the animals were euthanized.

2.5. Perfusion imaging by single-photon emission computed tomography (SPECT)

The blood perfusion of hindlimbs was evaluated by SPECT (NanoSPECT/CT, Bioscan Inc., Washington DC, USA) at the Molecular Imaging Center of Linkou Chang Gung Memorial Hospital (Taoyuan, Taiwan). Before imaging, all animals were maintained under a temperature-control scanning chamber with 1.5% isoflurane anesthesia for 10 min. Subsequently, test animals were placed in an image bed; scintigraphy was performed immediately after the tail injection of Tc-99m diethylenetriaminepentaacetic acid (Tc-99m-DTPA, 17.5 MBq/0.1 mL). Dynamic planar images were collected at 2-s intervals up to 20 min. Following the perfusion scan, planar X-ray was also acquired for anatomy correlations. All image frames were summed into one blood-pool image for quantification analysis by using the PMOD software workstation (version 3.2, PMOD Technologies Ltd., Zurich, Switzerland). The mean radioactivity counts (Kcounts/s) within the bilateral hindlimbs were then