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Intramuscular delivery of 3D aggregates of HUVECs and cbMSCs for cellular cardiomyoplasty in rats with myocardial infarction

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ABSTRACT

Cell-based therapeutic neovascularization is a promising method for treating ischemic disorders. In this work, human umbilical vein endothelial cells (HUVECs) were thoroughly premixed with cord-blood mesenchymal stem cells (cbMSCs) and cultivated to form three-dimensional (3D) cell aggregates for cellular cardiomyoplasty. In the *in vitro* study, tubular networks were formed at day 1 after the co-culturing of dissociated HUVECs and cbMSCs on Matrigel; however, as time progressed, the grown tubular networks regressed severely. Conversely, when 3D cell aggregates were grown on Matrigel, mature and stable tubular networks were observed over time, under the influence of their intensive cell–extracellular matrix (ECM) interactions and cell–cell contacts. 3D cell aggregates were transplanted into the peri-infarct zones of rats with myocardial infarction (MI) *via* direct intramyocardial injection. Based on our pinhole single photon emission computed tomography (SPECT) myocardial-perfusion observations, echocardiographic heart-function examinations and histological analyses, the engrafted 3D cell aggregates considerably enhanced the vascular densities and the blood flow recovery in the ischemic myocardium over those of their dissociated counterparts, thereby reducing the size of perfusion defects and restoring cardiac function. These results demonstrate that the intramuscular delivery of 3D cell aggregates of HUVECs/cbMSCs can be a valuable cell-based regenerative therapeutic strategy against MI.

1. Introduction

Massive cell death has commonly been observed following myocardial infarction (MI), as a result of the reduced or obstructed blood flow, resulting in the formation of fibrous non-contractile scars that ultimately lead to progressive heart failure [1,2]. Cell-based neovascularization is emerging as an option to ensure sufficient tissue perfusion and preserve the viability of ischemic tissues, thereby restoring heart functions [3–6]. Therapeutic neovascularization can be achieved by transplanting the cellular components of vessel walls — endothelial cells (ECs) and vascular smooth muscle cells (SMCs) — directly into the ischemic tissues [7]. Neovascularization depends on not only specific cell–extracellular matrix (ECM) interactions but also cell–cell contacts [8].

Typical cell transplantation involves the administration of dissociated cells *via* direct intramuscular injection. In this process, a large proportion of the transplanted cells are either washed away by local bleeding or squeezed out from the injected sites by cardiac contraction, due to their insufficiency in physical size [9,10]. Employing a thermo-responsive methylcellulose (MC) hydrogel system, we have previously developed a method in which human umbilical vein ECs (HUVECs) and cord-blood mesenchymal stem cells (cbMSCs) self-aggregate together in a three-dimensional (3D) organization. Our earlier study verified the potential of cbMSCs to differentiate into SMCs [11]. The developed 3D cell aggregates could reach adequate physical size to be trapped in the muscular interstices, thus enhancing the retention of the transplanted cells at the sites of the cell graft [12,13]. Additionally, by using a mouse model with hindlimb ischemia, we demonstrated that the transplantation of 3D HUVEC/cbMSC aggregates promoted ischemic neovascularization and the salvaging of limbs more effectively than their dissociated counterparts [11].

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This work extends these previous findings by documenting that exogenously transplanted 3D HUVEC/cbMSC aggregates may augment myocardial neovascularization and exert a beneficial impact on the restoration of heart functions following MI. Transplantation of 3D cell aggregates into the peri-infarct zones of immune-suppressed rats at 1 week after MI induction was conducted *via* direct intramyocardial injection. Four weeks later, their vascularization and cardiac functions were evaluated; rats that were treated with saline or dissociated HUVECs/cbMSCs were used as controls.

2. Materials and methods

2.1. Preparation and characterization of 3D HUVEC/cbMSC aggregates

The HUVECs and cbMSCs were obtained from the Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan. The cbMSCs that were utilized in this study were transfected with red fluorescent protein (RFP) and human telomerase reverse transcriptase [14]. Detailed methods used for cultivating the 3D aggregates of HUVECs/cbMSCs have been previously reported by our group [11]. Briefly, equal amounts of dissociated HUVECs and cbMSCs were thoroughly mixed and seeded in an MC-hydrogel-coated 96-well plate (2.5×10^3 cells each type per well); a cell aggregate was formed in each well within 24 h. The grown cell aggregates were then collected for the subsequent experiments. In a preliminary study, we found that the tubular networks produced by the cell aggregates with a HUVECs:cbMSCs ratio of 1:1 on Matrigel were the most robust among all studied cell ratios; therefore, the cell aggregates with a HUVECs:cbMSCs ratio of 1:1 were selected for the study.

The as-prepared 3D cell aggregates were fixed in 4% paraformaldehyde for 1 h and stained with rabbit anti-RFP (Invitrogen, Carlsbad, CA, USA) and mouse anti-von Willebrand factor (vWF, Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies in phosphate-buffered saline Tween-20 (PBST) that contained 5% suitable serum, before being incubated with proper Alexa Fluor secondary antibodies (Invitrogen) in PBST to magnify the fluorescent signals. Fluorescent images were then obtained by a confocal laser scanning microscope (CLSM, Zeiss LSM 780, Carl Zeiss, Jena GmbH, Germany).

2.2. Tube formation assay

Neovascularization or the assembly of the vascular wall from its cellular and ECM components depends on both cell–ECM interactions and cell–cell contacts [15,16]. ECM is known to provide a scaffold that is crucial for stabilizing the organization of ECs into blood vessels [17]. The effects of cell–ECM interactions on the formation of tubular networks on Matrigel, derived from the 3D cell aggregates, were investigated *in vitro*; their dissociated counterparts were utilized as a control. Briefly, μ-Slide Angiogenesis plates (ibidi, Munich, Germany) were coated with an ice-cold growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) and incubated at 37 °C for 1 h. The 3D HUVEC/cbMSC aggregates and their dissociated counterparts were then individually seeded on the Matrigel-coated μ-Slide plates and maintained in an MEM Alpha Medium (Invitrogen) that contained 20% fetal bovine serum (FBS, HyClone, Logan, UT, USA).

On days 1, 4 and 7, the tubular networks that had formed on Matrigel were observed under an inverted fluorescence microscope (Axio Observer Z1, Zeiss, Jena, Germany). Subsequently, the formed tubular networks were fixed in 4% paraformaldehyde for 1 h and stained with rabbit anti-collagen I, mouse anti-collagen III, or mouse anti-fibronectin primary antibodies (Abcam, Cambridge, MA, USA) in PBST that contained 5% suitable serum. Appropriate Alexa Fluor-conjugated secondary antibodies (Invitrogen) were then applied to obtain the fluorescent signals. The tubular networks were also co-stained by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich, St. Louis, MO, USA) to observe nuclei, followed by inspection with CLSM.

To elucidate the effect of cell–cell contacts in the stability of neovascularization, the tubular networks that were grown on Matrigel were stained with rabbit anti-RFP (Invitrogen), mouse anti-smooth muscle actin (SMA), anti-SM22 (Abcam), or mouse anti-vWF (Santa Cruz Biotechnology) in PBST that contained 5% suitable serum. Fluorescent signals were obtained by using suitable Alexa Fluor-conjugated secondary antibodies. To observe nuclei, the tubular networks were also co-stained by SYTOX Blue (Invitrogen).

2.3. Animal study

The use and care of experimental animals in this study conformed 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. Acute MI was induced in Lewis rats that weighed 250–300 g [18]. The animals accomplishing the echocardiographic inclusion criterion [left ventricular fractional shortening (LVFS) < 35%] were intramyocardially injected with saline, dissociated HUVECs/cbMSCs (1×10^6 cells each type per rat), or 3D HUVEC/cbMSC aggregates (400 cell aggregates per rat, equivalent to 1×10^6 cells each type) at the border zones of the infarct [19]. To prevent graft rejection, the rats receiving cell transplantation were treated with cyclosporine A daily *via* the intramuscular injection route (10 mg/kg/day) three days before transplantation until sacrifice [18].

2.4. Pinhole single photon emission computed tomography (SPECT) imaging

The SPECT imaging was performed on a NanoSPECT/CT small-animal scanner that was equipped with four 2.5-mm multi-pinhole collimators (Bioscan, Washington DC, USA) at both the baseline and at 4 weeks post treatment ($n = 6$ for each studied group). Test animals were intravenously injected with Tc-99m sestamibi (37 MBq) at 1 h before the SPECT image acquisition began. The SPECT images were acquired for 24 min. The images were displayed and analyzed by using an image analysis software (PMOD Technologies, Zurich, Switzerland). The PMOD Cardiac Modeling Tool was utilized to generate the LV myocardial contours and ensure their standardized delineation.

To measure the size of the perfusion defect, the tracer activity was normalized to its maximum value and displayed as a polar map with 17 segments or reconstructed in a 3D format, by using the PMOD image analysis software. The perfusion defect area was defined as the fraction of the polar map elements whose radio-activities were reduced to more than 50% of their maximum values [20].

2.5. Echocardiography

Echocardiography was carried out on test animals under light sedation (2% isoflurane in oxygen) at 5 days following surgery (as the baseline) and at 4 weeks after treatment ($n = 6$ for each studied group). A digital ultrasound system that was equipped with a 4–12 MHz phased-array transducer (Vivid E9, GE Healthcare, Wauwatosa, WI, USA) was used. Standard parasternal short-axis views in two-dimensional (2D) images or M-mode tracings were obtained at a frame rate of >200 frames per second and at an average depth of 20 mm. Analyses of both radial and circumferential strains, defined as the change in length divided by its original length, were conducted employing a speckle-tracking algorithm (EchoPAC, GE Healthcare) [21]. The LV end systolic dimension (LVESD) and end diastolic dimension (LVEDD) were acquired *via* M-mode tracings; the LVFS (%) was then calculated as $[(LVEDD - LVESD) / LVEDD] \times 100\%$ [18].