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Epicardial delivery of VEGF and cardiac stem cells guided by 3-dimensional PLLA mat enhancing cardiac regeneration and angiogenesis in acute myocardial infarction

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ABSTRACT

Congestive heart failure is mostly resulted in a consequence of the limited myocardial regeneration capacity after acute myocardial infarction. Targeted delivery of proangiogenic factors and/or stem cells to the ischemic myocardium is a promising strategy for enhancing their local and sustained therapeutic effects. Herein, we designed an epicardial delivery system of vascular endothelial growth factor (VEGF) and cardiac stem cells (CSCs) using poly(L-lactic acid) (PLLA) mat applied to the acutely infarcted myocardium. The fibrous VEGF-loaded PLLA mat was fabricated by an electrospinning method using PLLA solution emulsified VEGF. This mat not only allowed for sustained release of VEGF for 4 weeks but boosted migration and proliferation of both endothelial cells and CSCs *in vitro*. Furthermore, sustained release of VEGF showed a positive effect on *in vitro* capillary-like network formation of endothelial cells compared with bolus treatment of VEGF. PLLA mat provided a permissive 3-dimensional (3D) substratum that led to spontaneous cardiomyogenic differentiation of CSCs *in vitro*. Notably, sustained stimulation by VEGF-loaded PLLA mat resulted in a substantial increase in the expression of proangiogenic mRNAs of CSCs *in vitro*. The epicardially implanted VEGF-loaded PLLA mat showed modest effects on angiogenesis and cardiomyogenesis in the acutely infarcted hearts. However, co-implantation of VEGF and CSCs using the PLLA mat showed meaningful therapeutic effects on angiogenesis and cardiomyogenesis compared with controls, leading to reduced cardiac remodeling and enhanced global cardiac function. Collectively, the PLLA mat allowed a smart cargo that enabled the sustained release of VEGF and the delivery of CSCs, thereby synergistically inducing angiogenesis and cardiomyogenesis in acute myocardial infarction.

1. Introduction

Myocardial infarction (MI) is one of the most costly cardiovascular diseases, which often ultimately has a high mortality rate [1]. The existing MI treatments, including drugs, devices and cardiac transplants are limited in their ability to repair myocardial necrosis [2]. Numerous approaches to the application of stem cells and biological growth factor therapies for cardiac regeneration have been reported [3–6]. Despite therapeutic advances, clinical trials have reported inadequate success (approximately 3% improvement in left ventricle ejection fraction). This lack of success is related to the cell source, number of implanted cells, route of implantation and limited regeneration efficacy [7,8].

Many research groups have studied various types of cells including embryonic stem cells, bone marrow/adipose-derived mesenchymal stem cells, hematopoietic stem cells and endothelial progenitor cells for MI treatment [9–13]. Resident cardiac stem cells (CSCs) have emerged as a leading candidate for MI stem cell therapy due to their ability to self-renew, proliferate and highly differentiate into cardiomyocytes, endothelial cells and smooth muscle cells [2,14,15].

Stem cell therapies by coronary artery injection or intramuscular injection have been studied as strategies to replace the damaged cardiomyocytes and restore cardiac function. However, the damaged cardiac tissue is an unfavorable environment, which results in low cell survival rates *in vivo* [16].

To overcome these limitations, epicardial implantation of CSCs might be a promising route for cardiac tissue regeneration. The scaffolds carrying CSCs should be designed specifically to this purpose. Our previous study reported fibrous poly(L-lactic acid) (PLLA) scaffolds composed of unbound micromesh with three-dimensional (3D) structure [17]. The fibrous 3D PLLA can be fabricated to a type of mat in which CSCs are loaded in massive cell numbers that can survive in

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favorable environment. The mat can be sutured onto an infarcted site of the heart resulting in more effective epicardial implantation of CSCs than a conventional cell injection. Furthermore, the fibrous mesh of the mat may function as a reservoir for sustained release of growth factors, i.e., vascular endothelial growth factor (VEGF).

One of the major challenges facing stem cell therapy for MI is angiogenesis [2,11]. VEGF, which is frequently used in MI treatment, plays vital roles in both angiogenesis and vasculogenesis [18–20]. The lack of delivery efficiency to injured organ reduces the efficacy of the VEGF [21]. A bolus overdose of VEGF may induce the abnormal vessels, immature vessel or tumors [22]. Infusion of VEGF in a sustained manner could avoid the systemic side effects compared with a bolus injection [23]. The short half-life of VEGF is also a major concern to its use because a period of several weeks is required for neo-vessel formation [24–26].

Local sustained release of VEGF from the PLLA mat would thus be most beneficial to maintain the therapeutic concentration at the target damaged site of the heart. Additionally, VEGF released from the mat would induce early microvessel recruitment for the improved survival rates of the epicardially implanted CSCs.

In this study, the functions of PLLA mats as a vehicle to deliver the therapeutic dose of CSC and VEGF for the treatment of myocardial infarction will be discussed. The effects of VEGF on migratory, proliferative and angiogenic properties of CSC are described.

2. Materials and methods

2.1. Reagents

PLLA (Mw 1.1×10^5 Da) was kindly provided by Purac Biochem (Gorinchem, The Netherlands). Methylene chloride and acetone were purchased from Duksan Pure Chemicals Co. (Seoul, Korea). All media and regents used in culture were obtained from Gibco (Seoul, Korea) unless otherwise specified. Fetal bovine serum (FBS, Gibco, Nebraska, USA), Dulbecco's Modified Eagle Medium (DMEM, Gibco), nutrient mixture F-12 antibiotics (Gibco), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (Peprotech, Seoul, Korea) were dissolved in the growth media. Fibrinogen, thrombin and urokinase were purchased from Sigma (St. Louis, MO, USA). Recombinant human $VEGF_{165}$ and the Quantikine® VEGF ELISA kit were purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Fabrication and characterization of PLLA mats

To electrospin the PLLA mat, a 20% PLLA solution was prepared in methylene chloride/acetone (90:10 v/v) solution. The polymer solution was loaded into a 10 ml plastic syringe with a 23-G needle. The flow rate was 0.03–0.06 ml/min and the applied voltage was 10 kV. The electrospun fibers were freeze-dried to evaporate the organic solvent. VEGF-loaded mats were produced by using the w/o emulsion method. VEGF was dissolved in PBS, and the aqueous solution was dispersed into a 20% PLLA solution containing Span 80. FITC-conjugated BSA (Sigma-Aldrich, St. Louis, MO, USA) was used as a model protein for fluorescence microscopic evaluation of the VEGF distribution in the PLLA fibers.

The morphology of the electrospun fibers was examined using scanning electron microscope (SEM, JSM 5200, JEOL, Tokyo, Japan) at 10 kV. The samples were coated with gold using a mini sputter coater (Polaron SC-7620, Thermo VG Microtech, England).

2.3. In vitro release of VEGF

To determine the release kinetics of VEGF from the PLLA mat, the mats were cut into a uniform size ($6 \times 6 \times 1$ mm^3). The mats ($n = 6$) were immersed in 1 ml of PBS and placed in a shaking incubator at 37 °C. The cumulative release of VEGF from the PLLA mats was measured at predetermined interval. The levels of VEGF protein in PBS were determined using Quantikine® VEGF ELISA kit according to the manufacturer's instructions. The absorbance was measured on a microplate reader at 450 nm using a micro-plate reader.

2.4. Cell isolation and culture

All animal studies were approved by the Institutional Animal Care and Use Committee at the Inje University School of Medicine. CSCs were isolated by cardiac explant culture as described previously [27] with minor modification. Heart tissue was obtained from Sprague-Dawley (SD) rats (8-week old, male, Charles River Lab Inc., Seoul, Korea). After removal of pericardium and greater vessels, the myocardial fraction was carefully separated from the epicardium and endocardium under stereomicroscopic guidance, minced into 2–3 mm^3 fragments and suspended in 1 unit/mL of thrombin dissolved in DMEM containing 100 μg/mL of aminomethylbenzoic acid (Sigma-Aldrich). The myocardial fragments suspended in thrombin solution were mixed with the same volume of 0.5% fibrinogen solution dissolved in DMEM and then 10 mL of a mixture containing approximately 200 mg of the myocardial fragments was placed in a tissue culture dish. After incubation at 37 °C for 2 h, growth culture medium (90% DMEM–Ham's F12 (1:1) mixture, 10% FBS, 10 ng/mL EGF, 2 ng/mL bFGF, 10 ng/mL IGF and 10 g/mL gentamicin) was added. Organ culture was performed under dynamic conditions at 15 rpm or static conditions. After 14 days of culture, outgrown phase-bright cells in the fibrin were recovered by the selective degradation of the fibrin using 5000 units of urokinase for 30 min in the presence of 30% calf serum in DMEM. These collected cells were suspended in growth culture media, plated into a 150-mm tissue culture dish and then cultured under a conventional monolayer culture condition. When cells reached 80% confluence, cells were detached, seeded at a 10,000 cells/cm^2 and subcultured. This initial passage was referred to as passage 1. All *in vitro* assays and *in vivo* experiments used subcultured CSCs at passage 3 unless otherwise specified. Human umbilical vein endothelial cells (HUVECs) obtained from American Type Culture Collection (Manassas, VA, USA) were used as the control.

2.5. Growth kinetics, immunophenotype and in vitro differentiation potential of CSCs

To analyze the growth competency, colony forming unit-fibroblast (CFU-F) assay was performed. Briefly, CSCs at P_3 were seeded at 2 cells/cm^2 density and cultured for 7 days. After staining with Gentian violet, established CFU-F were counted using ImageJ (NIH, Bethesda, MD). The population doubling time (PDT) was determined until CSCs went into senescence during subsequent subcultures. To measure the PDT, one thousand cells were plated into a 48-well plate. The cells were then lysed and the cellular DNA content at 1 and 5 days of the culture was determined. The PDT was determined using the following formula: $PDT = \frac{\text{days in exponential phase}}{(\log N_2 - \log N_1)/\log 2}$, where N_1 or N_2 were the cellular DNA content at 1 or 5 days, respectively.

The immunophenotype of CSCs was analyzed with a FACSCalibur (Becton Dickinson, San Jose, CA, USA). The cells were harvested at 80% confluency and suspended in PBS containing 0.5% bovine serum albumin (Sigma-Aldrich) and 0.1% sodium azide at a concentration of 1×10^6 cells/ml. The cell viability was more than 98% as determined by trypan blue exclusion. The cells (1×10^5) were incubated with phycoerythrin-conjugated anti-rat CD31, CD34, or isotype-matched control antibodies for 30 min. All antibodies were purchased from BD Bioscience (San Jose, CA) unless otherwise specified. After incubation, the cells were washed with PBS and fixed in 2% paraformaldehyde in PBS. The cells were incubated with unconjugated antibodies specific for α-smooth muscle actin (SMA), α-sarcomeric actinin (α-SA; Abcam, Cambridge, MA, UK), CD105 (R&D Systems), GATA-4 (Abcam), Nkx-2.5 (Abcam), nestin (Sigma-Aldrich) or Sca-1 (Abcam). After incubation, cells were washed, incubated with FITC-conjugated secondary