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Enhancement of endothelial differentiation of adipose derived mesenchymal stem cells by a three-dimensional culture system of microwell

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ABSTRACT

Adipose derived mesenchymal stem cells (AdMSCs) have been demonstrated to have ability to differentiate into several cell lineages, including endothelial cells. The low endothelial differentiation efficiency, however, limits further clinical application of AdMSCs for therapeutic angiogenesis. This study was designed to investigate the feasibility to promote endothelial differentiation efficacy of AdMSCs using microwell array as a 3-D culture system. AdMSCs aggregates were prepared using photocrosslinkable polyethylene glycol dimethacrylate (PEGDM) derived microwell. AdMSCs aggregated and formed well defined 3-D aggregates following seeding. The microwell was effective in regulating the size of AdMSCs aggregates with low variation. AdMSCs within the 3-D aggregates maintained the cell surface epitopes of AdMSCs with high viability. Endothelial growth medium was used to induce the in vitro endothelial differentiation of AdMSCs. Both gene expression results from real time PCR and protein expression data from immunofluorescent staining revealed that 3-D cultured aggregates significantly promote the endothelial differentiation efficacy of AdMSCs. AdMSCs or AdMSCs aggregates were injected into the subcutaneous space of nu/nu mice to investigate the endothelial differentiation in vivo. The immunofluorescent staining data indicated promoted endothelial differentiation of 3-D aggregates compared with 2-D AdMSCs. Aggregates dissociated cells were obtained by transferring 3-D aggregates onto the adherent surfaces. Cells dissociated from induced aggregates were still positive for endothelial specific markers and were able to form endothelial-like tube structures on matrigel, indicating the endothelial properties. We conclude that microwell is an ideal 3-D culture system for promoting endothelial differentiation efficacy of AdMSCs.

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1. Introduction

Endothelial dysfunction is a prominent feature of a wide variety of diseases, including cardiovascular diseases [1], diabetes mellitus [2], and stroke [3]. One of the ideal strategies for endothelial dysfunction is surgical replacement of dysfunctional or diseased blood vessels with tissue engineered vascular grafts. However, the currently available vascular grafts cannot totally replace the physical functions of native healthy vessels due to short-term or long-term graft failures such as thrombosis or stenosis [4]. The presence of robust endothelial cells (ECs) has been already shown to improve the patency of synthetic vascular grafts [5]. However, it is difficult to obtain a sufficient number of autologous endothelial cells due to invasive surgery and limited expansional capacity of endothelial cells in vitro [6].

Mesenchymal stem cells (MSCs), firstly identified in bone marrow, have been demonstrated to be multipotent cells with great potential to differentiate into various mesodermal lineages [7].

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Adipose tissue represents an abundant and accessible source of mesenchymal stem cells (AdMSCs). Several comparison studies have shown that AdMSCs are similar in cell surface expression profiles, differentiation potential and therapeutic efficacy with MSCs derived from bone marrow [8–10]. Most importantly, sufficient number AdMSCs for clinical application could be obtained with minimal side effects under local anesthesia, making AdMSC an alternative cell source for therapeutic angiogenesis.

The endothelial differentiation potential of AdMSCs has been well demonstrated in several studies [11–14]. The main culture system for isolation and expanding of AdMSCs are cultural dishes or flasks which are defined as two-dimensional (2-D) cultural system because the single cells are separated from their neighbors and attached onto the 2-D surface of culture system [15]. AdMSCs have been demonstrated to lose replicative ability, colony-forming efficiency, and differentiation capacity with time in traditional 2-D culture system [16,17], which limits their potential use for therapeutic application. Recently, the capacity of AdMSCs to differentiate into ECs has also been reported limited in 2-D culture system [18]. Hence, the application of tree-dimensional (3-D) cell culture techniques which mimic the exact cellular microenvironment in vivo, has received increasing interests. The main 3-D cell culture techniques include porous scaffolds, hydrogel, and cellular aggregates. Among them, cellular aggregates have drawn rising attention because they are free of exogenous biomaterials. As a main technique of cellular aggregates, hanging drop has been widely used for stem cell culture. However, it's difficult to make scale production by using hanging drop method. Microwell has been reported as a useful technique in producing cell aggregates in our group [19]. The prominent superiority of microwell is the effectiveness of scale production.

Recently, 3-D culture system has been demonstrated to successfully increase MSCs differentiation efficacy over traditional 2-D flasks [20–22]. However, the effect of 3-D culture on the endothelial differentiation of AdMSCs is unknown. In the present study, a 3-D multicellular aggregates culture system based on microwell array methodology was firstly applied to explore endothelial differentiation efficiency of AdMSCs.

2. Materials and methods

2.1. Cell culture

AdMSCs were isolated from paratesticular fat of Sprague–Dawley rats according to the previously described protocol [12,23,24]. Briefly, adipose tissue was minced into small pieces, and then incubated with 0.075% type I collagenase (Sigma–Aldrich, St. Louis, MO, USA) for 1 h at 37 °C. After centrifuging, the remaining cells were suspended in Dulbecco's modified Eagle's medium (DMEM, Life Science Technology, Shanghai, China) supplemented with 10% fetal bovine serum (FBS, Life Science Technology), and plated at a density of 1×10^6 cells in a 10-cm dish. AdMSCs were passed under the same conditions through no more than five passages before being used for assays. All procedures were approved by Institutional Animal Care and Use Committee of Nanjing University.

Human umbilical vein endothelial cell (HUVEC), was purchased from Lonza Biologics Inc. (Portsmouth, NH) and cultured in endothelial cells growth medium (EGM-2) supplemented with growth factors and cytokines (Lonza Biologics Inc.). Unless otherwise indicated, all AdMSCs were cultured in DMEM supplemented with 10% FBS. Culture incubator was set at 37 °C with 5% CO_2.

2.1.1. Microwell assembly and generation of AdMSCs aggregates

Microwells were generated using micromolding on UV-photocrosslinkable polyethylene glycol dimethacrylate (PEGDM, MW = 1000, 20% in PBS) (Sigma–Aldrich) with 1% photoinitiator 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-L-propanone (Irgacure D2959, Ciba Specialty Chemicals Inc., Florham Park, NJ, USA) according to the previously described protocol [19,25]. Briefly, PEGDM macromer solution was pipetted on the 3-(trimethoxysilyl) propylmethacrylate (TMSPMA) (Sigma–Aldrich) coated glass slide. Thereafter, a patterned PDMS stamp was placed on PEGDM to make PEGDM distribute evenly between PDMS stamp and slide. The microwell was produced by photo-crosslinking (l = 350–500 nm, 10 s, 10 mW/cm^2; OmniCure Series 2000 curing station, EXFO, Mississauga, Canada). For generation of AdMSCs aggregation, AdMSCs were seeded into microwells by using a previously developed method [26]. Briefly, cell suspension (1×10^6 cells/ml) was pipetted on the surface of the microwell array (100 μl/cm^2). Two hours later, the array was immersed in media in a culture dish. Twenty-four hours post-seeding, the aggregated size was determined by imaging cells using phase contrast microscopy. A schematic summarizing the protocol is shown in Fig. 1.

2.2. Cell viability

A rapid, simultaneous double-staining procedure using fluorescein diacetate (FDA) and propidium iodide (PI) (Sigma–Aldrich) was used in the determination of cell viability [27]. In brief, cells were stained with 5 μg/ml PI and 4 μg/ml FDA and observed under the fluorescent microscopy with an appropriate barrier filter set.

2.3. Flow cytometry

Briefly, 1 × 105 AdMSCs were harvested and suspended in 500 μl PBS, incubated with anti-CD29 (Bio-Legend, San Diego, CA, USA), anti-CD45 (Millipore Corporation, Billerica, MA, USA) or anti-CD31(Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature in darkness. After washing with PBS for 3 times, AdMSCs were analyzed using FACSCalibur (BD Bioscience, Sparks, MD, USA). Data was analyzed with the Cell Quest software (BD Biosciences). AdMSCs incubated with fluorescence-conjugated IgG isotypes were used as negative controls.

2.4. In vitro endothelial differentiation

Twenty-four hours after AdMSCs were seeded in dishes (2-D) or microwell array (3-D), the culture medium were discarded, followed with the addition of fully supplemented EGM-2. Fully supplemented EGM-2 contained 0.02 ml/ml FBS, 22.5 μg/ml heparin, 0.2 μg/ml hydrocortisone, 5 ng/ml EGF, 10 ng/ml FGF2, 20 ng/ml IGF-I, and 0.5 ng/ml VEGF. Induction proceeded for 6–9 days, with the medium replenished every three days.

2.5. In vivo endothelial differentiation

For the cell tracking, AdMSCs were labeled with EdU (Life Science Technology) for 48 h. A total of 1×10^6 EdU labeled AdMSCs or AdMSCs derived aggregates in 200 μl Matrigel (BD Biosciences) were injected using a 26G needle into the subcutaneous space of nu/nu mice. Seven days later, implants were removed from euthanized mice, fixed with 4% paraformaldehyde over night, embedded in paraffin, and sectioned (5 μm thick).

2.6. Histology and immunocytochemistry

After rinsed with PBS and fixed with 4% paraformaldehyde for 10 min, cells or sections were rinsed with PBS and permeabilized with 0.05% triton X-100 for 10 min. After incubated with 5% horse serum for 30 min and then with primary antibody over night at 4 °C, the cells or sections were incubated with secondary antibodies for 1 h at room temperature. The primary antibodies included: anti-CD31 antibody (1:400, Santa Cruz Biotechnology), anti-collagen IV (1:100, Boster, Wuhan, China), anti-laminin (1:100, Boster), anti-fibronectin (1:100, Boster), anti-vimentin (1:100, Boster), or anti-KDR (1:200, Santa Cruz Biotechnology). The secondary antibodies include: Alexa-488- or Alexa-594- conjugated secondary antibodies (1:500; Life Science Technology). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Life Science Technology).

For Hematoxylin and eosin (HE) staining, the slides were stained using commercial available kits (Jiancheng, Nanjing, China) according to the instruments.

2.7. Real time polymerase chain reaction (PCR)

Gene expressions of endothelial cells specific markers were determined by real time PCR. Briefly, cells were lysed with TRIzol Reagent (Life Science Technology) for RNA extraction. RNA was reverse-transcribed to cDNA by using a commercial available Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR for detection of transcript was performed by using Power SYBR Green PCR Master Mix (Invitrogen); all experiments were performed in triplicate for each sample and each gene. The primer sequence was listed in Table S.1.

2.8. Matrigel-based capillary-like tube formation assay

The assay was initiated by coating a 4-well CultureSlide (BD Bioscinece) plate with 150 μl of growth factor-reduced Matrigel (BD Biosciences) per well. Approximate 5×10^4 cells in 500 μl medium were then seeded onto each well and incubated at 37 °C. Development of capillary-like tubes was examined by phase-contrast microscopy and photographed 16 h after seeding.

2.9. Image analysis and quantification

For tissue specimens, data were averaged on at least 6 randomly selected high power fields. Images were captured on a Nikon microscope with a Spot RT color digital camera, and digital histomorphometric analysis was performed using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

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