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Differentiation of mesenchymal stem cells from human amniotic fluid to vascular endothelial cells

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ABSTRACT

Endothelial dysfunction is a principle feature of vascular-related disease. Endothelial cells have been acquired for the purposes of the restoration of damaged tissue in therapeutic angiogenesis. However, their use is limited by expansion capacity and the small amount of cells that are obtained. Human amniotic fluid mesenchymal stem cells (hAF-MSCs) are considered an important source for vascular tissue engineering. In this study, hAF-MSCs were characterized and then induced in order to differentiate into the endothelial-like cells. Human amniotic fluid cells (hAFCs) were obtained from amniocentesis at the second trimester of gestation. The cells were characterized as mesenchymal stem cells by flow cytometry. The results showed that the cells were positive for mesenchymal stem cell markers CD44, CD73, CD90 and HLA-ABC, and negative for CD31, Amniotic fluid stem cells marker: CD117, anti-human fibroblasts, HLA-DR and hematopoietic differentiation markers CD34 and CD45. The hAF-MSCs were differentiated into endothelial cells under the induction of vascular endothelial growth factor (VEGF) and analyzed for the expression of the endothelial-specific markers and function. The expression of the endothelial-specific markers was determined by reverse transcriptase-quantitative PCR (RT-qPCR), while immunofluorescent analysis demonstrated that the induced hAF-MSCs expressed von Willebrand factor (vWF), vascular endothelial growth factor receptor 2 (VEGFR2), CD31 and endothelial nitric oxide synthase (eNOS). The network formation assay showed that the induced hAF-MSCs formed partial networks. All results indicated that hAF-MSCs have the potential to be differentiated into endothelial-like cells, while human amniotic fluid might be a suitable source of MSCs for vascularized tissue engineering.

1. Introduction

Cardiovascular disease, diabetes mellitus and stroke have been identified as major problems occurring in aging societies and are a worldwide cause of mortality. Risk factors for these health problems have been identified as smoking, hypertension, obesity and hyperlipidemia. Endothelial dysfunction is a response to certain cardiovascular risk factors and leads to the development of atherosclerosis. To restore dysfunction, endothelial cells are required in vascular tissue engineering or therapeutic angiogenesis (Boos et al., 2006; Qiu et al., 2015). However, an obstacle to the therapeutic use of endothelial cells is the difficulty involved with obtaining enough cells, limiting their proliferation and functions (Doan et al., 2014; Liu et al., 2007a). For these reasons, the stem cell-based therapy has shown potential for the treatment of vascular disease.

Recently, mesenchymal stem cells have been acknowledged as a promising agent in therapeutic applications developed for the purposes of restoring injured tissue. They can give rise to multiple lineages such as chondroblasts, osteoblasts and adipocytes (Suzdal'tseva et al., 2007). MSCs are found in the non-hematopoietic bone marrow stroma. Bone-marrow mesenchymal stem cells (BM-MSCs) have the potential to be differentiated into the mesodermal lineage (Rastegar et al., 2010). However, their use in clinical applications has been limited because the procedures used to obtain the mesenchymal stem cells are invasive, the number of cells is low and the differentiation potential may decrease with the increasing age of the donor (Fei et al., 2013; Wu et al.,

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2007). Therefore, it is necessary to search for new or alternative sources of mesenchymal stem cells for the purposes of therapeutic angiogenesis.

Amniotic fluid contains a heterogeneous population of cells that are of increasing interest in terms of their characterization, culture, and therapeutic applications. This fluid has been suggested to be a novel source of mesenchymal stem cells (Antonucci et al., 2011). Owing to the advantages of this source, the mesenchymal stem cells can be collected easily by the direct adherence method (Fei et al., 2013). In the culturing conditions, amniotic fluid mesenchymal stem cells (AF-MSCs) must adhere to the plastic dish, represent a fibroblast-like morphology and maintain an undifferentiated state with no trend of tumorigenicity (Zhou et al., 2014). The phenotypic characteristics of AF-MSCs are similar to MSCs from other sources (Chen et al., 2009; Kempa et al., 2005). Interestingly, AF-MSCs express Oct-4; a pluripotency marker of embryonic stem cells. Consequently, attention has been focused on the AF-MSCs at an intermediate stage between embryonic and adult stem cells (De Coppi et al., 2007; Prusa et al., 2003). Moreover, it has been reported that the AF-MSCs have greater potential than BM-MSCs due to the ability of these cells to be differentiated into multiple cell lineages of three embryonic germ layers, such as chondrocytes, endothelial cells, hepatocytes and neurons (De Coppi et al., 2007). Therefore, this study has attempted to culture human AF-MSCs from the second trimester of gestation and evaluate the potential of hAF-MSCs to be differentiated into the endothelial lineage *in vitro*.

2. Materials and methods

2.1. Human amniotic fluid cell (hAFC) culture

The human amniotic fluid samples were obtained during weeks 16–22 of gestation for routine prenatal diagnosis at the Human Genetics Laboratory of Anatomy Department, Faculty of Medicine, Chiang Mai University. The hAFCs were cultured with BIOAMF-3™ Complete Medium (Biological Industries, Kibbutz Beit Haemek, Israel, cat. No. 01-196-1) in a 25 cm^2 culture flask (Corning Incorporated, NY, USA, cat. No. 430372200) at 37 °C, 5% CO_2 and 95% humidity for 9 days; then, the cell colonies were observed. The adherent cells were washed by phosphate buffer saline (PBS) (Sigma-Aldrich, St Louis, MO, USA, lot No. SLBL3211V) and were cultured for expansion in Dulbecco's Modified Eagle Medium (DMEM) – high glucose (Gibco, NY, USA, lot No. 1645799) supplemented with 10% fetal bovine serum (Gibco, South America, NY, lot No. 41Q3022K), gentamycin (T.P. Drug Laboratories, lot No. 514204) and Pen Strep (penicillin and streptomycin) (Gibco, NY, USA, lot No. 1166825) (basal medium) in the same environment. The medium was changed every 3 days. After reaching 80% confluence, the cells were sub-cultured as passage 1 using 0.25% trypsin-EDTA (Gibco, NY, USA, lot No. 1316929). After the second passage, the cells were used for the experiments. Informed written consent was obtained after approval was granted by the Ethics Committee from the Faculty of Medicine, Chiang Mai University, April 21st, 2015 No. ANA-2558-2943.

2.2. Flow cytometry analysis

To characterize the phenotypes of hAFCs as MSCs, flow cytometry was performed. After trypsinization and centrifugation, the cell samples (n = 3) were incubated with monoclonal antibodies; fluorescein isothiocyanate (FITC) – conjugated mouse anti-human CD34 (Biolegend, San Diego, USA cat. No. 343504,), mouse anti-human CD90 (Biolegend, San Diego, USA cat. No. 328108), mouse anti-human HLA-ABC (Immuno Tools GmbH, Friesoythe, Germany, cat. No. 21159033) and mouse anti-human fibroblasts (EDM Millipore Crop, UK, lot No. 2476333), as well as phycoerythrin (PE)-conjugated mouse anti-human CD31 (Immuno Tools GmbH, Friesoythe, Germany, cat. No. 21270314), mouse anti-human CD44 (Pierce Biotechnology, Rockford, USA, cat. No. MA1-12128), mouse anti-human CD45 (Biolegend, San Diego, USA cat. No.304008), mouse anti-human CD73 (Life Technologies, California, USA, cat. No. A16356), mouse anti-human CD105 (Pierce Biotechnology, Rockford, USA cat. No. MA1-80944), mouse anti-human CD117 (Immuno Tools GmbH, Friesoythe, Germany, cat. No. 22151174), mouse anti-human HLA-DR (Immuno Tools GmbH, Friesoythe, Germany, cat. No. 21388994), and kept on ice for 1 h. FITC mouse isotype control (Biolegend, San Diego, USA, cat. No. 400110) and PE mouse isotype control (Biolegend, San Diego, USA, cat. No. 400114) were used as the control. Finally, the cells were washed with 1% BSA-PBS. The cell surface marker expression was detected by FACScan (Becton Dickinson, Lincon Park, NJ) and analyzed by CellQuest Pro 9.0 software (Becton Dickinson).

2.3. Alamar blue cell proliferation assay

Alamar blue cell viability reagent was used to quantitatively evaluate the proliferation of hAF-MSCs. With regard to the oxidation-reduction reaction of living cells, resazurin (blue color) is converted to resorufin (red color) according to the process of changing from an oxidized form to a reduced form. In terms of measurement, the absorbance was used to monitor fluorescence with an excitation wavelength at 540–630 nm. Briefly, the cells (n = 3) were trypsinized and centrifuged at 3700 rpm for 6 min. They were plated into 24 well-plates (Corning Incorporated, NY, USA cat. No. 3524) at a density of 2×10^3 cells/well with basal medium (DMEM + 10% FBS). After 24 h, the basal medium was removed and 100 ul of 10% (v/v) Alamar blue e (Sigm-Aldrich, St Louis, MO, USA, cat. No. 62758-13-8) in DMEM was added into the well plates containing cells. The cells were cultured for 4 h. The solution was also added into the wells without cells as a control. Then, the solution was removed and the fluorescence was measured with the excitation wavelength at 540–630 nm using a spectrophotometer plate reader (original Multiskan EK, Thermo Scientific, UK). Then, the cells were continuously cultured under the same conditions. Alamar blue cell proliferation assay was performed on days 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21.

2.4. Endothelial differentiation of hAF-MSCs

To differentiate hAF-MSCs into endothelial-like cells, the cells were divided into two groups (non-induced group and induced group). The third passage of hAF-MSCs (n = 5) involved the seeding at 1×10^4 cells/cm^2 on each well of the 24-well plate. The non-induced group was cultured in basal medium and the induced group was cultured in basal medium supplemented with 50 ng/ml vascular endothelial growth factor (VEGF) (Sigm-Aldrich, St Louis, MO, USA, cat. No. V7259) for 14 days. Human umbilical vein endothelial cells (HUVECs) (Life Technologies, California, USA, cat. no. C-003-5C) were used as a positive control.

2.5. Reverse transcriptase – quantitative PCR (RT-qPCR)

After endothelial differentiation, total RNA was extracted using Illutra RNAspin Mini RNA Isolation kit (GE Healthcare, Little Chalfont, UK, lot No. 1411/001). RNA was reverse transcribed into single-stranded cDNA using a Tetro cDNA synthesis kit (Bioline, USA, lot No. RA384-B032060) according to the manufacturer's protocol. Gene transcripts were measured by SsoFast™ EvaGreen® Supermix (Bio-Rad, Singapore, cat. No. 172–5201) with a Chromo4™ Real-Time PCR Detector (Bio-Rad, United States). PCR primers were used to target *von Willebrand Factor (vWF)*, *vascular*

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