Neuroscience 272 (2014) 229–239

COCULTURE WITH EMBRYONIC STEM CELLS IMPROVES NEURAL DIFFERENTIATION OF ADIPOSE TISSUE-DERIVED STEM CELLS

L. BAHMANI, M. F. TAHA AND A. JAVERI *

Department of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

Abstract—Embryonic stem (ES) cells secrete some soluble factors which may affect the differentiation potential of adult stem cells toward different lineages. In the present study, we evaluated neural differentiation of mouse adipose tissue-derived stem cells (ADSCs) following coculture with ES cells. For this purpose, ADSCs were induced in a medium supplemented with a synthetic serum replacement and various concentrations of retinoic acid (RA). Then, third-passaged ADSCs were indirectly cocultured with ES cells, and the expression levels of pluripotency markers, OCT4 and Sox2, mesenchymal stem cell markers, CD73 and CD105, and proliferating cell nuclear antigen (PCNA), were assessed in the cocultured ADSCs. Moreover, the control and cocultured ADSCs were differentiated with or without RA treatment. We showed here that 2-week differentiated ADSCs expressed several neuron-specific genes, and RA treatment improved neural differentiation of the ADSCs. The expression levels of OCT4, Sox2 and PCNA were upregulated in the cocultured ADSCs. Moreover, coculture with the ES cells significantly improved neural differentiation of the ADSCs. Treatment of the cocultured ADSCs with RA diminished the expression of neural maturation markers. Coculture with the ES cells efficiently improves neural differentiation of the ADSCs. Non-contact coculture with the ES cells may be used as an efficient strategy to improve differentiation potential of adult stem cells for developmental studies and regenerative medicine.

Key words: ADSC, embryonic stem cell, neural differentiation, coculture.

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stromal cells with the capability to differentiate into various cell types. They are useful candidates for basic research and cell replacement therapy. MSCs are present in different adult tissues. For many years, bone marrow-derived MSCs (BM-MSCs) have been the main focus of adult stem cell researchers. However, recent studies have identified adipose tissue as a rich source of stem cells with a close molecular resemblance to BM-MSCs (Zuk et al., 2002; Aust et al., 2004; Izadpanah et al., 2006; Peroni et al., 2008a). Adipose tissue-derived stem cells (ADSCs) can be isolated easily and propagated rapidly (Zuk et al., 2001a; Nakagami et al., 2006). They express several mesenchymal cell-specific markers (Schaffler and Buchler, 2007) and can be differentiated to osteogenic, adipogenic and chondrogenic lineages (Zuk et al., 2001b; Dicker et al., 2005; Lin et al., 2005; Guilak et al., 2006; Hachisuka et al., 2007). Neural differentiation of the ADSCs has also been reported (Safford et al., 2002; Zuk et al., 2002; Ashjian et al., 2003; Kang et al., 2003; Yang et al., 2004; Fujimura et al., 2005; Ning et al., 2006; Huang et al., 2007). In our previous study, we successfully induced neural differentiation of mouse ADSCs in a medium containing synthetic serum replacement (Taha et al., 2014).

One disadvantage regarding the application of MSCs over embryonic stem (ES) cells is the low efficiency of differentiation. Long passages compromise the differentiation capacity of MSCs. Therefore, it is beneficial to use proper strategies to improve differentiation capacity of MSCs after several passages. One strategy in this regard may be coculture of MSCs with ES cells.

Coculture is a way to cultivate different cell types in close proximity in the same culture environment. Coculture can be performed through direct cell-to-cell contact or indirect ways. Indirect coculture takes the advantage of culture inserts with porous membrane. In this technique, cocultured cell populations are kept physically separated, but they can affect each other through the secretion of soluble factors. It is clear that chemical components of the microenvironment, such as cytokines, hormones, ionic gradients and other soluble factors, have critical roles in determining the fate of developing cells (Rangappa et al., 2003).

In the current study, we first assessed the effect of retinoic acid (RA) on neural differentiation of the ADSCs. Then, we cocultured the ADSCs with ES cells

*Corresponding author. Address: Department of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Pajoohesh Boulevard, P.O. Box 161-14965, Tehran, Iran. Tel: +98-2144580474, mobile: +98-9128601243; fax: +98-2144580395.

E-mail address: arashj@nigeb.ac.ir (A. Javeri).

Abbreviations: β-ME, β-mercaptoethanol; ADSC, adipose tissue-derived stem cells; bFGF, basic fibroblast growth factor; BM-MSCs, bone marrow-derived mesenchymal stem cells; DMEM, Dulbecco's modified Eagle's medium; ES, embryonic stem; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; LIF, leukemia inhibitory factor; MSCs, mesenchymal stem cells; PCNA, proliferating cell nuclear antigen; PE, phycoerythrin; RA, retinoic acid; SDF1, stromal cell-derived factor 1; SVF, stromal vascular fraction; Syp, synaptophysin; VEGF, vascular endothelial cell growth factor.

http://dx.doi.org/10.1016/j.neuroscience.2014.04.063

using culture inserts and assessed neural differentiation of the cocultured ADSCs. Our results showed that the expression of some pluripotency markers was upregulated in the cocultured ADSCs. In addition, neural differentiation of the ADSCs was significantly improved after coculture with the ES cells.

EXPERIMENTAL PROCEDURES

Isolation and characterization of the ADSCs

All animal works conducted in this study were approved by the ethics committee of the National Institute of Genetic Engineering and Biotechnology. ADSCs were isolated and expanded as described previously (Taha and Hedayati, 2010). Briefly, inguinal adipose tissue of 8–10-week old NMRI mice was digested by 0.2% collagenase A (Roche Applied Science, Mannheim, Germany). After centrifugation, the stromal vascular fraction (SVF) was resuspended in fresh medium containing Dulbecco's modified Eagle's medium (DMEM) and 20% fetal bovine serum (FBS) and cultured.

For characterization of the expression of cell surface markers, third-passaged ADSCs were labeled with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated rat anti-mouse CD11b, CD45, CD31, CD29, CD44 and CD105 antibodies (all from Abcam, Cambridge, UK). The cells that stained with FITC or PE rat anti-mouse IgG were considered as negative controls. The cells were examined by an Attune® Acoustic Focusing Cytometer (Applied Biosystems, Life Technologies, Carlsbad, USA) and analyzed using FlowJo 7.6.1 software (Tree Star, Inc., Ashland, OR, USA).

Neural differentiation of the ADSCs

Third to fifth-passaged ADSCs were cultured in 0.1% gelatin-coated (Sigma–Aldrich, St. Louis, MO, USA) tissue culture plates with a density of 5×10^4 cell/ml. ADSCs were cultured to reach 90% confluency, and then neural differentiation was induced by a medium containing DMEM with 15% Knockout Serum Replacement (KoSR, Gibco, Life Technologies, Carlsbad, USA). To examine the effects of RA on neural differentiation, 10^{-8}, 10^{-7} or 10^{-6} M all-trans RA (Sigma–Aldrich) was added to the culture medium. Medium was changed every 2 days. After 2 weeks of neural induction, the expression of neural-specific genes and proteins was assessed by RT-PCR and quantitative real-time PCR (qPCR).

Culture of mouse ES cells

The mouse ES cell line Royan B1 (RSCB0001, Royan Stem Cell Bank, Royan Institute, Tehran, Iran) was used in the present study. ES cells were cultured on top of a feeder layer of mitomycin C inactivated mouse embryonic fibroblasts (MEFs) in a medium containing Knockout DMEM (Gibco), 15% FBS (ES qualified, Gibco), 2 mM L-glutamine (Gibco), 0.1 mM nonessential amino acids (Sigma–Aldrich), 0.1 mM 2-β-mercaptoethanol (2β-ME, Sigma–Aldrich) and 1000 IU/ml leukemia inhibitory factor (LIF, Chemicon, Millipore, Billerica, USA). The culture medium was renewed every day.

Coculture of the ADSCs and ES cells

Royan B1 mouse ES cells, with a density of 10^5 cells/ml, were cultured on top of six-well transwell clear inserts, 0.4-μm pore size (Greiner Bio-One, Kremsmünster, Austria). The wells of six-well plates were already gelatinized and seeded with 5×10^4 cells/ml ADSCs. After 48 h, culture inserts were removed, and the expression of proliferating cell nuclear antigen (PCNA), MSC markers, CD73 and CD105, and pluripotency markers, OCT4 and Sox2, was assessed in the control and cocultured ADSCs by RT-PCR and qPCR.

Neural differentiation of the cocultured ADSCs

Neural differentiation of the cocultured ADSCs was initiated in a medium containing DMEM and 15% KoSR with or without 10^{-8} M RA. Two weeks after neural induction, the expression of neural-specific genes and proteins were analyzed by RT-PCR, qPCR, immunocytochemistry and Western blot.

RT-PCR and qPCR

Total RNA was extracted using High Pure RNA Isolation Kit (Roche), according to manufacturer's instructions. Then, 1 μg of total RNA was transcribed into cDNA using oligo-dT primers and RevertAid Reverse Transcriptase (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA). The synthesized cDNA was used in PCR reactions with gene-specific primers (Table 1).

Quantitative real-time PCR reactions were performed on a Rotor-Gene 6000 real time analyzer (Corbett Research, Qiagen) using SYBR® Green Master (Exiqon, Vedbaek, Denmark) and specific primers for target genes (Table 1). β-Tubulin 5 (Tubb5) and eukaryotic translation elongation factor 2 (eEF2) were considered as internal controls for normalization of the acquired data. Relative gene expression was calculated by comparative method using REST 2009 (Relative Expression Software Tool, Qiagen, Germany) based on Pair Wise Fixed Reallocation Randomization Test© (Pfaffl et al., 2002).

Immunocytochemistry and western blotting

Two week-differentiated ADSCs were fixed using 4% paraformaldehyde, permeabilized using 0.2% Triton X-100, blocked by 10% goat serum (Gibco) and incubated with primary and secondary antibodies for 45 min at 37 °C. Antibodies used in this study included monoclonal antibodies for β-tubulin III, MAP2 and Neurofilament 68 (NEFL) and FITC-conjugated goat anti-mouse IgG (all from Sigma–Aldrich). Preparations were examined using an inverted fluorescence microscope (Eclipse TE 2000U) and photographs were taken by a DXM1200F high-resolution digital camera (both from Nikon, Tokyo, Japan).

For Western blot analysis, differentiated cells were homogenized in ice-cold RIPA lysis buffer containing