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Neural differentiation of mouse embryonic and mesenchymal stem cells in a simple medium containing synthetic serum replacement

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ABSTRACT

Neural differentiation of embryonic and adult stem cells has been reported previously. Several studies have used different proportions of serum or a cocktail of growth and differentiation factors for this purpose. In the present study, we examined neural differentiation of mouse embryonic stem (ES) cells in KoSR-containing media. We also investigated neural differentiation of mouse adipose tissue-derived stem cells (ADSCs) in a medium containing KoSR, a synthetic serum replacement, and compared it with neural differentiation in low-serum condition. Meanwhile, effect of β-ME on neural differentiation was investigated in both conditions. As revealed by RT-PCR and immunocytochemistry analyses, KoSR-containing medium induced neural differentiation of mouse ES cells. Moreover, under the culture conditions we tested, ADSCs were differentiated to neuron-like cells and expressed some neuronal markers. Low concentration of β-ME improved neuron-like differentiation of the ADSCs in the 4% FBS-supplemented medium, while addition of β-ME in KoSR condition decreased neural differentiation. KoSR-containing medium without any additional factor improved generation of neuron-like cells, upregulated the expression of mature neuronal markers and led to the formation of cytoplasmic processes. In summary, our findings are indicating that mouse embryonic and mesenchymal stem cells are capable of neural development in KoSR-containing media.

1. Introduction

Neurogenesis occurs continuously in several regions of the adult mammalian brain (Altman, 1969; Bai et al., 2003; Eriksson et al., 1998; Gould et al., 1999; McKay, 1997; Ming and Song, 2005). However, regenerative capacity of the nervous system is not sufficient for complete replacement of the lost neurons following neurodegenerative disorders or CNS injuries. Stem cell transplantation has provided a promising tool for replacement of damaged neurons in the CNS. There are two main groups of stem cells, embryonic and adult stem cells. Although ES cells have an excellent potential for differentiation, their clinical application has been confronted by several problems including immunorejection, tumorigenicity and ethical issues. To overcome these problems, transplantation of autologous adult stem cells can be implemented as a more practical and feasible choice (Taha, 2010).

During recent years, adipose tissue has been identified as an accessible and rich source of stem cells (Fujimura et al., 2005; Halvorsen et al., 2000; Zuk et al., 2001). ADSCs can be cultured at the presence of appropriate factors to generate osteogenic, adipogenic, myogenic or chondrogenic lineages (Dicker et al., 2005; Guilak et al., 2006; Hachisuka et al., 2007; Lin et al., 2005; Zuk et al., 2001). In addition, several groups have shown the differentiation of rat, mouse, piglet, Rhesus monkey and human ADSCs to neurons and glial cells (Ashjian et al., 2003; Fujimura et al., 2005; Huang et al., 2007; Kang et al., 2003; Ning et al., 2006; Safford et al., 2002; Yang et al., 2004; Zuk et al., 2002). ADSCs express nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF) which are known neurotrophic factors (Kang et al., 2003; McCoy et al., 2008; Rehman et al., 2004).

So far, several research groups have reported neural differentiation of ADSCs in low-serum or serum-free media. Most of these studies have used a cocktail of neural inducing factors. Safford et al. (2002) used butylated hydroxianisole (BHA), KCl, valporic acid, forskolin, hydrocortisone and insulin for neural differentiation

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of ADSCs. Fujimura and colleagues (Fujimura et al., 2005) induced neural differentiation of mouse ADSCs at the presence of insulin, indomethacin and isobutylmethylxanthin. A cocktail of β-mercaptoethanol (β-ME), GNDF, BDNF, retinoic acid (RA) and 5-azacytidine was used by Pavlova et al. (2012). Abdanipour and Tiraihi used selegiline, sonic hedgehog (SHH) and RA for differentiation of ADSCs into motoneuron-like cells (Abdanipour and Tiraihi, 2012). Neurogenic media containing β-ME, dimethyl sulfoxide (DMSO) and BHA or basic fibroblast growth factor (bFGF) and forskolin (Jang et al., 2010) have also been used by the other investigators (Qian et al., 2010; Woodbury et al., 2000). Moreover, spontaneous differentiation of ADSCs toward neuronal phenotype has been reported (Qian et al., 2010; Taha and Hedayati, 2010; Tseng et al., 2007; Yang et al., 2004).

In the present study, we evaluated the effectiveness of a medium containing a synthetic serum replacement (KoSR) for neural differentiation of mouse ES cells and ADSCs. We also compared potential of the ADSCs for neural differentiation between low-serum condition and KoSR-containing medium. The effect of β-ME, a known neurogenic factor (Woodbury et al., 2000), on neural differentiation of the ADSCs was also examined in these two conditions. We showed that KoSR-containing medium provides an efficient and simple condition for neural differentiation of mouse ES cells and ADSCs.

2. Material and methods

2.1. Neural differentiation of mouse ES cells

The mouse ES cell line Royan B1 (Royan Stem Cell Bank, Royan Institute, RSCB0001), obtained from C57BL/6 strain mouse blastocyst (Baharvand et al., 2004; Baharvand and Matthaei, 2003), was used in the present study. ES cells were cultured on top of a mitomycin C-treated mouse embryonic fibroblast (MEF) feeder layer at the presence of leukemia inhibitory factor (LIF, Chemicon, ESGRO), as explained previously (Taha et al., 2007). The ES cells were dissociated from the feeder layer, and differentiation was initiated by embryoid body formation using hanging drop method, as described previously (Taha and Valojerdi, 2008). Differentiation medium consisted of Knockout™ Dulbecco's Modified Eagle's Medium (Ko-DMEM, high-glucose, with sodium pyruvate, Gibco, Life Technologies, USA) supplemented with 15% Knockout™ Serum Replacement (KoSR, Gibco), 0.1 mM β-mercaptoethanol (Sigma–Aldrich, St. Louis, MO, USA), 1 mM L-glutamine, 1% nonessential amino acid stock and 1% penicillin–streptomycin (all from Gibco).

2.2. Isolation and culture of mouse ADSCs

All animal experiments were approved by ethical committee of the National Institute of Genetic Engineering and Biotechnology. ADSCs from the inguinal adipose tissue of 8 to 10-week old NMRI mice were isolated using 2 mg/ml collagenase A (Roche Applied Science, Germany) digestion, as previously described (Taha and Hedayati, 2010). Isolated cells were plated at 5×10^4 cells/ml seeding density in 6-cm tissue culture plates, and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 20% fetal bovine serum (FBS, Gibco). Cells were daily observed by an inverted phase-contrast microscope and were passaged after reaching 80–90% confluency. The culture medium was changed every 2 days.

2.3. Neural differentiation of the ADSCs

Third-passaged ADSCs were cultured in 0.1% gelatinized 6-well tissue culture plates with a density of 5×10^4 cell/ml. The cells were cultured to reach a confluence of 90%, and then culture media were replaced by the differentiation medium. Neural differentiation of the ADSCs was induced under two different culture conditions: DMEM plus 4% FBS and DMEM plus 15% KoSR. To determine the effects of β-mercaptoethanol (Sigma) on neural differentiation of the ADSCs, differentiation media were supplemented with the final concentrations of 0.1 or 1 mM β-ME. Two groups of ADSCs were cultured under the same conditions without β-ME as the controls. Medium was changed every two days. Morphological changes of the ADSCs were observed daily by an inverted microscope.

2.4. Proliferation/viability assay by MTT

Third-passaged ADSCs were plated in 96-well tissue culture plates at a density of 5000 cells/well. After 24 h, the growth medium was replaced by 4% FBS- or KoSR-containing media. The cells were treated with 0.1 or 1 mM β-ME and were cultured for two weeks. Every treatment group was performed in quadruplicate and the experiment was repeated three times.

For MTT assay, medium of each well was replaced by 100 μl RPMI 1640 (Gibco). Then 10 μl of 12 mM MTT stock solution was added to each well, and the plates were incubated for 4 h at 37 °C. Next, 75 μl of the medium was removed, and 50 μl of DMSO was added to solubilize the MTT tetrazolium crystals. Finally, the plates were shaken, and light absorbance was read at 490 nm by a microplate reader (Labsystem Multiskan MS).

2.5. RNA isolation and RT-PCR

Total RNA was extracted using High Pure RNA Isolation kit (Roche), according to the manufacturer's instructions. Then, 1 μg of total RNA was transcribed to cDNA using oligo-dT primers and RevertAid H Minus MMuLV Reverse Transcriptase (Fermentas, Thermo Fisher Scientific Inc., USA). Obtained cDNA was amplified by 35 cycles of denaturation at 94 °C for 30 s, annealing at the optimum temperature for 30 s and extension at 72 °C for 45 s using specific primers (Table 1). PCR products were size fractionated by 1.5% agarose gel electrophoresis and visualized on a UV transilluminator.

2.6. Quantitative real-time PCR

For quantitative real-time PCR (qPCR) analysis, specific primers for Nestin, NSE, NeuN and Synaptophysin (Syp) genes and β-tubulin 5 (Tubb5) as the housekeeping gene were used (Table 1). Reactions were performed using RealQ PCR Master (Ampliqon A/S, Denmark) on a Rotor-Gene™ 6000 (Corbett Research, Qiagen, Germany) real-time analyzer with cycling parameters of a hot start at 95 °C for 15 min, then 45 cycles of 15 s at 95 °C and 40 s at 61 °C. Comparative quantitation was performed between selected groups using REST 2009 (Relative Expression Software Tool, Qiagen). For quantitative analysis, the experiments were repeated four times.

2.7. Immunocytochemistry

For immunostaining, differentiated ADSCs were fixed using 4% paraformaldehyde, permeabilized by 0.5% Triton X-100 (Sigma), blocked by 10% goat serum (Gibco) and incubated with primary and secondary antibodies, each for 45 min at 37 °C. Antibodies used in this study included monoclonal antibodies for β-tubulin III (Sigma), microtubule associated protein 2 (MAP2, Sigma), neurofilament-68, synapsin I, PCNA (all from Millipore, USA) and anti-mouse FITC-conjugated IgG antibody (Sigma). Preparations were examined and photographed by a Nikon fluorescence microscope (Nikon, Elipse TE 2000U, Japan).