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Culture and neural differentiation of rat bone marrow mesenchymal stem cells *in vitro*

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Abstract

Adult bone marrow mesenchymal stem cells (MSCs) can differentiate into several types of mesenchymal cells, including osteocytes, chondrocytes, and adipocytes, but can also differentiate into non-mesenchymal cells, such as neural cells, under appropriate experimental conditions. Until now, many protocols for inducing neuro-differentiation in MSCs *in vitro* have been reported. But due to the differences in MSCs' isolation and culture conditions, the results of previous studies lacked consistency and comparability. In this study, we induced differentiation into neural phenotype in the same MSCs population by three different treatments: β-mercaptoethanol, serum-free medium and co-cultivation with fetal mouse brain astrocytes. In all of the three treatments, MSCs could express neural markers such as NeuN or GFAP, associating with remarkable morphological modifications. But these treatments led to neural phenotype in a non-identical manner. In serum-free medium, MSCs mainly differentiated into neuron-like cells, expressing neuronal marker NeuN, and BME can promote this process. Differently, after co-culturing with astrocytes, MSCs leaned to differentiate into $GFAP^+$ cells. These data confirmed that MSCs can exhibit plastic neuro-differentiational potential *in vitro*, depending on the protocols of inducement.

Keywords: Rat; Mesenchymal stem cells; Cell culture; Neural differentiation

1. Introduction

In adult bone marrow, there are two distinct populations of stem cells. One is hematopoietic stem cells (HSCs) that can renew circulating blood elements, such as red cells, monocytes, platelets, granulocytes and lymphocytes. The other is mesenchymal stem cells (MSCs), which can differentiate into osteoblasts, chondroblasts, adipocytes, and hematopoiesis-supporting stroma (Prockop, 1997; Pittenger et al., 1999; Bianco et al., 2001). Besides originating the mesenchymal tissue, many studies demonstrated that MSCs could differentiate into various non-mesenchymal tissue lineages under appropriate experimental conditions *in vitro* and *in vivo*, such as hepatocytes (Jiang et al., 2002; Schwartz et al., 2002), cardiomyocytes (Makino et al., 1999; Toma et al., 2002), lung alveolar epithelium (Kotton et al., 2001), even neuron and glia (Woodbury et al., 2000; Sanchez-Ramos et al., 2000; Azizi et al., 1998; Kopen et al., 1999; Muñoz-Elias et al., 2004). The amazing neuro-differentiational potential of MSCs attracts intense interest in the possible applications of MSCs in cell and gene therapy for neurological disease, because MSCs can be obtained from bone marrow easily and expanded rapidly *in vitro* (Colter et al., 2000; Javazon et al., 2001).

Until now, it has been reported that MSCs could induce neuro-differentiation through many ways *in vitro*, such as

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chemical inducers (Woodbury et al., 2000), cytokines (Jiang et al., 2002, 2003; Hermann et al., 2004), co-culture with nerval cells (Wislet-Gendebien et al., 2003, 2005), chemical inducers plus cytokines (Sanchez-Ramos et al., 2000; Kohyama et al., 2001) and transfect plus cytokines (Dezawa et al., 2004), etc. But unfortunately, due to the differences in MSCs' isolation and culture conditions, the results of these previous studies lacked consistency and comparability. On the other hand, the mechanism and the crucial regulative factor of neuro-differentiation in MSCs are still unclear. In our experiment, we induced differentiation into neural phenotype in the same MSCs population by three different treatments: β-mercaptoethanol, serum-free medium and co-cultivation with fetal mouse brain astrocytes. We also evaluated the diversity of differentiational patterns in the three treatments. It will be helpful for us to further understand the trait of neuro-differentiation in MSCs.

2. Methods and materials

2.1. Preparation and culture of MSCs

For isolation of rat MSCs, tibias and femurs were dissected from adult Sprague–Dawley rat (200–300 g; Animal Research Center, Medical Department, Peking University). The ends of the bones were cut, and the marrow was extruded with 5 ml D-Hank's solution by using a needle and syringe. The marrow was resuspended in 0.84% NH_4Cl solution. After 5 min, about 20×10^6 marrow cells were plated on 25 cm^2 plastic flask in α-MEM (Hyclone), supplemented with 15% fetal bovine serum (FBS, PAA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. All of the cells were incubated at 37 °C with 5% humidified CO_2. After 24 h, the nonadherent cells were removed by replacing the medium. The medium was added and replaced every 3 or 4 days for about 2 weeks. When the cells grew to confluent, they were harvested with 0.25% trypsin and 1 mM EDTA (Hyclone) for 5 min at 37 °C, replated on 25 cm^2 plastic flask, again cultured to next confluence, and harvested. Before being used in inducing neuroectodermal differentiation, the MSCs had been expanded for about 20 passages.

2.2. Chemical induction for neuronal differentiation

Sub-confluent cultures of MSCs (approx. 3×10^5 cells/25 cm^2 plastic flask) were maintained in α-MEM plus 15% FBS. Twenty-four hours prior to neuronal induction, medium was replaced with preinduction medium consisting of α-MEM, 15% FBS, and 1 mM β-mercaptoethanol (BME; Sigma). To initiate neuronal differentiation, the preinduction medium was removed, and the cells were washed twice with D-Hank's solution and transferred into neuronal induction medium composed of DMEM/F12 (Hyclone) and 5 mM BME. Cells were fixed for immunocytochemistry at 3 h postinduction.

2.3. Neuro-glial differentiation in serum-free medium

Passage 20–25 MSCs were plated on either poly-L-lysine-coated (100 μg/ml, Sigma) coverslips in six-well plate or 25 cm^2 plastic flask, and grew in α-MEM plus 15% FBS. When the cells became sub-confluent (approx. 3×10^5 cells/25 cm^2 plastic flask), to initiate differentiation, they were washed three times with D-Hank's solution. Then the medium was replaced with serum-free DMEM/F12, containing N2 and B27 supplements (Invitrogen, which are two multicomponent cell culture supplements). From 6 to 48 h postinduction, the cells on the coverslips were fixed for immunocytochemistry, and the cells in plastic flasks were harvested for RT-PCR analysis.

2.4. Preparing and culture of fetal mouse brain astrocytes

Fetal mouse brains were dissected from embryonic day 15 (E15) CD-1 mice fetuses (Animal Research Center, Medical Department, Peking University). These brains were minced into small fragments in Hank's balanced salt solution (HBSS) and incubated in 0.125% trypsin plus 0.05% DNase (Sigma) at 37 °C for 20 min. The further dissociation was achieved mechanically by up-and-down passage through a sterile narrowed Pasteur pipette. The resulting cell suspension was filtered with a 70-μm-pore filter. Then the cells were centrifuged at 800 rpm for 5 min and resuspended in DMEM (Hyclone) plus 10% FBS. Cells were plated onto 100 mm tissue culture dishes (precoated with poly-L-lysine overnight) at a density of about 6×10^5 cells/cm^2. The astrocytes were cultured in DMEM plus 10% FBS until confluent, and then were harvested with 0.25% trypsin and 1 mM EDTA for co-culture experiment.

2.5. Co-culture of rat MSCs and fetal mouse brain astrocytes

When MSCs grew to 40–60% confluent (approx. $1–2 \times 10^5$ cells/25 cm^2 plastic flask) in α-MEM plus 15% FBS, bromodeoxyuridine (BrdU; Sigma) at 6 μg/ml was added to the medium to label dividing MSCs for 3 days. Then the BrdU-labeled MSCs were trypsinized and co-cultured with fetal mouse brain astrocytes (about 1×10^4 MSCs and 1×10^6 astrocytes per milliliter) on poly-L-lysine-coated 24-well plate in DMEM/F12 containing N2 and B27 supplements. After 5 days, the mixed cultures were fixed for immunofluorescent double-labeling as described below.

2.6. Immunocytochemistry (ICH) and immunofluorescence

The expression of each antigen was examined in separate experiments at least three times. After each treatment, cells were fixed with 4% (v/v) paraformaldehyde for 15 min at room temperature and washed three times with 0.01 M phosphate-buffered saline (PBS). They were then permeabilized in 1% Triton X-100 (v/v) for 15 min and washed three times in PBS. Non-specific binding was blocked by a 1-h treatment in 3% normal horse serum–PBS. The cells were incubated with primary antibodies at 4 °C overnight and then 2 h at room temperature. Sources and dilution of primary antibodies were as follows: mouse anti-nestin (1:250; Chemicon, Temecula, CA, USA; monoclonal antibody to label neuroepithelial stem cells), mouse anti-neuron-specific nuclear protein (anti-NeuN, 1:800; Chemicon, Temecula, CA, USA; monoclonal antibody to label neurons), mouse anti-glial fibrillate acid protein (anti-GFAP, 1:1000; Novacastra Lab. Ltd; monoclonal antibody to label astrocytes). After three-time washes in 0.1 M PBS, cells were incubated with biotinylated horse anti-mouse IgG (1:500; Jackson ImmunoResearch) for 2 h at room temperature, followed by 2 h of incubation in avidin-biotinylated peroxidase complex (1:150; Vector Elite Kit) at room temperature. Diaminobenzidine (DAB; Sigma; 0.05%) was used as chromagen, with reactions sustained for 30 min at room temperature and in the dark. For negative controls, replacing the primary antibodies with normal horse serum or staining without secondary antibody was investigated in each experiment, and in either case, no specific positive staining was detected.

For BrdU/NeuN or BrdU/GFAP immunofluorescent double-labeling, the fixed cells were incubated in 2 N HCl at 37 °C for 30 min, followed by incubation in borate buffer, pH 8.4, for 20 min. The cells were subsequently incubated overnight at 4 °C with primary antibodies: rat anti-BrdU (1:3000; Accurate Chemicals, Westbury, NY, USA), mouse anti-NeuN and mouse anti-GFAP as mentioned above. After rinsed with 0.1 M PBS for three times, the cells were incubated with secondary antibodies: AlexaFluor 488-conjugated rabbit anti-rat IgG (1:500; Molecular Probes, Eugene, OR, USA) and TRITC-conjugated donkey anti-mouse IgG (1:50; Jackson ImmunoResearch) for 2 h at room temperature. Labeled cells were observed on an Olympus optic microscope (IX 70, Tokyo, Japan) using appropriate fluorescence filters, and imaged using a SPOT II camera (Diagnostic Instruments, Sterling, MI, USA).

2.7. RT-PCR analysis

Total RNA from MSCs (normal, BME-induced for 5 h and treated with serum-free medium) was extracted by TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions. Subsequently, 5 μg of total RNA was converted into cDNA by using Moloney-murine leukemia virus (M-MLV) Superscript II reverse transcriptase (Promega) and random primers (Oligo dT_{18};