Generation of Neuronal-Like Cells from Umbilical Cord Blood-Derived Mesenchymal Stem Cells of a RFP-Transgenic Cloned Cat

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(Received 20 November 2007/Accepted 14 March 2008)

ABSTRACT. Umbilical cord blood (UCB)-derived mesenchymal stem cells (MSCs) are multipotent adult stem cells, which can differentiate into cells of connective tissue and neural lineages. This study investigated the potential for neuronal differentiation of red fluorescent protein (RFP)-transgenic cat UCB-derived MSCs. The cells were cultured in pre-induction medium for 24 hr and in neuronal-induction medium for 72 hr. Immunofluorescent staining showed that 6.85% of the total cells were β III-tubulin-positive, 3.37% were neurofilament light (NF-L)-positive and 7.04% were neurofilament medium (NF-M)-positive. A β III-tubulin band was detected by western blot analysis. Our results demonstrate that RFP-transgenic UCB-derived MSCs can be differentiated into neuronal cells in vitro. Thus, RFP-transgenic MSCs could provide alternative tracing material for stem cell transplantation.

KEY WORDS: differentiation, mesenchymal stem cell, neuronal-like cell.

NOTE Laboratory Animal Science

Umbilical cord blood (UCB)-derived mesenchymal stem cells (MSCs) are one alternative source for the treatment of central nervous system disorders. Since these cells can not only differentiate into cells of connective tissue and neural lineages [3, 7, 13, 18], but also possess many advantages involving immaturity, less immunogenicity, without ethical problems and without harm to the mother or infant. Initial studies by Ha et al. [3] have shown that neural markers are expressed in cultured human UCB monocytes. In vitro studies have further demonstrated the neural differentiation potential of UCB-derived MSCs [7, 13, 18]. Some in vivo experiments have shown functional improvements in corresponding animal model systems following UCB-derived stem cell transplantation [4, 5, 17, 19].

The domestic cats are phylogenetically close to humans. They have been used extensively in studies of normal physiology and disease which is associated with human, particularly of the nervous system [1, 20]. Park et al. [12] have demonstrated that reporter genetic modification does not affect the self-renewal and neuronal differentiation ability of neural stem cells in transgenic mice. In the present study, we examined whether UCB-derived MSCs of RFP-transgenic cloned cat emit red fluorescence and remain the potential of neuronal differentiation.

All experimental procedures were conducted according to the Guidelines for Animal Experiments of Gyeongsang National University. RFP-transgenic cloned cat have been produced using a plasmid (pLHCRW)-infected cat fibroblast cells (RFP-transfected cells) for somatic cell nuclear transfer (BOR papers in press). Cat UCB samples were obtained from the unborn umbilical cord of full-term deliveries of two cloned cats with RFP transgenes and processed within 2 hr of collection. Red blood cells were removed by adding 1 ml of 1 M NH₄Cl to the UCB pellet and centrifuging for 10 min at 2000 rpm. The cell pellet was plated at density of 1 × 10⁶ cells per cm² in 24-well plates in 1 ml medium consisting of low-glucose Dulbecco’s Modified Eagle’s medium (DMEM; Gibco-BRL), 10% fetal bovine serum (FBS; Gibco-BRL), 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma). The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ until they reached 90% confluence. For passage 1 and subsequent passages, the cells were replated at a mean density of 2 × 10⁵ cells per cm² in 90-mm dishes. Cells were harvested from the second passage.

To detect cell-surface antigens, the cells were immunolabeled with cat-specific antibodies against CD9 (Serotec, Raleigh, NC), CD18, CD44, and CD45-like (VMRD, Pullman, WA), followed by FITC-conjugated secondary antibodies. Labeled cells were analyzed by flow cytometry.

For neural differentiation, the cells were seeded at a density of 2 × 10⁴ cells per cm² in 6-well plates with glass cover slips and cultured in pre-induction medium, consisting of DMEM, 10% FBS, 10 ng/ml basic fibroblast growth factor (bFGF; Roche Applied Science), and 1 mM β-mercaptoethanol (Sigma), 24 hr prior to neural induction. Then, the cells were transferred to a neurogenic medium, composed of DMEM, 2% dimethyl sulfoxide (DMSO; Sigma), 200 mM butylated hydroxyanisole (BHA; Sigma-Aldrich), 10 μM forskolin (Sigma), 5 μg/ml insulin-transferrin-sodium selenite (Roche Applied Science), 10 ng/ml epidermal growth factor (EGF; Roche Applied Science), and 20 ng/ml bFGF, for 72 hr. The bFGF and EGF were added to the neurogenic medium daily during neuronal induction. Non-induced con-
control cells were cultured in DMEM supplemented with 10% FBS for 96 hr.

To further identify MSCs, the primary chicken anti-vimentin antibody (1:5,000; Chemicon) was employed for 2 hr; followed by FITC-conjugated anti-chicken IgG (1:320; Sigma) for 1 hr. To identify neuronal differentiation, the cells were processed using primary antibodies anti-β III-tubulin (1:400, mouse monoclonal; Sigma), anti-NF-L (1:500, rabbit polyclonal; Chemicon), and anti-NF-M (1:40, mouse monoclonal; Sigma) for 2 hr, followed by FITC-conjugated anti-mouse IgG (1:64; Sigma) for 1 hr. Nuclei of cultured cells were stained using Hoechst 33342 (1 µg/ml; Sigma) for 5 min at room temperature. To exclude the possibility of non-specific staining, secondary antibodies were used at the same concentrations as the primary antibodies. Labeled cells were analyzed with a fluorescent microscope (Olympus, Japan). Total and positively stained cells were counted in 10 random identical visual fields (10× magnification) on four cover slips for each marker in three different experiments.

After neuronal induction for 72 hr, the differentiated cells were homogenized in lysis buffer (10 mM Tris-HCl, 5 mM EDTA, 0.15 M NaCl, and 10% Triton X-100). Total protein (10 µg) of each sample was separated by SDS-PAGE and transferred electrophoretically to nitrocellulose filters. Immunoblotting was carried out with antibodies against β III-tubulin (1:800, monoclonal; Sigma) or GAPDH (1:300, monoclonal; Chemicon), followed by horseradish peroxidase-conjugated anti-mouse IgG (1:5,000, Chemicon). Blots were developed by the enhanced chemiluminescence method (Amersham Biosciences, UK). Non-induced cells and PC12 cells acted as negative and positive control, respectively.

Initially, the MSCs from passage 0 comprised a morphologically heterogeneous population of cells that included fibroblast-like, spindle-shaped, and small round morphologies. Later, MSCs began to display a broadened, flat morphology. The cat MSC-surface antigen profile was ascertained by staining with cat-specific monoclonal antibodies followed by flow cytometry as shown in Fig. 1. Cat MSCs were strongly positive for CD9 and CD44 and negative for CD18 and CD45-like. Thus, these cells had retained the MSC phenotype [10]. Furthermore, the MSCs were further identified by anti-vimentin antibody (data not shown). At 24 hr post-neuronal induction, the majority of the MSCs displayed a typical neuronal shape. Cell bodies became increasingly spherical and refractile, exhibiting a neuronal perikaryal appearance. Some cells were in contact through their long branches with evident varicosities. Undifferentiated MSCs retained a flattened morphology. Cell bodies became increasingly spherical and refractile, exhibiting a neuronal perikaryal appearance. Some cells were in contact through their long branches with evident varicosities. Undifferentiated MSCs retained a flattened morphology. At 72 hr post-neuronal differentiation, immunocytochemical staining showed that 6.85% were β III-tubulin-positive, 3.37% were NF-L-positive, and 7.04% were NF-M-positive (Fig. 2). Non-induced cells were negative with all of the above antibodies (data not shown). Western blots of the cultures confirmed the presence of β III-tubulin protein in induced, but not in non-induced cells (Fig. 3).

Here we describe for the first time the neuronal differentiation of UCB-derived MSCs from RFP-transgenic cats. Some studies have demonstrated that neural-cell differentiation is significantly increased by EGF plus bFGF [7, 8, 21]. bFGF and EGF are very important growth factors in the central nervous system and play significant roles in its developmental processes [2, 6, 15]. Different concentrations of bFGF can induce cortical neural precursor cells to proliferate or differentiate into the three fundamental neuronal lineages (neurons, astrocytes, and oligodendrocytes), but EGF can promote the later developing phase of neural precursor cells to proliferate and differentiate [14, 16]. Therefore, we
added bFGF (20 ng/ml) and EGF (10 ng/ml) to the neurogenic medium daily during the neuronal induction stage. Our results differ from those reported by Lu et al. [9] and Neuhuber et al. [11]. They differentiated BM-derived MSCs using chemical inducing agents (DMSO and BHA) for 24 hr and conclude that the phenomenon of neuronal differentiation is an artifact or pseudoneuronal phenotype. Thus, it will be important to determine whether our differentiated cells possess functional and electrophysiological characteristics of neurons.

In conclusion, our results show that RFP is expressed in RFP-transgenic MSCs, and the MSCs do not lose their potential for neural differentiation. Thus, RFP-transgenic MSCs should be a viable alternative material for stem cell transplantation.

ACKNOWLEDGEMENTS. This work was supported by a grant from the Korea Science and Engineering Foundation (KOSEF; #M105250 10001–05N25 01–001 10), funded by the Korean government (MOST). Guang Zhen Jin, Eugene Choi, Young Soo Lee, and Su Jin Cho were supported by scholarships from the Post BK21 Program.

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Fig. 3. β III-tubulin and GAPDH expression in RFP-transgenic MSC-derived neuronal cells by western blot analysis. (A) Non-induced cells. (B) PC12 cells as a positive control. (C) Neuronally-induced cells. The β III-tubulin band was observed at approximately 55 kDa in neuronally-induced cells, but not in non-induced cells.