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Biochemical and Biophysical Research Communications 326 (2005) 425–434
BBRC
www.elsevier.com/locate/ybbrc

Isolation and characterization of neural stem cells from human fetal striatum

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Received 2 November 2004
Available online 19 November 2004

Abstract

This paper described that neural stem cells (hsNSCs) were isolated and expanded rapidly from human fetal striatum in adherent culture. The population was serum- and growth factor-dependent and expressed neural stem cell markers. They were capable of multi-differentiation into neurons, astrocytes, and oligodendrocytes. When plated in the dopaminergic neuron inducing medium, human striatum neural stem cells could differentiate into tyrosine hydroxylase positive neurons. hsNSCs were morphologically homogeneous and possessed high proliferation ability. The population doubled every 44.28 h and until now it has divided for more than 82 generations in vitro. Normal human diploid karyotype was unchanged throughout the in vitro culture period. Together, this study has exploited a method for continuous and rapid expansion of human neural stem cells as pure population, which maintained the capacity to generate almost fifty percent neurons. The availability of such cells may hold great interest for basic and applied neuroscience.

Keywords: Neural stem cells; Striatum; Adherent; Expansion; Proliferation; Differentiation

Self-renewing multi-potential neural stem cells (NSCs) can be isolated from both developing and adult central nervous system (CNS) [1–3], such as subventricular zone (SVZ), subgranular layer of hippocampus, striatum, and other brain regions [4–7]. Long-term culture systems allow continuous propagation of potential heterogeneous populations of neural stem and progenitor cells either as a monolayer on substrate-coated plates or as suspended clonal aggregates of cells known as neurospheres [8,9]. Mahendra S. Rao isolated adherent cultures of E10.5 rat neuroepithelial cells "termed neuroepithelial (NEP) stem cells" from the caudal neural tube at early stages of development [10], while most previous reports showed only more restricted precursors could be isolated as adherent culture on fibronectin and polyornithine [11,12]. On the other hand, the routine selection of neurospheres has been reported from high concentration suspension culture supplemented with basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and leukemia inhibitory factor (LIF) [13–15]. Although neurospheres possess the capability of self-renewing and multi-differentiation into mature neuronal and glial progeny [14,16,17], many studies demonstrated an inter-clonal heterogeneity in the expression of neural lineage-specific markers [9,18,19]. Recently, neurospheres were found to consist of a mixed population of neural stem and more restricted progenitor cells, whereby every 10–20 thousand cell sphere contains <1% NSCs and >99% progenitor cells [20]. Moreover, neurospheres were passaged about every 7–30 days [2,21] and it was reported that the number of

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.11.044

cultured NSCs derived from the embryonic human forebrain could be expanded at most to a 10^7-fold of this heterogeneous population in 1 year in vitro [22].

The problem of studying neural stem cells in general has been compounded by the difficulty in continuous and rapidly propagating quantities of a pure population of neural stem cells in vitro. Based on the findings mentioned above, in this study, we concentrated on whether human fetal neural stem cells could be isolated and expanded rapidly as pure population in monolayer and differentiate into neurons and glia.

Materials and methods

Cultivation and enrichment of human fetal striatum neural stem cells (hsNSCs). Human fetal natural aborted samples were obtained with mother's consent from maternity hospital. Permission to use human embryonic tissue was granted by *Peking University Health Science Center's Ethical Committee*. The human fetal striatum tissue fragment at 12–20 weeks gestational age was dissected, washed with sterile PBS and mechanically separated into small pieces of size about 1 mm^3, and triturated gently with a fire-polished, siliconized Pasteur glass pipette. Finally, the cell suspension was passed through a 70-μm strainer and cultivated in uncoated 75 cm^2 cell culture flasks at 1×10^6 cells/ml density. The primary culture medium was Dulbecco's modified Eagle's medium/nutrient mixture F12 (DMEM/F12, Gibco-BRL) supplemented with 10% fetal bovine serum (FBS, Hyclone), EGF (20 ng/ml, Gibco-BRL), bFGF (20 ng/ml, Gibco-BRL), and LIF (10 ng/ml, Chemicon). After 48–72 hours (h), no more than 10 adherent clones (10–20 cells/clone) appeared in each 75 cm^2 flask. The clones were washed with fresh sterile PBS for five times to discard the suspended cells and replaced with fresh culture medium in the end. After about a period of 1–2 weeks, the initial adherent small clones began to show their unique proliferation ability and maintained good proliferation feature. They were cultured continuously to get human fetal striatum-derived NSCs named hsNSCs. When cells reached 95% confluence, passage was carried out. Cells were trypsinized with 0.25% trypsin (Gibco-BRL) and passaged at a 1:3 ratio. Cells from passage 5 to 20 were used in the following experiments.

The proliferation studies of hsNSCs. Cells for cell cycle analysis were trypsinized into single cell suspensions and then fixed with 70% ethanol for 30 min on ice. RNA was degraded by incubation with 100 μg/ml RNAase A (Sigma–Aldrich, St. Louis, MO) for 1 h at 37 °C. DNA was labeled with 20 μg/ml propidium iodide (Sigma). Cell cycle was analyzed by FACScalibur flow cytometry with the software ModiFit.

Growth curve was obtained by counting cells manually. hsNSCs were plated in 60 mm culture dishes supplemented with 4 ml culture medium at a density of 2×10^5 cells/dish. The numbers of cells on each of the three dishes were counted three times every day. The population doubling time of hsNSCs was estimated based on the logarithmic growth phase of cell growth curve.

Cells for proliferating cell nuclear antigen (PCNA) analysis were trypsinized into single cell suspension, permeated with PBS containing 0.3% Triton X-100 at 4 °C for 15 min, and then stained with fluorescein (FITC) labeled anti-PCNA antibody (Becton–Dickinson). Mouse IgG2ak-FITC (Becton–Dickinson) was used as isotype control. Stained cells were analyzed by FACScalibur flow cytometry (Becton–Dickinson). Result was shown as the overlaid histograms of PCNA with its isotype control.

G-banding chromosome analysis. hsNSCs chromosome analysis was performed by conventional Giemsa staining. Microscopic images of metaphases were acquired by a video camera on a Leica microscope (Optronic cooled CCD, MDE1850). The karyotypic analysis was performed with an Olympus microscope (Optronic cooled CCD). The karyotypic analysis was performed by the dedicated cytoscan-karyotyping FISH & CGH system (United Biotechnology USA).

Phenotype analysis by FACS. For membrane antigen staining, cells were trypsinized into single cell suspension and stained with phycoerythrin (PE) labeled antibodies anti-CD34, CD44, and CD90, or fluorescein (FITC) labeled antibodies anti-CD14, CD45, CD71, CD147, and HLA-DR (Becton–Dickinson). Mouse IgG2a-FITC, IgG1-FITC, IgG2a-PE, and IgG2b-PE (Becton–Dickinson) were used as isotype control. For cytoplasm antigen staining, cells were fixed with 4% paraformaldehyde in PBS and then permeated with 0.3% Triton X-100. Fixed cells were incubated with normal goat serum at 4 °C for 1 h and then incubated with primary antibodies (mouse normal serum was used as negative control) including mouse anti-nestin (1:100, Chemicon), Map2a, b (1:100, Neomarker) at 4 °C for 2 h and finally with secondary antibodies, FITC labeled secondary antibody goat anti-mouse IgG (1:200, Santa Cruz), at 4 °C for 40 min. Stained cells were analyzed by FACScalibur flow cytometry (Becton–Dickinson). Results were shown as the overlaid histograms of analyzed markers with their isotype controls.

Neuronal differentiation of hsNSCs in vitro. hsNSCs were plated on poly-L-lysine-coated 12 mm coverslips and 25 cm^2 uncoated cell culture flasks in various conditions to promote differentiation. To induce neuronal differentiation, cells were exposed to 10% FBS supplemented with isobutylmethylxanthine (IBMX, 0.5 mM, Sigma), forskolin (10 μM, Sigma), bFGF (100 ng/ml, Gibco), and dibutyryl cyclic AMP (dbcAMP, 1 mM, Sigma). After 3 days differentiation, fixed cells were stained with anti-β-tubulin III, Map2a, b, and E-NCAM antibodies to assess their capacity of differentiation into neurons. Cells were then plated in a medium containing 10% FBS and pituitary adenylate cyclase-activating polypeptide (PACAP, 100 nM, Sigma) for 5 days and checked with anti-GFAP, A2B5, and O4 antibodies to identify astrocytes and oligodendrocytes. In addition, under both aforementioned induced conditions, co-expression of neuron and glia markers was also examined. To investigate whether hsNSCs possessed the potential to differentiate into special phenotype neurons such as dopaminergic (DAergic) neurons, hsNSCs were plated in conditioned medium including sonic hedgehog (SHH, 100 ng/ml, Petrotech), fibroblast growth factor (FGF) 8 (100 ng/ml, Petrotech), glia-derived neurotrophic factor (GDNF, 10 ng/ml, Petrotech), all-*trans* retinoic acid (RA, 0.5 μM, Sigma), dbcAMP (1 mM), and 5% FBS termed DA neuron inducing medium for 7 days and then were checked with anti-TH antibody to identify DAergic neurons. Experiments were performed in triplicate, respectively.

Immunofluorescence. To stain cell membrane proteins, the living cells were rinsed with PBS, blocked with normal goat serum at room temperature for 30 min, and then incubated with anti-O4 monoclonal antibody (1:50, Santa Cruz) or PE labeled anti-CD44 (1:100, Becton–Dickinson), CD 90 (1:100, Becton–Dickinson) monoclonal antibodies. To stain cytoplasm and nuclear proteins, cells were rinsed, fixed and permeated with 0.3% Triton X-100 (Sigma) for 15 min, and then blocked with normal goat serum at room temperature for 30 min. Cells were incubated with primary antibodies at 4 °C overnight and subsequently incubated with secondary antibodies either TRITC labeled goat anti-rabbit IgG or FITC labeled goat anti-mouse IgG (1:200, Santa Cruz) according to the primary antibodies. Mouse or rabbit normal serum was used as negative control. The primary antibodies include mouse anti-Ki67 (1:100, Chemicon), nestin (1:100, Chemicon), β-tubulin III (1:200, Chemicon), Map2a, b (1:100, Neomarker), E-NCAM (1:50, Chemicon), TH (1:250, Sigma), and oligophrenin-4 (O4, 1:50, Santa Cruz) and rabbit anti-nestin (1:100, Neomarker), pax6 (1:100, Chemicon), sox2 (1:100, Chemicon), A2B5 (1:50, Santa Cruz), and glial fibrillary acidic protein (GFAP, 1:100, Neomarker) antibodies. Photographs were taken on an Olympus Immunofluorescent Microscope (Model BX51TR) and Apogee Instruments Microscopy Fluorescence System (Model KX85). For cell counts, three coverslips