Biochemical and Biophysical Research Communications 430 (2013) 604–609

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Improvement in protocol to generate homogeneous glutamatergic neurons from mouse embryonic stem cells reduced apoptosis

Ryosuke Fujiki [a,b], Akinobu Sato [a,b], Katsuhiko Hata [a,b], Fumi Tashiro [c], Noriko Yasuhara [d], Jun-ichi Miyazaki [c], Yoshihiro Yoneda [d], Masashi Fujitani [a,b,*], Toshihide Yamashita [a,b,*]

[a] Department of Molecular Neuroscience, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] JST, CREST, 5, Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan
[c] Division of Stem Cell Regulation Research, G6, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[d] Biochemistry, Medical School, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 17 November 2012
Available online 5 December 2012

Keywords:
ES-derived glutamatergic neuron
Neural progenitor cell
Survival
BDNF
bFGF

ABSTRACT

Obtaining a homogenous population of central nervous system neurons has been a significant challenge in neuroscience research; however, a recent study established a retinoic acid-treated embryoid bodies-based differentiation protocol that permits the effective generation of highly homogeneous glutamatergic cortical pyramidal neurons from embryonic stem cells. We were able to reproduce this protocol regarding the purity of glutamatergic neurons, but these neurons were not sufficiently healthy for long-term observation under the same conditions that were originally described. Here, we achieved a substantial improvement in cell survival by applying a simple technique: We changed the medium for glutamatergic neurons from the original complete medium to commercially available SBM (the Nerve-Cell Culture Medium manufactured by Sumitomo Bakelite Co. Ltd.) and finally succeeded in maintaining healthy neurons for at least 3 weeks without decreasing their purity. Because SBM contains glial conditioned medium, we postulated that brain-derived neurotrophic factor or basic fibroblast growth factor is the key components responsible for pro-survival effect of SBM on neurons, and examined their effects by adding them to CM. As a result, neither of them had pro-survival effect on pure glutamatergic neuronal population.

1. Introduction

A significant problem in neuroscience had been the lack of relevant culture system that allows unlimited generation of defined populations of post-mitotic, process-bearing central nervous system (CNS) neurons. Embryonic stem (ES) cells [1,2] have created the possibility of generating infinite numbers of any types of CNS neurons, and several protocols have been developed for differentiation of ES cells into neurons [3,4]. However, neuronal cultures derived from ES cells contain a variety of neuronal subtypes as well as neural precursor cells (NPCs) and non-neural cells, including glial cells.

Recent advances in culture techniques have overcome this issue. Bibel and co-workers revealed that their retinoic acid-treated embryoid bodies based protocol permits generation of a homogeneous population of glutamatergic cortical pyramidal neurons from ES cells [5,6]. The purity of this population reaches 90–95%, which is the highest for differentiation of glutamatergic neurons ever reported [7,8]. In our hands, this protocol was highly reproducible regarding the purity of ES cell-derived glutamatergic neurons, but these neurons did not survive longer than 2 weeks under the same conditions that were originally described [6]. In addition, we found that low-density culture of neurons resulted in low survival rate, but in order to assess axonal properties, synaptic transmission, and dendritic arborization of glutamatergic neurons, long-term low-density culture is required. Therefore, it is necessary to improve survival of these ES-derived neurons.

Here, we made a substantial improvement in cell survival by using a simple technique: We changed the medium for glutamatergic neurons from the original medium (complete medium, CM) to a commercially available medium (the Nerve-Cell Culture Medium from Sumitomo Bakelite Co. Ltd., SBM) and finally succeeded in

Abbreviations: CNS, central nervous system; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; ES cell, embryonic stem cell; NPC, neural progenitor cell; CA, cellular aggregate; N_2M, N_2 medium; CM, complete medium; SBM, the Nerve-Cell Culture Medium from Sumitomo Bakelite Co. Ltd.; ICC, immunocytochemistry; Tuj1, neuronal class III β-tubulin; VGLUT1, vesicular glutamate transporter 1; DAPI, 4′,6-diamidino-2-phenylinodole; PBS, phosphate-buffered saline.

* Corresponding authors at: Department of Molecular Neuroscience, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 3669.

E-mail addresses: masashi.fujitani@molneu.med.osaka-u.ac.jp (M. Fujitani), yamashita@molneu.med.osaka-u.ac.jp (T. Yamashita).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.11.106

keeping neurons healthy for at least 3 weeks without decreasing their purity below 98%.

2. Materials and methods

2.1. Antibodies and reagents

The following reagents were used for culture of glutamatergic neurons: the Nerve-Cell Culture Medium (SBM, Sumitomo Bakelite Co. Ltd., Tokyo, Japan), recombinant human brain-derived neurotrophic factor (BDNF, Pepro Tech Inc., Rocky Hill, NJ, USA), and recombinant human basic fibroblast growth factor (bFGF, BD Bioscience, Bedford, MA, USA). All other reagents have been described previously [5,6]. For immunocytochemistry, the following antibodies were used: mouse monoclonal antibody to neuronal class III β-tubulin (Tuj1, 1:1000; Covance Laboratories, Inc., Berkeley, CA, USA); rabbit polyclonal antibodies to vesicular glutamate transporter 1 (VGLUT1, 1:1000, Synaptic System, Goettingen, Germany), and cleaved caspase-3 (1:200, Cell Signaling Technology, USA). Fluorescent Mounting Medium was purchased from DakoCytomation Inc., (USA). We used the following fluoroscence-conjugated secondary antibodies: Alexa Fluor 488- or 568-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (1:400, Invitrogen, USA).

2.2. Cell culture

The ES cell line we selected was E14TG2a (CRL-1821, American Type Culture Collection, Manassas, VA, USA). ES-cell derived glutamatergic neurons were differentiated as previously described [5,6], until the stage where NPCs differentiated into neurons. We adjusted the CO_2 content in the incubators to maintain the pH of each culture medium at about 7.4, which made cells a little healthier. Specifically, we used the following conditions: ES cells in ES medium, cellular aggregates (CAs) in CA medium, neurons in complete medium (CM) with 10% CO_2, NPCs in N_2 medium (N_2M) with 7% CO_2, and neurons in SBM with 5% CO_2. The temperature of the incubators was kept constant at 37 °C.

We always once froze all of dissociated CAs, which were NPCs, and started all of our experiments from NPCs by thawing and plating them as originally described [5,6]. We plated the NPCs on glass coverslips in 24-well plates at a density of 0.45×10^6 cells/well in all experiments, except to examine the purity of glutamatergic neurons, where we plated them at a density of 0.9×10^6 cells/well. Glass coverslips were double coated with poly-dl-ornithine and laminin before use as originally described [6]. After culturing of NPCs in N_2M for 2 days, we changed the medium to either CM, as used originally, or to SBM. The time schedule for treating cells either with CM or SBM and fixing treated cells for immunocytochemistry (ICC) is described in Fig. 1a. Because SBM consists of glial conditioned medium and might contain BDNF and bFGF, and CM does not, we hypothesized that these factors were responsible for the improved growth of cells in SBM. To test this hypothesis, we added BDNF and bFGF to CM and measured the effect on longevity and purity. The time schedule for treating cells with BDNF or bFGF and fixing the cells for ICC is described in Figs. 2a and 3a. We tested most of our cells for the presence of mycoplasma as originally recommended [6].

2.3. Immunocytochemistry and nuclear staining

Cells cultured on glass coverslips in 24-well plates were washed with phosphate-buffered saline (PBS). After transferring coverslips in new 24-well plates, we fixed cells with 4% paraformaldehyde in 0.1 M phosphate buffer (Wako Pure Chemical Industries, Japan) for 10 min. Cells were washed with PBS and incubated for 10 min in permeabilizing solution (PBS containing 0.2% Triton X). After three washes with PBS, cells were incubated for 1 h in blocking solution (PBS containing 5% bovine serum albumin and 0.05% Tween). Subsequently, cells were incubated with primary antibodies for 1 h at room temperature or overnight at 4 °C. After three washes with PBS, cells were incubated with fluorescence-conjugated secondary antibodies for 1 h at room temperature or overnight at 4 °C. After three washes with PBS, nuclei were counterstained with Hoechst 33342 (Invitrogen) or 4′,6-diamidino-2-phenylinodole (DAPI, Dojindo, Japan). Coverslips were then rinsed three times with PBS and mounted on glass slides. The samples were viewed under an inverted light microscope equipped with epifluorescence and dry condenser for phase-contrast microscopy (DP70, Olympus, Tokyo, Japan) using a 10× objective.

2.4. Detection of apoptosis

Hallmarks of apoptotic cell death include activation (cleavage) of caspases, condensation and fragmentation of nuclei, and formation of apoptotic bodies. We investigated caspase-3 activation by using ICC and examined extensive chromatin condensation and nuclear fragmentation using Hoechst staining. Neurons were analyzed for immunofluorescent labeling for neuronal marker Tuj1 (green) and for apoptotic marker cleaved caspase-3 (red), and nuclei were counterstained with Hoechst 33342 or DAPI (blue). We obtained 4 representative images per well randomly under the microscope with a 10× objective, and counted all of the cells in those images for one experiment. We quantified the percentage of cleaved caspase-3-positive neurons (cleaved capase-3^+ cells per all Tuj1^+ neurons) in each culture. More than 1500 cells were counted in each of three separate independent experiments to quantify cleaved caspase-3-positive cells.

2.5. Statistical analysis

The quantitative data are expressed as mean ± SEM of three independent experiments. Statistical analysis of these values was performed using Student's t test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. SBM remarkably prolonged the survival of ES cell-derived glutamatergic neurons without decreasing their purity

Bibel and co-workers recently established a differentiation protocol that generates highly homogeneous glutamatergic cortical neurons from ES cells [5,6]. This protocol was highly reproducible regarding the purity of ES cell-derived glutamatergic neurons in our hands, but these neurons did not survive longer than 2 weeks under the same conditions that were originally described [6]. We quantified apoptotic neurons by investigating caspase-3 activation by using ICC and found that 60% of the neurons underwent apoptosis within 6 d after plating (Fig. 1b, left). These apoptotic cleaved caspase-3-positive neurons also showed other hallmarks of apoptosis, including extensive chromatin condensation and nuclear fragmentation, as assessed by Hoechst staining (data not shown). In order to improve neuronal survival, we modified this protocol by changing the medium for glutamatergic neurons from CM to SBM 48 h after plating (SBM protocol, Fig. 1a). We continued to culture neurons in CM or SBM for 4 d and then examined caspase-3 activation by using ICC. SBM significantly decreased the percentage of cleaved caspase-3-positive cells from 60% to 20% (Fig. 1b and c); in addition, we verified that neurons remained healthy for at least