Comparison of different culture modes for long-term expansion of neural stem cells

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Abstract Neural stem cells (NSCs) can be cultured in two modes of suspension and monolayer in vitro. The cultured cells are different in both the ability to proliferate and heterogeneity. In order to find the appropriate methods for large-scale expansion of NSCs, we systematically compared the NSCs cultured in suspension with those cultured in monolayer. The forebrain tissue was removed from embryonic day 14 (E14) mice, then the tissue was dissociated into single-cell suspension by Accutase and mechanical trituration. The cells were cultured in both suspension and monolayer. The NSCs cultured in suspension and in monolayer were compared on viability, ability to proliferate and heterogeneity by fluorescent dyes, immunofluorescence and flow cytometry on DIV21 (21 days in vitro), DIV56 and DIV112, respectively. The results indicated that the NSCs cultured in both suspension and monolayer represented good viability in long-term cultures. But they displayed a distinct ability to proliferate in long-term cultures. The NSCs cultured in monolayer preceded those cultured in suspension on the ability to proliferate on DIV21

and DIV56, but no obvious difference on DIV112. The NSCs population cultured in suspension displayed more nestin-positive cells than those in monolayer during the whole process of culture. The NSCs population cultured in monolayer, however, displayed more β III tubulin-positive cells than those in suspension in the same period. The suspension culture mode excels the monolayer culture mode for large-scale expansion of NSCs.

Keywords Cell culture · Monolayer · Neural stem cells · Neurospheres · Suspension

Introduction

The use of neural stem cells (NSCs) has the potential to revolutionize the treatment of neurodegenerative diseases such as Parkinsonism and Alzheimer's disease, but a large number of cells are required for this clinical application. Therefore, limited NSCs must be expanded in large-scale in vitro. The cultured cells must be nontransformed, well characterized and able to be differentiated into the appropriate cell types.

Neural stem cells can be cultured in two modes of suspension and monolayer in vitro. Reynolds and most other groups cultured NSCs as neurospheres in suspension (Reynolds and Weiss 1996; Svendsen et al. 1998; Gritti et al. 1996; Cai et al. 2002;

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Johansson et al. 1999). But there are several disadvantages to culturing NSCs as neurospheres in suspension. The morphology of cells in the neurospheres is difficult to determine visually and if the size of a neurospheres exceeds a certain critical value, the cells inside the neurosphere would die due to the lack of nutrients needed for their survival and hence, cell yield will decline with time and passage (Milosevic et al. 2004). In this case Ray and Gage cultured NSCs in monolayer (Ray et al. 1993; Ray and Gage 1999). Cells cultured in monolayer can obtain abundant nutrients from the medium and it is easy to use for morphology observation, immunology and biochemical assays (Ostenfeld and Svendsen 2004). However, the monolayer culture mode may induce cell differentiation (Ma et al. 2001; Ostenfeld et al. 2000; McLaren et al. 2001). There is little study to date to systematically compare the properties of NSCs cultured as neurospheres and monolayers for long periods. In order to find the appropriate methods for large-scale expansion of NSCs, we systematically compared the NSCs cultured in suspension with those cultured in monolayer in this experiment.

Materials and methods

Cell culture

Neural stem cells were dissociated from the forebrain tissue of embryonic 14-day (E14) mouse

as Ray and Gage (1999) described. Briefly, following trituration, the cell suspensions were plated onto 35 cm² T-flasks at 0.8×10^5 cells/ml in suspension and at 2×10^4 cells/cm² in monolayer, respectively. The medium used was based on the one applied by Reynolds and Weiss (1996) and improved by our laboratory (Zhao 2004) with its contents shown in Table 1. The NSCs cultured in both suspension and monolayer were passaged every 5–7 days by dissociation of the neurospheres or the adhered cells to a single-cell suspension with AccutaseTM solution (Sigma, USA). The media were replaced by half every 3 days.

Immunocytochemistry

As described by McLaren et al. (2001), NSCs cultured in suspension and monolayer were dissociated to a single-cell suspension by Accutase. The single cells were plated on glass coverslips that had been coated with poly-L-ornithine (PORN, 0.01%, Sigma, USA)/laminin (0.01%, Sigma). One or two days after plating, the cells were fixed with 4% paraformaldehyde in PBS buffer (0.01 M, pH 7.3) for 20 min and then incubated in goat monoclonal antibodies to nestin (1:1,000, Chemicon, USA), to β III tubulin (1:500, Chemicon, USA) and to glial fibrillary acidic protein (GFAP, Chemicon, USA), respectively, at 4°C overnight. After a further three washes in PBS, the cells were then incubated in rabbit anti-goat-IgG-FITC (1:200, Santa Cruz, USA) at 20°C

Table 1 The components in 50 ml culture media

The stocks and concentration	Volume	Final concentration
DMEM (Sigma) (2.5×)	6.66 ml	–
Ham's F12 (Gibco) (2.5×)	6.66 ml	–
RPMI 1640 (Sigma)	8.33 ml	–
NaHCO ₃ (Sigma) (7.5)	3.5 ml	5.25 g/l
HEPES (Roche, Germany) (1 M)	250 μ l	5 mM
D-Glucose (Gibco) (30%)	1.35 ml	9.151 g/l
GlutaMax-1 (Gibco) (200 mM)	1 ml	4.5 mM
EGF (Invitrogen) (10 μ g/ml)	100 μ l	20 ng/ml
bFGF (Invitrogen) (10 μ g/ml)	50 μ l	10 ng/ml
Heparin (Biochemical Institute of Changzhou Guangming, Jiangsu, China) (5 g/l)	40 μ l	4 mg/l
Lipid (Gibco)	50 μ l	1/1,000
B27(Gibco) (50×) or N2(Gibco) (100×)	1 or 0.5 ml	–
Antibiotics, penicillin and streptomycin (Dalian Merro, China)	100 μ l	80–100 U/ml
Millipore-Q water	The left	–

for 1 h, washed three more times to remove the unbound antibody.

For the analysis of the ability to proliferate, the NSCs cultured in suspension and monolayer was plated on glass coverslips coated with PORN/laminin and kept in growth medium containing 5-bromo-2-deoxyuridine (BrdU, 30 µg/ml, Sigma) for 24 h. As described by Bez et al. (2003), the cells were fixed with 4% paraformaldehyde (Sigma, USA) in PBS for 20 min and incubated in formamide at 100°C for 5 min; the cells were then incubated with goat anti-BrdU antibody (1:500, Sigma, USA) for 1.5 h at 37°C. The cells were then incubated in rabbit anti-goat-IgG–rhodamine (1:200, Santa Cruz, USA) at 20°C for 1 h and washed three more times to remove the unbound antibody.

Hoechst33342/propidium iodide fluorescent stain

Neurospheres and single-cell suspension obtained from suspension and monolayer culture respectively, were incubated with Hoechst33342 (10 µg/ml, Sigma, USA) in PBS at 37°C for 10 min. The neurospheres and cells were then incubated with propidium iodide (PI, 10 µg/ml, Sigma, USA) in PBS at 4°C for 20 min and fixed with 4% paraformaldehyde in PBS for 10 min.

Cellular growth curve

For the cellular growth curve, NSCs in suspension and monolayer were incubated from 0 to 9 days, and the number of viable cells was determined by cck-8 kit (Dojindo Laboratories, Japan) each day. Briefly, for the WST-8 assay, 10 µl of the cell counting kit solution was applied to each well in the assay plate and incubated for 4 h at 37°C. Absorbance was measured at 450 nm using a microplate reader (Tecan, Austria) with a reference wavelength at 655 nm (Kanemura et al. 2002).

Flow cytometry

Neurospheres and NSCs cultured in monolayer were dissociated into single-cell suspensions, respectively. All the cells were then resuspended

in PBS to reach a concentration of 10^6 cells/ml. The single-cell suspensions were incubated with 10 µl of Annexin V (20 µg/ml, Sigma, USA) in 100 µl of binding buffer at room temperature for 30 min, then the cells were suspended in PI diluted to a concentration of 2 µg/ml at room temperature for 5 min. Cell fluorescence was analyzed immediately after staining with the flow cytometer (FACS Vantage SE, BECTON DICKINSON, USA).

The NSCs were also analyzed by flow cytometry for their heterogeneity. As described by McLaren et al. (2001), neurospheres and NSCs cultured in monolayer were dissociated into single-cell suspension, respectively. The cells were incubated in primary antibody (goat anti-nestin, goat anti-βIII tubulin) at 4°C for 1 h with continuous shaking. The cells were washed three times and incubated in the rabbit anti-goat-IgG–FITC at 0°C for 1 h. Cell fluorescence was analyzed immediately after staining.

Results and discussion

Comparison of cellular viability in different culture modes

Observation by microscope

Neurospheres were observed every day via a microscope (IX70, Olympus, Japan) and they increased in size to 150–200 µm in diameter 5–6 days after plating. When mice NSCs were plated in monolayer, proliferating cells could be seen after 24 h. They represented the morphology of bright cellular bodies and many processes. The morphology of NSCs cultured in both suspension and monolayer did not change obviously with the elapse of the days in vitro (Fig. 1). The results indicated that cells cultured in both suspension and monolayer can maintain stability of morphology in long-term expansion in vitro.

Hoechst33342/PI fluorescent stain and Annexin V/PI analysis by flow cytometry

Neurospheres and single-cell suspension obtained from suspension and monolayer cultures were,

Fig. 1 Morphology of mouse NSCs from E14 mice forebrain tissue cultured in vitro in suspension and monolayer in the presence of bFGF and EGF. **(A–C)** Neurosphere cultured in suspension DIV21, DIV56 and DIV113, respectively; **(D–F)** NSCs cultured in monolayer DIV21, DIV56 and DIV113, respectively

respectively, stained with Hoechst33342/PI (Fig. 2). The results illustrated that the cells positive for PI were hardly seen on the external layer of neurospheres, whereas the cells positive for Hoechst33342, a dye capable of labeling nuclei of viable cells, were demonstrated on the

neurosphere surfaces in different culture stages. The single cells dissociated from neurospheres and the cells cultured in monolayer were both positive for Hoechst33342.

Flow cytometry analysis of single cells cultured in suspension and monolayer revealed that more

Fig. 2 Hoechst33342/PI assay of the cells cultured in suspension and monolayer. Most cells and neurospheres were labeled with Hoechst33342 (*blue*), suggesting that the most of the cells cultured in both suspension and monolayer were healthy. **(A–C)** Cells derived from neurospheres cultured in suspension DIV21, DIV56 and DIV113, respectively; **(D–F)** neurospheres cultured in suspension DIV21, DIV56 and DIV113, respectively; **(G–I)** cells cultured in monolayer DIV21, DIV56 and DIV113, respectively

Fig. 3 Annexin V/PI assay by flow cytometry. Results indicated that the most of the cells cultured in both suspension and monolayer were healthy. (A–C) Cells derived from neurospheres cultured in suspension DIV21, DIV56 and DIV113, respectively; (D–F) cells cultured in monolayer DIV21, DIV56 and DIV113, respectively

than 95% of the cells cultured in suspension and monolayer were healthy cells on DIV21, DIV56 and DIV113 (Figs. 3 and 4) with Annexin V/PI staining. Annexin V is usually applied to detect the initial apoptosis of cells, since it can characteristically combine the phosphatidylserine on the cellular membrane of early apoptotic cells. The NSCs cultured in this investigation represented good viability in long-term culture. Milosevic et al. (2004), however, indicated that NSCs underwent spontaneous apoptosis in vitro, on average $30.7 \pm 3.4\%$ cells of midbrain-derived neural precursors and $32.1 \pm 2.5\%$ of forebrain-

derived neural precursors were found to be apoptotic within neurospheres. However, we found much fewer apoptotic cells in NSC populations cultured in suspension and monolayer. In our study, NSCs were passaged by Accutase, a mild enzyme for dissociating cells. It is different from the method applied by Milosevic et al., who passaged NSCs with mechanical triturating. The Accutase could reduce mechanical damage in dissociating neurospheres and accordingly, could reduce necrosis and apoptosis in NSC populations. Additionally, we passaged NSCs whenever the neurospheres reached 150–200 μm in diameter while Milosevic et al. did not mention the diameter of the neurospheres in their process of passage. In our study we found the appropriate diameter was an important parameter in the process of harvesting. The big neurospheres would induce necrosis and apoptosis in NSC populations, whereas small neurospheres could not expand NSCs efficiently. Therefore, we can avoid or reduce apoptosis through choosing the adaptive methods and time for passage.

Fig. 4 Percentage of healthy cells in NSCs population cultured in suspension and monolayer on different days in vitro. Most of the cells were healthy and represented good viability

Ability of proliferation of NSCs

Neural stem cells cultured in suspension and monolayer were stained for BrdU to reveal their proliferation ability (Fig. 5). The results illustrated that most of the cells cultured in

Fig. 5 NSCs cultured in suspension and monolayer were treated with BrdU for 24 h prior to plating onto PORN/laminin-coated dishes. Cells positive to BrdU were visualized with BrdU immunostaining (*red*). (**A, B**)

Suspension culture, cells under fluorescence and differential interference microscope, respectively; (**C, D**) monolayer culture, cells under fluorescence and differential interference microscope, respectively

suspension and monolayer had the ability to proliferate.

In order to compare the ability to proliferate of cells with different culture modes quantitatively, we obtained the cellular growth curves by WST-8 assay (Fig. 6). They displayed distinct ability to proliferate in long-term culture. The NSCs cultured in monolayer preceded those cultured in suspension in that ability to proliferate on DIV21 (Fig. 6B) and DIV56 (Fig. 6C) but no obvious difference was detected on DIV113 (Fig. 6D). The ability of NSCs to proliferate cultured in both suspension and monolayer decreased obviously on DIV56. In this stage the tissue-specific precursors and the differentiated cells from these precursors died gradually, which resulted in the number of viable cells decreasing obviously. After this stage the proportion of the stem cells

increased and the NSCs population could gain good ability to proliferate again.

Comparison of the heterogeneity of NSCs cultured in suspension and monolayer

Nestin, β III tubulin and GFAP are the characterized markers for neuroepithelial stem cells, neurons and astrocytes, respectively. The results of immunocytochemistry showed that the NSCs cultured in suspension and monolayer were both composed of a heterogeneous cellular population, including immature neurons and glia as shown in Fig. 7. It can be seen from the figure that the morphology and size of nestin-positive cells cultured in monolayer on DIV21 and 56 were different from those on DIV113. This resulted from the gradual differ-

entiation of NSCs after a long period of culture in monolayer. When the stem cells begin to differentiate into neurons and glia, their morphology changes and their size becomes larger, although they still express nestin during their early differentiation.

Flow cytometry analysis revealed that the nestin-positive cells in suspension and monolayer culture were 82.38 and 74.53%, 76.11 and 36.77%, 84.5 and 58.32% on DIV21, DIV56 and DIV113, respectively. The β III tubulin-positive cells in suspension and monolayer culture were 7.74 and 11.34%, 3.51 and 13.42%, 6.09 and 36.78% (Figs. 8 and 9). The NSCs population cultured in suspension displayed more nestin-positive cells than those in monolayer in all the processes of culture. The NSCs population cultured in monolayer, however, displayed more β III tubulin-positive cells than those in suspension for the same period.

The above results indicated that most of the NSCs cultured in suspension may undergo symmetric cell division when they had a strong ability to proliferate and most of the

cells cultured in monolayer may undergo asymmetric cell division. Symmetric cell division maintains the high proportion of stem cells in the NSC population cultured in suspension. The tissue-specific precursors derived from asymmetric cell division differentiated into β III tubulin-positive cells in the monolayer cultured mode. Moreover in the monolayer culture mode, the PORN and laminin coated on the glass coverslips may induce the differentiation of NSCs even in serum-free conditions.

Conclusions

The results of this study indicate that the NSCs in long-term suspension culture represented good viability. Most of the cells in the population can maintain the characteristics of stem cells. The monolayer culture can induce partial NSCs differentiation. In order to expand NSCs in the long-term, the suspension culture mode excels the monolayer culture mode.

Fig. 6 Growth curves for mouse NSCs cultured in both suspension and monolayer. (A) Correlation between the total number of cells and the absorbance obtained by using the WST-8 assay; (B–D) growth curves for NSCs cultured in both suspension and monolayer DIV21, DIV56 and DIV113, respectively

Fig. 7 Immunocytochemistry assay of the heterogeneous NSCs cultured in suspension and monolayer, respectively. The results indicated that the NSCs cultured in both suspension and monolayer consisted of different types of

cells. (A–C) Suspension culture, nestin-positive, GFAP-positive and β III tubulin-positive cells, respectively; (D–F) monolayer culture, nestin-positive, GFAP-positive and β III tubulin-positive cells, respectively

Fig. 8 Analysis by flow cytometry of cell type and composition in NSCs populations. (A, B) Suspension culture, nestin-positive and β III tubulin-positive cells, respec-

tively; (C, D) monolayer culture, nestin-positive and β III tubulin-positive cells, respectively

Fig. 9 Percentage of cells in NSC populations for nestin⁺ and β III tubulin⁺. Graphs show a variational percentage of nestin-positive cells cultured in both suspension and monolayer and the cells cultured in suspension displayed

more nestin-positive cells than those in monolayer. (A) Percentage of nestin-positive cells cultured in suspension and monolayer; (B) percentage of β III tubulin-positive cells cultured in suspension and monolayer

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