Co-transfection with cDNA encoding the Bcl family of anti-apoptotic proteins improves the efficiency of transfection in primary fetal neural stem cells

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Abstract

Inefficiency in primary neural stem cell transfection is a major obstacle in gene expression research aimed at determining the mechanisms underlying brain development. Following our initial finding that liposome-mediated transfection procedures are fairly toxic to neural stem cells, we further examined whether transfection efficiency could be improved by preventing cells from undergoing apoptosis. Transfection efficiencies were markedly enhanced by co-transfection of cells with prototypic anti-apoptotic genes, such as bcl-2 and bcl-xL, and supplementing the culture medium with B27 Supplement. This combination of anti-apoptotic gene co-transfection and B27 Supplement resulted in approximately 5% transfection efficiency of primary neural stem cells, compared to less than 0.2% in control transfections. Therefore, this procedure and other similar approaches employed to enhance the efficiency of transfecting neural stem cells may facilitate the understanding of mechanisms underlying self-renewal of neural stem cells and their differentiation into various cell lineages. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The neuroepithelium gives rise to neural stem cells that proliferate, survive and differentiate into neurons and glia in the developing brain. Neural stem or precursor cells have been isolated from embryonic and adult brain and cultured in vitro (Gage et al., 1995; Palmer et al., 1997; Chiasson et al., 1999; Kukekov et al., 1999). Cultured neural stem cells retain the potential to expand and differentiate into various types of neurons and glial cells, comparable to those in vivo (Johe et al., 1996; McKay, 1997). Thus, in vitro neural stem cell cultures provide an appropriate model system to study brain development. The manipulation of genes of interest in neural stem cells represents an important strategy to evaluate the fundamental mechanisms of developmental processes, including the proliferation of neural stem cells and their subsequent differentiation into specific cell lineages. However, current methods of transfection of primary neural stem cells, such as calcium-phosphate co-precipitation, electroporation, liposome and cationic lipids, are mostly inefficient. The use of viral infection to induce exogenous gene expression has been shown to be more effective (Robert et al., 1997; Cot et al., 1998), but these procedures are often time-consuming and the proteins synthesized in viral systems may be processed differently.

Transfection procedures are often toxic to cells, resulting in poor transfection efficiency and consequently hampering further study. We speculated that the transfection of neural stem cells could be improved if
the toxic effects of these procedures could be ameliorated. In this study, we demonstrate that the transfection efficiency of primary neural stem cells is greatly improved by co-transfection with anti-apoptotic genes and supplementation of the cell culture medium with B27 Supplement.

2. Materials and methods

2.1. Cell culture

The culture for neural stem or precursor cells was performed as described previously (Johe et al., 1996). Embryonic CNS tissues were dissected from rat cortices, ventral mesencephalons and lateral ganglionic eminences between gestation days 12 (E12) and 15 (E15). Cells were isolated by mechanical dissection in Ca2+/Mg2+-free Hank’s balanced salt solution (CMF-HBSS) and plated on 12 mm glass coverslips (Bellco, Vineland, NJ) pre-coated with polyornithine-fibronectin. Coated coverslips were prepared by initial incubation with poly-L-ornithine (15 μg/ml, Sigma, St. Louis, MO) at 37 °C overnight, followed by fibronectin (1 μg/ml, Sigma) for at least 2 h before use. Cells were allowed to proliferate in the presence of 20 ng/ml basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, MN) in serum-free defined medium (N2) for 4 days at 60–80% confluency, following which differentiation was induced by withdrawal of the mitogen. Cell cultures were maintained at 37 °C in a 5% CO2 atmosphere and the medium was changed every 2 days. B27 Supplement or B27 Supplement Minus AO (Life Technologies, Gaithersburg, MD) was added to the culture medium as required.

2.2. Construction of expression vectors

To generate a vector expressing the gene of interest under control of the EF1α promoter (pcDNAEF1α), we substituted the human cytomegalovirus immediate early promoter of the pcDNA3.1(+) plasmid (Invitrogen, La Jolla, CA) with the 1.2 kb human EF1α promoter (Gene bank accession number J04617). A reporter plasmid substituted the human cytomegalo virus immediate early promoter of the pcDNA3.1(+) plasmid (Invitrogen, La Jolla, CA) with the 1.2 kb human EF1α promoter (Gene bank accession number J04617). A reporter plasmid (pcDNAEF1αEGFP) under the control of the EF1α promoter was constructed by engineering EGFP cDNA (Clontech, Palo Alto, CA) into the multiple cloning site of the pcDNAEF1α plasmid using NotI restriction enzyme. The pcDNAEF1αBcl-2 and pcDNAEF1αBcl-xL vectors expressing the anti-apoptotic genes, bcl-2 (Oh et al., 1995) and bcl-xL (Kim et al., 1999), respectively, were similarly generated.

2.3. Transfection procedures

The transfection efficiency of neural stem cells was assessed using the reporter plasmid, pcDNAEF1αEGFP. Transfection was performed using SuperFect™ Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer’s protocol, with minor modifications. Briefly, after cells were grown to 60–80% confluency in 24-well plates, the medium in each well was replaced with 350 μl fresh N2 containing 20 ng/ml bFGF. The pcDNAEF1αEGFP DNA (0.5 μg) was mixed with 1.0 μl of SuperFect™ reagent per well in serum-free DMEM (Invitrogen, final volume of 60 μl), and DNA-lipid complexes were formed by incubation for 15 min at room temperature. Complexes were added drop-wise to the cells. Plates were gently swirled to ensure the uniform distribution of complexes to the cells. The medium in each well was replaced with fresh N2 after 16–24 h of incubation. Transfection efficiency was assessed 48 h after transfection, either by direct microscopy using GFP filter (GFP(B)-BP, Nikon, Tokyo, Japan) or immunocytochemistry against GFP. Transfections with other reagents, such as LIPOFECTIN™, LIPOFECTAMINE™, LIPOFECTAMINE™ Plus (Life Technologies) and Effecten™ (Qiagen) were performed according to manufacturer’s instructions.

To co-express anti-apoptotic and the reporter genes in transfected cells, 1.5 μg pcDNAEF1αBcl-2 or pcDNAEF1αBcl-xL was mixed with 0.5 μg reporter vector and introduced into cells using the transfection procedure described above. As a control transfection experiment, the same amount of pcDNAEF1α was mixed with the reporter vector, following which the transfection procedure was performed.

2.4. Immunocytochemical staining

Cells on glass coverslips were fixed in 4% (w/v) paraformaldehyde/0.15% picric acid in phosphate buffered saline (PBS) and blocked with 10% (v/v) normal goat serum (NGS)/0.3% (v/v) Triton X-100 in PBS for 20 min at room temperature. The following primary antibodies were used: GFP at 1:400 dilution (mouse monoclonal, Roche Molecular Biochemicals, Basal, Switzerland), Nestin #130 at 1:50 dilution (rabbit polyclonal, kindly provided by Dr Ron McKay, National Institutes of Health, Bethesda, MD), β-tubulin type III (TuJ1) at 1:2000 dilution (rabbit polyclonal, Babco, Richmond, CA) and glial fibrillary acidic protein (GFAP) at 1:100 dilution (rabbit polyclonal, ICN Biochemicals, Costa Mesa, CA). Incubations with primary antibodies were carried out overnight in 10% NGS in PBS at 4 °C and cells were then washed three times in PBS. For rabbit primary antibodies, biotinylated anti-rabbit IgG (Vector Laboratories, Burlington,
CA) at 1:200 dilution in PBS was applied for 45 min at room temperature and followed by three time washes in PBS. The biotinylated complexes were then detected by Fluorescein (DTAF)-conjugated streptavidin (Jackson Immunoresearch Laboratories, West Grove, PA) at 1:400 dilution. Cells labeled with mouse primary antibodies were directly visualized by incubating with Cy3-conjugated anti-mouse antibody (Jackson) at 1:200 dilution. The fluorescent-labeled antibodies were incubated at room temperature for 2 h and the cells were then washed three times in PBS. Cells on coverslips were mounted in VECTASHIELD® with DAPI (Vector Laboratories) mounting medium for fluorescence and photographed using a fluorescent microscope (Nikon, Tokyo, Japan).

2.5. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling analysis

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) reactions (Boehringer Mannheim, Mannheim, Germany) were performed according to manufacturer’s instructions. FITC-labeled TUNEL-positive cells were visualized by direct microscopy.

2.6. Cell counting and statistical procedure

The nuclei of cells were stained with DAPI using VECTASHIELD®. Transfection efficiencies were calculated by measuring the ratio of GFP-immunoreactive (ir) cells/DAPI+ cells counted under a fluorescence microscope (Nikon). GFP-ir and DAPI+ cells were counted per well at a magnification of 100 × . Statistical comparisons were made by Tukey post hoc analysis of one-way ANOVA using spss computer software (spss Inc., Chicago, IL).

3. Results and discussion

The transfection efficiencies of various methods using liposomes and cationic lipids as DNA carriers were assessed using the reporter plasmid pEF1αEGFP expressing EGFP in primary cultures of stem cells isolated from E14 rat cortices. Cells were detected by microscopy with the GFP filter or immunocytochemical analysis using a specific anti-GFP antibody, 48 h after transfection. Although transfection using the SuperFect™ reagent was the most effective method tested, only 0.2±0.1% (n=6) cell transfection was achieved with this procedure.

There are two main possibilities for the poor transfection efficiency. Firstly, the DNA carriers may inefficiently introduce DNA into the cell through the cell membrane. Secondly, although the carriers may efficiently transfer DNA into the cells, most transfected cells subsequently die as a result of toxicity of the transfection procedure and therefore, only the remaining living cells are detected as transfection-positive. TUNEL analysis performed at 24 h following transfection revealed that over 40% of cells underwent apoptosis (Fig. 1A and C). However, ~100% of GFP-ir cells were TUNEL-negative (Fig. 1B and C). These findings strongly support the second possibility, implying that the transfection efficiency of primary neural stem cells may be improved if apoptosis occurring during the procedure is minimized. Bcl-2 and bcl-xL are well-known anti-apoptotic genes that protect cells from various apoptotic stimuli (Shimizu et al., 1995; Tsujimoto et al., 1997; Chao and Korsmeyer, 1998; Tatton, 1999). Accordingly, we attempted to induce bcl-2 or bcl-xL expression along with the reporter gene to protect cells against toxicity associated with the transfection procedure. The percentage of EGFP-expressing cells was markedly higher in cultures co-transfected with the bcl-2 vector, pcDNAEF1zBcl-2 (molar ratio of pcDNAEF1zEGFP: pcDNAEF1zBcl-2 is 1:3), com-
pared to cultures co-transfected with the control vector, pcDNAEF1α. Only 0.07 ± 0.03% (n = 4) cells were GFP-ir in cultures co-transfected with the control vector versus 2.5 ± 0.75% (n = 6, P < 0.001) in those co-transfected with the bcl-2 vector (Fig. 2B and E). Transfection efficiency with the bcl-xL vector was similar to that with bcl-2 (2.8 ± 0.65%, n = 6, Fig. 2C and E).

The B27 Supplement has a pro-survival effect on neural stem cells (Brewer et al., 1993; Svendsen et al., 1995). The addition of B27 to the culture medium led to a 4.3-fold increase in transfection efficiency (0.85 ± 0.11%, P < 0.001). Interestingly, the lack of improvement in transfection efficiency in cultures supplemented with B27 Minus AO (B27 Supplement without the antioxidant components) indicates that B27-mediated enhancement of transfection efficiency is mainly the effect of antioxidants. Moreover, transfection efficiency following co-transfection with anti-apoptotic genes was...
further increased upon B27 treatment. Overall, transfection efficiency was improved to $4.36 \pm 1.10\%$ on cotransfection with bcl-2 in conjunction with B27 (Fig. 2D and E). These effects were not limited to neural stem cells derived from E14 cortical tissues. A similar improvement in transfection efficiency was noted to those reported here for E14 cortical stem cells (data not shown), regardless of embryonic days and CNS regions from which neural stem cells were isolated.

Immunocytochemical staining for Nestin (a marker for neural stem cells), TuJ1 (a neuronal marker) and GFAP (an astrocytic marker) was performed 2 days after differentiation (3 days post-transfection). GFP-ir co-localized well in the different cell types (Fig. 3), suggesting that transfection occurs in stem cell cultures without any preference for cell type.

The enhancement of efficiency of neural stem cell transfection is a significant advance in gene expression research aimed at elucidating mechanisms controlling the self-renewal of neural stem cells and their differentiation into various cell lineages. Increased knowledge of the genes specific for these fundamental brain developmental processes should provide further insights into the pathogenesis of brain diseases, including brain tumors and neurodegenerative disorders, and facilitate the development of novel therapeutic strategies.

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References


