Multi-lipofection efficiently transfected genes into astrocytes in primary culture

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Abstract

This study demonstrated that liposome-mediated transfection — lipofection — is suitable for delivering genes into astrocytes. By repeatedly lipofecting the same astrocyte cultures, a process we call multi-lipofection, the transfection efficiency of the β-galactosidase (β-gal) gene was improved from 2.6 ± 0.6 to $17.4 \pm 1.1\%$. This is the highest efficiency ever reported in gene-transfer with Lipofectin® in a primary culture of mouse cerebral cortical astrocytes. Furthermore, multi-lipofection did not cause observable disturbance to astrocytes as indicated by insignificant changes in the glial fibrillary acidic protein content in the cultures. In order to demonstrate that the transfected gene achieved a physiologically relevant expression level, a plasmid containing the pEF-hsp70 protein gene was lipofected into astrocytes. This produced colonies of astrocytes showing an increased resistance to heat-induced cell death. A similar experiment was performed with the glial-derived neurotrophic factor (GDNF) gene. Control astrocytes had no detectable GDNF. In the transfected astrocytes, the GDNF protein could be identified intracellularly by immunocytochemistry. Western blot analysis revealed, as compared to astrocytes with one lipofection, a 2.9-fold increase of GDNF with four lipofections. GDNF remained detectable in astrocytes 2 weeks after four lipofections. Thus, multi-lipofection provides a mild and efficient means of delivering foreign genes into astrocytes in a primary culture, making astrocytes good candidate vehicle cells for gene/cell therapy in the CNS. © 2000 Published by Elsevier Science B.V.

Keywords: Astrocytes; GDNF; Liposome; Heat shock protein; β-Galactosidase; Multi-lipofection

1. Introduction

An efficient and non-invasive method for transferring foreign genes into cells is of great importance for basic research and the development of gene/cell therapy. For example, cultured brain cells efficiently transfected with specific genes could potentially be used in the treatment of neurological disorders. Over the past few years extensive emphasis has been placed on the development of non-invasive transfection methods and the selection of suitable vehicle cells to carry therapeutic genes to the central nervous system (CNS). Previous studies have shown that fibroblasts (Tuszynski et al., 1996; Schinstine et al., 1997; Griffitt et al., 1998), myoblasts (Jiao et al., 1993; Lisovoski et al., 1997) and human bone marrow stromal cells (Azizi et al., 1998) can be used as vehicle cells for delivering genes into the CNS. However, these cells are far from ideal since they are not normally present in the CNS. Astrocytes are a better option since they are supportive cells in the brain. After being transplanted they have been shown to survive (Quintana et al., 1998) and to incorporate well into host brain tissues (Emmett et al., 1991; Jacque et al., 1992; Lundberg et al., 1996). However, it is generally very difficult to transfect foreign genes into primary cultured astrocytes, which makes it difficult to use them as vehicle cells for gene/cell therapy. Another limitation is their rapid reactivation under harsh transfection conditions (Eng et al., 1992).

Gene transfer strategies in brain cells involve viral and nonviral techniques. The concerns with viral techniques are that some viruses might disturb the host

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DNA transcription and synthesis, regain their pathogenic activity, and generate immune responses (Flotte and Carter, 1995; Hermens and Verhaagen, 1998; Parr et al., 1998). Nonviral gene transfer techniques are attractive alternatives because they produce fewer side effects (Roth and Cristiano, 1997). Lipofection, which is liposome-mediated transfection, is one such technique. Using lipofection, we previously transferred antisense oligonucleotides of glial fibrillary acidic protein (GFAP) into astrocytes in primary cultures and effectively inhibited the GFAP synthesis in astrocytes treated with dBcAMP (Yu et al., 1991, 1993a,b). Cationic liposome-mediated transfection was first developed by Felgner et al. (1987). In Felgner's technique, a cationic liposome-DNA complex is used to deliver DNA plasmid across the plasma membrane via a fusion process involving endosomes. However, the technique's application is limited by low transfection efficiency. Felgner tried to improve the efficiency by using higher concentrations of liposomes. Since that time, it has been reported that lipofection has efficiently delivered genetic materials to *Xenopus* brain cells (Holt et al., 1990), mammalian brain cells (Holt et al., 1990; Cao et al., 1995; Thorsell et al., 1996; Mizuguchi et al., 1997), a primary culture of astrocytes (Bochelen et al., 1992; Yu et al., 1991, 1993a,b), oligodendrocytes (Guo et al., 1996), and septo-hippocampal cells (Le et al., 1996; Yang et al., 1996). However, the transfection efficiency remains low. In this study, we experimented with multiple lipofection, or what we call multi-lipofection in a primary culture of astrocytes with a β-galactosidase (β-gal) gene. We studied the expressions of a functional gene, the heat shock protein 70 (hsp70) cDNA, and a therapeutic gene, glial-derived neurotrophic factor (GDNF) cDNA introduced into astrocytes by multi-lipofection.

2. Materials and methods

2.1. Primary cultures of cerebral cortical astrocytes

Primary cultures of cerebral cortical astrocytes were prepared from newborn ICR mice (Animal Care Facility, The Hong Kong University of Science and Technology, China) as described previously (Yu et al., 1986, 1993a,b; Yu and Lau, 2000) with minor modifications. Briefly, neopallia free of meninges were cut into small cubes (< 1 mm^3) in Dulbecco's modified Eagle medium (DMEM) (GibcoBRL, Life Technologies, NY, USA). The tissue was mechanically dissociated by vortex for 1.5 min and the cell suspension was sieved through 70 and 10 μm sterile mesh nylon filters (Spectrum Medical Industries, TX, USA). A cell suspension containing about 4.5×10^5 cells was seeded in a 35-mm tissue culture dish (Becton Dickinson, NJ, USA) supple-

mented with 10% fetal bovine serum (FBS) (Gibco-BRL, Life Technologies, NY, USA). Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. The medium was changed twice weekly. Only cultures more than 3 weeks old were used for the experiment.

2.2. Plasmids

Transfection efficiency was monitored by the reporter vector pCMVβ (Clontech, CA, USA). This vector encoded β-gal from *Escherichia coli*. The plasmid also contained CMV, an immediate early gene promoter with an intron and polyadenylation signal from SV40.

pEF-hsp70 was a gift from Dr J.B. Uney (Department of Anatomy, University of Cambridge, UK). In this plasmid the inducible hsp70 protein (HSP70) cDNA (2.3 kb) was cloned into pEF-BOS, a powerful expression vector that used the promoter of a human elongation factor 1-α (EF1-α) chromosomal gene (Mizushima and Nagata, 1990).

pcDNA-GDNF was a gift from Professor J.S. Han (Neuroscience Research Institute, Beijing Medical University, China). Cloned human GDNF cDNA (696 bp) was inserted into the mammalian expression vector pcDNA (Invitrogen, USA) at the site of *Hin*dIII and *Bam*HI. All plasmids for gene transfection were amplified and purified by the Wizard Maxi-prep DNA purification system (Promega, USA). The DNA concentration was determined by GeneQuant®II RNA/DNA calculator (Pharmacia, Sweden).

2.3. Lipofection

Prior to transfection, cultures were rinsed twice with a serum-free medium. DNA was mixed with Lipofectin® according to the protocol provided by the manufacturer (GibcoBRL, Life Technologies, NY, USA). Briefly, for each transfection, 1–20 μl Lipofectin® (1 mg/ml) was diluted with 100 μl serum-free Opti-MEM (GibcoBRL, Life Technologies, NY, USA) and kept at room temperature for 35 min. It then was mixed with 100 μl serum-free Opti-MEM containing 1–40 μg plasmid DNA and the mixture (0.2 ml DNA–Lipofectin® complex) was left at room temperature for 10 min before being diluted with 0.8 ml serum-free Opti-MEM. The diluted DNA–Lipofectin® complex was added to the astrocytes. After incubation at 37°C for 12 h the transfection medium was replaced with a fresh culture medium containing 10% FBS. Transfection efficiency was assessed 48 h after transfection.

Multi-lipofection was performed by exposing astrocytes to the DNA–Lipofectin® complex several times for 12 h each time. In between each lipofection the transfection medium was replaced with fresh DMEM containing 10% FBS for 12 h. In some cultures multi-lipofection was performed up to eight times.

2.4. Identification of transfected cells

Activity of β-gal was detected histochemically as previously described by Jiao et al. (1993). Briefly, after transfection the astrocytes were rinsed with PBS three times before being fixed in 2% paraformaldehyde and 0.2% glutaldehyde in PBS for 2 min at room temperature. They were then rinsed with PBS three times and incubated in a detection solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM magnesium chloride in PBS) at 37°C for 12 h. The cells were rinsed three times with PBS and mounted in a 70% glycerol solution. The number of positive cells was estimated from pictures taken from nine different random fields from each culture under an inverted microscope. Some of the cultures were counter-stained with neutral red to facilitate cell counting. Transfection efficiency was determined as the ratio between the number of positively stained cells and the total number of cells counted.

2.5. GFAP measurement

Two days after multi-lipofection, the astrocytes were washed twice with 0.9% NaCl and dissolved in 0.5 ml of a sample buffer containing 0.15% sodium dodecyl sulfate (SDS). The cell solution was collected in a 1.5 ml Eppendorf tube and dispersed at 4°C for 5 min with a sonicator. After centrifugation the cellular extracts (5 μg of protein per lane) were separated by one-dimensional 12% SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to a nitrocellulose membrane following the method of Towbin et al. (1979). The membranes were incubated with a monoclonal antibody against GFAP (Sigma, MO, USA) for 1 h at room temperature. After washing, horseradish peroxidase-conjugated anti-mouse was added and GFAP were detected by the enhanced chemilumines-

cence (ECL) procedure described by the supplier (Amersham, Buckinghamshire, UK). The films were quantified by an IS-1000 image system (Alpha Innotech, CA, USA), and protein levels were expressed as the relative values of the controls without transfection.

2.6. Heat shock treatment

Astrocyte cultures after four lipofections with pEF-hsp70 were obtained. At 48 h after the four lipofections, cultures were incubated in a 46°C water bath for 20 min and then returned to a 37°C incubator for 24 h. Untransfected and pCMVβ-lipofected cultures were the controls. These cultures were then rinsed three times with PBS and fixed with 4% paraformaldehyde for 30 min. After neutralization with 0.1 M glycine in PBS (pH 7.4) for 30 min, cultures were permeabilized with 0.1% Triton X-100 for 10 min and then washed three times with PBS. Cultures were labeled with mouse monoclonal antibody against heat shock protein 70 (HSP70) (1:100 dilution) (Santa Cruz Biotechnology, CA, USA) followed by FITC-conjugated anti-mouse antibody labeling.

2.7. GDNF detection

To observe the cellular location of GDNF expression after GDNF multi-lipofection, immunohistochemistry was performed on GDNF transfected astrocytes. The cell cultures were treated as same as those for heat shock treatment except with an anti-rabbit GDNF antibody (Santa Cruz Biotechnology, CA, USA) in 1:100 dilution.

In Western blot analysis, the cell lysates equivalent to 10 μg protein were used and the protein was resolved by 12.5% SDS-PAGE. GDNF was labeled with anti-GDNF antibody (Santa Cruz Biotechnology, CA, USA) and detected by the ECL chemiluminescent detection system (Pharmacia, Sweden). The levels of GDNF were estimated by an IS-1000 digital imaging system (Alpha Innotech, CA, USA).

2.8. Statistical analysis

Data was computed to give means ± S.E.M. of at least three separate experiments with at least three cultures per experiment. Statistical significance was calculated by Student's t-test with $P < 0.05$ being considered significant.

3. Results

3.1. Lipofection

Different amounts of DNA and Lipofectin® were

Table 1
Effects of ratios and amount of DNA and Lipofectin® in the complex on β-gal gene transfection in primary astrocytes[a]

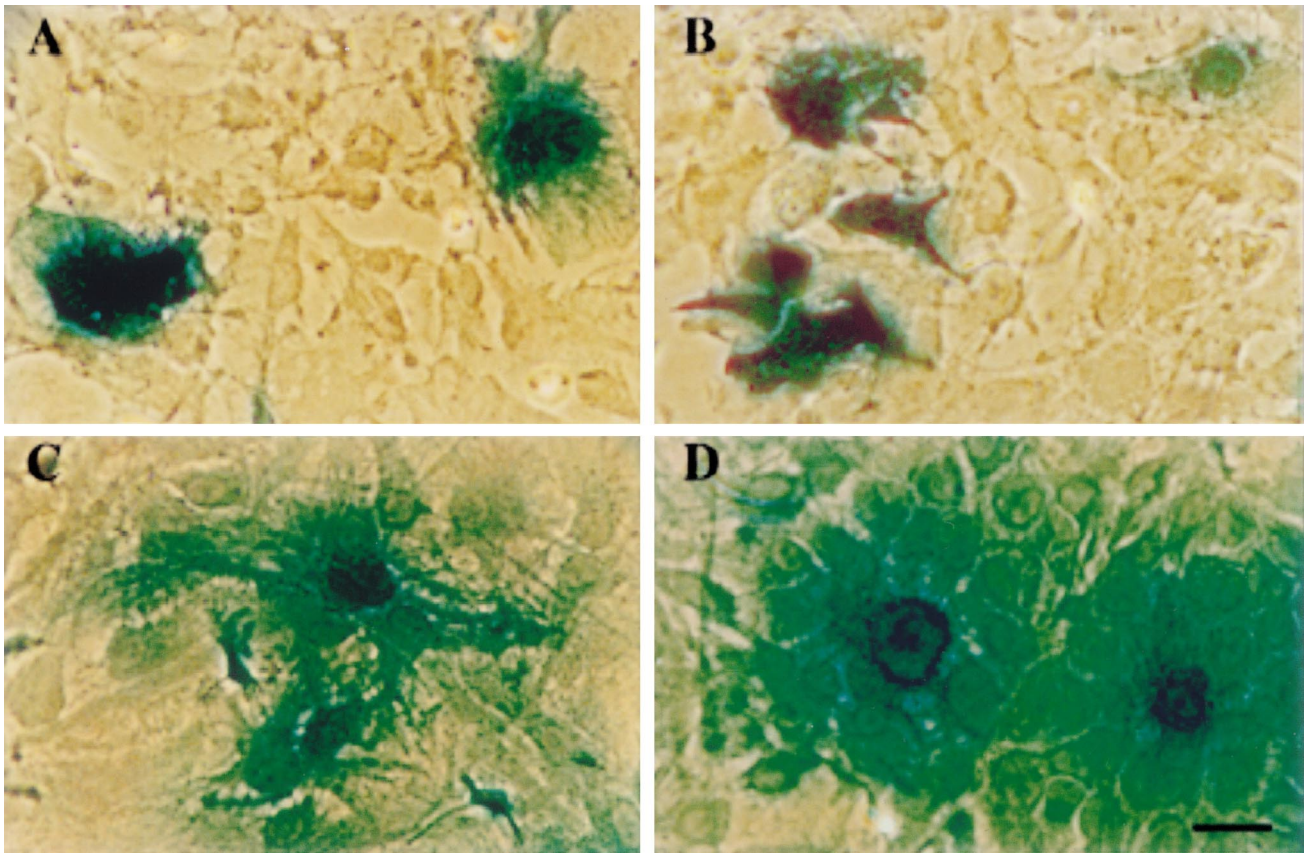| DNA:Lipofectin®		Efficiency
Ratios	μg:μl	(β-gal positive cells /total cells) × 100%
1:20	1:20	0.50 ± 0.30
1:10	1:10	0.91 ± 0.28
1:5	1:5	0.88 ± 0.39
1:1	1:1	1.43 ± 0.42
8:1	40:5	0.16 ± 0.12
4:1	20:5	1.67 ± 0.53
2:1	10:5	2.12 ± 0.61
2:1	8:4	1.91 ± 0.56
2:1	6:3	2.38 ± 0.58
2:1	3:1.5	1.76 ± 0.63

[a] $N = 9$ from three separate experiments.

Fig. 1. Expression of β-gal gene in primary astrocytes after transfection with lipofection method. Transfection conditions: Lipofectin®, 3 μg; DNA, 6 μg; transfection time, 12 h; transfection medium, Opti-MEM; cell age, 4 weeks; recovery time between each lipofection, 12 h. (A), (B), (C), and (D) represented one, two, three, and four lipofections, respectively. Cells with blue staining were being count for estimation of the transfection efficiency, disregard of their staining intensity. Scale bar = 170 μm.

combined to form complexes (Table 1). Among all the complexes tested, the one prepared with a ratio of two portions of DNA to one portion of Lipofectin® achieved the highest transfection efficiency, ranging from $1.76 \pm 0.63\%$ $(n = 9)$ to $2.38 \pm 0.58\%$ $(n = 9)$. Increasing the amount of DNA and Lipofectin® in the complex did not produce any improvement. In terms of specific amounts, the highest efficiency was obtained with a complex prepared from a combination of 6 μg DNA and 3 μl Lipofectin®. For unknown reasons, in cultures that had been treated with this complex for longer than 12 h, cells detached during the X-gal staining. The transfection efficiency did not differ significantly when lipofection was performed in either 1-week-old cultures or 4-week-old cultures. Based on the above observation the highest transfection efficiency of $2.6 \pm 0.6\%$ was obtained on a 4-week-old primary culture of astrocytes with a complex of 6 μg DNA and 3 μl Lipofectin® and a lipofection period of 12 h.

3.2. Multi-lipofection of astrocytes

Fig. 1 shows that the number of β-gal positive cells in a primary culture of astrocytes increased with the number of lipofections. Fig. 1A shows a culture with one lipofection. The number of β-gal positive cells increased after two lipofections (Fig. 1B). From Fig. 1C–D, the number of cells with β-gal staining clearly increased. The transfection efficiencies under multi-lipofection are summarized in Fig. 2. Single lipofection achieved an
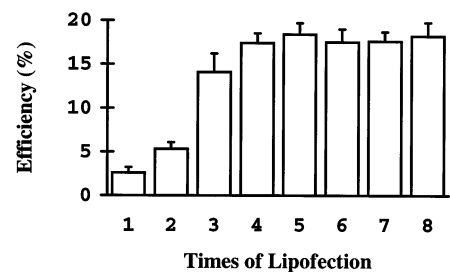

Fig. 2. Effects of frequency of lipofection on transfection efficiency in primary astrocytes. Transfection conditions: DNA, 6 μg; lipofectin, 3 μg; medium, Opti-MEM. Results are expressed as means ± S.E.M. of three separated experiments.
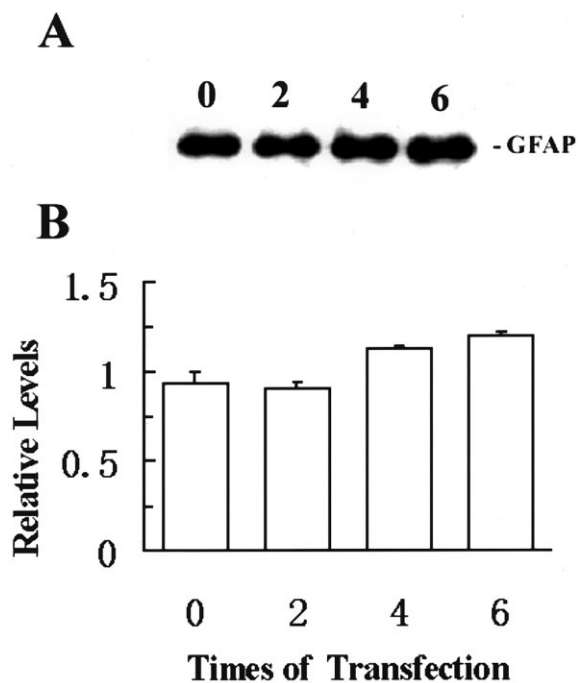
A

B

Fig. 3. The levels of GFAP in astrocytes after multi-lipofection: 0 represents the control; 2, 4, and 6 represent two, four, and six lipofections. The transfection condition was the same as mentioned in Fig. 1. (A) Western blot analysis of GFAP content. (B) Densitometric analysis of GFAP band intensity from (A). The intensity of the bands was expressed as a ratio to the control. Data represents the mean $\pm$ S.E.M. taken from three different experiments.

efficiency of $2.6 \pm 0.6\%$. Two lipofections achieved a $5.3 \pm 0.8\%$ efficiency. The efficiency was increased to $14.1 \pm 2.1\%$ after three lipofections and $17.4 \pm 1.1\%$ after four lipofections. There was no further significant increase in the transfection efficiency with more than four lipofections. No detachment of cells was observed in cultures after multi-lipofection.

3.3. GFAP measurement

The change of GFAP content in a primary culture of astrocytes after increasing the frequency of the lipofection is summarized in Fig. 3. The levels of GFAP in astrocytes did not change as a result of the multi-lipofection. Although four and six lipofections slightly increased the GFAP content in the culture, the increases were statistically insignificant.

3.4. HSP and GDNF transfection

Forty-eight hours after four lipofections with pEF-hsp70 or pCMVβ the astrocytes were examined by immunocytochemical staining and Western blot analysis. Immunostaining showed fluorescent signals of HSP70 in normal astrocytes and astrocytes transfected with pEF-hsp70 or pCMVβ. Due to the high basal level of HSP70 in a normal culture it was very difficult to

distinguish whether the cells transfected with hsp70 or pCMVβ had a higher HSP70 than the controls. Western blot analysis indicated that HSP70 was constitutively expressed in control astrocytes, and heat shock stress could increase the level of HSP70 by 2-folds. After four lipofections of pEF-hsp70 the band for HSP70 did not show any obvious change in intensity. This outcome was consistent with the result from the immunocytochemistry study.

Cells transfected with pEF-hsp70, or pCMVβ, and untransfected cells were exposed to severe heat shock at 46°C for 20 min. Fig. 4 shows the number of surviving cells 24 h after the heat shock treatment. In the untreated culture all cells were severely injured and cells appeared as debris in the culture (Fig. 4A). The fluorescent staining did not show any intact cells with HSP70 staining (Fig. 4D). In pCMVβ treated-cultures, a few cells remained in the culture dish but appeared to be severely damaged (Fig. 4B). Fluorescent staining did not show any HSP70 staining in these cells (Fig. 4E). In the pEF-hsp70 transfected cultures there were many colonies of astrocytes that appeared to be intact (Fig. 4C). They showed a strong fluorescent staining for HSP70 (Fig. 4F).

In the primary culture of astrocytes without transfection of the GDNF, no positive immunostaining of GDNF was detected (Fig. 5A). After the cultures were lipofected with GDNF/pcDNA3, some astrocytes were positive to GDNF staining (Fig. 5B and C). The staining appeared as packages in the cytoplasm, and no staining was located in the nucleus. The amount of intracellular GDNF was semi-quantified by Western blotting (Fig. 6). Astrocytes in cultures transfected with GDNF yielded a product with a size of 20 kDa. GDNF was not detectable in the negative controls. Recombinant human GDNF was used as the positive control. Its band runs remarkably (ca. 15 kDa) lower than GDNF expressed in the transfected cultures (ca. 20 kDa). The level of GDNF in the cell lysate increased with the number of lipofection, with a 2.9-fold increase from one lipofection to four lipofections. GDNF was still detectable in cell lysate in cultures 2 weeks after four lipofections.

4. Discussion

We have previously reported that 11–17 µg Lipofectin® per ml of medium did not cause toxicity in astrocytes in a primary culture if the transfection time was less than 5 h (Yu et al., 1993a,b). In this study, we optimized several parameters including the ratio of DNA to Lipofectin® and the type of transfection medium. Due to the low amount of Lipofectin® (3 µg) used, we were able to extend the transfection period to 12 h without causing observable toxicity.

The 2.6% efficiency in one lipofection was not high enough to produce sufficient transfected cells for any meaningful application such as gene/cell therapy and neurotransplantation. Using the new strategy of multi-lipofection, the transfection efficiency was multiplied by over six times. The correlation between the frequency of lipofection and the efficiency of transfection seemed to be linear up to four lipofections. The reason for the lack of increased efficiency, thereafter, is not clear. In general it is very difficult to transfect foreign genes into primarily cultured astrocytes using non-viral tech-niques. The transfection efficiency accomplished by multi-lipofection was the highest ever reported in a primary culture of astrocytes using Lipofectin®.

Multi-lipofection thus provided an efficient means for gene transfer in a primary culture of astrocytes. It was easy to carry out and was able to deliver large-size fragments of DNA molecules into the target cells. It has been shown that liposome-mediated transfection re-sulted in an increase of hsp72 expression in HeLa cells (Andrews et al., 1997), indicating that lipofection might induce cellular stress. Due to the gliotic nature of
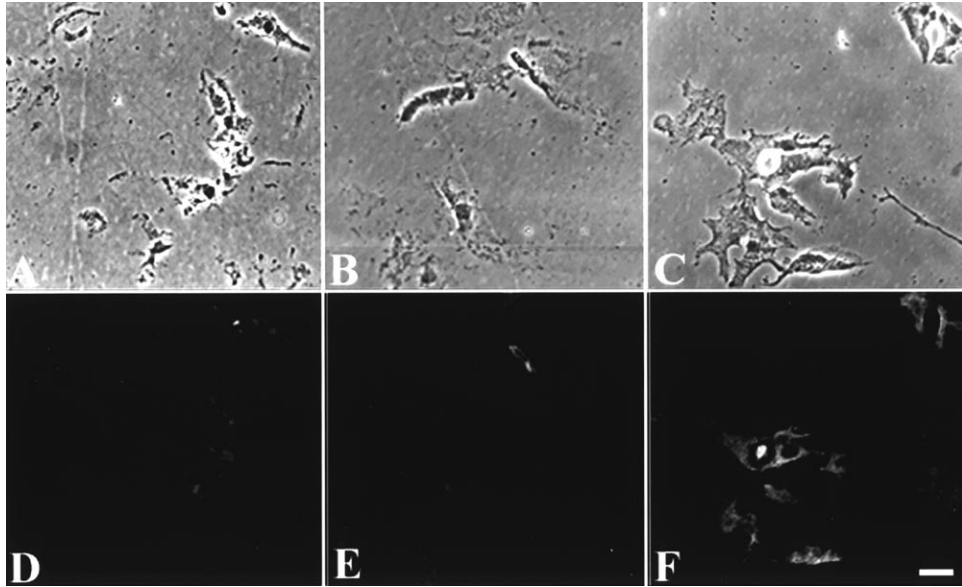

Fig. 4. Primary cultures of astrocytes immunocytochemically stained for HSP70 after being exposed to severe heat shock injury. Compared with untransfected control cells (A, D) and cells transfected with pCMVβ (B, E), more cells with intensive positive staining for HSP70 remained in the cultures lipofected 4 times with pEF-hsp70 cDNA (C, F). Upper panels are phase contrast micrographs and lower panels are fluorescent micrographs. Scale bar = 40 μm.
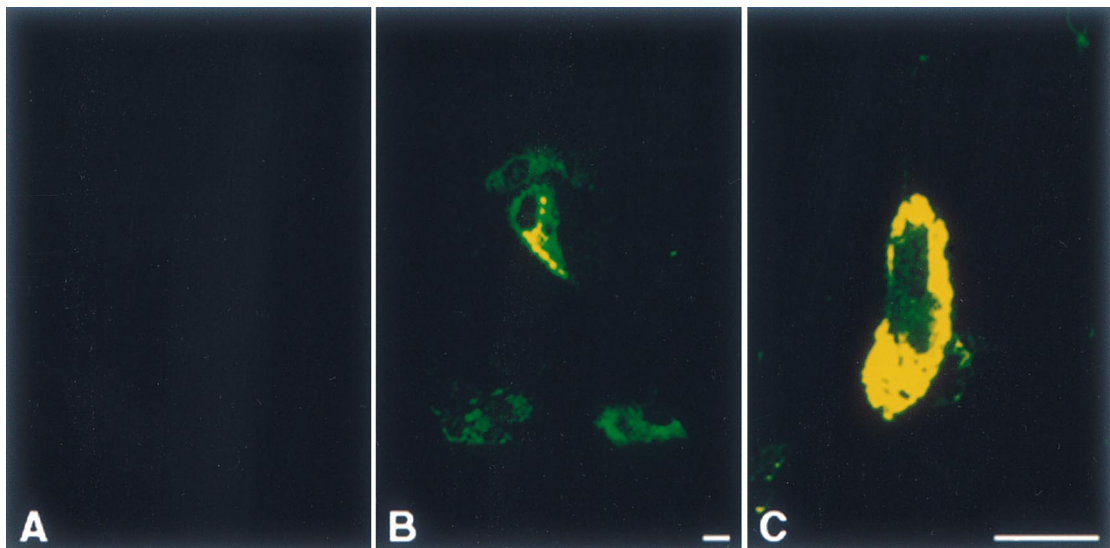

Fig. 5. Fluorescent micrographs of primary cultures of astrocytes immunocytochemically stained for GDNF. (A) Control. (B, C) Primary cultures of astrocyte stained immunochemically for GDNF after four lipofections with pcDNA-GDNF. Scale bar = 25 μm.
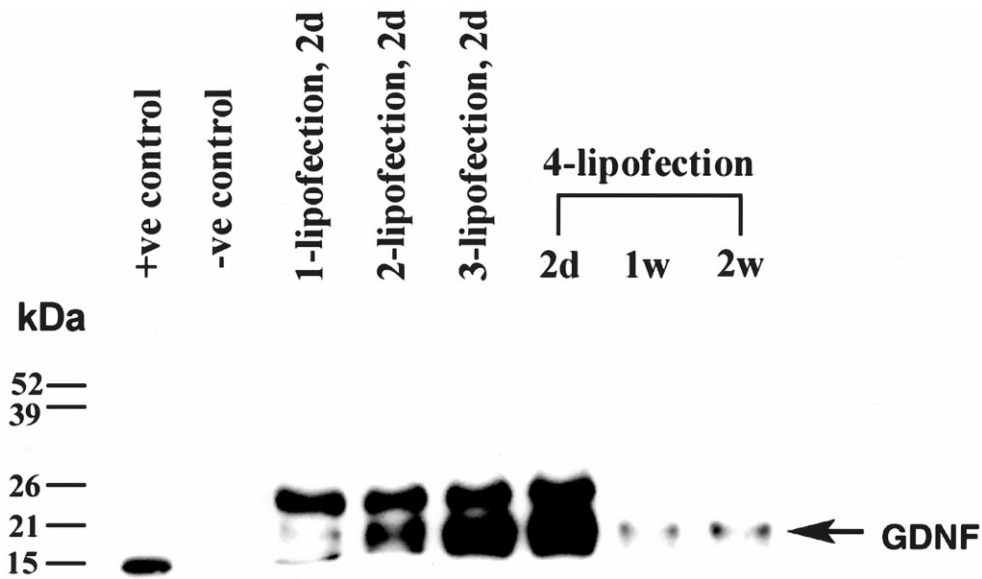
Fig. 6. Measurement of GDNF expression in astrocyte lysates by Western blot analysis. GDNF levels increased with the frequency of lipofection, decreased with the time after four lipofections. The pre-stained protein ladder was loaded to indicate the molecular mass. Recombinant GDNF protein (2 μg/well) was the positive control. For each lane 10 μg of proteins was loaded.

astrocytes (Eng et al., 1992; Eng and Ghirnikar, 1994), astrocytes might be reactivated by lipofection. Reactivated astrocytes have a different gene expression profile that affects their ability to migrate into and optimally integrate with the host tissue. Furthermore, such cells would disturb the normal functioning of the recipient CNS. This reactivation would jeopardize the usefulness of the transfected astrocytes for gene/cell therapy and transplantation. We showed that the multi-lipofection did not cause an observable measurable disturbance to the astrocytes as reflected by an insignificant change in GFAP content in the transfected astrocytes. GFAP is a marker of gliosis (Eng and Ghirnikar, 1994) and is used by many investigators to assess neurotoxicity and astrocytic reactivation after injury (Gainetdinov et al., 1998).

In order to clarify the cell specificity of multi-lipofection, we also performed multi-lipofection of the β-gal gene on primary cultures of cerebral cortical neurons and a culture of PC12 cells (data not shown). Unlike with astrocytes, the multi-lipofection did not transfect the cerebral cortical neurons successfully and many neurons were killed during the process. Multi-lipofection on PC12 cells could only transfect a small number of cells. This suggests that the multi-lipofection conditions designed in this study might be specific for delivering genes to astrocytes in primary cultures, but not to neurons or neuronal cell lines.

To demonstrate that the obtained gene transfer efficiency is sufficient to achieve physiologically relevant levels of expression, we first tried to transfect astrocytes with a plasmid containing the hsp70 under control of the EF1α promoter. Heat shock proteins contribute to cellular thermotolerance, which is the ability to survive

severe heat stress (Morimoto et al., 1992; Parsell et al., 1993). It was surprising that after efficient transfection, no increased HSP70 was detected. It was even more surprising that heat shock treatment did not mediate a higher induction of HSP expression in the pEF-hsp70 transfected cells than in mock transfected cells. No conclusion could be drawn based on these observations. In cultures transfected with the pEF-hsp70, many colonies of astrocytes survived the severe heat shock treatment. The surviving astrocytes displayed a strong HSP70 staining. The result was consistent with the observation by Uney et al. (1993). Despite the fact that no elevated HSP70 level was detected after transfection in a normal culture, many colonies of astrocytes had increased resistance to heat-induced cell damage.

A 2.9-fold increase in GDNP in cell lysates from one to four lipofections has further proven that multi-lipofection could enhance the transfection efficiency in primary culture of astrocytes. GDNF is known for its therapeutic effect on dopaminergic neuronal survival. Although there is a reduction in GDNP from 2 days to 1 week, our data showed that GDNF was detectable in cell lysates of cultures two weeks after four lipofections. This suggests that if gene therapy were being used in treatment, permanent treatment might not be necessary and transient expression of therapeutic genes would be useful. Whether the multi-lipofection would permanently transfect a gene into astrocytes is not the scope of this study and will need further clarification.

Multi-lipofection has the advantages of simplicity, safety of use, biological inertness easy for automation and non-immunogenicity. Primary astrocytes are a normal component of the CNS and they were shown to

survive well after transplantation. Our work documents a mild and efficient nonviral gene transfer method with high specificity for delivering genes to astrocytes in a primary culture. The experiment of multi-lipofection of the pEF-hsp70 and pcDNA-GDNF validated the idea that gene transfer through multi-lipofection could express into a functional protein. The mild nature and high specificity of multi-lipofection makes astrocytes candidate vehicle cells for CNS gene/cell therapy and a better alternative to viral transfection, tumorogenic cell lines and non-CNS cells.

Acknowledgements

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