Stable Expression of the *hBDNF* Gene in CHO Cells

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Abstract: objective. To transfect the *hBDNF* (human brain-derived neurotrophic factor) gene into CHO cells, establish a stable expression system, and to detect the biological activity of the expressed hBDNF protein. **Methods.** Liposomes were used to mediate the transfection, and RT-PCR, Western-blotting and MTT method were to detect. **Results.** *hBDNF* mRNA was detected in the transfected CHO cells, and hBDNF protein, promoting PC12 cells' growth, can be detected in the supernatant. **Conclusion.** The stable expression system of hBDNF-CHO was successfully established, which could produce hBDNF protein with biologic activity. [Life Science Journal. 2006;3(3):49-52] (ISSN: 1097-8135).

Keywords: hBDNF; eucaryon transfection; stable expression; function

Abbreviations: BDNF: brain derived neurotrophic factor; CGM: complete growth medium; CHO cell: Chinese hamster ovary cell; COS-7 cell: African green monkey SV40-transformed kidney fibroblast cell; CS: calf serum; SM: screening medium

1 Introduction

The brain derived neurotrophic factor (BDNF) belongs to the neurotrophin family[1], and plays important roles in the development and maturation process of nervous system. It is good for the regeneration, recovery and protection of neurocytes from degeneration after trauma. The most recent researches show that BDNF has high biologic activities upon the survival and development of many types of neurons, including the septal cholinergic neuron[2], mesencephalic dopaminergic neuron[3], and motor neuron in cornu anterius medullae spinalis[4]. They are potential in the treatment of nervous system disease. Our department successfully introduced the *hBDNF* gene into Chinese hamster ovary cell (CHO) cells by gene-engineering technology and cell-engineering technology; the hBDNF protein secreted by the hBDNF-CHO cells has a certain biologic activity, which establishes the experimental base of biologic hBDNF.

2 Materials and Methods

2.1 Materials

The plasmid of pTracer^{TM}-EV/V5-His-hBDNF was constructed by our department, CHO cells PC12 cells and *E. coli* DH5α all were from our department. Calf serum (CS) and cation liposome were purchased from GIBCO (USA); zeocin was from Invitrogen (America); thiazolyl blue was from Sigma (USA). Primers were synthesized by Shenggong Shanghai. Rabbit polyclonal antibodies against hBDNF were purchased from Santa Cruz Biotechnology, and the goat anti-rabbit antibodies together with its substrate were purchased from Shanjing, Shanghai.

2.2 Methods

2.2.1 *hBDNF* gene's introduction into CHO cells: The day before transfection, 2×10^5 CHO cells were seeded per well of a 6-well plate in 2 ml complete growth medium (CGM) with serum and incubated at 37 ℃ in a 5% CO_2 incubator until cells were 40% - 60% confluent overnight. Solution A: diluting 10 μg DNA (plasmids pTracer^{TM}-EV/V5-His or plasmids pTracer^{TM}-EV/V5-His) to 100 μl with medium DMEM without serum; solution B: diluting 15 μg cation liposomes to 100 μl with the same medium as above. Solution A and B were gently mixed and incubated at the room temperature for 30 min to form DNA-liposome complexes. For each transfection, 0.8 ml medium without serum was added to the tube containing the complexes, then mixed gently and overlaid onto the rinsed CHO cells. The cells with complexes subsequently were incubated at 37 ℃ with 5% CO_2. After 18 - 24 hours the medium was replaced with fresh CGM containing 10% CS.

2.2.2 Screening for positive clones: seventy-two hours after transfection we began to screening the positive clones by replacing the CGM with screening medium (SM), which was made up with 10% CS, DMEM and 800 μg/ml *zeocin*. After most cells were killed we changed the SM into main

medium, which was made up with CGM containing 10% CS and 200 μg/ml *zeocin*. Forty days later we got the two cloned lines i. e. CHO-pTracer™-EV/V5-His and CHO-pTracer™-EV/V5-His-hBDNF.

2.2.3 RT-PCR: The total RNA of the two cloned lines of CHO-pTracer™-EV/V5-His and CHO-pTracer™-EV/V5-His-hBDNF were respectively extracted, then the first-strand cDNA was synthesized from the mRNA template using reverse transcriptase and subsequently were amplified by PCR with above-mentioned primers as the following program: 95 ℃ for 5 min; degeneration 95 ℃ for 30 sec, primers annealing 52 ℃ for 40 sec, extension 72 ℃ for 45 sec for 35 cycles and a final extension 72 ℃ for 5 min. Finally 10 μl PCR products and DNA Marker were electrophoresed on 1.5% agarose gel.

2.2.4 Concentration dialysis and filtration the supernatants of these CHO cells: The above two cell lines were cultivated on large scale, and 72 hours later their supernates were collected, and concentrated 20 – 50 times by Polyethylene glycol 6000. Afterwards, the concentrated solution in bag filters were dialyzed with PBS and filtrated sterilization.

2.2.5 Western-blot analysis: 100 μl concentrated supernatants of the two kinds of CHO cells were respectively mixed with 100 μl 2 × loading buffer, and the two mixtures were boiled for 5 min, then followed with SDS-PAGE electrophoresis, incubation of the blot with primary antibody in the antibody binding buffer overnight at 4 ℃, washing the blot 5 times in TBST buffer, incubate the blot with second antibody, washing the blot 5 times in TBST buffer again in order, at last the blot was developed following DAB (p-dimethylaminoazobenzene) substrate instruction.

2.2.6 Biological activity detection

Promote PC12 cells' growth: The density of PC12 cells was adjusted to 4×10^5/ml by 2% DMEM, then 1 ml of such cells' suspension and 1.7 ml 2% DMEM were added to every small square bottle, subsequently we added 0.3 ml concentrated supernatant of pTracer™-EV/V5-His-hBDNF-CHO cells into the experimental group, 0.3 ml concentrated supernatant of pTracer™-EV/V5-His-CHO cells into the control group and 0.3 ml 2% DMEM into the blank group. All these small bottles of cells were cultivated at 37 ℃ with 5% CO_2. Seventy-two hours later the PC12 cells were observed and counted.

Activity detection by MTT assay: A 96-well-plate was divided into a blank group, a control group and an experiment group. Every well in the blank group contained 50 μl PC12 cells at the density of 1.2×10^5/ml and 50 μl 2% DMEM complete medium in each well; each well in the control group contained 50 μl PC12 cells at the density of 1.2×10^5/ml and 50 μl pTracer™-EV/V5-His-CHO cells' concentrated supernatant, which was diluted with 1:2, 1:4, 1:8, 1:16, 1:32, 1:64; and every well in the experiment group contained 50 μl PC12 cells also at the density of 1.2×10^5/ml and 50 μl pTracer™-EV/V5-His-hBDNF-CHO cells' concentrated supernatants, which was also diluted by 1:2, 1:4, 1:8, 1:16, 1:32, 1:64. Then the plate was put into a incubator at 37 ℃ with 5% CO_2. 72 hours later, 10 μl 5 mg/L thiazolyl blue was added into each well, and 3 – 4 hours later 100 μl 10% acidation SDS were added into all wells to terminate reaction, then the A_{570} value of each well was measured after 12 – 14 hours. Finally, all these date were analyzed by SPSS statistics software.

3 Results

3.1 *hBDNF* gene's introduction into CHO cells

72 hours after transfection CHO cells were observed under fluorescence microscope, and sporadic cells with green fluorescence could be seen (Figure 1). After 40 days' screening the CHO cells were again observed under fluorescence microscope, all cells were found to emit green fluorescence (Figure 2). This proved that the plasmids had been transfected into CHO cells and the GFP (green flurescence protein) gene in the plasmid of pTracer™-EV/V5-His could normally be expressed.

3.2 RT-PCR analysis

The products of RT-PCR were electrophoresed on 1.5% agarose gel, and a band can be seen near 750 bp. This demonstrated the gene *hBDNF* introduced into CHO cells could be effectively transcribed into mRNA (Figure 3).

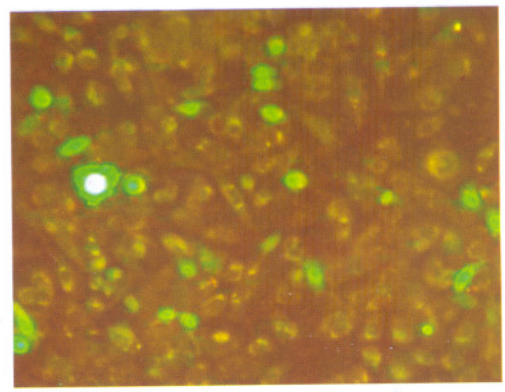

Figure 1. CHO cells 72 h after transfection

3.3 Westen-blot analysis

A brown band appeared on the lane of experiment group (EG), but no strap appeared on the control group's (CG), which illustrated that the target protein had been expressed in pTracer^{TM}-EV/V5-His-hBDNF-CHO cells, but not in the pTracer^{TM}-EV/V5-His-CHO cells (Figure 4).

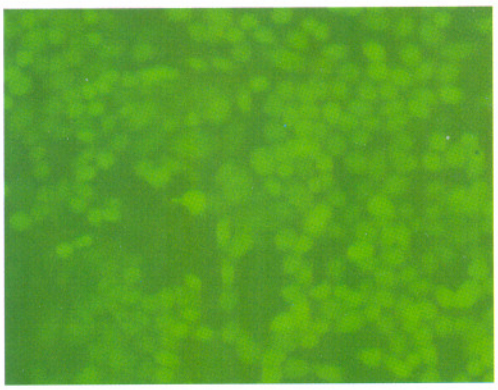

Figure 2. CHO cells after 40 days' screening

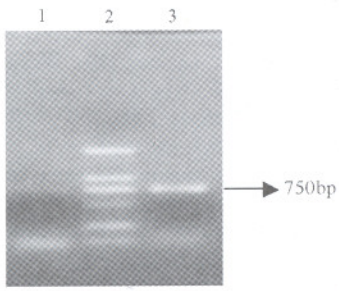

Figure 3. Lane 1 and 3 respectively showed the RT-PCR results of cells pTracer^{TM}-EV/V5-His-CHO and pTracer^{TM}-EV/V5-His-hBDNF-CHO; Lane 2 showed DNA Marker

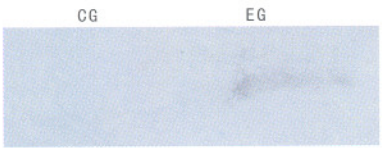

Figure 4. CG and EG respectively showed the Western-blot results of enriched supernatants of the two kinds of CHO cells: pTracer^{TM}-EV/V5-His-CHO and pTracer^{TM}-EV/V5-His-hBDNF-CHO

3.4 Activity detection for the eukaryotic expression product of *hBDNF* gene

3.4.1 Promoting PC12 cells' growth: After incubation for 72 hours, cells in the blank group (BG) adhered and stretched, but in small number. Cells in vacant plasmid group adhered, stretched were a little more than blank group. Cells in experiment group adhered, stretched were in high density (Figure 5A, B, C). The total number in each bottle was 1.8×10^5, 2.5×10^5 and 4.0×10^5, respectively.

3.4.2 Activity detection by MTT assay: The A_{570} value of the blank group was 0.137 ± 0.009, the A_{570} values of the vacant plasmid group (VG) and the experiment group (EG) in different dilute strength were shown in Table 1.

3.4.3 Statistics analysis results: The t test of the A_{570} value between two groups of EG and VG shows $P<0.01$, ($\bar{x}\pm s, n=3$).

4 Discussion

BDNF, a kind of protein, which was first found and isolated from a pig's brain in 1982 by German neurobiologist Barde and his colleagues, can promote neurons' growth; generally its active form exists as a dimeride combined by non-covalent bonding. The binding of BDNF to its receptor tyrosine kinase (TrkB) leads to the dimerization and autophosphorylation of tyrosine residues in the intracellular domain of the receptor and subsequent activation of cytoplasmic signal transmission[5-6]. At present, we get BDNF mainly from tissue's isolation and purification or recombined gene's expression. Large-scale preparation of these natural hBDNF proteins directly isolated from tissues is very difficult, in spite of their better activities.

The BDNF proteins, expressed by prokaryocytes through the technology of recombination *in vitro*, have relatively lower activities because these synthetic polypeptides cannot properly fold. However, proteins expressed in eukaryocytes are more approximate to the natural hBDNF protein and have higher activities. There are some merits for those proteins expressed in eukaryocytes than in prokaryocytes: first, acquiring more elaboration, e.g. α-helix and β-pleated sheet, glycosylation and phosphorylation; second, acquiring mature mRNA by recognizing and eliminating the introns of exogenous genes; third, eukaryocytes transfected with target genes can stably express target proteins even after frezeeing and revivals.

We have constructed the plasmid pTracer^{TM}-EV/V5-His-hBDNF. By comparing the difference of promoting PC12 cells' growth between the concentrated supernatants of the two kinds of CHO cells, pTracer^{TM}-EV/V5-His-hBDNF-CHO and pTracer^{TM}-EV/V5-His-CHO, we concluded that CS and hBDNF could promote PC12 cells' growth synergistically, for CS in both kinds of enriched supernatants were in some higher concentration. And PC12 cells grow better in VG than in BG, but worse

Table 1. Comparison between the EG and VG in different dilute strength

1/64(A)	1/32(B)	1/16(C)	1/8(D)	1/4(E)	1/2(F)	
EG	0.273±0.009	0.27±0.037	0.283±0.021	0.310±0	0.350±0.014	0.493±0.0286
VG	0.163±0.009	0.173±0.005	0.207±0.009	0.227±0.009	0.247±0.025	0.363±0.024

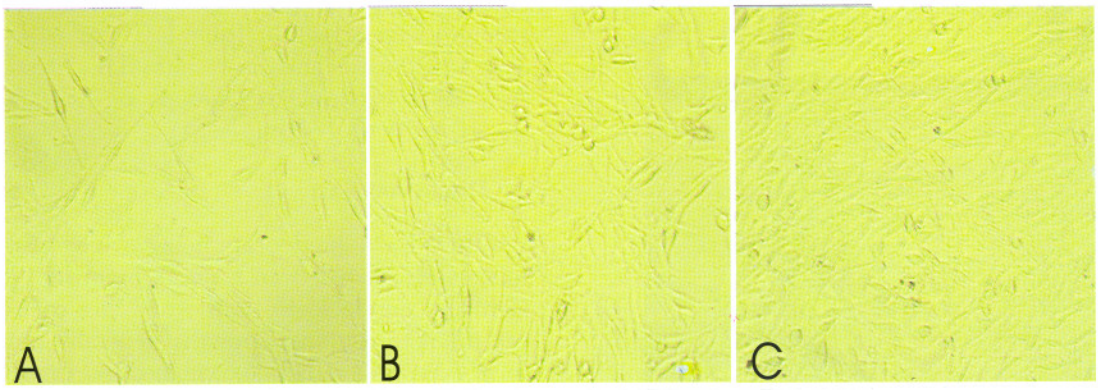

Figure 5. A: cells in the blank group B: cells in the vacant plasmid group C: cells in the experiment group

than in EG. In addition, we selected CHO cells as host cells but not African green monkey SV40-transformed kidney fibroblast cell (COS-7) cells, it was because that exogenous genes' expressions in CHO cells are stable and long-term after screening with G418, but not in COS-7 cells, which are only used to be the transient expression host cells. As a result, gene-engineering pharmacy prefers CHO cells than COS-7 cells.

Our subject establishes the experiment base for further research on developing these kind bioengineered medicines and treatments for some nervous system problems.

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