Transfer and Expression of VEGF Gene in Neural Stem Cells

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Abstract: Objective. To construct retroviral vector containing vascular endothelial growth factor (VEGF) and observe its expression in rat neural stem cells. **Methods.** The recombinant retroviral vector pLXSN-VEGF containing VEGF gene was established and transferred to packaging cell line-pA317 to acquair virus. The titer was calculated by infection of NIH3T3 cells, then the recombinant virus was to infect neural stem cells (NSCs) isolated from the whole brain of embryonic day 14 (E14) SD rats. Transduced cells were cloned in G418 and expanded for analysis of VEGF transgene expression, VEGF mRNA, VEGF expression and its expression curve were detected and worked by using RT-PCR, immunocytochemistry and enzyme-linked immunosorbent assay (ELISA), respectively. **Results.** Retroviral vector pLXSN-VEGF was successfully established and it was proved by enzyme cutting and sequence analysis. The titer of retrovirus was determined to be 6.5×10^5 CFU/ml. RT-PCR and immunochemistry verified the expression of VEGF in neural stem cells. The peak of VEGF expression was showed 6-8 days after transfection by ELISA. **Conclusion.** Retroviral vector containing VEGF were successfully constructed. VEGF gene can be stably expressed in rat neural stem cells. [Life Science Journal. 2006; 3(2): 50 - 54] (ISSN: 1097 - 8135).

Keywords: NSCs; VEGF; retrovirus; transfection; transgene

Abbreviations: NSC: neural stem cell; VEGF: vascular endothelial growth factor

1 Introduction

Neural stem cells (NSCs) are the primary cell element for generation and maintenance of nervous system. Their essential biologic characteristics are: undifferentiation, deficiency of differentiation signal, self-renewal, potency to differentiate into neurons, oligodendrocyte and astrocyte[1]. NSCs may provide for cell replacement or gene delivery vehicles in neurodegenerative disease therapies[2], and target delivery and expression of growth factor transgenes such as vascular endothelial growth factor (VEGF) in NSCs to construct genetic engineering[3]. NSCs is an important direction in gene therapy of nervous system disease. The secreted glycoprotein VEGF is a potent and specific mitogen for vascular endothelial cells that is capable of stimulating angiogenesis during embryonic development and adult neurogenesis *in vivo*[4]. It has been reported that VEGF stimulated the expansion of neural stem cells. VEGF also acts as a trophic factor for neural stem cells *in vitro* and for sustained neurogenesis in the adult nervous system[5]. This experiment plans to construct a retroviral vector containing VEGF gene, transfer the gene into NSCs and detect the expression of it; and to provide experimental evidence for transgenic neural stem cell transplantation therapy of neurodegenerative diseases.

2 Materials and Methods

2.1 Materials

The plasmid pcDNA 3.1-VEGF contains a cDNA copy of the human VEGF gene, constructed in our laboratory using a pcDNA3.1 plasmid backbone (Invitrogen, Carlsbad, CA, USA). Pregnant SD rats were offered by Experimental Animal Center of Henan Province of China (Zhengzhou, China). Main reagents: restriction endonuclease *EcoR*I and *Xho*I, T4 DNA Ligase (TaKaRa); retroviral vector pLXSN (clontech); *E. coli* JM109 (TaKaRa); plasmid miniprep kit, Agarose Gel DNA fragment recovery kit, DNA extraction kit (Watson Biotechnologies, INC); Lipofectamine 2000 Liposome, B27, bFGF (Invitrogen); DMEM/F12, FBS (Hyclone); Nestin antibody, VEGF antibody (Boster).

2.2 Methods

2.2.1 Constuction of recombinant vector pLXSN-VEGF: According to compatible restriction sites in pcDNA 3.1-VEGF and pLXSN, *EcoR*I and *Xho*I were to digest the plasmid pcDNA 3.1-VEGF and pLXSN. Agarose electrophoresis and gel extraction were performed to acquire VEGF cDNA fragment and digeste pLXSN, then T4 DNA ligase was used to connect cDNA and plasmid (ratio: 3:1). After 16 h reaction, ligation mixture was transformed into *E. coli* JM109, and 6 separate colonies were harvested next day. After propagation plasmids were extracted to perform restriction analysis to identify the construction.

2.2.2 Packaging virus and determining viral titer: The procedures were performed following the User Manual[6]. Briefly, 5×10^5 PA317 packaging cells were seeded on 35 mm culture dish and cultured in 37℃, 5% CO_2 incubator for 18 h. When cells grew to 75% confluence, removed the culture, rinsed the cells with serum-free medium, used Lipofectamin to mediate recombinant plasmid transfecting pA317 packaging cells: 2 μg plasmid and 10 μL Lipofectamin diluted to 100 μL with serum-free and antibody-free DMEM, and slightly mixed together. The mixture were tiled to the layer of pA317 cells, placed in 37℃, 5% CO_2 incubator for 15 h. Next added 1 mL DMEM containing 20% FBS into it, and next day replaced the culture with normal solution. After 48 h, began screening using culture solution containing 800 mg/L G418. This process lasted for 3 weeks. Placed NIH 3T3 cells in 6-well plates at a density of $0.5-1\times10^5$ cells per well. Add 2 mL medium per well. Prepare 20 mL of complete medium and added 60 μl of 4 mg/ml polybrene. Collect virus-containing medium from packaging cells. Prepare six 10-fold serial dilutions. Infected NIH 3T3 cells by adding 1 mL of the diluted virus medium to the wells. Final polybrene concentration will be 4 μg/ml. The viral titer corresponded to the number of colonies present at the highest dilution that contains colonies, multiplied by the dilution factor.

2.2.3 Rat embryonic NSCs isolating culture and identification: NSCs were isolated from embryonic mice (day 16 - 18) essentially as described[7]. Briefly, embryonic brain was cleared of meninges, then separated the whole brain, rinsed with HBSS solution and sheared to small tissue pieces, digested with 0.125% pancreatic enzyme at 37 ℃ water bath. Filtered and centrifuged, removed supernatant, cultured by DMEM/F12 containing 2% B27, EGF (20 ng/ml), bFGF (20 ng/ml). Changed the liquid every 3 to 4 days and proliferated every 7 days. Limiting dilution assay was used to prepare monoclonal cell, immunocytochemistry was used to identify Nestin expression in NSCs. DMEM/F12 with 5% FBS was to induce NSCs differentiation. Immunocytochemistry was used to identify MAP2, GFAP expression in differentiated cells.

2.2.4 Infecting NSCs: Secondary generation neurospheres were separated into two sets: one was added with virus, and the other was treated with an insert-free virus as control cells. All were infected at a multiplicity of infection and cultured for 20 h. Transduced cells were cloned in G418 (200 μg/ml) and expanded for analysis of VEGF transgene expression. Immunocytochemistry was used to identify Nestin expression in transgenic NSCs; DMEM/F12 containing 5% FBS was used to induce NSCs differentiation and immunocytochemistry was used to identify MAP2, GFAP expression in differentiated cells.

2.2.5 RT-PCR: Total RNA was isolated from transgenic NSCs according to the protocol recommended by the manufacturer (TRI reagent; Sigma, USA), then a One-Step RT-PCR kit was used to amplify VEGF cDNA fragment. Samples were treated for 30 min at 56°C, then 5 min at 94°C and then 30 cycles of amplification were performed as follows: 45 sec at 94°C, followed by 45 sec at the annealing temperature, and 90 sec at 72°C, with final extension at 72°C for 10 min. Forward primer (5′-GCAAATGGGCGGTAGGCGTG-3′) and the reverse primer (5′-ATA GGA TCC TCA CCG CCT CGGCTT-3′) amplified a 570-bp pLXSN: VEGF specific gene transcript. β-actin transcripts were amplified from each RNA sample with β-actin-specific primers (upstream: 5′- TACAA CCTCC TTGCA GCTCC-3′, downstream: 5′-GGATC TTCAT GAGGT AGTCA GTC-3′) used as an internal control (620 bp). The RT-PCR amplification products were gel electrophoresed, stained with ethidium bromide.

2.2.6 Immunocytochemistry: Transgenic NSCs were fixed in 4% formaldehyde and the membrane was ruptured with 0.1% Triton for 10 min. After blocking cells for 30 min in diluent containing 10% goat serum, samples were incubated with primary antibodies overnight at 4°C. After rewarmed and rinsed biotinylated goat anti-mouse IgG was added, rinsed with PBS, added with SABC, rinsed with PBS again and stained with DAB. After stained with hematoxylin and alcoholic dehydrating and mounting, the samples were observed by light microscope. NSCs treated with an insert-free virus were as control cells.

2.2.7 Enzyme-Linked Immunosorbent Assay (ELISA): The supernatant of transgenic NSCs was collected and ELISA was performed as User Mannal described. According to the experimental data, curve of VEGF secretion state at different time was drawn.

3 Results

3.1 Restriction analysis and sequencing of pLXSN-VEGF

pLXSN-VEGF was digested by *EcoR*I and *Xho*I, then Agarose electrophoresis was performed. The results showed a 570 bp product and a 6,000 bp product, corresponding to VEGF and pLXSN. Linker region and VEGF cDNA were sequenced. There were no mutations generated from the plasmid construction, and the VEGF gene remained in frame.

3.2 Viral titer

After infected by retroviral, NIH 3T3 cells formed resistant cell colony after 3 weeks under the selective medium. The average viral titer was 6.5×10^5 cfu/ml, the highest was 1.8×10^6 cfu/ml.

3.3 Rat embryonic NSCs identification

Cells obtained from embryonic rats grew in suspension in DMEM/F12 and showed propagation manifest (Figure 1). The third generation of NSCs were fixed 2 hours after adherence, and most expressed the neuron specific protein Nestin. After differentiation the astrocyte marker GFAP and neuron marker MAP2 were expressed.

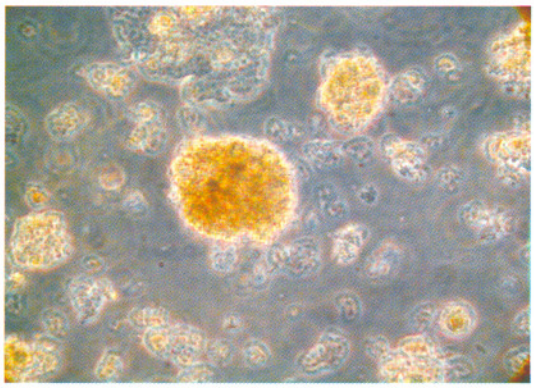

Figure 1. Neural stem cell globe (×400)

3.4 Infection of NSCs

Transgenic NSCs still remained in undifferentiated state. Immunocytochemistry results showed that the transgenic NSCs expressed the neuron specific protein Nestin (Figure 2). 7 days after adherence, most cell colonies differentiated into neurons, astrocytes and oligodendrocyte of typical shape. Immunocytochemistry showed that there were MAP-2 positive expression and GFAP positive cells (Figures 3,4).

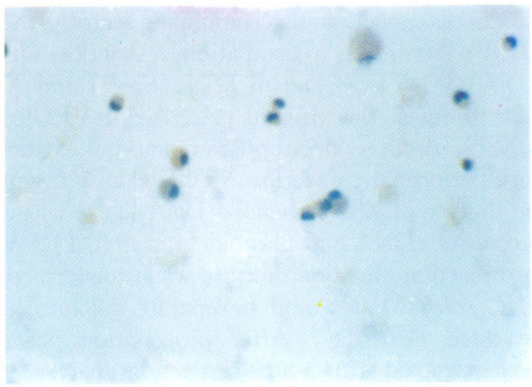

Figure 2. The positive expression of nestin antigen (×400)

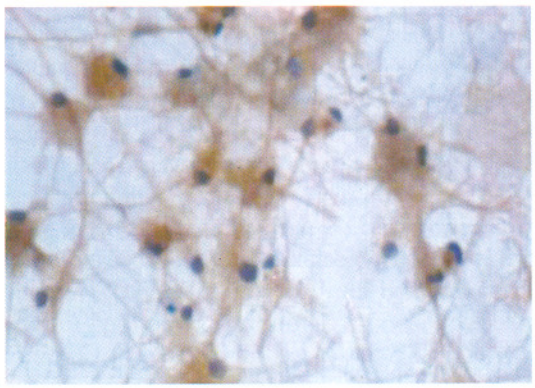

Figure 3. Identification of the expression of MAP-2 in glial cells by immunohistochemistry method (×400)

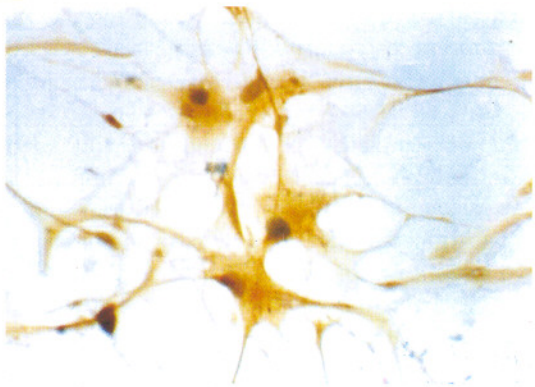

Figure 4. Identification of the expression of GFAP in neurons by immunohistochemistry method (×400)

3.5 RT-PCR and immunocytochemistry

RT-PCR studies verified transgenic VEGF mRNA in transduced clones (Figure 5), immunocytochemistry showed that the transgenic NSCs expressed VEGF protein (Figure 6), and NSCs treated with an insert-free virus were VEGF negtive.

3.6 ELISA

After transfection, concentration of VEGF in

supernatant gradually increased, and achieved peak in 6－8 days, then gradually descended until stabilization (Figure 7). The expression was maintained for at least 1 month (the last time point tested). After freeze-thaw, the transgenic NSCs were still able to stably secrete VEGF. In contrast, normal NSCs kept to secrete VEGF at a lower level.

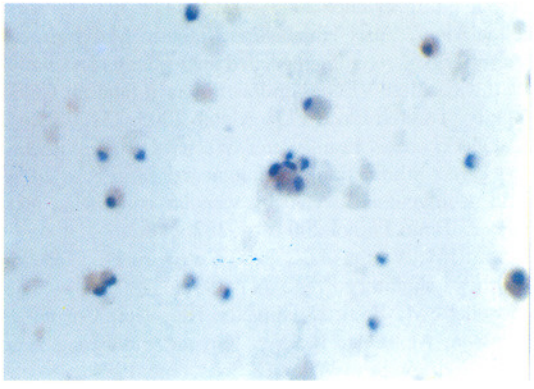

Figure 5. Identification of the expression of VEGF gene in transgenic neural stem cells by immunohistochemistry (×400)

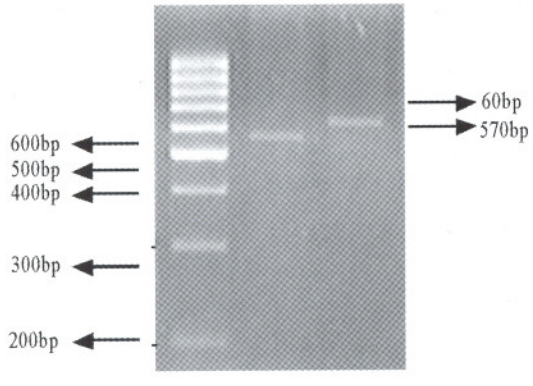

Figure 6. RT-PCR result of transgenic neural stem cells.
1: Maker 2: VEGF 3: β-actin

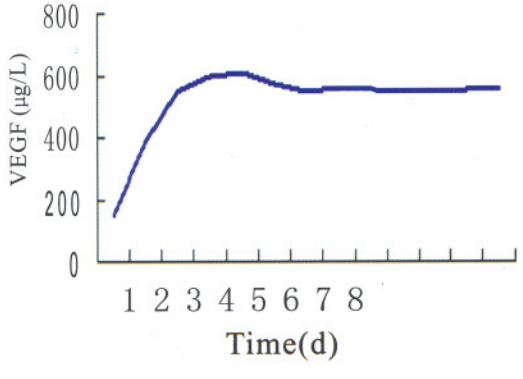

Figure 7. VEGF expression curve determined by ELISA

4 Discussion

NSCs are considered a heterogeneous population of mitotically active, self-renewing, multipotent, immature progenitor cells[4], making them uniquely situated to restore nervous system and therapy of nervous degenerating diseases. They may also present an ideal route for cell-mediated gene therapy as well as offer new possibilities for the replacement of neurons lost by injury or disease[8].

VEGF is a potent and specific mitogen for vascular endothelial cells. Anne has reported that hypoxia induced VEGF expression in clonally-derived adult rat neural stem cells *in vitro*, and low dosage of VEGF (2.4 ng/d) could stimulate adult neurogenesis *in vivo*[5]. He observed that VEGF could reduce neural stem cells apoptosis without altering its proliferation. This suggests that VEGF has survival promoting effect in neural progenitor cells[9]. We propose that VEGF acts as a trophic factor for neural stem cells *in vitro* and for sustained neurogenesis in the adult nervous system. The long-term and stable expression of VEGF by NSCs through retrovirus mediated gene transfer would enhance the concentration of VEGF maybe useful for the therapy of neurodegenerative diseases.

Retroviral gene transfer can efficiently introduce stable, heritable genetic material into the genome of any dividing cell type. pLXSN contains elements derived from Moloney murine leukemia virus (MoMuLV) and Moloney murine sarcoma virus (MoMuSV), and is designed for retroviral gene delivery and expression[10]. Upon transfection into a packaging cell line, pLXSN can transiently express, or integrate and stably express, a transcript containing viral packaging signal, the gene of interest, and a selectable marker. The 5′ viral LTR in this vector contains promoter/enhancer sequences that control expression of the gene of interest in the multiple cloning site. The SV40 early promoter (P_{SV40e}) controls the expression of the neomycin resistance gene (Neo^r), which allows antibiotic selection in eukaryotic cells. pLXSN also includes the Col E1 origin of replication and *E. coli* Amp^r gene for propagation and antibiotic selection in bacteria.

pLXSN does not contain the structural genes (*gag*, *pol*, and *env*) necessary for particle formation and replication. However, these genes are stably integrated into pA317 packaging cells. Subsequent introduction of pLXSN, containing the extended viral packaging signal (*psi*), transcription and processing elements, and the gene of interest

produces high-titer, replication incompetent infectious virus. That is, these retroviral particles can infect target cells and transmit the gene of interest (which is cloned between the viral LTR sequences), but cannot replicate within these cells since the cells lack the viral structural genes. Transforming NIH3T3 cells showed the average viral titer was 6.5×10^5 cfu/ml, in which the highest was 1.8×10^6 cfu/ml.

After transfection of NSCs, we detected the VEGF concentration every 3 days. The data showed peak of VEGF concentration was on the 6th – 8th day, then descended slightly, and reached its stabilization. After freeze-thaw, the transgenic NSCs were still able to stably secrete VEGF.

In this experiment, VEGF gene stably expressed in NSCs and the transfected NSCs still maintained its multipotency and self-renewal. These results hold promise for the use of genetically manipulated stem cells for CNS therapies.

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References

1. Ronald McKay. Stem cells in the central nervous System. Science 1997; 276(5309): 66 – 71.
2. Ramalho-Santos M, Yoon S, Matsuzaki Y, et al. "Stemness": transcriptional profiling of embryonic and adult stem cells. Science 2002; 298(5593): 597 – 600.
3. Ivanova NB, Dimos JT, Schaniel C, et al. Response to Comments on "'Stemnes': transcriptional profiling of embryonic and adult stem cells" and "A stem cell molecular signature. Science 2003; 302(5644): 393 – 9.
4. Nina Mani, Alfia Khaibullina, Janette M Krum, et al. Activation of receptor-mediated angiogenesis and signaling pathways after VEGF administration in fetal rat CNS explants. J Cereb Blood Flow Metab 2003; 23: 1420 – 9.
5. Anne Schänzer, Frank-Peter Wachs, Daniel Wilhelm, et al. Direct stimulation of adult neural stem cells *in vitro* and neurogenesis in vivo by vascular endothelial growth factor. Brain Pathol 2004; 14: 237 – 48.
6. Coffin JM, Varmus HE, Eds. Retroviruses. Cold Spring Harbor Laboratory Press, NY 1996.
7. Ausubel FM, Brent R, Kingston RE, et al. Current Protocols in Molecular Biology. Greene Publishing Associates, Inc. & John Wiley & Sons, Inc. 1994.
8. Sondell M, Lundborg G, Kanje M. Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. J Neurosci 1999; 19: 5731 – 40.
9. Samii A, Unger J, Lange W. Vascular endothelial growth factor expression in peripheral nerves and dorsal root ganglia in diabetic neuropathy in rats. Neurosci Lett 1999; 262: 159 – 62.
10. Miller AD, Rosman GJ. Improved retroviral vectors for gene transfer and expression. BioTechniques 1989; 7(9): 980 – 90.

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