腺病毒载体介导的VEGF基因转染对C17.2神经干细胞凋亡的影响

Neural stem cells have been recognized as promising seed cells for genetically engineering cells with great potential in the treatment of cerebrovascular disease, especially in cell transplantation for therapy of cerebral infarction. But more often than not, the implanted cells are subjected to unfavorable, typically hypoxic, local microenvironment in vivo, which almost inevitably affect their survival. Experimental evidence has demonstrated that hypoxia induces massive apoptosis of neural stem cells in vitro[1]. Looking for effective measures to ensure the survival of the implanted stem cells against ischemia-induced hypoxia becomes therefore a major concern in the research of cell transplantation therapy for cerebral infarction. Vascular endothelial growth factor (VEGF) is one of the secretary proteins capable of inducing the proliferation of endothelial cells, thereby promotes neovasculari-zation, but of greater interest to researchers, VEGF can also protect the neurons and neural progenitor neural cells from apoptosis[2][3]. In this study, we observed the effects of human VEGF gene transfer on hypoxia- induced apoptosis of neural stem cells in vitro, attempting to provide theoretical basis for neural stem cell transplantation for therapy of cerebral infarction.

MATERIAL AND METHODS

Materials

Recombinant adenovirus pAdCMV $VEGF_{165}$ was constructed in our lab. C17.2 neural stem cells were kindly provided by Prof. Snyder, Harvard Medical University, USA.

Trypsin and DMEM/F12 was purchased from Gibco Company (USA), and fetal cattle serum (FCS) was the product of Sijiqing Biological Engineering Materials Co. Ltd (Hangzhou, China). Polylysine was supplied by Sigma Co. (USA). TUNEL assay kit and FuGENE kit were from Roche Molecular Biochemicals Co. (Swiss), and S-P immunohistochemical detection kit and DAB reaction kit from Mycine Biological Engineering Co. (Fujian, China). Rat anti-human mono-clonal nestin antibody and rabbit anti-human polyclonal VEGF antibody were manufactured by Santa Cruz Co. (USA), and VEGF anti-sense oligodeoxynucleotide (ODN, sequence: 5'-AGAGAGCAGAAAGTTCT-3'[3]) was synthesized by Shanghai Biological Engineering Co. (China).

Methods

Cell culture C17.2 neural stem cells were cultured in DMEM/F12 medium (containing 10% FCS, 5% horse serum, 1×10^6 U/L penicillin, and 1×10^5 U/L streptomy- cin) at 37 ℃ with 5% CO_2. After the cells had spread on the slides, immunohistochemical staining for nestin and X-gal was performed for identification of the stem cells[4].

Recombinant adenovirus infection

After the C17.2 neural stem cells were cultured to over 90% confluency, the supernatant was discarded and the cells were infected with pAdCMV $VEGF_{165}$ with a multiplicity of infection (MOI) of 20. Following incubation of the cells at 37 ℃ with 5% CO_2 for 3 h, DMEM/F12 medium was added and another 48 h incubation was performed. Fluorescence microscopy was then performed for observing the infection rate of the cells, represented by the percentage of green fluorescent protein (GFP)-positive cells among the total cells.

Grouping

The cells were assigned randomly into 4 groups (6 wells in each group), namely the blank control group (Group A), hypoxic group (Group B, in which the cells were cultured at 37 ℃ with 95% N_2 and 5% CO_2), hypoxia + pAdCMV $VEGF_{165}$ infection group (Group C, where the cells were cultured at 37 ℃ with 95% N_2 and 5% CO_2 after pAdCMV $VEGF_{165}$ infection for 48 h), and hypoxia + pAdCMV $VEGF_{165}$ infection + anti-sense VEGF ODN group (Group D, where the cells were infected by pAdCMV $VEGF_{165}$ and anti-sense VEGF ODN followed by incubation at 37 ℃ with 95% N_2 and 5% CO_2 for 48 h). After incubation at 37 ℃ with 95% N_2 and 5% CO_2 for 8 h, the cells were further cultured at 37 ℃ with 5% CO_2 for another 24 h before collected for examination. The entire procedure was repeated twice.

Western blotting

Western blotting was performed to study the expression of VEGF in C17.2 neural stem cells. Briefly, the total protein of the cells was extracted and the concentration of protein determined by bicinchoninic acid (BCA) assay.

From each group, 15 μg of the total protein was mixed with 2×SDS loading buffer (1∶1) and incubated at 100 ℃ for 5 min followed by discontinuous electrophoresis on 12% SDS-PAGE gel (containing 12% separation gel and 5% stacking gel) at 100 V for 3 h. The gel was transferred at 100 mA for 4 h onto a membrane which was then blocked for 1 h followed by incubation with polyclonal rabbit anti-human VEGF antibody (1∶400) at 4 ℃ overnight. Finally, the positive staining was detected chemically after incubation with horse-radish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature.

TUNEL staining

TUNEL staining was performed for detection of apoptotic cells following the manufacturer's instruction of TUNEL detection kit. The positive staining was examined by DAB method. The cells were then observed under microscope, and the positive cells defined as brown staining in the nuclei. The apoptosis index (AI) was calculated in 5 randomly selected visual fields at high-power magnification (×400).

Flow cytometry analysis

After hypoxia treatment, the cells were incubated at 37 ℃ with 5% CO_2 again for 12 h and collected for staining with Annexin V-PI double staining according to the instruction of the kit. Flow cytometry detection was then performed for detecting cell apoptosis and necrosis, and 1×10^4 cells were counted each time to calculate the apoptotic rate.

Fluorescence microscopy

When they had sufficiently spread on the slides, the cells were washed with cold phosphate buffer sodium (PBS, pH 7.0) solution and fixed in 4% methanol for 30 min. After washing with PBS for another 5 min, the cells were incubated with 0.1% acetic acid for 30 s, washed again with PBS, thoroughly air-dried at room temperature, and stained with Hoechst33342 for 10 min, followed by washing with distilled water for 1 min and again air-drying at room temperature. Finally, the apop- totic bodies (AB) were observed under fluorescence microscope and photographs were taken.

Statistical analysis

Statistical analysis was performed with SPSS 11.0 software. The data were presented as Mean±SD. F test was performed for examining the difference between the groups (α= 0.05).

RESULTS

Identification of C17.2 neural stem cells

Under microscope, normal C17.2 neural stem cells exhibited irregular or triangular morphology, and about 95% of the cells showed positivity for nestin immuno- histochemical staining and over 90% were positive for X-gal staining, shown by blue stains in the cytoplasma with a clear normal morphology, suggesting stable expression of LacZ gene in cultured C17.2 neural stem cells.

Effect of recombinant adenovirus infection on C17.2 neural stem cells

Forty-eight hours after infection with recombinant adenovirus, the cells were observed under fluorescence microscope. The infected C17.2 neural stem cells showed green fluorescence, and the infection rate exceeded 95%. The infected cells diffused evenly in the medium and showed a normal morphology without any visible pathological changes.

Exogenous VEGF gene expression in C17.2 neural stem cells after pAdCMV $VEGF_{165}$ infection

As expected, a specific band (molecular weight of 22 000) was detected by Western blotting in pAdCMV $VEGF_{165}$-infected cells, suggesting successful expression of VEGF gene. Interestingly, the cells infected with both pAdCMV $VEGF_{165}$ and anti-sense VEGF ODN also showed the specific band, but with much less density, indicating that anti-sense VEGF ODN could effectively inhibit the expression of pAdCMV $VEGF_{165}$. However, this band was not presented in either the blank control groups (Fig.1).

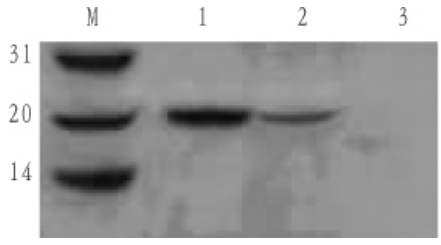

Fig.1 Expression of VEGF in the C17.2 neural stem cell
M: Protein marker; Lane 1: pAd $VEGF_{165}$ infected group; Lane 2: pAd $VEGF_{165}$- and antisense VEGF ODN-infected group; Lane 3: Normal control group

AI of hypoxic C17.2 neural stem cells following transfection

Following TUNEL staining, the C17.2 neural stem cells in groups B and D showed very high and com- parable AI

(21.45±2.47 and 19.05±2.78, respectively, P>0.05), whereas the AI in Group C (8.42±1.28) was significantly lower than that in the above two groups (P<0.01), but still higher than that of group A (0.98±0.12, P<0.01), suggesting that VEGF gene transfer could attenuate apoptosis at some level.

Flow cytometry for apoptotic rate

Flow cytometry revealed that the cells in groups B and D had similarly significant increase in the apoptotic rate (19.98%±0.55% and 19.06%±0.64%, P>0.05) as compared with group A (P<0.01). The rate of group C (10.38%±0.48%) was significantly lower than that of groups B and D (P<0.01) but still higher than that of group A (P<0.01, Fig.2).

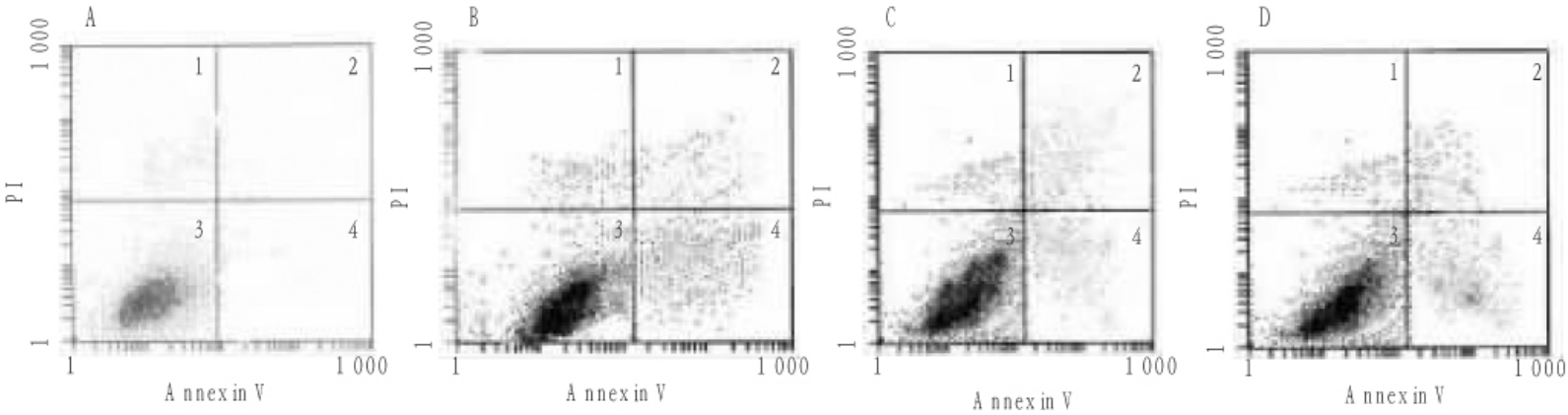

Fig.2 Apoptotic rate of C17.2 neural stem cells detected by flow cytometry

Fluorescence microscopic observation of apoptotic bodies

After Hoechst33342 staining, only a few cells in group A showed strong staining in the nuclei, whereas a large number of cells in groups B and D exhibited such changes as strong staining in the nuclei and nuclear condensation with typical apoptotic bodies in the cytoplasma (Fig.3). In group C, most of cells showed light staining in the nuclei with only occasional strong staining, and no typical apoptotic bodies were found (Fig.4).

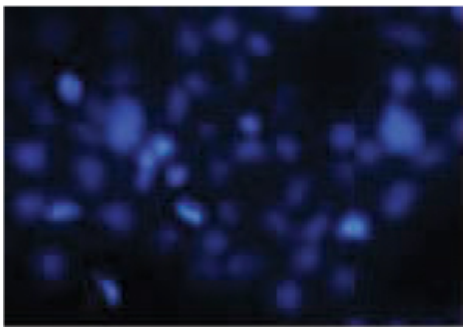

Fig.3 C17.2 neural stem cell stained with Hoechst33342 after hypoxia showing strong staining in the nuclei or apoptotic bodies

Fig.4 C17.2 neural stem cell infected by pAdCMV $VEGF_{165}$ and stained with Hoechst33342 after hypoxia showing strong staining in only a few nuclei without apoptotic bodies

DISCUSSION

Highly prevalent as it is among the aged, cerebral ischemia remains a difficult clinical entity that causes neuronal death, for which no effective interventions were currently available. Amelioration of blood supply and improving the survival of neurons around the ischemic foci have been the major strategies in therapy of cerebral ischemia, but they often fail to prevent the occurrence of brain injury or induce the regeneration of the brain tissue following cerebrovascular accident. Exogenous neural stem cell transplantation, a currently attractive strategy for management of cerebral ischemia, has provided new possibility for replacing the lost neurons and promoting the neurological function recovery[5][6]. The neural stem cells, however, almost inevitably encounter hypoxic or ischemic conditions in vivo after transplantion to endanger their survival. Recent reports indicated that hypoxia could induce massive neural stem cell death in the manner of apoptosis[1]. Therefore, how to inhibit or attenuate the apoptosis of the transplanted neural stem cells and improve their survival in hypoxic conditions becomes the key issue in neural stem cell transplantation for therapy of cerebral ischemia. VEGF belongs to the family of glycoproteins that plays an essential role in the development of blood vessels, the generation of new vascular networks from existing vessels, and hemato- poiesis[7][8]. But of greater importance, VEGF has recently proved to possess immediate neurotrophic and neuroprotective effects as in enhancing the growth of axons of the dorsal root ganglion neurons in the upper cervical segments[9] and promoting the survival of neurons and endothelia in the central nervous system[10], which also protects brain neurons and rat hippocampal HN33 cells from hypoxia-induced apoptosis[11]. Studies indicate that VEGFR2/FLK-1 receptors exist in the neurons and progenitor neurons, and activation of VEGFR2 induces the differentiation of the progenitor neurons into mature neurons [12]. Further- more, it protects the neurons from hypoxic injury[11] and glutamate toxicity[13], and enhances neurogenesis in the subventricular zone in vivo as well as in vitro[14].

In this study, for the first time we developed the apoptotic model of C17.2 neural stem cells induced by hypoxia, and observed the effects of recombinant adenovirus containing VEGF gene on the apoptosis of infected cells. Our results indicated that hypoxia induced significant apoptosis of C17.2 neural stem cells as compared with the normal control group, and this high apoptotic rate was markedly reduced by pAdCMV $VEGF_{165}$ infection, suggesting the efficacy of $VEGF_{165}$ gene transfer in inhibiting hypoxia-induced apoptosis. Co-treatment with VEGF anti-sense ODN obviously canceled the anti-apoptotic effect of VEGF gene transfer, resulting in the apoptotic rate of the cells comparable to that of only hypoxia-treated cells, which further verifies that VEGF gene transfer could attenuate neural stem cell apoptosis after hypoxia. It was noted that the apoptotic rate of cells treated with hypoxia+ pAdCMV $VEGF_{165}$ was still much higher than that of the normal control cells, indicating that VEGF gene transfer could not totally block the apoptosis-inducing effect of hypoxia, which might be attributed to the possible involvement of other apoptotic factors or pathways.

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