Introduction

Ischemic stroke is caused by narrowing of the arteries in the neck or brain, or heart attack, or abnormalities of the heart valves. It is one of the common neurological diseases which brings heavy burden on individual and family, as well as medical cost, and it is a global problem. Patients suffering from stroke may develop motor deficiency, and memory loss etc, depending on the region of the brain affected. One of the hallmarks in ischemic stroke is that massive cell death in infarct area which could include many brain sub-regions such as cortex, hippocampus, striatum and hypothalamus, thus stroke patients display differentially clinical symptoms.

Currently, there are two well-characterized animal models used for ischemia study including forebrain global ischemia and middle cerebral artery occlusion (MCAO). And MCAO is more prone to mimic clinical features of stroke than global ischemia. It is reproducible and controllable, which could be achieved by withdrawing the suture at defined time points.

Neurotrophic factors have long been implicated to play important roles in both physiological and pathological conditions such as neuronal development, survival and differentiation, synaptogenesis and synaptic plasticity, as well as in nerve injury, ischemic insults and epileptogenesis [1-4]. Brain-derived neurotrophic factor (BDNF) is a well-characterized neurotrophic factor which exerts its function through binding to the high-affinity receptor tyrosine kinase (TrkB) thus activating its downstream signaling pathways. BDNF is expressed ubiquitously in the central nervous system during development and shows trophic effects on neuronal cells challenged with serum deprivation [5, 6]. In vivo, BDNF could be able to rescue neuronal cells from neurodegeneration resulted by central nervous system injuries [7-12]. BDNF and trk...
B mRNAs were found to increase in a widespread region of the ipsilateral cortex outside the infarct following MCA occlusion [13]. Prior reports employing intraventricular injection of BDNF reduced the infarct size after focal ischemia [14]. Application of BDNF could be able to protect ischemic cell damage and enhance post-stroke sensorimotor recovery [3, 15]. These findings suggest that BDNF potentially plays a neuroprotective role in ischemia.

With the rapid development of gene therapy, the notion that virus-mediated gene delivery in treating neurodegenerative disease has been accepted. Post-ischemic administration of GDNF and VGF by sendai virus-mediated gene delivery prevents delayed neuronal death in gerbils [16]. Mmesenchymal stem cells modified with BDNF gene by virus-mediated gene delivery promoted functional recovery and reduce infarct size in the rat middle cerebral artery occlusion model [17].

Here, we generated recombinant adeno-associated virus (rAAV) containing BDNF gene. Overexpression of BDNF by rAAV-mediated gene delivery could be able to increase both BDNF mRNA and protein level in cultured cells infected with viral particles. The increased expression level of BDNF was later found to be functional because it promoted dendrite outgrowth in neuronal cultures and protected neuron from apoptosis by serum deprivation. Further more, we demonstrated that the rAAV mediated BDNF delivery was sufficient to prevent neuronal death induced by focal ischemia. Thus our study suggested that rAAV-mediated delivery of BDNF is functional as that of application of BDNF protein, which results in promoted dendrite outgrowth as well as protects neuronal loss induced by serum deprivation in vitro and focal ischemia in vivo. This piece of work also provided the evidence that the future application of virus-mediated gene therapy in neurodegenerative disease.

**Material and methods**

**Generation of recombinant adeno-associated virus (AAV)-mediated BDNF overexpression**

A cDNA encoding rat BDNF was amplified and subcloned in an AAV plasmid backbone containing the 1.1 kb CMV enhancer/chicken β-actin (CBA) promoter, the woodchuck post-transcriptional regulatory element (WPRE) and the bovine growth hormone polyA (bGH) to yield the construct pAAV-BDNF. The same pAAV-CBAWPRE-bGH backbone carrying no cDNA (pAAV-empty) were used as controls [18, 19]. rAAV mosaic vectors containing a 1:1 ratio of AAV1 and AAV2 capsid proteins with AAV2 inverted terminal repeats (ITRs) were generated by crosspackaging as described [20]. Briefly, HEK293 cells were transfected with the AAV cis plasmid, the AAV1 and AAV2 helper plasmids and the adenovirus helper plasmid by standard calcium phosphate transfection methods. 48 h after transfection, cells were harvested and the vector purified using heparin affinity columns (Sigma, St. Louise, MO). Genomic titers were determined using the ABI 7300 real time PCR cycler (Applied Biosystems) with primers designed to BDNF.

**rAAV vector administration**

Briefly, male Sprague-Dawley rats weighing 300 to 360 g were anaesthetized with an intraperitoneal injection of 400 mg/kg chloral hydrate, and 10 µl of either rAAV-empty or rAAV-BDNF (3 × 10^11 viral genomes/ml) was injected bilaterally into the cerebral ventricle using a stereotaxic frame (Kopf Instruments, Tujunga, CA). Vectors were infused at a rate of 200 nl/min using a microprocessor controlled mini-pump (World Precision Instruments, Sarasota, FA). TTC staining began 14 days after vector infusion when transgene protein expression has peaked to remain at stable levels [21].

**Primary hippocampal neuron cultures**

Hippocampal neurons were prepared from E18 rats embryos and maintained for 10–12 days in vitro (DIV) as described. Primary cultures of hippocampal neurons were plated on poly-D-lysine-coated 6-well plates at 1,500,000 per well for biochemical experiments or on coverslips at 5,000 per coverslip for morphology experiments. All cell culture reagents were from Gibco. The neurons were infected with AAV-BDNF at DIV 1 for dendrites growth and apoptosis assay, and at DIV 7 for RT-PCR and Elisa assay. Control cultures received a similar amount of PBS, which was the solvent for all adenovirus. All experimental procedures were under the approval of the Animal Experiment Committee of Shanghai Institutes for Biological Sciences.
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**Immunoblotting and antibodies**

Cultured cells were lysed in an ice-cold RIPA lysis buffer after different treatments, as described elsewhere. The lysates were mixed with sample buffer for SDS-PAGE. We used the following antibodies: rabbit anti-BDNF (1:1,000; Cell Signaling Technology), and mouse anti-actin (1:5000; Chemicon). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:10,000, Pierce) were used as secondary antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Pierce). Densitometric analysis was conducted using Molecular Analyst software (Version 1.4, Bio-Rad laboratories) and analyzed with Quantity. The same experiments (with multiple experiment conditions) were repeated at least three times (n > 3).

**RT-PCR reactions**

TRIZOL reagent kit (Invitrogen) was used to isolate mRNA from cultured neurons. The first-strand synthesis and PCR reaction were performed using M-MLV Reverse Transcriptase and Tag polymerase (Promega) according to the manufacturer’s instructions. The sets of primers are: BDNF primers, 5'-CATCCAGTTCACCAGCT-3', and 5'-CCATGGGTCCGCACAGCT-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, 5'-ACCACAGTCCTGCCATCAC-3' and 5'-TCCACCACCTGGTGCTGTA-3'.

**Cell survival assay**

Propidium iodide (PI) was added to a final concentration of 5 μg/ml 30 min before the end of the induction of apoptosis to label dying cells. Data are presented as the means and SEM of determinations made in four to eight wells per condition of a representative experiment. Each experiment was performed at least three times (n > 3).

**Immunocytochemical staining and fluorescence microscopy**

Hippocampal neurons grown on glass coverslips (Corning) for 5 days were infected with AAV-BDNF. The infected neurons were transferred to MEM without serum at DIV 7. 48 hours later, neurons were fixed, and then incubated with mouse anti-actin (1:200; Santa Cruz) in PBS containing 0.2% goat serum overnight at 4 °C. The cultures were washed, and incubated with Alexa Fluor 546 (Molecular Probes)-conjugated secondary antibodies diluted in PBS containing 0.2% goat serum for 1 h at room temperature. Images were acquired with Zeiss microscope (40X). For dendritic growth assay, infection of hippocampal neurons with AAV-BDNF was carried out immediately after dissociation. Anti-MAP2 (1:1000; Chemicon) were used in the following immunocytochemical procedures.

**Assay for dendritic growth**

Cultured hippocampal neurons at 3 days in vitro were grown in neurobasal medium containing B27 supplements. Cells were stained with mouse anti-MAP2 (1:1,000; Chemicon). Images were obtained using a Nikon microscope (20X, 546 nm laser). After taking images of MAP2-positive neurons with cell body for diameter (15-20 μm), the number of the primary dendrites per neuron was counted and the dendritic length was determined by tracing all dendritic processes in LSM software. The identity of processes as dendrites was verified based on MAP2 immunofluorescence. At least 30 neurons were captured per condition in each experiment, and each experiment was repeated multiple times (n > 3).

**MCAO ischemic model**

All procedures used in this study were in accordance with the approval of the Animal Experiment Committee of Shanghai Institutes for Biological Sciences. Seventy-two male Sprague-Dawley rats weighing 300 to 360 g were injected with AAV-BDNF or PBS into cerebral ventricle. Fourteen days later, the rats were anesthetized with an intraperitoneal injection of 400 mg/kg chloral hydrate. Focal brain ischemia was induced by the intraluminal suture MCAO method as previously described [22]. Briefly, the right CCA, ICA, and ECA were exposed through a midline incision of the neck. A 4-0 silicone-coated nylon suture was used as an occluder and was inserted via the CCA (CCA route) or ECA (ECA route). For the CCA occlusion route, the proximal portions of the right CCA and the ECA were ligated with 5-0 surgical sutures, and the occluder was inserted through an arteriotomy of the right CCA 3 mm below the carotid bifurcation. For the ECA occlusion route, the occluder was inserted through a stump of the ECA, and the CCA was kept open and intact. The occluder...
was advanced into the ICA 17 to 19 mm beyond the carotid bifurcation. Mild resistance indicated that the occluder was properly lodged in the anterior cerebral artery and thus blocked blood flow to the middle cerebral artery (MCA). For temporary MCAO, reperfusion was obtained by withdrawing the suture approximately 10 mm. For hypothalamic occlusion, the suture was inserted through an arteriotomy of the right CCA 3 mm below the carotid bifurcation and advanced into the ICA only 15 to 15.5 mm above the carotid bifurcation.

**Assessment of necrosis**

We used histochemical staining with triphenyl tetrazolium chloride solution (TTC method) to assess necrosis. Rat brain slices about 1 cm thick were placed in triphenyltetrazolium chloride (TTC) buffered in 0.2 mol/L Tris (pH 7.8) at approximately 37°C for 15 minutes for demarcation of the area of necrosis [23]. They were subsequently photographed. The TTC-positive areas (dark red; viable brain region) and the TTC-negative areas (white or pale; necrotic brain region) were outlined and measured by planimetry. The extent of necrosis was expressed in percentage of the brain area: TTC-negative area x 100/(TTC-negative+TTC-positive area) [24].

**Results**

**Generation and functional characterization of AAV vector carrying BDNF**

The generation of rAAV-BDNF expression construct was illustrated in Figure 1A. Briefly, the open reading frame (ORF) of rat bdnf gene was amplified from rat cDNA library, and was inserted into rAAV backbone. The characterization of two rAAV-BDNF clones was achieved by PCR (Figure 1A, right lane 2 and 3). In order to test if the rAAV-BDNF is functional and infectious, we infected HEK293 cells with high titer rAAV-BDNF viral particles. Compared with non-infected HEK293 cells, we found that high titer of rAAV-BDNF damaged cellular architecture 48 hr after infection assessed by morphological observation under phase microscopy; this was largely due to the toxin effect of rAAV (Figure 1B). To further study if overexpressing BDNF using rAAV-mediated gene delivery was functional, we infected Hela or HEK293 cells with rAAV-BDNF particles and assess the both mRNA and protein level of BDNF in the cultured cells. The endoge-
nous bndf mRNA was undetectable in non-infected HeLa cells (Figure 1C, lane 2) whereas the bdnf mRNA was increased in rAAV-BDNF infected HeLa cells in a dose-dependent manner because high MOI group had relative high bdnf mRNA compared with low MOI group (Figure 1C, lane 3, 4). This result showed that our rAAV-BDNF was able to increase bdnf mRNA level in cultured cell lines. Furthermore, we infected HEK293 cells with rAAV-BDNF (MOI=10) and also could be able increase bdnf mRNA (Figure 1C, lane 5).

In order to detect if the infection could be able to produce BDNF protein, we undertook both ELISA and Western blotting to valuate the BDNF protein level. Cultured neuronal cells were infected with rAAV-BDNF viral particles at two MOIs (MOI=100, 200) respectively. Cultured medium were collected at different time points (48 h, 72 h, 5 d, 7 d and 10 d) and subjected to ELISA. We found that overexpressing BDNF mediated by rAAV could be able to produce mature BDNF in a both time-dependent and dose-dependent fashion (Figure 1D). The concentration of BDNF in cultured medium increased with the infection time because at the time point of 10 d, it reached peak. High MOI-infected (MOI=200) group secreted about 2-fold of BDNF than that of low MOI-infected group (MOI=100) (Figure 1D). Moreover, we lysated both two cell lines infected with rAAV-BDNF and subject the lysates to immunoblotting to test the BDNF protein level. We found that BDNF protein level was dramatically increased in virus-treated Hela and HEK293 cells and this increment was also in a MOI-value dependent manner as high MOI group produced more BDNF than that of from low MOI group (Figure 1C). These results suggest a successful delivery of rAAV vector carrying BDNF into both cell lines and primary cultured, which could be able to produce mature BDNF.

rAAV-mediated gene delivery promotes BDNF expression and neurite outgrowth in cultured neurons

Previous study has shown that endogenous application of BDNF in cultured neurons could be able to promote neurite outgrowth and enhance neurite regeneration [25-28]. In order to achieve the functional study of overexpressing BDNF using rAAV-mediated gene delivery, we infected neuronal cultures with rAAV-BDNF and assayed the mRNA level of bdnf. Neuronal cultures infected with rAAV carrying BDNF increased bdnf mRNA at the time point of 72 hr after infection in both MOI=50 and MOI=100 groups (Figure 2A, lane 2 and 4) compared with control cultures (Figure 2A, lane 1 and 3). GAPDH served as loading control. In order to investigate whether rAAV-mediated overexpression of BDNF functions similar as mature BDNF, we infected cultured hippocampal cultures at DIV 3 with rAAV-BDNF. At 48 h after infection, cells were fixed with 4% PFA and an immunocytochemistry staining against MAP2 was performed to visualize neuronal morphology. We counted both the primary neurite number and total dendrite length, two parameters to evaluate neurite growth in cultured neurons. We found that rAAV-BDNF promoted neurite outgrowth in cultured hippocampal neurons because both primary neurite number and total dendrite length increased 48 h after infection at approximately 2 folds compared with non-infected group. The representative neuronal morphology was illustrated in Figure 2B and the statistical analysis was shown in Figure 2C.

rAAV-mediated BDNF expression protects neurons from apoptosis in vitro and neuronal loss in MCAO model

There have been reports showing that BDNF functions as a protective factor against cell death signaling [5, 15, 17]. To investigate whether rAAV-mediated BDNF delivery is able to protect neurons from apoptosis, we employed both in vitro assay and in vivo study to address this question.

First, we used serum deprivation model, a classic apoptotic trigger in cultured cells. Neuronal cell cultures were first infected with or without rAAV-BDNF viral particles at DIV 5 and then were deprived of serum at DIV 8 for 48 hr. Neuronal morphology was visualized by immunostaining using anti-actin antibody and survival was assess by PI/DAPI staining. rAAV-BDNF significantly protected neurons from apoptosis because cultured cells infected with rAAV-BDNF maintained normal morphology upon serum deprivation whereas in non-infected group, most of the cells collapsed and lost the cellular architecture and displayed the atrophy of neurites (Figure 3A). These cells were also positive for PI staining while in rAAV-BDNF group, few apoptotic cells were seen (Figure 3B).
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To further elucidate if overexpressing BDNF by rAAV-mediated gene delivery could protect cell death in vivo, we used MCAO model, an animal model mimicking clinical stroke and in which massive neuronal death is observed in neocortex, hippocampus, striatum and hypothalamus after reperfusion and could be assessed by TTC staining. Adult male SD rats were received intracerebroventricular (i.c.v.) injection of either rAAV-BDNF viral particles or rAAV empty vector viral particles serving as control. After recovering for 14 days, MCAO were conducted to both control and experimental groups for 0.5 hr and reperfusion was achieved by withdrawing the suture 10 mm and lasted for 2 hr. TTC staining on coronal brain slices at the level of 8 mm post frontal pole indicated that the infarct areas included several brain regions such as neocortex, hippocampus, striatum and hypothalamus in control group. However, in rAAV-BDNF group, the volume of the infarct area was dramatically decreased because only a mild damage in neocortex and few necrosis/apoptosis was seen in hippocampus, striatum or hypothalamus (Figure 3C). Taken together, rAAV-BDNF was able to protect neurons from apoptosis/necrosis both in vitro and in vivo, suggesting that the potentially functional relevance of rAAV-mediated BDNF overexpressing in treating neurological diseases such as ischemia and stroke.

Discussion

In our study, three major points were presented: 1) rAAV vector carrying BDNF gene could be able to sufficiently increase mature BDNF expression at both mRNA and protein levels. 2) rAAV-mediated BDNF overexpression in cultured neurons promoted dendritic outgrowth and inhibited neuronal loss induced by serum deprivation. 3) In vivo injection of rAAV-BDNF into brains challenged with ischemic insults had neuroprotective effect on neurodegeneration.

Initial treatment for neurodegenerative disease is largely dependent on diagnosis of underlying disorder. Medication usually relieves the symptoms but could not cure the disease thoroughly. For example, treatment with L-dopa can inhibit symptoms of Parkinson's disease for a short time, but this could also be able to accelerate the symptom. And most of the efforts are being made to stabilize cognitive function at the level existing at time of diagnosis and treatment.

Gene therapy is the introduction of genetic material into cells for therapeutic purposes. The two classic delivery systems include viral vector systems and non-viral techniques. Gene therapy based on adeno-associated virus (AAV) is widely...
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Accepted and with most potential to be applied in clinical use concerning its non-pathogenic property compared with other viruses. Clinical trials have been initiated in which rAAV vectors are used to deliver genes, some trials are currently in phase I or II.

Neurotrophic factors are key regulatory proteins in central nervous system that modulate neuronal survival, axonal growth, synaptic plasticity and neurotransmission. The dysregulation of neurotrophic factors has long been demonstrated participating in multiple aspects including neuronal development and plasticity as well as in neuropathogenic processes. Thus manipulation of neurotrophin receptors as well as its downstream signaling pathways has been tried to be applied in clinical, i.e. the epidermal growth factor receptor (EGFR) inhibitor in treating certain types of cancer [29-32].

Currently, the application of gene therapy in neurobiological diseases is hindered due to its inherent difficulties. Apparently, direct injection into the target tissue may be required for most such diseases since blood-brain barrier (BBB) is a substantial obstacle. Concerning that most of the neuronal cells in adult are non-dividing cells, neural tissues can not be treated with retroviruses. Therefore, adenov associated vectors (AAV) are developed. The combination of AAV carrying neurotrophic factor genes would be of great potential because of the vector’s non-pathogenic property as well as the wide spectrum functions of neurotrophic factors. One of the key steps in rAAV-mediated gene delivery is the integration of virus DNA into host cells and continuously transcribing functional products and finally producing sufficient neurotrophines to overcome the insults-induced cell damage.

We successfully generated rAAV vector carrying BDNF, and the increase of BDNF at both mRNA and protein levels were observed in infected cell lines as well as in cultured neurons assessed by RT-PCR and western blotting. Further more, this high level BDNF could dramatically promote neurite outgrowth and inhibit apoptosis induced by serum deprivation. This result illustrated that rAAV mediated overexpression of BDNF functions at similar extent with application of matured BDNF protein [25]. Meanwhile, the infec-
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...tion of viral particles showed little toxicity on cell survival (data not shown). In vivo study showed that single injection of rAAV-BDNF had neuroprotective effect against cell death by significantly reducing the infarct size after middle artery occlusion. Taken together, these results demonstrated the functional relevance of rAAV mediated BDNF delivery in protecting cell from death induced by ischemic insults. However, whether other neurotrophic factors or the combination of multiple neurotrophin genes delivered by rAAV would potentiate the neuroprotection by BDNF is required to be further explored.

Another important issue which would also be the advantages of using rAAV-mediated gene delivery into central nervous system to achieve gene therapy is that most of the neuronal cells are post-mitotic cells which means that they are non-dividing. This brought the notion that single injection of viral particles would be sufficient to reverse cell death and the related behavioral consequence. The identification of effects on post-traumatic injection of rAAV-BDNF and the time from the injury to injection of rAAV vectors is needed.

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References

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