Brain-targeting delivery for RNAi neuroprotection against cerebral ischemia reperfusion injury

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ABSTRACT

Nanoparticles (NPs) with modification of brain-targeting molecules have been extensively exploited for therapeutic gene delivery across the blood--brain barrier (BBB). As one of the effective RNA interference (RNAi) approaches, short hairpin RNA (shRNA) has been proved to be promising in the field of gene therapy. Apoptosis signal-regulating kinase 1 (Ask1) has been reported to be an important target for gene therapy against cerebral ischemia reperfusion injury. In this study, dendrigraft poly-L-lysine (DGL) was decorated by dermorphin (a μ-opiate receptor agonist) through PEG for efficient brain-targeting, then complexed with anti-Ask1 shRNA plasmid DNA, yielding the DGL-PEG-dermorphin/shRNA NPs. The DGL-PEG-dermorphin/shRNA NPs were characterized and estimated the brain-targeting ability. In vitro, increased cellular uptake and transfection efficiency were explored; in vivo, preferable accumulation and gene transfection in brain were showed in images. The DGL-PEG-dermorphin/shRNA NPs also revealed high efficiency of neuroprotection. As a result of RNAi, corresponding mRNA was distinctly degraded, expression of Ask1 protein was obviously suppressed, apoptotic cell death was apparently decreased and cerebral infarct area was significantly reduced. Above all, DGL-PEG-dermorphin/shRNA NPs were proved to be efficient and safe for brain-targeting RNAi neuroprotection against cerebral ischemia reperfusion injury.

1. Introduction

RNA interference (RNAi) has been extensively researched and proved to hold great potential for revolutionizing the traditional medicine of many cerebral diseases, especially cerebral ischemia reperfusion injury [1]. As one of the RNAi strategies, short hairpin RNA (shRNA) has been widely reported. Specific sequences are designed and cloned into plasmid vectors, generating corresponding gene expression cassettes, which are delivered into nucleus [2]. As a result, shRNA is expressed in the nucleus and takes effect by silencing the expression of specific genes via distinct mRNA degradation pathways. Compared with other strategies of RNAi, shRNA has been considered to be more like endogenous interfering RNA, leading to more stable gene down-regulation [3].

Abundant researches have shown that the mitogen-activated protein kinase (MAPK) cascade plays an important role in cerebral ischemic cell death [4]. According to reports, MAPK cascade signaling pathway can be activated by the overproduced reactive oxygen species (ROS) after ischemia and reperfusion, and induce remarkable cell apoptosis in brain (Scheme 1). Apoptosis signal-regulating kinase 1 (Ask1) is an important member of mitogen-activated protein kinase kinase kinase (MAPKKK) family [5,6]. Based on the extensive expression in cerebral neurocytes and the significance for cell apoptosis, Ask1 has been considered as a prospective target for RNA interference (RNAi) therapy on ischemia reperfusion injury. In this study, the anti-Ask1 shRNA was exploited, in order to effectively silence the expression of Ask1 in brain. Without enough Ask1, when ischemia reperfusion happened, the MAPK cascade signal pathway has been considered hard to work properly as usual, resulting in neuroprotection against cerebral ischemia reperfusion injury.

However, the blood--brain barrier (BBB) has been proved as the main obstacle for therapeutic agents to be delivered into brain parenchyma after systemic administration [7]. The BBB is mainly comprised of brain capillary endothelial cells (BCECs). The BCECs are connected with each other by tight junctions (TJs), strictly controlling the exchange of substances between blood and brain [8]. Even so, there are still opportunities for shRNA therapeutic agents to cross...
the BBB and enter the neurocytes (Scheme 2). It has been reported that many receptors are expressed on the surface of BCECs as well as the neurocytes in CNS. Therefore, the specific ligands to these receptors hold a certain promise for brain-targeting [9].

The specific receptor exploited in this study was \(\mu\)-opioid receptor (MOR), which has been reported to be extensively expressed in CNS, including BCECs and cerebral neurocytes [10]. In this study, a novel ligand to MOR, dermorphin, was selected as the brain-targeting functional molecule. Dermorphin is a MOR agonist with high potency and distinct selectivity [11,12]. It is about 30–40 times more potent than morphine for binding MOR, but less likely to produce drug tolerance and addiction. Among the opioid peptides selective to MOR, dermorphin exhibits the highest brain influx rate. Nearly 90% of free dermorphin can reach the brain parenchyma, but not be held up on the surface of BCECs [13]. Therefore, dermorphin can probably act as a brain-targeting functional molecule for shRNA delivery system, facilitating this system to cross the BBB.

Thus, the RNAi therapeutic system constructed here demonstrated targeting attributes in three levels: firstly, binding to the \(\mu\)-opioid receptor on the surface of BCECs and then cross the BBB; secondly, entering the cerebral neurocytes by binding to the receptor specifically; thirdly, degrading the specific mRNA in nucleus of the neurocytes.

In this study, dendrigraft poly-L-lysine (DGL) generation 3 was exploited as the vector, because of its superb biodegradability and vast numbers of positive charges [14]. DGL are modified with PEG and targeting ligand for long circulation, low toxicity and active targeting [15], yielding the dermorphin-PEG-DGL polymer. Plasmid DNA, which is able to express shRNA in nucleus, is prone to being condensed by dermorphin-PEG-DGL through electric interactions [16]. Dermorphin-PEG-DGL/DNA nanoparticles (NPs) were constructed as the brain-targeting shRNA therapy system to explore the ability to cross the BBB and suppress the neurocytes apoptosis (Scheme 3).

2. Materials and methods

2.1. Materials

DGL (Generation 3, MW 22000) were purchased from Colcom, France. \(\alpha\)-Malemidyl-\(\omega\)-N-hydroxysuccinimidyl polyethyleneglycol (NHS-PEG-MAL, MW 3500) was obtained from JenKem Technology Co., Ltd (Beijing, China). The peptide dermorphin with a cysteine on C-terminal (Tyr-DAla-Phe-Gly-Tyr-Pro-Ser-...
The construction of the DGL-PEG-dermorphin/shRNA system and the process of therapy.

- **Ethidium monoazide (EMA)**: Fresh plasmid DNA solution (1 mg/mL 0.05M Tris was inserted into the internal carotid artery (ICA) through the ECA, causing a blockage of 0.32 mm diameter monofilament.

- **Animals**: Male ICR mice of 20–25 g body weight, male nude mice of 18–20 g body weight and male SD rats of 200–240 g body weight were supplied by the Experimental Animals Department of Fudan University, and carried out in accordance with the guidelines evaluated and approved by the ethics committee of Fudan University.

2.2. Cell lines

Brain capillary endothelial cells (BCECs) were kindly gifted by Prof. J. N. Lou (the Clinical Medicine Research Institute of the China-Japan Friendship Hospital). BCECs were expanded and maintained in special Dulbecco's modified Eagle medium (DMEM) (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 20% heat-inactivated fetal calf serum (FCS), 100 μg/ml epidermal cell growth factor, 2 mM/L-glutamine, 20 μg/ml heparin, 40 μg/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin and cultured at 37 °C under a humidified atmosphere containing 5% CO₂.

U87 glioblastoma cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured at 37 °C in a humidified 5% CO₂ atmosphere.

Growth medium was supplemented with fetal bovine serum (FBS) (10%), streptomycin (100 μg/ml) and penicillin (100 units/ml).

All the cells used in this study were in the logarithmic phase of growth.

2.3. Animals

Male ICR mice of 20–25 g body weight, male nude mice of 18–20 g body weight and male SD rats of 200–240 g body weight were supplied by the Experimental Animals Department of Fudan University, and carried out in accordance with the guidelines evaluated and approved by the ethics committee of Fudan University.

To build the transient cerebral ischemia model, SD rats were anesthetized with 10% chloral hydrate. The four legs of rat were fastened and the external carotid artery (ECA) was exposed with an incision of the median neck. Then a MCAO monofilament with silica gel coated distal end (diameter of the silica gel coated end: 0.32 ± 0.02 mm; diameter of the monofilament body: 0.24 mm; length: 40 mm) was inserted into the internal carotid artery (ICA) through the ECA, causing a blockage of blood flow in the middle cerebral artery (MCA). The MCAO monofilament was removed 1 h later for reperfusion.

The operation of MCAO was conducted under the kind assistance and direction of the Experimental Animals Department of Fudan University. The confirmation method for successful building of transient cerebral ischemia model has been described in papers published before [17].

2.4. Synthesis and characterization of DGL derivatives

DGL was reacted with NHS-PEG3500-MAL at a molar ratio of 1:5 (DGL: PEG) in PBS (pH 8.0) for 2 h at room temperature. The primary amino groups on the surface of DGL were specifically reacted with the NHS groups of the bifunctional PEG. The resulting polymers, DGL-PEG, were purified by ultrafiltration through a membrane (cutoff × 5 kDa) and the buffer was changed to PBS (pH 7.0). Then DGL-PEG were reacted with dergmorphin at a molar ratio of 1:2 (DGL-PEG: dergmorphin) in PBS (pH 7.0) for 24 h at room temperature. The MAL groups of DGL-PEG were specifically reacted with the thiol groups of dergmorphin, yielding the DGL-PEG-dermorphin polymer.

DGL-PEG-dermorphin was freeze-dried, redissolved in D₂O and analyzed in a 400 MHz spectrometer (Varian, Palo Alto, CA, USA).

2.5. Preparation of dendrimers/DNA nanoparticles (NPs)

Dendrimers (DGL, DGL-PEG, and DGL-PEG-dermorphin) were freshly prepared and diluted to appropriate concentrations in PBS (pH 7.4). DNA solution (100 μg/ml, 50 μM sodium sulfate solution) was added to obtain specified weight ratios. Then the mixture was immediately vortexed for 30 s at room temperature to form the corresponding nanoparticles (NPs).

When it is necessary, plasmid DNA was covalently labeled with fluorescent dye ethidium monoazide (EMA). Fresh plasmid DNA solution (1 mg/ml) in Tris-HCl buffer, pH 8.0) was diluted with aqueous solution of EMA (0.1 mg/ml) and incubated for 30 min in dark. The complex was then exposed to UV light for 1 h, and the
resulting solution was precipitated by addition of ethanol to a final concentration of 30% (v/v). The precipitate was collected by centrifugation and further dissolved in 50 mM sodium sulfate solution to obtain EMA-labeled plasmid DNA.

Other times, plasmid was labeled by a DNA fluorescence intercalator, YOYO-1 and iodide, through vortexing for 2 min. After 8 min in dark, plasmid DNA emits green fluorescence.

2.6. Characterization of dendrimers/DNA nanoparticles (NPs)

The DGL-PEG-dermorphin/DNA NPs were freshly prepared at weight ratios (DGL:DNA) of 0.05:1, 0.1:1, 0.5:1, 1:1, 3:1, 6:1 and 10:1. 0.7% agarose gel electrophoresis was performed to evaluate the DNA encapsulation effect of DGL in the NPs compared with naked DNA. The optimal weight ratio for efficient encapsulation and low toxicity was also confirmed.

The DGL-PEG-dermorphin/DNA NPs were freshly prepared with a specified ratio (6:1, DGL to DNA, w/w). The mean diameter and zeta-potential of the NPs were determined by dynamic light scattering using a Zeta-Potential/Particle Sizer Nicomp™ 380 ZLS (PPS Nicomp Particle Size System, USA). The morphology of the DGL-PEG-dermorphin/DNA NPs was examined under a high-resolution transmission electron microscope (TEM) (JEM-2010HT, Japan).

The stability of NPs was also tested. NPs including DGL/DNA, DGL-PEG/DNA, DGL-PEG-dermorphin/DNA (6:1, DGL to DNA, w/w) were freshly prepared. The DNA used here were pORF-TRAIL plasmid. To determine the stability, NcoI (Promega, USA) and XhoI (Promega, USA) were added to each kind of NPs. After incubation at 37 °C for 2 h, the enzymolysis was terminated by changing the incubation condition to 65 °C for 10 min. All the samples were analyzed by 0.7% agarose gel electrophoresis. The integrity of plasmid was compared with untreated naked DNA and NcoI, XhoI co-treated naked DNA.

2.7. Cellular uptake

BCECs were seeded at a density of 2 × 10^4 cells/well in 24-well plates (Corning-Coaster, Tokyo, Japan), incubated for 72 h, and checked under the microscope for confluency and morphology. Then BCECs were incubated with YOYO-1-labeled dendrimers/DNA NPs at the concentration of 30 μg/well measured by DGL for 30 min at 37 °C and 4 °C respectively. After that, the solutions were removed. Cells were washed three times with Hank’s solution and observed under an IX2-RFCA fluorescent microscope (Olympus, Osaka, Japan).

BCECs were also tested with several cellular uptake inhibitors. Cells were seeded as described previously. After checking the confluency and morphology, U87 cells were incubated with dendrimers/pEGFP-N2 NPs respectively at the concentration of 30 μg/mL, filipin complex (4 μg/mL), colchicines (2 μg/mL), colchicine (2 μg/mL), or noloxone (2 μg/mL) was added into each group of wells separately and BCECs were incubated with them at 37 °C for 10 min. Then the solution was removed, and YOYO-1-labeled DGL-PEG-dermorphin/DNA NPs with different inhibitor (the concentration of the inhibitors was same with that for pre-incubation) was added into the corresponding group of wells. After incubation at 37 °C for 30 min, the solution was removed again. The cells were washed three times with Hank’s solution and observed under the fluorescent microscope.

After observation, BCECs were trypsinized, diluted by Hank’s solution and centrifugated at 1500 rpm for 8 min. The supernate was discarded. The precipitates were redissolved with Hank’s solution and analyzed by Flow Cytometry (FACSCalibur, BD, USA).

2.8. In vitro transfection experiment

U87 cells were seeded at a density of 4 × 10^4 cells/well in 24-well plates and incubated for 48 h. After checking the confluency and morphology, U87 cells were incubated with dendrimers/pEGFP-N2 NPs respectively at the concentration of 30 μg/well measured by DGL at 37 °C for 2 h. Then cells were further incubated for with fresh medium 48 h for transfection. The fluorescent images were acquired with a fluorescence microscope.

2.9. In vivo imaging analysis

The dendrimers/DNA NPs (6:1, to DNA, w/w) labeled by EMA were injected into the tail vein of nude mice at a dose of 50 μg DNA/mouse. Then the mice were anesthetized by 10% chloral hydras. Images were taken by CRI in vivo imaging system.
system (Maestro, USA) 60 min after injection. Brains were extirpated 2 h after injection to check the relative accumulation.

2.10. Qualitative gene expression in vivo

The dendrimers/pRFP NPs (6:1, DGL to DNA, w/w) were injected into the tail vein of mice at a dose of 50 μg DNA/mouse. About 48 h later, animals were anesthetized with diethyl ether and killed by decapitation. The brains were removed, fixed in 4% paraformaldehyde for 48 h, dehydrated in 15% sucrose solution until subsidence (6 h), then in 30% sucrose until subsidence (24 h). After this, brains were frozen in OCT embedding medium (Sakura, Torrance, CA, USA) at –80 °C. Frozen sections of 20 μm thickness were prepared with a cryotome Cryostat (Leica, CM 1900, Wetzlar, Germany) and stained with 300 nM DAPI for 10 min at room temperature. After washing twice with PBS (pH 7.4), the staining slices were immediately examined under the fluorescence microscope.

2.11. Quantitative gene expression in vivo

The dendrimers/gPL3 NPs (6:1, DGL to DNA, w/w) were injected into the tail vein of mice at a dose of 50 μg DNA/mouse. 48 h later, the mice were humanely decapitated and the principal organs (including brain, heart, liver, spleen, lung, and kidneys) were extirpated. The organs were carefully washed with distilled water and homogenized in 1 mL lysis reagent (Promega, Madison, WI, USA) using a JY92-HN tissue homogenizer (Ningbo, Jiangsu, China). The homogenate was centrifuged at 14,000 g for 20 min at 4 °C, and the supernatant was reserved for the following tests. Luciferase activity was quantified by a luciferase assay system from Promega (Madison, WI, USA). The density of Ask1 was analyzed with an imaging analysis program and compared with GAPDH.

2.12. RT-PCR for expression measurement

The dendrimers/shRNA NPs (6:1, DGL to shRNA, w/w) were injected into the tail vein of rats at a dose of 150 μg shRNA/rat, and the dendrimers/scramble NPs were taken as control. After expression for 48 h, rats underwent the transient cerebral ischemia operation. About 24 h later, rats were sacrificed and brains were extracted. The RT-PCR detection was carried out by Sangon Biological Engineering Co., Ltd (Shanghai, China). The density of Ask1 was analyzed with an imaging analysis program and compared with GAPDH.

2.13. Fluorescence staining of brain slices

The dendrimers/shRNA NPs (6:1, DGL to shRNA, w/w) were injected into the tail vein of rats at a dose of 150 μg shRNA/rat and the dendrimers/scramble NPs were taken as control. After expression for 48 h, rats underwent the transient cerebral ischemia operation. About 24 h later, animals were anesthetized with diethyl ether and killed by decapitation. The brains were removed, fixed in 4% paraformaldehyde for 48 h, dehydrated in 15% sucrose solution until subsidence (6 h), then in 30% sucrose until subsidence (24 h). After this, brains were frozen in OCT embedding medium (Sakura, Torrance, CA, USA) at –80 °C. Frozen sections of 20 μm thickness were prepared with a cryotome Cryostat (Leica, CM 1900, Wetzlar, Germany) and stained with 300 nM DAPI for 10 min at room temperature. After washing twice with PBS (pH 7.4), the staining slices were immediately examined under the fluorescence microscope.

2.14. TTC staining for measuring the infarct size

The dendrimers/shRNA NPs (6:1, DGL to shRNA, w/w) were injected into the tail vein of rats at a dose of 150 μg shRNA/rat and the dendrimers/scramble NPs were taken as control (n = 6). After expression for 48 h, rats underwent the transient cerebral ischemia operation. Then after 24 h, the rats were sacrificed and brains were extracted. The anterior brain area was sliced into sections coronally for 2 mm. Each section was stained by immersion in 1% TTC solution and incubated for 20 min. Stained sections were recorded with a camera. Afterwards, the infarct size of different groups was measured with the software ImageJ and calculated respectively.

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\text{Percentage of infarct area} \% = \frac{\text{infarct area}}{\text{total area}} 
\]

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\text{Percentage of TUNEL positive cells} \% = \frac{\text{amount of TUNEL positive cells}}{\text{amount of total cells}} 
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3. Results

3.1. Characterization of DGL derivatives and dendrimers/DNA NPs

In 1H NMR spectra (Fig. 1A), the solvent peak of D2O was found at 4.65 ppm. Compared with 1H NMR spectra of DGL-PEG (data not shown), the sharp peak of PEG’s MAL group at 6.7 ppm disappeared in the 1H NMR spectra of DGL-PEG-dermorphin, whereas the repeat units of PEG (–O–CH2–CH2–O–) still presented a sharp peak at 3.6 ppm (Fig. 1A), implying that the MAL group had reacted with the thiol group of derrmophin. Furthermore, there were an extra cluster of peaks between 7.4 and 6.6 ppm in the 1H NMR spectra of DGL-PEG-dermorphin (Fig. 1A), which represented the benzene ring in derrmophin. Thus, the 1H NMR spectra proved the successful synthesis of DGL-PEG-dermorphin.

The mean diameter of DGL-PEG-dermorphin/DNA NPs (6:1, DGL to DNA, w/w) was detected to be 104 ± 12 nm (Fig. 1C), which was benefit for crossing the BBB. The mean zeta-potential was tested to be 10.31 ± 2.29 mV, contributing to serum stability.

The transmission electron micrograph (Fig. 1B) showed that DGL-PEG-dermorphin/DNA NPs were of well-formed spherical shape and had a compacted structure.

Fig. 1D showed the electrophoresis results of DGL-PEG-dermorphin/DNA NPs at weight ratios (DGL: DNA) ranging from 0.05:1 to 10:1. Compared with naked DNA (Fig. 1D lane 2), NPs at the weight ratio of 3:1 and above (Fig. 1D lane 7–9) encapsulated DNA completely with no electrophoresis shift. Consequently, we made it 6:1 for all the following experiments.

To investigate the protective effect of DGL derivatives for DNA against enzymatic degradation, naked DNA and the dendrimers/DNA NPs were incubated with Ncol and Xhol. Naked DNA without endonucleases served as control (Fig. 1E, lane 2). The naked plasmid DNA was completely digested by Ncol and Xhol (Fig. 1E, lane 3), whereas all the dendrimers provided good protection for DNA from digestion (Fig. 1E, lane 4–6).

3.2. Cellular uptake

BCECs were incubated with YOYO–1–labeled dendrimers/DNA NPs under different conditions for cellular uptake experiments. Results were shown qualitatively by fluorescent images. The fluorescence intensity increased obviously when cells were treated with DGL-PEG-dermorphin/DNA NPs at the temperature of 37 °C (Fig. 2A.a), compared with DGL/DNA (Fig. 2A.b) and DGL-PDG/DNA (Fig. 2A.c) groups. However, when cells were incubated at the temperature of 4 °C, the fluorescence intensity turned to be quite low (Fig. 2A.d). Different endocytosis pathway inhibitors, excessive free dermorphin and MOR antagonist naloxone were also used to clarify the endocytosis process of DGL-PEG-dermorphin/DNA NPs. Compared with control, excessive free dermorphin (Fig. 2A.e) and naloxone (Fig. 2A.f) lowered the uptake efficiency of BCECs obviously. PhAsO (Fig. 2A.g), flilpin (Fig. 2A.h) and colchicine (Fig. 2A.i) also decreased the fluorescent intensity to some extent.

Flow cytometry was carried out for more direct visualization of the cellular uptake with shift of curves (Fig. 2B).

3.3. In vitro transfection experiment

The transfection result in U87 cells was shown in Fig. 3. Green fluorescence represented the expression of pEGFP-N2 plasmid. Cells treated with DGL/pEGFP-N2 and DGL-PEG-dermorphin/pEGFP-N2 demonstrated stronger fluorescence signal the DGL-PDG/pEGFP-N2 group. However, all the fluorescence intensity was very weak.
Fig. 2. Cellular uptake of dendrimers/DNA NPs. A) a) DGL-PEG-dermorphin/DNA at 37°C. b) DGL-PEG/DNA at 37°C. c) DGL/DNA at 37°C. d) DGL-PEG-dermorphin/DNA at 4°C. e) DGL-PEG-dermorphin/DNA at 37°C with dermorphin. f) DGL-PEG-dermorphin/DNA at 37°C with Naloxone. g) DGL-PEG-dermorphin/DNA at 37°C with PhAsO. h) DGL-PEG-dermorphin/DNA at 37°C with filipin. i) DGL-PEG-dermorphin/DNA at 37°C with colchicine. Green: YOYO-1-labeled DNA. Original magnification: ×200. B) Flow cytometry curves of A) a–i. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3.4. In vivo imaging analysis

Nude mice were injected with the EMA-labeled DGL-PEG-dermorphin/DNA NPs, or DGL-PEG/DNA NPs as control. In vivo fluorescent images were shown in Fig. 4. EMA-labeled DNA was obviously accumulated in brain of the mouse injected with the DGL-PEG-dermorphin/DNA NPs. But the fluorescence of the DGL-PEG/DNA group was not so significant. The picture below in Fig. 4 showed that the brain uptake of DGL-PEG-dermorphin/DNA NPs (right) was relatively much higher than that of DGL-PEG/DNA NPs (left).

3.5. Qualitative gene expression in vivo

RFP expression in the ventricle, hippocampus, cortical layer, substantia nigra and corpus striatum were shown in Fig. 5. For the mouse injected with DGL-PEG-dermorphin/DNA NPs, gene expression was observed in all the five regions and was more significant than the DGL-PEG/DNA group, especially in ventricle, hippocampus and cortical layer.

3.6. Quantitative gene expression in vivo

The transfection efficiency of DGL-PEG/pG3 and DGL-PEG-dermorphin/pG3 NPs in the principal organs was measured 48 h after tail vein injection (Fig. 6). The luciferase expression in brain of the mouse injected with DGL-PEG-dermorphin/pG3 NPs was significantly higher than the DGL-PEG/pG3 group (Fig. 6A). There was no obvious difference in other organs (Fig. 6B).

3.7. RT-PCR for expression measurement

RT-PCR was performed to determine the suppression effect of shRNA NPs for Ask1 expression. Compared with scramble NPs, shRNA NPs significantly suppressed the expression of Ask1 (Fig. 7). For DGL-PEG-dermorphin/shRNA group, the brain tissue expressed even less Ask1 protein than those of DGL-PEG/dermorphin group.

3.8. Fluorescence staining of brain slices

To explore the relationship between Ask1 expression and DNA fragmentation in neuronal cells and assess the brain-targeting efficiency of dermorphin, brain slices were labeled with different fluorescences (Ask1-red, TUNEL-green and nuclei-blue) (Fig. 8). In the brain slices of rats which were injected with scramble NPs, the fluorescence signals of Ask1 and TUNEL had a precise colocalization (Fig. 8A). However, for shRNA groups, there were significant decreases in the fluorescence integrity of Ask1 (red), as well as in TUNEL (green) (Fig. 8A). Especially for DGL-PEG-dermorphin/
shRNA group, there was hardly any fluorescence signal for Ask1 or TUNEL in the staining slices (Fig. 8A). There was a significant difference in percentage of Ask1 positive cells between the rats injected with DGL-PEG-dermorphin/shRNA and DGL-PEG/shRNA (Fig. 8B), as well as in percentage of TUNEL positive cells (Fig. 8C).

3.9. TTC staining of brain slices

The mice injected with scramble NPs still had serious cellular apoptosis in brain, and the injury area was obvious in staining slices (Fig. 9A). However, NPs with anti-Ask1 shRNA had an outstanding neuroprotection effects on brain, especially the ones decorated with dermorphin (Fig. 9). After injection with DGL-PEG-dermorphin/shRNA, the injury area in brain reduced markedly. This result was in good consistence with the fluorescence staining experiment. There was a significant difference in brain infarct sizes between the rats injected with DGL-PEG-dermorphin/shRNA and DGL-PEG/shRNA (Fig. 9B).

4. Discussion

In this study, dermorphin was linked to DGL by PEG, enhancing the brain-targeting capability of the gene delivery system. Among all the dendrimers/DNA NPs used here, DGL-PEG-dermorphin/DNA NPs possessed a favorable brain-targeting quality, which were displayed by experiments both in vivo and in vitro. Moreover, PEGylation rendered them longer circulatory time in bloodstream and better biocompatibility [18]. With the specifically designed therapeutic gene, the delivery system built up in this study, DGL-PEG-dermorphin/shRNA, successfully achieved RNAi neuro-protection against cerebral ischemia reperfusion injury.

According to previous researches, the infarct area caused by ischemia/reperfusion was very large, which indicated severe damage to brain [19]. Therefore, an efficient carrier with the ability to transport the therapeutic gene across the BBB freely was badly needed. In this study, the gene delivery system, which was modified by dermorphin, demonstrated efficient brain-targeting capability. As the specific receptor of dermorphin, MOR mainly locates in the CNS, especially in brain [10]. The expression of MOR can be found in various regions in brain, such as cerebellum, nucleus accumbens, caudate nucleus, cerebral cortex, hippocampus, substantia nigra, corpus striatum and so on [20]. However, the expression of MOR in different structures has been proved to be in different levels. According to the detection, MOR is expressed in cerebral cortex at a high level, in substantia nigra together with hippocampus at a middle level, and, in corpus striatum at a low level [21]. The results in this study (Fig. 5) were in complete agreement with the aforementioned researches. According to these reports, dermorphin has an outstanding brain influx rate among all the opioid peptides, which are specific to MOR. And nearly 90% of dermorphin can get across the BBB freely, with little retention by BCECs [22]. Moreover, the mean particle size of NPs in this study was detected as 104 nm (Fig. 1C), which was optimal for crossing the BBB; and the mean zeta-potential turned to be +10 mV, which were favorable for the fusion of NPs with negatively charged membranes. Taking all these factors, DGL-PEG-dermorphin/DNA NPs proved to be efficient brain-targeting gene delivery system.

Despite of the expression in brain, MOR has been detected in several peripheral tissues as well [21]. According to report, MOR was expressed in heart, spleen and lung at middle or even low level, compared with the high level in brain [23]. Whereas, the results in this study (Fig. 6) were not exactly the same. The high expression level of luciferase in these three organs was probably due to the properties of DGL but not dermorphin, since there was no significant difference in gene expression quantity between DGL-PEG/DNA NPs and DGL-PEG-dermorphin/DNA NPs. Furthermore, the NPs were much easier to enter the high-perfusion organs. Similar results can be obtained in the early researches of DGL with other ligand modifications [15]. Significantly higher gene expression of DGL-PEG-dermorphin/DNA NPs than DGL-PEG/DNA NPs in brain could be observed in this study (Fig. 6A), which proved that the peripheral expression of MOR did not influence the brain-targeting efficiency of dermorphin modified gene delivery system at all. Meanwhile, when the system was used for neuro-protection against cerebral ischemia reperfusion injury, the sequence-defined shRNA was specifically designed for the suppression of Ask1...
expression in brain, preventing the other primary organs from being influenced, achieving the third level of targeting.

Since the DGL-PEG-dermorphin/DNA gene delivery system had been proved to possess great brain-targeting efficiency by qualitative and quantitative brain uptake measurements (Figs. 4–6), it was further exploited for RNAi delivery to protect the neurocytes against cerebral ischemia reperfusion injury.

In this research, sham-operated group was conducted. However, no infarct injury was observed at all (Figure not shown). Therefore, it was meaningless to set sham-operated group for evaluating the RNAi neuroprotection against cerebral ischemia reperfusion injury.

In the fluorescence staining experiments (Fig. 8), it was obviously exhibited that the Ask1 protein played an important role in the apoptotic cell death process caused by transient cerebral ischemia. This was in good accordance with the findings before, that overexpression of Ask1 contributed to cellular apoptosis [5]. From the results of RT-PCR (Fig. 7), it was demonstrated that the anti-Ask1 shRNA had a significant suppressive effect on the expression of Ask1 protein. Because of the specific sequences in shRNA, relevant mRNA were disturbed and degraded, resulting in a great decrease in protein expression. Therefore, after injection with shRNA NPs, the
apoptotic cell death in brain was reduced obviously (Fig. 8), the Ask1 proteins were seldom expressed (Figs. 7 and 8), and quite milder infarct injury symptoms in brain were observed (Fig. 9). TTC staining has been in common use for assessing ischemia/reperfusion injury. TTC was able to react with the succinate dehydrogenase in mitochondria of living cells, yielding a red product to confirm the cell vitality. When cellular death happened, the tissue remained white. According to researches, neurons are the most sensitive cells to cerebral ischemia/reperfusion injury. After ischemia/reperfusion, free radicals did great harm to neurons. Apoptosis has been reported as the primary way to die for neurons. When ischemia/reperfusion injury happened, neurocyte apoptosis would be the majority of cellular death. As a result, in cerebral ischemia reperfusion, TTC could be further used to evaluate the neurological damage after ischemia/reperfusion. Therefore, in TTC staining experiment, DGL-PEG-dermorphin/shRNA provided effective neuroprotection against cerebral ischemia/reperfusion injury (Fig. 9).

Compared with DGL-PEG/shRNA NPs, DGL-PEG-dermorphin/shRNA NPs were more effective in neuroprotection against cerebral ischemia reperfusion injury, thanks to the further decoration of the brain-targeting molecular dermorphin (Figs. 8 and 9). The NPs with dermorphin bound with the µ-opiate receptors on the surface of cells specifically, and entered the cells more easily than DGL-PEG/shRNA NPs. Consequently, the accumulation of DGL-PEG-dermorphin/shRNA NPs in nucleus was much more abundant for RNAi. Therefore, more significant neuroprotection effect of DGL-PEG-dermorphin/shRNA NPs against cerebral ischemia reperfusion injury was demonstrated by qualitative, semi-quantitative and quantitative measurements (Figs. 7–9).

The mechanism of cellular internalization by BCECs was also explored in this study. The uptake process showed a temperature depending manner as the fluorescence intensity decreased significantly at 4 °C compared with physiological 37 °C (Fig. 2A.a, d). Naloxone, which is a nonspecific opiate receptor agonist, as well as free dermorphin, restrained the cellular uptake of DGL-PEG-dermorphin/DNA NPs (Fig. 2A.e, f). It suggested that dermorphin promoted the brain-entry effect through the specific binding to the opiate receptors on BCECs, which was in accordance with the fact that MOR was extensively located in brain [20]. These two results indicated that the cellular internalization of dermorphin modified NPs was the energy-dependent and competitively inhibited active transport progress.

Other inhibitors involved in different endocytosis processes were also applied in this study. PhAsO is an inhibitor of the clathrin-mediated endocytosis, which is most related to specific receptor–ligand interaction [24]. Treated with PhAsO, weaker fluorescence was shown in Fig. 2A.g. The uptake of DGL-PEG-dermorphin/DNA NPs by BCECs were inhibited by PhAsO, therefore clathrin-mediated endocytosis was involved in its mechanisms. Caveolaemediated endocytosis is another major pathway for cellular uptake, which can be inhibited by Filipin. This kind of endocytosis process is associated with the surface charge of the NPs, which turns to be adsorption-mediated endocytosis [25]. The cellular internalization were inhibited by Filipin (Fig. 2A.h), suggesting that the caveolaemediated endocytosis process is also responsible for the uptake of DGL-PEG-dermorphin/DNA NPs by BCECs. Meanwhile, macropinocytosis was also under investigation, by use of colchicine as the inhibitor. This kind of endocytosis process was associated with the particle size. Treated with colchicine, less fluorescence-labeled DGL-PEG-dermorphin/DNA NPs was observed (Fig. 2A.i) in BCECs. Obviously, clathrin-mediated endocytosis, caveolaemediated endocytosis and macropinocytosis make up the cellular uptake mechanism system of DGL-PEG-dermorphin/DNA NPs by BCECs.

The cellular uptake was intuitively compared in the Flow Cytometry shift curves (Fig. 2B). Weaker fluorescence intensity of DGL-PEG-dermorphin/DNA NPs could be observed at a low temperature or with different inhibitors. The %Parent of control group was about 37%, whereas at other conditions, the value decreased to about 24% or even less. The data was in good agreement with the fluorescent images (Fig. 2A), which well contributed to the exploration of the cellular uptake mechanism. Moreover, the uptake efficiency of DGL/DNA NPs seemed to be the most highest, which probably due to the maximum exposed amino groups. However, high toxicity and large particle size
set a limit for DGL to be used as efficient gene vectors, and its electronegativity induces a rapid clearance in vivo.

5. Conclusion

In this study, DGL-PEG-dermorphin/shRNA NPs were successfully synthesized as brain-targeting shRNA delivery system for RNAi neuroprotection against cerebral ischemia reperfusion injury. The brain-entry capability was evaluated in vitro and in vivo, and the system proved to be promising for brain-targeting therapy. NPs with anti-Ask1 shRNA demonstrated significant suppressive effect on the expression of Ask1, which played an important part in the process of cellular apoptosis induced by cerebral ischemia reperfusion. Injection with DGL-PEG-dermorphin/shRNA NPs in advance successfully reduced the apoptotic cell death as well as the infarct area in brain, preventing the rats from severe cerebral ischemia reperfusion injury. Therefore, we hold the belief that DGL-PEG-dermorphin/shRNA has great potential for safe and efficient brain-targeting RNAi neuroprotection against cerebral ischemia reperfusion injury.

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