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Polybutylcyanoacrylate nanoparticles for delivering hormone response element-conjugated neurotrophin-3 to the brain of intracerebral hemorrhagic rats

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ABSTRACT

Hypertensive intracerebral hemorrhage (ICH) is a rapidly evolutional pathology, inducing necrotic cell death followed by apoptosis, and alters gene expression levels in surrounding tissue of an injured brain. For ICH therapy by controlled gene release, the development of intravenously administrable delivery vectors to promote the penetration across the blood–brain barrier (BBB) is a critical challenge. To enhance transfer efficiency of genetic materials under hypoxic conditions, polybutylcyanoacrylate (PBCA) nanoparticles (NPs) were used to mediate the intracellular transport of plasmid neurotrophin-3 (NT-3) containing hormone response element (HRE) with a cytomegalovirus (cmv) promoter and to differentiate induced pluripotent stem cells (iPSCs). The differentiation ability of iPSCs to neurons was justified by various immunological stains for protein fluorescence. The effect of PBCA NP/cmvNT-3-HRE complexes on treating ICH rats was studied by immunostaining, western blotting and Nissl staining. We found that the treatments with PBCA NP/cmvNT-3-HRE complexes increased the capability of differentiating iPSCs to express NT-3, TrkC and MAP-2. Moreover, PBCA NPs could protect cmvNT-3-HRE against degradation with EcoRI/PstI and DNase I *in vitro* and raise the delivery across the BBB *in vivo*. The administration of PBCA NP/cmvNT-3-HRE complexes increased the expression of NT-3, inhibited the expression of apoptosis-inducing factor, cleaved caspase-3 and DNA fragmentation, and reduced the cell death rate after ICH *in vivo*. PBCA NPs are demonstrated as an appropriate delivery system for carrying cmvNT-3-HRE to the brain for ICH therapy.

1. Introduction

In hypertensive intracerebral hemorrhage (ICH), a hemorrhagic stroke belongs to the primary injury resulting from various hematomatic effects. The secondary events include diverse cellular and molecular disruptions, such as calcium homeostasis disturbance, generation of free radicals and excitotoxic neurotransmitters, lipid peroxidation, inflammatory response and apoptotic activation, in the neighboring brain parenchyma [1–4]. Poor outcomes are always associated clinically with hematoma expansion and edema formation in peri-ICH zone, which responds poorly to surgical clot evacuation, even though no further bleeding occurs [5]. Therefore, the evaluation and management of the peri-ICH microenvironment are important for ICH treatment. In addition, the blood–brain barrier (BBB) is a complex cellular system comprises mainly endothelia, astrocytic end-feet, pericytes and basement membrane and maintains the normal physiological characteristics of the central nervous system (CNS) [6]. ICH-induced cellular variations in the brain can be identified by BBB disruption, local generation of osmotically active substances and edema formation [7]. All these factors are essential to ICH prognosis.

Neurotrophin-3 (NT-3), a member of the neurotrophin family, was cloned in 1990. Its primary structure is very similar to neuron growth factor and brain-derived neurotrophic factor with high affinity to the TrkC receptor and low affinity to the p75 receptor [8]. The neurotrophin expression in cells depends on these receptors [9]. In addition, NT-3 plays a crucial role in the regulation of neurogenesis in the CNS [10] and in the promotion of neuronal survival, outgrowth of nerve fiber and neuronal differentiation of stem cells *in vitro* [11–13]. NT-3 has been shown to mediate neuroprotective effects in various models of apoptosis *in vitro* [14–17] and *in vivo*

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[18,19]. Moreover, the neurovascular unit, constituting endothelial cells, astrocytes, pericytes and neurons, can collaborate through neurotrophins for neuronal survival under hypoxic conditions [20,21]. Furthermore, an accumulated body of evidence has proved that brain ischemia decreases the NT-3 mRNA expression in rat hippocampus; maintaining the NT-3 level can stabilize calcium and prevent neurons from excitotoxic injury [22–25]. Thus, the enhancement of local NT-3 concentration is a promising strategy to repair neurologic disorders.

Polybutylcyanoacrylate (PBCA) nanoparticles (NPs) are composed of *n*-butyl-2-cyanoacrylate (BCA) with high stability, good biocompatibility, low cytotoxicity and non-immunogenicity. In recent years, PBCA NPs became a commonly applied excipient for delivering pharmaceuticals to the brain [26–33]. PBCA NPs have also been employed to enhance the transport of large-molecule horseradish peroxidase (HRP; 44 kDa) and green fluorescent protein (29 kDa) to the brain with trauma [34]. This suggests that PBCA NPs are efficacious vectors for delivering large molecules to the injured brain. In addition, it has been drawn that PBCA NPs can be gene carriers to avoid digesting deoxyribonucleic acid (DNA) with lysosomal enzymes and to transfer oligonucleotides to mammalian cells [35,36]. Therefore, PBCA NPs are justified as an appropriate non-viral system for gene delivery.

The aim of this study was to demonstrate the capability of a colloid-mediated gene delivery system comprising PBCA NPs, cytomegalovirus (cmv) promoter, NT-3 and hormone response element (HRE) to treat ICH rats. The rats were subjected to collagenase-induced ICH and mimicked hypertensive ICH in human [37–40]. We constructed a plasmid NT-3 with HRE, controlled with cmv promoter. PBCA NP-mediated plasmid NT-3 containing HRE was used to trigger the hypoxia-inducible factor-1/HRE system for gene regulation and to produce NT-3 in ICH rats. The characteristics of PBCA NP/cmvNT-3-HRE complexes, transfection efficiency and neuronal differentiation ability of induced pluripotent stem cells (iPSCs) during hypoxic insults *in vitro* were evaluated. After administration of PBCA NP/cmvNT-3-HRE complexes, we evaluated the expression of NT-3, apoptosis-inducing factor (AIF), cleaved caspase-3 and fragmented DNA and assessed the nerve repair effect of the gene vector in ICH rats.

2. Materials and methods

2.1. Cell isolation and culture

2.1.1. Rat brain-microvascular endothelial cells (RBMECs)

The method for isolating and cultivating primary RBMECs was modified from a previous study [41]. The meninges were eliminated from the brain cortices of 4-week-old male Sprague-Dawley (SD) rats (BioLasco, Taipei, Taiwan). The brain cortices were minced in Hank's balance salt solution (Gibco, Grand Island, NY, USA) in an ice bath. The homogenate was digested with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA; Gibco) at 37 °C for 30 min. After adding Dulbecco's modified Eagle's medium (DMEM; Gibco) replenished with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) to trypsin-EDTA-treated suspension, the homogenate was filtered through a 100-μm and a 70-μm nylon mesh (BD Falcon, Bedford, MA, USA). The remnants on the 70-μm nylon mesh were collected and digested with 0.1% type II collagenase (Sigma, St. Louis, MO, USA) at 37 °C for 30 min. Afterward, the digested mixture was washed with DMEM containing FBS and centrifuged at 200 ×*g* for 5 min. The pellet was resuspended in DMEM/F12 (1:1) medium (Gibco) containing 20% FBS, endothelial cell growth supplement (0.2 mg/mL, Sciencell, Corte Del Cedro Carlsbad, CA, USA), basic fibroblast growth factor (10 ng/mL, Invitrogen, Grand Island, NY, USA) and antibiotics (100 U/mL penicillin G and 100 mg/mL streptomycin sulfate, Invitrogen). RBMECs were seeded in culture dishes (10 cm, Corning, Lowell, MA, USA) pre-coated with 2% gelatin (type A from porcine skin, Sigma) and cultured in an incubator (Forma 310, Thermo Fisher Scientific, Wilmington, MA, USA) with a gas phase controlled at 5% CO_2 and 95% relative humidity at 37 °C. When the cultured colony reached 80–90% confluence, adherent cells were detached with 0.25% trypsin-EDTA and equally distributed into four 10-cm culture dishes. RBMECs of passage 4–7 were used in the subsequent experiments. The optical images of proliferated RBMECs were obtained using an inverted phase-contrast fluorescence microscope (Eclipse TE300, Nikon, Tokyo, Japan). RBMECs were identified by immunostaining with a polyclonal rabbit anti-human von Willebrand factor antibody (anti-factor VIII; 1:200, Sigma) at room temperature for 1 h. For characterizing tight junction (TJ), RBMECs were immunostained with a polyclonal rabbit anti-ZO-1 (1:100, Zymed, South San Francisco, CA, USA) at room temperature for 1 h. The cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:100, Millipore, Billerica, MA, USA) at room temperature in darkness for 1 h. The fluorescent images of RBMECs were visualized using a fluorescent microscope (Eclipse E600, Nikon).

2.1.2. CTX TNA2 cells

CTX TNA2 cells (rat astrocytes; RAs) were purchased from Bioresource Collection and Research Center (BCRC; no. 60547, passage 27, Hsin-Chu, Taiwan) and cultured in DMEM replenished with 10%FBS. RAs were cultivated in the humidified CO_2 incubator at 37 °C. When the adherent cells reached 80–90% confluence, RAs were detached with 0.25% trypsin-EDTA and equally distributed into four tissue culture flasks (75 cm^2, BD Falcon, Franklin Lakes, NJ, USA). RAs of passage 28–32 were used in the subsequent experiments. The optical images of proliferated RAs were obtained using the inverted phase-contrast fluorescence microscope. RAs were identified by immunostaining with a polyclonal rabbit anti-glial fibrillary acidic protein (anti-GFAP; 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. The cells were incubated with an FITC-conjugated secondary antibody (1:100, Millipore) at room temperature in darkness for 1 h. The fluorescent images of RAs were visualized using the fluorescent microscope.

2.1.3. Mouse iPSCs

Mouse iPSCs (SC201A-iPSCs, passage 3) were obtained from System Biosciences (Mountain View, CA, USA) and cultured without feeder layer by a method described in literature [42–44]. The multiplication of iPSCs required ESGRO Complete Plus clonal grade medium (Millipore) in the humidified CO_2 incubator at 37 °C. The multiplied iPSCs at 80–90% confluence were detached with ESGRO Complete Accutase (Millipore) and expanded in four gelatin-coated 75-cm^2 tissue culture flasks. The propagated iPSCs of passage 7–9 were used in the subsequent experiments and were identified by immunocytochemistry through incubating with Milli-Mark anti-SSEA-1 cloning MC-480 PE conjugate (1:100, Millipore) at 4 °C in darkness overnight. The fluorescent images of iPSCs were visualized using the inverted phase-contrast fluorescent microscope.

2.2. Construct of cmvNT-3-HRE

The domain of NT-3 with amino acids 164–282 was cloned and sequenced by the following method. Based on rat genomic DNA, the DNA fragment was amplified by polymerase chain reaction with the restriction enzyme *Bam*HI (Fermentas, Vilnius, Lithuania) attached to the recognition sites of the forward primer 5′-GGATCCTATGCAGAGCATAAGAGTCACC-3′ and the restriction enzyme *Hind* III (Fermentas) attached to the recognition sites of the reverse primer 5′-AAGCTTTGTTCTTCCGATTTTTCTTGACA-3′. After amplification, the DNA fragment was purified, sequenced for determining precision and cloned into the *p*SV-β-galactosidase control vector (Promega, Madison, WI, USA), where a cmv promoter in the *p*GEM base (Promega) replaced the SV40 promoter. The *p*SV-β-galactosidase control vector was also digested with *Bsa*A I (New England BioLabs, Ipswich, MA, USA) and *Ava* I (New England BioLabs). HREs (P18 × 3) with the P18 sequence TGTCACGTCCTGCACGAC were inserted into a *Sal* I (New England BioLabs) site. *Escherichia coli* JM 109 cells (ECOS™ 9–5, Yeastern Biotech, Taipei, Taiwan) were applied to transform the constructed cmvNT-3-HRE (plasmid DNA; *p*DNA). A quick plasmid preparation (Mini *Plus*™ Plasmid DNA Extraction System, Viogene, New Taipei City, Taiwan) with enzyme digestion was used to select the positive clones by DNA sequence verification for the precision of the positive clones. The Maxi *Plus*™ Ultrapure Plasmid DNA Extraction System (Viogene) produced large-scale *p*DNA. An ND-1000 spectrophotometer (NanoDrop, Thermo Fisher Scientific) with a wavelength at 260 nm determined the concentration of *p*DNA, where the optical density at 260–280 nm ranged between 1.8 and 1.9.

2.3. Synthesis of PBCA NPs and FITC-labeled PBCA NPs

The method for synthesizing PBCA NPs was described in a previous study [45]. The emulsified solution for polymerization contained 0.1% BCA (Sicomet, Sichel Werke, Hanover, Germany) and 0.1% dextran 70000 (Sigma) in hydrochloric acid (0.01 N, Hayashi, Osaka, Japan) at 400 rpm and room temperature for 3.5 h. To terminate reaction, sodium hydroxide (0.1 N, Showa, Tokyo, Japan) was added into the medium and centrifuged at 5250 ×*g* for 10 min. The polymeric suspension was filtrated through a polyethylene terephthalate membrane filter (mean pore diameter of 0.22 μm, Millipore). Replacing dextran 70000 with FITC-dextran (Fluka Biochemika, Buchs, Switzerland) in the reaction system yielded FITC-labeled PBCA NPs (FITC-PBCA NPs). PBCA NP/cmvNT-3-HRE complexes were prepared by mixing cmvNT-3-HRE with PBCA NPs in 2 μL of Dulbecco's phosphate-buffered saline (DPBS; Sigma) at room temperature for 1 h and centrifuging at 16100 ×*g* for 30 min.