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Therapeutic effects of a reducible poly (oligo-D-arginine) carrier with the heme oxygenase-1 gene in the treatment of hypoxic-ischemic brain injury

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ABSTRACT

Non-viral carriers for gene therapy have been developed to minimize carrier cytotoxicity and to enhance transfection efficiency. Previously, we synthesized a 9-arginine-based reducible high molecular weight peptide for gene delivery. For the reducible poly(oligo-D-arginines) (rPOA), 9-arginine oligopeptides are connected by internal disulfide linkages to produce a high molecular weight peptide. In this study, rPOA was evaluated as a carrier of the heme oxygenase-1 (HO-1) gene for the treatment of ischemia/reperfusion (I/R) -induced brain stroke. An *in vitro* transfection assay showed that rPOA had higher transfection efficiency and lower toxicity than polyethylenimine (PEI). For *in vivo* evaluation, I/R rat models were produced by middle cerebral artery occlusion (MCAO). rPOA/HO-1 expression plasmid (pHO-1) polyplexes were injected into the brain at 1 h before MCAO, and HO-1 expression levels in the brain were then measured by ELISA. The results indicated that rPOA/pHO-1 polyplexes had higher transfection efficiencies than PEI/pHO-1 polyplexes. The rPOA/pHO-1 polyplexes significantly reduced infarct volumes. In addition, tumor necrosis factor-alpha (TNF-α) was reduced in the rPOA/pHO-1 polyplex injection group, suggesting that HO-1 had an anti-inflammatory effect, while the PEI/pHO-1 polyplex did not show this effect. These results suggest that rPOA is a potential non-viral vector for HO-1 gene therapy to protect brain cells from I/R-related neuronal injury including stroke.

1. Introduction

Various non-viral vectors have been developed for gene therapy. Compared with viral vectors, non-viral vectors have advantages such as low immunogenicity, no integration into host chromosomes and low cost [1]. However, they have relatively low transfection efficiency and exhibit some toxicity, which limits their applications to clinical gene delivery. To overcome the limitations of non-viral vectors, efforts have been made to develop carriers with high transfection efficiency and low cytotoxicity.

Protein transduction domains (PTDs) have the ability to effectively but non-specifically transduce a wide variety of cargoes including proteins and genes into cells [2]. Various kinds of PTDs have been reported such as TAT, penetratin, transportan and polyarginine; among which, TAT and polyarginine have shown high transduction efficiency. Recent studies have demonstrated the usefulness of TAT and polyarginine as gene carriers [2]. However, short PTDs are not sufficient to condense, protect and transfect genes, due to the relatively weak cooperative effect between nucleic acids and PTDs [3]. On the other hand, high molecular weight cationic peptides have strong binding affinity resulting in poor dissociation rates of the polyplex [4]. Several polycations have therefore been developed to overcome these drawbacks by introducing environment-responsive reducibility to enhance the transfection efficiency and minimize the cytotoxicity resulting from the controlled release of gene in response to the external stimulus [5].

Reducible cationic peptides connected by internal disulfide bonds have the potential for use in gene therapy due to their environment-sensitive reducibility [6]. The stable polyplexes of the reducible peptides with nucleic acids spontaneously dissociate via the reduction of the internal disulfide linkages upon exposure to the reductive environments of cytoplasm. The site-specific reduction of peptides can facilitate the release of nucleic acids in a short period of time. Therefore, the reduction of polycations is a unique characteristic of disulfide bond-based polypeptides as gene carriers for

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enhancing transfection efficiency. Recently, 9-arginine-based reducible high molecular weight peptide, reducible poly(oligo-D-arginine) (rPOA), was developed as a gene carrier with high transfection efficiency by intracellular reduction and low cytotoxicity [7].

Heme oxygenase-1 (HO-1) is an antioxidant enzyme that catalyzes the degradation of heme. HO-1 is overexpressed under hypoxia. HO-1 produces carbon monoxide (CO), iron and biliverdin [8]. Although the mechanisms of HO-1 and its products are unclear, it has been shown that HO-1 down regulates the inflammatory reaction by attenuating the expression of adhesion molecules and leukocyte recruitment [9,10] or by repressing the induction of cytokines and chemokines [11,12]. Therefore, the overexpression of HO-1 gene can reduce tumor necrosis factor-alpha (TNF-α)-mediated inflammation [13] and protect cells from apoptosis under hypoxia [14]. Thus, HO-1 has been suggested as a therapeutic target of gene therapy for the treatment of ischemic disease including stroke. In addition, recent studies have reported that HO-1 protects cells from reactive oxygen radical species in acute lung injury [15–18].

I/R-related neuronal injury following stroke causes an infarction in the core region. In addition, the neighboring penumbra tissues are subjected to damage through apoptosis and activation of inflammation that leads to cell death. There is a strong correlation between cerebral I/R-related neuronal injury and inflammation [19]. Therefore, studies of therapeutic approaches for neuroprotection have focused on the protection of the penumbra region from delayed damage and inflammation. Also, stimulation of the endogenous antioxidant system is an alternative strategy to prevent neuronal damage.

In this study, the HO-1 expression plasmid, pHO-1, was delivered to the focal ischemic brain of stroke animal models using rPOA as a gene carrier. The effects of pHO-1 delivery were evaluated in various methods.

2. Materials and methods

2.1. Materials

Cys-(D-R9)-Cys (CRRRRRRRRRC, Mw 1628) was purchased from Peptron (Daejeon, Korea). PEI (Branch form, average molecular weight 25 000) was obtained from Sigma (St. Louis, MO). Plasmid DNA (pGL3 promoter, 5010 bp) and the luciferase assay kit were from Promega (Madison, WI). All other reagents were analytical grade.

2.2. Synthesis of rPOA

rPOA was prepared by DMSO-mediated oxidative polycondensation as previously described [7]. In brief, Cys-(D-R9)-Cys was reacted in PBS (pH 7.4) containing 30% DMSO at a final concentration of 57 mM. After 6 days, the reaction was terminated by adding 5 mM HEPES buffer. Impurities were removed by dialysis (MWCO: 10 000), and thereafter the purified peptides were collected and lyophilized using a vacuum-freeze dryer (Freezone 4.5; Labconco, Kansas City, MO).

2.3. Agarose gel electrophoresis assay

Various amounts of rPOA and PEI were incubated with 1 μg of HO-1 for 20 min at room temperature, and then the samples were electrophoresed in 0.8% (w/v) agarose gel for 30 min at 100 V in 0.5% TBE buffer. Ratios were expressed as weight ratios of the rPOA or PEI to DNA for all data.

2.4. Size and zeta potential determination

The mean diameter and surface zeta potential of the polyplexes were measured using Zetasizer-Nano ZS (Malvern Instruments, Worcestershire, UK). HO-1/rPOA or HO-1/PEI polyplexes were prepared at ratios of 1:0.5, 1:1, and 1:2 in PBS. The mean diameters and zeta potentials of the polyplexes were measured in triplicate.

2.5. DNA stability in serum

For the protection of DNA in mouse serum, rPOA/pHO-1 or PEI/pHO-1 polyplexes were prepared at ratios of 1:1 and 1:2 in PBS. After a 20-min incubation, mouse serum was added to the polyplexes at a final concentration of 50% (v/v). The DNA stability was tested by incubating in the serum for 2 h under heating at 37 °C and shaking at 150 rpm. After the incubation, heparin was added to the polyplexes at a final heparin to rPOA or PEI weight ratio of 300 in the presence of 0.01 M EDTA. After 1 h of incubation, the mixtures were electrophoresed under the same conditions described above.

2.6. In vitro transfection efficiency

Mouse N2a neuroblastoma cells were obtained from ATCC (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum, and penicillin (100 IU/ml)/streptomycin (100 μg/ml). Cells were incubated to 80% confluence at 37 °C under 5% CO_2. N2a cells were seeded on 24-well plates at a density of 5.0×10^4 cells/well. After 24 h incubation, the culture media was replaced with fresh media containing rPOA/pLuc or PEI/pLuc polyplexes. The polyplexes were prepared by mixing 2 μg DNA and rPOA or PEI at ratios of 1:1 and 2:1 in DMEM. After 24 h of incubation, cells were washed twice with PBS, and then treated with 150 μl of 1× reporter lysis buffer reagent (Promega) for 20 min. The cell lysates were scraped, harvested, and transferred to 1.5 ml microtubes, and centrifuged for 3 min at 13,000 rpm. The luciferase RLU of the cell lysates was measured on a 96-well plate luminometer (Berthold Detection Systems, Pforzheim, Germany) with 20-s integration, and the results were expressed as RLU/mg protein determined by the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin standard. N2a cells were seeded on 24-well plates at a density of 5.0×10^4 cells/well and incubated in the absence or presence of 50, 100 and 200 μM buthionine-sulfoximine (BSO) for 24 h prior to transfection. The transfection and the measurement of transgene expression were performed as described above. In all cases, the transfection efficiencies were measured three times with four replicates.

2.7. Cytotoxicity

For cytotoxicity measurement, N2a cells were seeded on 24-well plates at a density of 5.0×10^4 cells/well. After 24 h incubation, the culture media was replaced with fresh media containing rPOA/pLuc or PEI/pLuc at a ratio of 1:1 or 1:2. After 24 h, 30 μl of the CCK-8 (Dojindo Laboratory, Tokyo, Japan) solution was added in place of the media, and the cells were incubated for 2 h. The absorbance of each well was measured at 540 nm using a UV/Vis spectrophotometer. The relative cell viability was calculated and expressed as percent cell viability. The viability was measured three times with four replicates.

2.8. Surgical procedure for middle cerebral artery occlusion (MCAO) model

All experimental procedures were carried out as previously described [8]. Male Sprague–Dawley (SD) rats (280–320 g) were anesthetized with 5% isoflurane in a mixture of 30% oxygen and 70% nitrous oxide for about 2 min, which was maintained using