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Doxorubicin augments rAAV-2 transduction in rat neuronal cells

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ABSTRACT

Doxorubicin, an approved drug for cancer therapy, was recently found to be a potent agent to augment adeno-associated virus (AAV)-mediated transgene expression, especially in airway cells. Recombinant AAV type 2 (rAAV-2) has been shown to preferentially transduce neural tissues and is considered as the primary viral vector for the treatments of various neurodegenerative diseases including Parkinson's disease (PD). The goal of this study is to investigate whether doxorubicin can be applied to increase the efficacy of rAAV-2 transduction in the central nervous system. We co-administrated doxorubicin with AV2.luc/EGFP into the rat striatum, a preferred target site for PD gene therapy, and found that doxorubicin augmented rAAV-2 transduction dramatically without significant cytotoxicity and alteration of rAAV-2 tropism. By evaluating the effects of doxorubicin on rAAV-2 transduction in PC12 and MN9D neuronal cells, we found that doxorubicin appeared to promote the nuclear accumulation of rAAV-2, but did not affect viral binding or uptake. Our data suggested that doxorubicin might play an important role in modulating rAAV-2 intracellular trafficking in neuron-like cells. Our study also provided the initial *in vivo* evidence to facilitate AAV-mediated gene expression in the midbrain with the treatment of doxorubicin.

1. Introduction

Doxorubicin is an anthracycline antibiotic commonly used to treat cancers (Soloman and Gabizon, 2008). Recently, doxorubicin was reported to significantly augment adeno-associated virus (AAV) transduction in airway cell lines (Yan et al., 2004), which was similar to tripeptidyl aldehyde proteasome inhibitors that have been shown to effectively increase viral capsid ubiquitination and transduction of rAAV-2 and rAAV-5 serotypes (Yan et al., 2002), but through a distinct mechanism.

AAV is a non-pathogenic parvovirus with a 4.7-kb single-stranded DNA genome. It has been effectively developed into a highly promising vector system for gene therapy with various advantages. rAAV-2 is the emerging standard vector for the replacement type of approaches (Doroudchi et al., 2005) and the non-detectable vector-associated adverse events in more than 30 subjects were very encouraging (Kaplitt et al., 2007). As a vector with the potential for persistent expression following integration, it efficiently transduces a wide range of host cells and is a non-pathogenic virus with minimal cell-mediated immune responses (Mandel et al., 2006; Van Vliet et al., 2008). However, the transduction efficiency is unsatisfactory to produce sufficient transgene expression (Gao et al., 2005).

AAV second-strand synthesis was essential for AAV transduction and considered to be a limiting step in earlier studies. Therefore, self-complementary AAV (scAAV) was developed to test the strategies of bypassing this step to increase AAV transduction. Recent studies, however, suggested that scAAV contributed more to faster onset of the transgene expression rather than long-term expression (Natkunarajah et al., 2008). Meanwhile, growing evidences have shown that the intracellular trafficking appeared to be more rate-limiting in various cell types. Hence, searching for compounds that overcome the trafficking barrier became an attractive approach, among which proteasome inhibitors were thought to be most effective. Although the exact mechanism is unknown, an alteration of viral trafficking as reflected by the nuclear accumulation of AAV was observed after the treatments of proteasome inhibitors (Johnson and Samulski, 2009).

With the goal of transitioning the application of proteasome inhibitors to CNS with rAAV-2, we sought to investigate whether doxorubicin could increase rAAV-2 transduction, and to exploit its

Abbreviations: AAV, adeno-associated virus; CNS, central nervous system; DA, dopamine; EGFP, enhanced green fluorescent protein; GFAP, glia fibrillary acidic protein; MOI, multiplicity of infection; MPP^+, 1-methyl-4-phenylpyridinium ion; NeuN, neuronal nuclei; N.S., normal saline; PBS, phosphate-buffered saline; PD, Parkinson's disease; rAAV-2, recombinant adeno-associated virus type 2; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling.

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potentials to facilitate AAV-mediated gene expression in CNS. In the present study, we evaluated the ability of doxorubicin to augment rAAV-2 transduction in neuronal cell lines, primary cultures and *in vivo* animal models. We demonstrated that doxorubicin could similarly enhance rAAV-2 transduction significantly under appropriate conditions, without significant cytotoxicity and alteration of rAAV-2 tropism. Our findings have provided the first evidence for a proteasome modulating reagent to facilitate AAV-mediated gene expression in animal models, suggesting its future application in CNS, especially in neuronal cells.

2. Experimental procedures

2.1. Materials

All tissue culture reagents used in this study were purchased from Invitrogen (GIBCO). The primary antibodies for immunofluorescence or protein blotting assays were obtained from Chemicon, except for the anti-histone 3 antibody which was from Invitrogen (Molecular Probes). TRITC-labeled Goat anti-mouse IgG was purchased from Jackson Labs. The DeadEnd™ Fluorometric TUNEL System, luciferase assay reagents and DNase I were purchased from Promega. NE-PER® Nuclear and the Cytoplasmic Extraction Reagents were from PIERCE. The Alexa Fluor® 647 Microscale Protein Labeling Kit was supplied by Invitrogen. The NAG detection kit was manufactured by Ding Guo Biological Technology Co., Ltd., China. The qPCR reagents were from Strategene. Other chemicals, except for specially indicated, were supplied by Sigma–Aldrich.

The Microplate reader (Varioskan, Thermo Electron Corporation) and the Odyssey Infrared Imaging System (LI-COR Biosciences) were deployed for luciferase activity measurements and slot-blot scanning, respectively, in this study. The Mx3000P qPCR system (strategene) was used for viral genome quantification.

2.2. Tissue culture

PC12 (established from a rat pheochromocytoma) and MN9D (a fusion of neuroblastoma with embryonic ventral mesencephalic cells) cell lines were used for rAAV-2 transduction studies *in vitro*. PC12 cells were cultured as monolayer in RPMI-1640, supplemented with 100 U/ml penicillin, 100 U/ml streptomycin and 5% fetal bovine serum, and maintained in a 37 °C incubator with 5% CO_2. MN9D cells were cultured as monolayer in penicillin–streptomycin containing DMEM-F12 with 10% fetal bovine serum, and grown in a 37 °C incubator at 5% CO_2, until they reached the desired confluence.

Primary neuron cultures were prepared as following. Pregnant Sprague–Dawley rats were euthanized by decapitation under CO_2 anesthesia. The E14.5 embryos (the identification of vaginal plug was defined as E0.5) were collected in Ca^{2+}–Mg^{2+}-free HBSS. Small pieces of dissected ventral midbrain (without the underlying mesenchyme and meninges) were mechanically dissociated by gentle trituration using fire-polished Pasteur pipettes. The dissociated cells were centrifuged at 1000 × *g* for 5 min and resuspended in Neurobasal (Invitrogen product, containing 2% B27, 0.5 mM L-glutamine, 25 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 U/ml streptomycin). The obtained cells were cultured for 24 h with a change of fresh medium, and maintained in Neurobasal.

2.3. Recombinant rAAV-2 production and viral transduction

The rAAV-2 used in this study, AV2.EGFP and AV2.luc, were reporter vectors driven by CMV promoters. The viral package and purification were contracted to Gene Technology Company Limited (VGTC, China) using BHK21 as producer cells. The obtained viruses were tested to be clinical grade with the purity of 99.5% or above (as also demonstrated in supplemental Fig. 1). The viruses were aliquoted into 1×10^{12} v.g./ml in 100 μl Ca^{2+}–Mg^{2+} containing 10 mM HEPES buffer.

PC12 or MN9D cells were grown in 96-well plates to 70% confluence prior to infection. The rAAV-2 infections were performed at a multiplicity of infection (MOI) equal to 10^5 v.g./cell in 100 μl of 1% FBS RPMI-1640 or DMEM-F12 with the absence or presence of doxorubicin. At 24 h post-infection, luciferase assays were performed following manufacturer's standard protocol.

27 adult male Sprague–Dawley rats with 180–200 g body weight were used for *in vivo* rAAV-2 transduction studies. The animal subjects were maintained on a 12/12 h light/dark cycle at constant temperature and humidity, and provided with food and water ad libitum. Rats were anesthetized by *i.p.* with chloraldurat at 350 mg/kg body weight prior to viral injections. The rAAV-2 vectors were pre-diluted with PBS and supplemented without or with 200 μM doxorubicin. The rats were stereotaxically injected with normal saline (N.S.), rAAV-2 vectors (3×10^9 v.g. in 4 μl per site) in the absence or presence of doxorubicin in the striatum (the coordinates relative to the bregma and dura, AP +0.7 mm; ML 3.0 mm; DV −5.0 mm). The injections were carefully controlled as a constant rate of 1 μl/min. The injected rats were sacrificed at 2, 3 or 8 weeks post-infection for fluorescent observations or luciferase assays to determine the expression levels of AAV-mediated transgenes. Manual counting was performed to determine the number of AV2.EGFP infected cells in rat striatum sections. A total of 12 images from the sections at the same anatomic location were randomly selected from 3 individual rats in each group. The numbers of EGFP positive cells were averaged and presented with accorded statistics. All experimental procedures received approval from the Peking University on Animal Care and Use Committee.

2.4. Tissue preparation and luciferase assay

Sprague–Dawley rats were sacrificed 3 weeks post-infection, and the striatum tissues were collected and subjected for luciferase assays. Briefly, tissues were wrapped in aluminum foil, snap-frozen in liquid nitrogen and stored at −70 °C until homogenization. The frozen samples were thawed in 1× CCLR (Cell Culture Lysis Reagent, Promega) in 1.5 ml tubes. Tissues were homogenized for 30 s with tubes in ice, and then spun for 10 min at 3000 rpm at 4 °C. Immediately, 20 μl of supernatants were transferred into a luminometer plate in duplicate for each sample. Luciferase and substrate reaction was carried out by using the Promega Luciferase assay system. Luciferase activity was quantified and expressed as relative luciferase activity/per mg total tissue proteins.

2.5. Fluorescent confocal microscopy

The AAV-injected rats were lethally overdosed with chloraldurat and then perfused with N.S. and 4% paraformaldehyde (PFA) in PBS. The brain tissues were post-fixed in the same fixative solution overnight and then placed in a graded series of sucrose–PBS solutions as cryoprotective measures. The fixed tissues containing the striatum were then cut coronally into 30 μm sections and then subjected to standard immunofluorescent staining procedures. Briefly, the free-floating sections were washed twice in PBS and incubated with 0.5% Triton X-100 containing 1% BSA in 0.1 M PBS for 30 min, then incubated with normal serum block. Subsequently, sections were incubated at 4 °C overnight with mouse polyclonal antibodies against NeuN, GFAP or CD11b at a dilution of 1:400, 1:400 or 1:200, respectively. After extensive wash, the sections were incubated with goat anti-mouse IgG/TRITC at a dilution of 1:500 for 2 h. And finally, the stained sections were examined under a Zeiss LSM 5 confocal system, and DAPI was used to stain the nuclei.

2.6. MTT assay

The PC12 cells treated with different concentrations of doxorubicin for 24 h were rinsed with 100 μl PBS per well and immediately incubated with 0.5 mg/ml MTT dissolved in PBS for 4 h at 37 °C. Then 100 μl Triton X-100/ethanol/H_2O (1:5:4, volume ratio) was added per well to help dissolve the intracellular formazan crystals and kept for 6 h in the dark. The absorption at 570 nm was measured on a spectrophotometer. The cell viability was converted and expressed as the percentage of the control (naïve PC12 cells).

2.7. TUNEL assay

The *in situ* detection of the apoptotic cells was performed in doxorubicin treated PC12 cells or brain sections of doxorubicin injected rats with the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL) technique using a DeadEnd™ Flurometric TUNEL System. The procedures were carried out according to the manufacturer's instructions. The samples were stained with a DAPI-containing anti-fadant and then examined using a Leica DM-IRB fluorescent microscope. The positive control samples received the same treatments but were pretreated with DNase I (10 U/ml) for 30 min at 37 °C prior to TUNEL staining.

2.8. Viral fluorescent labeling

The labeling of rAAV-2 was performed using the Alexa Fluor® 647 Microscale Protein Labeling Kit according to the manufacturer's instructions. In brief, the appropriate amounts of Alexa Fluor® 647 in staining solution and AV2.luc were well mixed for 30 min, and then subjected to column chromatography using a costumed column prepared with Bio-Gel P6 fine resin. The fractions containing labeled viruses were separated, collected and then combined after characterizations (Jia et al., 2008).

2.9. Subcellular fractionation for the detection of rAAV-2 capsid proteins

PC12 cells were seeded at 5×10^5 per well in 24-well plates the day before rAAV-2 infection. Alexa Fluor® 647 labeled AV2.luc at MOI = 10^5 v.g./cell with or without doxorubicin (0.8 μM) was administrated. At 24 h post-infection, the cells were trypsinized, washed twice and resuspended in 1 ml PBS, and then transferred to a microcentrifuge tube. The cytoplasmic and nuclear fractions were isolated from the cell pellet with NE-PER® Nuclear and Cytoplasmic Extraction Reagents. The purities of each fraction were tested by immunoblot for nuclear antigen histone 3 or cytoplasmic β-glucosidase activities with the NAG detection kit. The virus-containing cytoplasmic or nuclear samples were transferred to a PVDF membrane using a 48-well slot-blot apparatus. The fluorescence scanning was performed