Original Paper

Single Tyrosine Mutation in AAV8 Vector Capsid Enhances Gene Lung Delivery and Does Not Alter Lung Morphofunction in Mice

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Key Words

Adeno-associated virus • Tyrosine mutant • Gene therapy • Lung function

Abstract

Background/Aims: Vectors derived from adeno-associated viruses (AAVs) are important gene delivery tools for treating pulmonary diseases. Phosphorylation of surface-exposed tyrosine residues from AAV2 capsid targets the viral particles for ubiquitination and proteasome-mediated degradation, and mutations of these tyrosine residues lead to highly efficient vector transduction *in vitro* and *in vivo* in different organs. We evaluated the pulmonary transduction efficiency of AAV8 vectors containing point mutations in surface-exposed capsid tyrosine residues. **Methods:** Male C57BL/6 mice (20–25 g, *n*=24) were randomly assigned into three groups: control group animals received intratracheal (i.t.) instillation of saline (50 µl), wild-type AAV8 group, and capsid mutant Y733F AAV8 group, which received (i.t.) AAV8 vectors containing the DNA sequence of enhanced green fluorescence protein (eGFP). Four weeks after instillation, lung mechanics and morphometry, vector transduction (immunohistochemistry and mRNA expression of eGFP), and inflammatory cytokines and growth factor expression were analyzed. **Results:** Tyrosine-mutant AAV8 vectors displayed significantly increased transduction efficiency in the lung compared with their wild-type counterparts. No significant differences were observed in lung mechanics and morphometry between experimental groups. There was no evidence of inflammatory response in any group. **Conclusion:** AAV8 vectors may be useful for new therapeutic strategies for the treatment of pulmonary diseases.

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Introduction

Adeno-associated virus (AAV) is a nonpathogenic parvovirus composed of a 4.7-kb single-strand DNA genome within a nonenveloped, icosahedral capsid [1]. Recombinant vectors based on AAV have been used extensively as gene delivery tools for the treatment of many respiratory diseases, including cystic fibrosis, α 1-antitrypsin deficiency, asthma, and acute lung injury [2–5].

The AAV vector seems to be the most advantageous for gene therapy due to a number of favorable features including lack of pathogenicity, low immunogenicity, lack of viral coding sequences, broad tropism, and the ability to support strong and persistent transgene expression [6].

To date, 12 AAV serotypes and more than 100 naturally occurring primate AAV variants have been described [7, 8]. Although different serotypes of AAVs have been shown to share a common genome structure, they have unique capsid proteins that can be recognized by different cell-surface receptors [9]. Adeno-associated virus 2 (AAV2) was the first AAV serotype to be made into vectors for gene transfer in preclinical and clinical applications [10, 11]. However, the use of AAV2 vectors in clinical trials in humans has two important limitations: (a) the vectors are inefficient at transducing cells of organs under therapy such as the liver, muscle cells and lungs; (b) gene transfer might be hindered by neutralizing anti-AAV2 antibodies, which are highly prevalent in the human population (up to 80% of all humans are estimated to be seropositive) [9, 12].

It is well known that anti-AAV neutralizing antibodies can completely prevent transduction of a target tissue, resulting in lack of efficacy, especially when it is necessary to readminister the vector to maintain gene expression. Repeated administration has been correlated with the generation of a neutralizing immune response that inhibits successive rounds of transduction [13]. Our group has shown that repeated administration of AAV2 intratracheally causes an inflammatory response that affects airway and lung parenchyma and a reduction of gene transfer [14]. To overcome such drawbacks, an increasing number of researchers are now focusing on other serotypes of AAV that are structurally and functionally different from AAV2, such as AAV8.

Among the various serotypes, AAV8 has features that make it particularly attractive as a vector for pulmonary gene therapy. The 37/67-kDa laminin has been identified as a cellular receptor for AAV8 and it is highly expressed in respiratory epithelial cells [15]. Furthermore, Boutin et al. [16] demonstrated that seroprevalence of antibodies against AAV8 is only moderate in the human population compared with AAV2, potentially facilitating immune escape of AAV8 vectors *in vivo*. Although some studies have already demonstrated a decrease in AAV2 efficiency after vector readministration [14, 17], no data have been reported for AAV8 vector. Therefore, these findings lend additional support to the use of the AAV8 serotype to maximize the efficiency of gene transfer and minimize immunologic reaction.

The effectiveness of AAV transduction is dependent on the interaction of the virus with the host cell, which includes receptor binding, cell entry, intracellular trafficking, uncoating, second-stranded synthesis, and vector genome stabilization [18]. In addition, previous studies have determined that the viral capsid is an essential element that influences cellular tropism and the efficiency of transgene expression [7]. Recently, it was shown that epidermal growth factor receptor protein tyrosine kinase-mediated phosphorylation of surface-exposed tyrosine residues from AAV2 capsid targets the viral particles for ubiquitination and proteasome-mediated degradation [19].

New advances in molecular biology have enabled the design of site-mutated AAV in an attempt to modulate AAV tropism to increase its transducing efficiency [20]. Site-directed tyrosine to phenylalanine (Y-F) mutagenesis of surface-exposed tyrosine residues on AAV2 capsids circumvents the ubiquitination step, thereby avoiding proteasome-mediated degradation and resulting in high-efficiency transduction of mutant vectors relative to the wild-type AAV2 vector both in tissue culture and in animals [19]. Furthermore, Petrs-Silva et al. [21] reported a 10- to 20-fold increase in transduction efficiency in mouse retina using

Fig. 1. (A) Schematic flow chart and (B) timeline of the study design. Animals received an intratracheal instillation of saline (CTRL, 50 μ l of saline), wild-type AAV8 (WT-AAV8, 10^{10} vg/50 μ l of saline), or tyrosine-mutant AAV8 (M-AAV8, 10^{10} vg/50 μ l of saline). Animals were harvested on day 28.

mutated AAV2, AAV8, and AAV9 compared with their wild-type counterparts. In addition, as found for AAV2, the proteasomal inhibitor (MG132) enhanced gene transfer efficiency mediated by AAV8 in embryonic kidney cells [22]. Although, AAV8 vectors hold promise for human gene therapy, no data are available on the transduction efficiency and possible functional and histologic damage in lungs subjected to tyrosine-mutant AAV8.

In the current study, we investigated whether tyrosine mutation could enhance the gene transfer efficiency of AAV8 to the lung after intratracheal administration of the vectors. Moreover, we evaluated if the administration of AAV8 vector caused an inflammatory response and lung damage, thus altering pulmonary morphofunction.

Materials and Methods

This study was approved by the Ethics Committee of the Carlos Chagas Filho Institute of Biophysics, Health Sciences Centre, Federal University of Rio de Janeiro. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences, USA.

Generation of rAAV vectors

Site-directed mutagenesis of surface-exposed tyrosine residues on AAV2 capsids has been described recently [19]. Similar strategies were used to generate AAV serotype 8 vectors containing tyrosine to phenylalanine mutations.

Vector preparations were produced using the plasmid co-transfection method [23]. The crude iodixanol fraction, as described, was further purified and concentrated by column chromatography on a 5-ml HiTrap Q Sepharose column using a Pharmacia AKTA FPLC system (Amersham Biosciences, Piscataway, NJ). The vector was eluted from the column using 215 mM NaCl, pH 8.0, and the rAAV peak was collected. Vector-containing fractions were then concentrated and buffer exchanged in Alcon BSS with 0.014% Tween 20, using a Biomax 100K concentrator (Millipore, Billerica, MA). Vector was then titrated for DNase-resistant vector genomes by real-time polymerase chain reaction (PCR) relative to a standard. The purity of the vector was validated by silver-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis, assayed for sterility and lack of endotoxin, and then aliquoted and stored at -80°C .

Treatment groups

Twenty-four male C57/BL6 mice (20–25 g) were randomly assigned to three groups ($n=8/\text{group}$). In the control group (CTRL), 50 μ l of saline were instilled intratracheally. Wild-type capsid AAV8 (WT-AAV8 group) or the capsid tyrosine-mutant Y733F AAV8 (M-AAV8 group, 10^{10} vg in 50 μ l of saline/mouse) containing the DNA of enhanced green fluorescence protein (eGFP) was delivered to mice lungs (Fig. 1).

The AAV8 vectors were provided by Dr Hilda Petrs-Silva, Laboratory of Neurogenesis, Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro.

Experimental protocol

Mice were anesthetized with sevoflurane, a 1-cm-long midline cervical incision was made to expose the trachea, and either AAV-8 or vehicle was instilled intratracheally with a microsyringe (intratracheal aerosolizer, IA-1C S/M-551 Model, Penn-Century, Inc., Philadelphia, PA) attached to a FMJ-250 high-pressure syringe (Penn-Century, Inc., Philadelphia, PA). After instillation, the cervical incision was closed with 5.0 silk suture and the mice were returned to their cage. Animals recovered rapidly after surgery.

Mechanical parameters

Twenty-eight days after the intratracheal instillation, animals were sedated (diazepam 1 mg intraperitoneally (i.p.), anesthetized (thiopental sodium 20 mg/kg i.p.), tracheotomized, and paralyzed (vecuronium bromide, 0.005 mg/kg intravenously). Mice were connected to a computer-controlled small-animal ventilator and quasi-sinusoidally ventilated (FlexiVent; SCIREQ, Montreal, Canada), as previously described [24], with a tidal volume of 7.5 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H₂O. Mice were allowed to acclimate to the ventilator for 2–3 min before initiation of readings, and three to four total lung-capacity functions were performed during this acclimation period to prevent atelectasis and to ensure maximum airway and alveolar recruitment. Static elastance was calculated from pressure–volume curves by the FlexiVent software (version 5.1). Thirty seconds after each pressure–volume curve, a 2-s perturbation at a frequency of 2.5 Hz was applied to generate data using the single-compartment model of respiratory mechanics. Only measurements with a coefficient of determination of 0.95 or greater were used, and measurements were repeated until a total of three pressure–volume curves and three single-compartment perturbations, each with acceptable coefficients of determination, were obtained. The averages of these three measurements were determined for each mouse and then averaged for each experimental group.

Lung histology

A laparotomy was done immediately after the determination of lung mechanics and heparin (1000 IU) was injected intravenously into the vena cava. The trachea was clamped at end expiration, and the abdominal aorta and vena cava were sectioned, leading to a massive hemorrhage that quickly killed the animals. The right lung was then removed, fixed in 3% buffered formaldehyde and embedded in paraffin. Slices were cut (4-μm thick) and stained with hematoxylin–eosin. Lung histology analysis was performed with an integrating eyepiece using a coherent system consisting of a grid with 100 points and 50 lines (known length) coupled to a conventional light microscope (Olympus BX51, Olympus Latin America-Inc., Brazil). The volume fraction of collapsed and normal pulmonary areas and the number of mononuclear and polymorphonuclear cells in pulmonary tissue were determined using the point-counting technique across ten random, noncoincident microscopic fields [25].

Immunohistochemistry

The left lung was removed after measurement of lung mechanics and fixed in 4% paraformaldehyde in sodium phosphate buffer at pH 7.4 for 24 h. The tissue was then washed in sodium phosphate buffer followed by 30% sucrose in the same buffer for 24 h. The lung was oriented under a dissection microscope in an aluminum chamber filled with optimal cutting temperature embedding medium, and 10-μm-thick transverse sections were cut in a cryostat. Tissue sections were first washed in phosphate buffered saline (PBS) for 5 min, incubated in 50 mM ammonium chloride for 30 min, washed in PBS for 5 min, then incubated with 0.5% Triton X-100 for 15 min, washed three times with PBS for 5 min each, followed by incubation with a blocking solution of 1% albumin for 30 min. The sections were then incubated with an antibody raised against the green fluorescent protein (Invitrogen, Molecular Probes, Carlsbad, CA) at 1:400 in blocking solution overnight at 4°C. Fluorescent staining was done with an anti-rabbit IgG secondary antibody, Alexa Fluor 488 (Molecular Probes) by incubating sections for 2 h at room temperature. The nuclei of the tissue cells were labeled with 1 μM of TO-PRO-3 dye (Invitrogen, Molecular Probes) in PBS for 15 min, then washed three times for 5 min in PBS. The specimens were mounted in ProLong Gold antifade reagent (Invitrogen, Molecular Probes). As a negative control of the technique, we used frozen tissue sections from all experimental groups incubated just with secondary antibody. The results were examined by fluorescence microscopy using an Axiophot microscope (Zeiss, Thornwood, NY). Digital pictures were taken with a camera connected to the Axiophot microscope. Only intensities above the threshold, above the intensity of the negative controls, were considered positive.

Real-time PCR

Quantitative real-time reverse transcription (RT)-PCR was performed to measure the relative levels of mRNA transcription of eGFP, vascular interleukin (IL)-1 β , IL-6, IL-13, interferon (IFN)- γ , and transforming growth factor (TGF)- β . Central slices of left lung were cut, collected in cryotubes, quick frozen by immersion in liquid nitrogen and stored at -80°C . Total RNA was extracted from the frozen tissues using an SV Total RNA Isolation System kit (Promega, Madison, WI) according to the manufacturer's recommendations. The concentration of RNA was measured by spectrophotometry in Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE). First-strand cDNA was synthesized from total RNA using the GoTaq 2-Step RT-qPCR system (Promega, Madison, WI). PCR primers for the target gene were purchased (Invitrogen, Carlsbad, CA). Relative mRNA levels were measured with an SYBR green detection system using Mastercycler ep realplex (Eppendorf, Hamburg, Germany). All samples were measured in triplicate. The relative RNA level of each gene was calculated as a ratio of the studied gene to control gene (acidic ribosomal phosphoprotein P0; 36B4). The following PCR primers were used: eGFP: forward, 5'-CAC ATG AAG CAG CAG GAC TT-3'; reverse, 5'-GGT GCG CTC CTG GAC GTA-3'. IL-1 β : forward, 5'-GTT GAC GGA CCC CAA AAG-3'; reverse, 5'-GTG CTG CTG CGA GAT TTG-3'. IL-6: forward, 5'-TCT CTG GGA AAT CGT GGA A-3; reverse, 5'-TCT GCA AGT GCA TCA TCG T-3'. IL-13: forward, 5'-GGA GCT GAG CAA CAT CAC A-3; reverse, 5'-TCC GGG CTA CAC AGA ACC-3'; IFN- γ : forward, 5'-GCT CTT CCT CAT GGC TGT TT-3'; reverse, 5'-GTC ACC ATC CTT TTG CCA GT-3'. TGF- β : forward, 5'-ATA CGC CTG AGT GGC TGT C-3'; reverse, 5'-GCC CTG TAT TCC GTC TCC T-3'. 36B4-Rplp0: forward, 5'-CAA CCC AGC TCT GGA GAA AC-3'; reverse, 5'-GTT CTG AGC TGG CAC AGT GA-3'.

Statistical analysis

The normality of the data was tested using the Kolmogorov-Smirnov test with Lilliefors' correction. The Levene median test was used to evaluate the homogeneity of variances. The effects of lung mechanics and morphometry were assessed using one-way analysis of variance followed by the Tukey post-hoc test. All data are expressed as the mean $\pm$ standard error of the mean. SigmaStat 3.1 statistical software package (Jandel Corporation, San Raphael, CA) was used and significance was established at $P < 0.05$.

Results

Lung cell transduction

In order to enhance the potential of AAV vectors for lung gene therapy, we analyzed the transduction of WT-AAV8 and M-AAV8 into mice lung 4 weeks after infection. Initially, we evaluated transgene expression by immunohistochemistry to determine the cellular pattern of transduction and to visualize cells with eGFP. WT-AAV8 showed sparse eGFP expression in the tissue (Fig. 2a-c), whereas the M-AAV8 showed high-intensity eGFP fluorescence throughout the tissue cells compared with WT-AAV8 (Fig. 2d-f). Clearly many more cells were transduced with the M-AAV8 vector.

Analysis of eGFP transcription

To establish the transduction efficacy of AAV8 vectors, eGFP transcription in the lung was quantified by real-time PCR. The analysis showed that the eGFP level was significantly higher (2.069 ± 0.76 , $n=6$, $p < 0.05$) in the M-AAV8 group compared with the WT-AAV8 group (Fig. 2g).

Lung histology

To determine whether the administration of AAV8 vectors to the airways induced histologic changes in the lung, we analyzed lung morphometry and tissue cellularity. The fractional area of collapsed alveoli, as well as the number of polymorpho- and mononuclear cells did not differ between the groups (Fig. 3A-C), suggesting that the administration of wild-type and capsid-mutant AAV8 vectors was safe and well tolerated.

Fig. 2. (a–f) Representative photomicrographs of immunohistochemistry for eGFP. Analysis of eGFP expression 4 weeks after intratracheal instillation of AAV vector at an equal dose of 10^{10} per mouse of wild-type (WT-AAV8) (a–c) or its 733 tyrosine-mutant (M-AAV8) (d–f) and saline (CTRL, 50 μ l of saline) (g–i). Immunohistochemistry for eGFP (a, d and g); nucleus of tissue cells stained with TO-PRO-3 (b, e and h); double staining eGFP and TO-PRO-3 (c, f and i). All pictures of eGFP immunostaining were taken using the same exposure time in order to compare eGFP intensity. Calibration bar=50 μ M. (j) Real-time PCR analysis of eGFP transcription. Animals received intratracheal instillation of saline (CTRL, 50 μ l of saline), wild-type AAV8 (WT-AAV8, 10^{10} vg/50 μ l of saline), or tyrosine-mutant AAV8 (M-AAV8, 10^{10} vg/50 μ l of saline). The y axis represents the fold increase compared with WT-AAV8. Values are means $\pm$ SEM of 6 animals per group. *Significantly different from the WT-AAV8 group ($P<0.05$).

saline), or tyrosine-mutant AAV8 (M-AAV8, 10^{10} vg/50 μ l of saline). The y axis represents the fold increase compared with WT-AAV8. Values are means $\pm$ SEM of 6 animals per group. *Significantly different from the WT-AAV8 group ($P<0.05$).

Cytokine and growth factor mRNA levels in lung tissue

To identify if a single dose of AAV8 vectors to the airways elicited inflammatory processes, some immune response mediators were analyzed. Our findings demonstrated that the instillation of AAV8 vectors did not result in changes in pro-inflammatory cytokines (IL-1 β and IL-6), IL-13 (a Th2 response marker), IFN- γ (a Th1 response marker), and TGF- β (a profibrotic agent) mRNA levels in the CTRL, WT-AAV8, and M-AAV8 groups (Fig. 4).

Lung mechanics

Lung mechanics were examined to assess whether pulmonary function was altered by administration of AAV8 vectors. Our results demonstrate that static lung elastance, a resistive parameter, was similar in the three groups (Fig. 5).

Discussion

In order to improve AAV vector lung transduction efficiency, different capsid serotype or capsid mutants with greater affinity for airway epithelial cells were used [15]. Although different viral capsid proteins and their receptors could contribute to differential infectivity

Fig. 3. (A) Representative photomicrographs of lung stained with hematoxylin-eosin on day 28. (B) Lung morphometry. (C) Lung tissue cellularity. Animals received intratracheal instillation of saline (CTRL, 50 µl of saline), wild-type AAV8 (WT-AAV8, 10^{10} vg/50 µl of saline) or tyrosine-mutant AAV8 (M-AAV8, 10^{10} vg/50 µl of saline). Values are means $\pm$ SEM of 8 animals/group.

Fig. 4. Real-time PCR analysis of cytokine and growth factor mRNA expression. (A) IL-1 β , IL-6, IL-13, IFN- γ , and TGF- β . Data are normalized to housekeeping gene acidic ribosomal phosphoprotein P0 expression (36B4). Animals received intratracheal instillation of saline (CTRL, 50 µl of saline), wild-type AAV8 (WT-AAV8, 10^{10} vg/50 µl of saline) or tyrosine-mutant AAV8 (M-AAV8, 10^{10} vg/50 µl of saline). The y axis represents the fold increase compared with CTRL. Values are means $\pm$ SEM of 6 animals per group.

of the viral vectors, distinct intracellular fate also seems to be a decisive element for determining the efficiency of infection [26].

The tyrosine to phenylalanine mutation was proposed to overcome one of the rate-limiting steps in AAV infection, that is, intracellular trafficking. The goal of this strategy is to

Fig. 5. Static lung elastance on day 28. Animals received intratracheal instillation of saline (CTRL, 50 μ l of saline), wild-type AAV8 (WT-AAV8, 10^{10} vg/50 μ l of saline), or tyrosine-mutant AAV8 (M-AAV8, 10^{10} vg/50 μ l of saline). Values are means $\pm$ SEM of 8 animals per group (10 determinations per animal).

reduce ubiquitination sites on the viral particles to minimize proteasome-mediated degradation, thus more AAV can survive and emerge from the trafficking pathway and enter the nucleus for transgene expression [27]. Because the Y-to-F mutant of the AAV8 serotype has been reported to increase gene expression in the eye and brain [21, 28], in the present study we investigated the impact of administration of wild-type and capsid-mutant AAV8 vectors on the quality of lung cell transduction and lung function in mice.

This is the first study that reveals the efficiency of tyrosine-mutant and wild-type AAV8 vectors in mice lung. The data presented in this article show that the administration of the M-AAV8 vector significantly increased the transduction efficiency compared with WT-AAV8 (approximately two times greater). The expression of eGFP in the lungs was analyzed by real-time PCR and immunohistochemistry (Fig. 2). Our data corroborate the study by Petrs-Silva et al. [21] who demonstrated increased transduction of the ganglion cell layer by the AAV serotype 8 mutant vectors. The tyrosine-mutant AAV8 showed improved transduction, with more cells transduced and eGFP intensity 10-fold higher, analyzed by immunofluorescence, than its wild-type counterpart [21].

AAV vectors are thought to exhibit less inflammatory and immune reactions than other viral vectors, such as adenovirus. However, it has been reported that AAV2 treatment stimulated an inflammatory response in peripheral blood mononuclear cell cultures from human donors and elicited IFN- γ and IL-13 responses [29].

It is well known that IL-6 and IL-1 β are increased in general inflammatory states and the increase in the level of mRNA of these cytokines in lung tissue is correlated with the development of pulmonary inflammation [30]. Our study demonstrated that intratracheal administration of AAV8 vectors did not elicit an increase in the mRNA levels of important inflammatory cytokines (IL-1 β , IL-6, IL-13, and IFN- γ) in lung in the experimental groups (Fig. 4).

Taking into consideration that inflammatory processes can cause lung injury and consequently lung remodeling, we quantified the critical chemical mediator of lung remodeling, TGF- β [31]. However, our results do not show any change in TGF- β mRNA levels in all experimental groups, suggesting that the AAV8 does not cause lung damage (Fig. 4). Corroborating these findings, the number of polymorpho- and mononuclear cells in the lung parenchyma was similar in our experimental groups (Fig. 2c).

Moreover, immunological reactions can lead to changes in lung function [31]. In the present study, pulmonary mechanics were examined and static lung elastance was similar in all experimental groups (Fig. 5). In addition, the results of the pulmonary histology were in agreement with the pulmonary mechanics, showing no alterations in lung parenchyma (Fig. 3A).

In conclusion, the present study analyzed the transduction capacity of AAV8 vectors and its impact on lung function. The M-AAV8 vectors improved transduction of the transgene (eGFP) in the lung, observed both by real-time RT-PCR and immunofluorescence, showing that this vector is more efficient at gene transfer than its wild-type counterpart. In addition, the administration of both AAV8 vectors (WT-AAV8 and M-AAV8) did not cause inflammation and morphometric changes in the lungs, thus keeping lung mechanics unaltered.

Our results show that the tyrosine-mutant AAV8 is safe and has improved transgene delivery capacity. These findings motivate the further development of viral-based gene ther-

apies for safe and effective delivery of therapeutic genes for the treatment of respiratory diseases such as cystic fibrosis, asthma, α 1-antitrypsin deficiency, and acute respiratory distress syndrome.

Disclosure Statement

The authors have no competing interests to declare.

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