Journal of Pediatric Surgery 51 (2016) 879–884

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

AAP Articles

Vector serotype screening for use in ovine perinatal lung gene therapy

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ARTICLE INFO

Article history:
Received 9 February 2016
Accepted 26 February 2016

Key words:
AAV
Screening
Gene therapy
Sheep
Lung

ABSTRACT

Purpose: Successful in utero or perinatal gene therapy for congenital lung diseases, such as cystic fibrosis and surfactant protein deficiency, requires identifying clinically relevant viral vectors that efficiently transduce airway epithelial cells. The purpose of the current preclinical large animal study was to evaluate lung epithelium transduction of adeno-associated viral (AAV) vector serotypes following intratracheal delivery.
Methods: Six different AAV vector serotypes (AAV1, AAV5, AAV6, AAV8, AAV9, and AAVrh10) expressing the green fluorescent protein (GFP) as the transgene were injected into the right upper lobe of perinatal sheep via bronchoscopy. At 1 week, samples were harvested, analyzed by fluorescent stereomicroscopy and immunohistochemistry, and quantified using a radial grid and quantitative real-time polymerase chain reaction (qPCR).
Results: Fluorescent stereomicroscopy demonstrated GFP expression in the right upper lobe following injection of all AAV serotypes assessed except AAV5. Immunohistochemistry analysis confirmed GFP expression in small- and medium-sized airways following intratracheal injection of AAV1, 6, 8, 9, and rh10. However, only AAV8 and AAVrh10 resulted in transgene expression in large airways. These results were confirmed by qPCR, yet, after 40 cycles, AAV1 did not show GFP gene amplification.
Conclusion: Adeno-associated viral vector serotypes 6, 8, 9, and rh10 demonstrated efficient GFP transgene expression at early time points, and AAV8 demonstrated efficient transduction of all airway sizes with high pulmonary GFP expression tested using qPCR.

Gene therapy holds considerable promise for the treatment of a number of congenital genetic disorders including those involving the lung epithelium, such as cystic fibrosis, surfactant protein B deficiency, and α1-antitrypsin deficiency. A number of these disorders, including surfactant protein B deficiency, result in significant morbidity and mortality in the newborn period [1]. Thus successful gene therapy prior to or shortly after birth has the ability to significantly reduce or eliminate the detrimental impact of these genetic conditions. Intrinsic to successful gene therapy is the ability to deliver the corrective gene to the target organ and obtain efficient transduction of the target cells. Gene therapy in the newborn period takes advantage of the small size of the recipient to maximize the dose of the corrective gene delivered per weight of the recipient. Although this increases the efficiency of transduction, the viral vector system in which the corrective gene is delivered is often the most important factor in the ability to successfully transduce cells of the target organ.

Adeno-associated viral (AAV) vectors are characterized by their safety, low toxicity, and ability to confer stable expression [2–4]. Also, because AAV can transduce nondividing cells [5], using AAV to transduce the lung epithelium is justified because <1% of airway epithelial cells are actively dividing [6]. Many AAV serotypes have been isolated and evaluated in various model systems [7–9]; however, no studies have looked at the large animal ovine neonatal model. In this study, we evaluated the ability of 5 AAV serotypes carrying the green fluorescent protein (GFP) transgene to transduce the respiratory epithelium following direct intratracheal injection in the preclinical neonatal ovine model.

1. Materials and methods

Experiment protocols were reviewed and approved by the Institutional Animal Care and Use Committee at The Children's Hospital of Philadelphia and followed the guidelines of the National Institute of Health's *Guide for the Care of Laboratory Animals*.

1.1. Adeno-associated viral vectors

All AAV serotypes were generated as previously described [10,11]. Briefly, recombinant AAV flanked with AAV2 inverted terminal repeats

Abbreviations: LGT, Lung gene therapy; AAV, Adeno-associated virus; C_T, Cycle threshold; DW, Deionized water; GFP, Green fluorescent protein; qPCR, Quantitative polymerase chain reaction; RUL, Right upper lobe.

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http://dx.doi.org/10.1016/j.jpedsurg.2016.02.048
0022-3468/

contained enhanced GFP under transcriptional control of the cytomegalovirus promoter. Recombinant AAV genomes equipped with AAV2 inverted terminal repeats were packaged by triple transfection of Human Embryonic Kidney 293 cells with cis-plasmid and adenovirus helper plasmid and a chimeric packaging construct in which the AAV2 rep gene is fused with cap genes of novel AAV. Vectors screened for ovine lung transduction included the following: AAV1 (AAV1.CB7.CI.eGFP.WPRE.rBG 2.04e13 GC/mL), AAV5 (AAV5.CB7.CI.eGFP.WPRE.rBG 4.21e13 GC/mL), AAV6 (AAV6.CMV.PI.eGFP.WPRE.bGH 6.55e12 GC/mL), AAV8 (AAV8.CB7.CI.eGFP.WPRE.rBG 1.78e13 GC/mL), AAV9 (AAV9.CB7.CI.eGFP.WPRE.rBG 1.48e13 GC/mL), and AAVrh10 (AAVrh10.CB7.CI.eGFP.WPRE.rBG 1.53e13 GC/mL). Small aliquots (0.1 mL) of each vector were diluted 1:1 with sterile phosphate-buffered saline to a final volume of 200 μL and kept on ice.

1.2. Animal model and delivery technique

Time-dated pregnant ewes were acquired and newborn lambs delivered in our facility. Intratracheal injection of six viral vectors was performed in six healthy lambs, one vector per lamb, at 1 week. Specifically, lambs were endotracheally intubated and maintained on isoflurane anesthesia. A 3.5-mm OD Storz (El Segundo, CA) bronchoscope with a working sideport was advanced through the endotracheal tube, and the right upper lobe (RUL) bronchus was cannulated. A 2.5-mm OD single lumen catheter was then introduced into the RUL bronchus (approximately 1 cm distal to the tip of the bronchoscope) under direct vision. Respirations were held, and 200 μL of the diluted vector was administered through the catheter into the RUL bronchus. The catheter was flushed with air to ensure delivery of the entire volume of vector, and the site of injection was monitored for 30 s postinjection to ensure no efflux of injectate. The lambs were euthanized 1 week later (2 weeks old), and bilateral lungs, heart, and liver were harvested for further analysis.

1.3. Stereomicroscopy

Fresh specimens were examined for GFP expression using fluorescent stereomicroscopy (MZ16FA; Leica, Heerbrugg, Switzerland) with a GFP bandpass filter (excitation 546/10 nm; emission 590 nm) and a triple (rhodamine, Alexa Fluor 546, and Texas Red) red-channel filter set for detection of autofluorescence. Whole-organ fluorescent images are composites of the GFP and red channels. The lungs were then pressure-fixed via the trachea to a distending pressure of 20 cmH_2O with 10% formalin. If the specimen showed no GFP expression, as in AAV5, the transduction was not deemed adequate for further investigations.

1.4. Immunohistochemistry

Paraffin sections (4 μm) of RUL lung specimens from each animal were deparaffinized in serial xylene washes followed by rehydration through a graded series of alcohol to deionized water (DW). The slides were placed in Uni-Trieve solution (Innovex Biosciences, Inc., Richmond, CA) for heat-induced epitope retrieval, at 65° for 30 minutes, followed by rinsing in DW. For blocking endogenous peroxidase activity, the slides were incubated in 3% H_2O_2 for 15 minutes at room temperature and rinsed in DW. They were then transferred to 0.1-mol/L Tris-buffered saline with Tween (TBST) prior to application of the primary antibody (Ab). We used rabbit anti-GFP (Invitrogen, Carlsbad, CA) at a 1:400 dilution as our primary antibody. Positive control slides were stained with rabbit anti-surfactant protein B (an endogenous protein expressed by pulmonary epithelial cells; Seven Hills Bioreagents, Cincinnati, OH) at a 1:400 dilution. After incubating the primary antibody overnight at 4 °C, the slides were rinsed in TBST for 10 minutes at room temperature and incubated for 20 minutes with SuperPicture Polymer horseradish peroxidase (Invitrogen cat. no. 87-8963). After rinsing in TBST at room temperature for 10 minutes, the slides were developed using DAB chromogen (Vector Lab cat. no. SK-4100, Burlingame, CA 94010). Finally, tissues were then lightly counterstained using hematoxylin (Vector Lab cat. no. H-3404). Negative controls underwent processing and incubation with horseradish peroxidase without primary anti-GFP antibody staining.

1.5. Quantification of the number of transduced lung epithelial cells

Airway epithelial GFP expression of immunoperoxidase-stained lung sections was evaluated via light microscopy (Leica DMRD, Leica Microsystems, IL) at a final original magnification between ×10 and ×40. A radial grid [12] was used to calculate the percentage of GFP-positive epithelium as previously described [9]. The internal diameter of the airway was calculated using a standardized microscopy legend and digital measuring tools within the IPLab scientific image processing software, and airways were divided into small (80–100 μm), medium (200–400 μm), and large (500–1000 μm) airways. Airways were excluded from analysis if measurements of shortest and longest diameter differed by more than 25%; thus, only airways that were approximately round in shape were included. At least 3 airways were counted from each size category per specimen, and a mean number of GFP-positive epithelial cells was calculated.

1.6. Real-time quantitative polymerase chain reaction

In addition to quantitative stereology, the efficiency of transduction was assessed by quantitative polymerase chain reaction (qPCR) for the GFP transgene. Each specimen was sectioned to obtain an 80 μm × 2 cm × 2 cm formalin-fixed, paraffin-embedded sample. The section was taken from an area shown to have GFP expression on immunohistochemistry from a sample immediately prior to the section. DNA was then extracted using the RecoverAll Nucleotide Extraction Kit for formalin-fixed, paraffin-embedded sections (Life Technologies, Thermo Fisher Scientific, Grand Island, NY). Reactions contained 100 ng template DNA in a 20-μL reaction mix containing 10 μL SYBR Green Master Mix (Life Technologies), 0.5 μL of forward/reverse primer, and the remaining volume of sterile molecular grade water. Cycling conditions were as follows: 50 °C for 20 s, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 60 s on an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies). Primers were synthesized by Integrated DNA Technologies (San Jose, CA). The GFP sequences were amplified with forward, 5′-CGA CCA CTA CCA GCA GAA CAC-3′, and reverse, 5′-ACC ATG TGA TCG CGC TTC TC-3′, primers. For each sample, the reference control gene for ovine β-actin was amplified in a separate well with forward primer 5′-ACT GGC ATC GTG ATG GAC TC-3′ and reverse primer 5′-GCT CCG TGA GGA TCT TCA TGA G-3′. The quantity of GFP was normalized to β-actin for each sample and compared to a positive control containing all of the serotypes to determine the relative GFP quantity (RQ) (comparative cycle threshold [C_T] method). Reactions were performed in triplicate.

2. Results

2.1. GFP expression after neonatal ovine lung gene transfer

Green fluorescent protein expression was evaluated by fluorescent stereomicroscopy on fresh whole-organ specimens from animals who received intratracheal injections of AAV serotypes carrying the GFP transgene (Fig. 1). GFP expression was noted in clusters of cells throughout the RUL of recipients of AAV serotypes 1, 6, 8, 9, and rh10. AAV5 showed no visible evidence of GFP expression on stereomicroscopy. Heart and liver specimens were also taken from each animal and screened for GFP expression. None of the vector serotypes showed GFP expression in heart or liver specimens, and GFP expression was not seen beyond the RUL of the injected lung.