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Vector Serotype Screening for Use in Ovine Perinatal Lung Gene Therapy

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Abstract

Purpose—Successful in utero or perinatal gene therapy for congenital lung diseases, like 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 vector (AAV) serotypes following intratracheal delivery.

Methods—Six different AAV serotypes (AAV1, AAV5, AAV6, AAV8, AAV9, AAVrh10) expressing the green fluorescent protein (GFP) as the transgene were injected into the right upper lobe (RUL) of perinatal sheep via bronchoscopy. At one-week samples were harvested, analyzed by fluorescent stereomicroscopy and immunohistochemistry, and quantified using a radial grid and quantitative real time PCR.

Results—Fluorescent stereomicroscopy demonstrated GFP expression in the RUL 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—AAV 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.

Keywords

AAV; screening; gene therapy; sheep; lung

INTRODUCTION

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 α 1-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] [3] [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] [8] [9] however no studies have looked at the large animal ovine neonatal model. In this study, we evaluated the ability of five AAV serotypes carrying the green fluorescent protein (GFP) transgene to transduce the respiratory epithelium following direct intratracheal injection in the preclinical neonatal ovine model.

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*.

Adeno-associated Viral Vectors

All AAV serotypes were generated as previously described [10] [11]. Briefly, recombinant AAV flanked with AAV2 inverted terminal repeats (ITRs) contained enhanced green fluorescent protein (GFP) under transcriptional control of the cytomegalovirus (CMV) promoter. Recombinant AAV genomes equipped with AAV2 ITRs were packaged by triple transfection of HEK 293 cells with cis-plasmid, 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: 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 (0.1mL) of each vector were diluted 1:1 with sterile phosphate buffered saline (PBS) to a final volume of 200 μ L and kept on ice.

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 one week of age. Specifically, lambs were endotracheally intubated and maintained on Isoflurane anesthesia. A 3.5mm 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 2.5mm 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 μ L 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 seconds post injection 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.

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 (Rhodamin, Alexa 546, 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 20cmH₂O with 10% formalin. If the specimen showed no GFP expression, as in AAV5, the transduction was not deemed adequate for further investigations.

Immunohistochemistry

Paraffin sections (4 μ m) 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-Bioscience) 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% H₂O₂ for 15 minutes at room temperature (RT), and rinsed in DW. They were then transferred to 0.1M Tris buffered saline with tween (TBST) prior to application of the primary antibody (Ab). We used rabbit anti-gfp (Invitrogen) 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 Hill Bioreagents) 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 Super-Picture Polymer HRP (horseradish peroxidase) (Invitrogen cat# 87-8963). After rinsing in TBST at RT for 10 minutes the slides were developed using the Chromogen DAB (Vector Lab cat SK-4100 Burlingame, CA 94010). Finally, tissues were then lightly counter stained using hematoxylin (Vector Lab cat# H-3404). Negative controls underwent processing and incubation with HRP without primary anti-gfp antibody staining.

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) at a final 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 (80–100µm), medium (200–400µm) and large (500–1000µm) airways. Airways were excluded from analysis if measurements of shortest and longest diameter differed by >25%; thus, only airways that were approximately round in shape were included. At least three airways were counted from each size category per specimen and a mean number of GFP-positive epithelial cells was calculated.

Real-time Quantitative Polymerase Chain Reaction

In addition to quantitative stereology, the efficiency of transduction was assessed by qPCR for the GFP transgene. Each specimen was sectioned to obtain an 80µm × 2cm × 2cm 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 FFPE sections (Life technologies, ThermoFisher Scientific, Grand Island, NY, USA). 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: 50° C for 20 sec, 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 C_T method). Reactions were performed in triplicate.

RESULTS

GFP expression after neonatal ovine lung gene transfer

GFP expression was evaluated by fluorescent stereomicroscopy on fresh whole organ specimens from animals who received intratracheal injections of AAV serotypes carrying the GFP transgene (Figure 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.

Immunohistochemical analysis and quantification of pulmonary GFP transduction

We sought to use immunohistochemistry to confirm pulmonary cell transduction visualized on stereomicroscopy and further evaluate the efficiency of transduction as well as the cell type transduced following neonatal intratracheal delivery of different AAV serotypes. All AAV serotypes, which demonstrated GFP expression on stereomicroscopy, were GFP positive on immunohistochemical analysis (Figure 2). The right upper lobe specimens demonstrated highly specific transduction of the respiratory epithelium without significant GFP expression elsewhere in the lung tissue. Additionally airways were categorized by size and subsequently analyzed. The small (80–100 μm) and medium (200–400 μm) airways representing terminal and non-cartilaginous bronchioles respectively demonstrated GFP expression following intratracheal injection of AAV serotypes 1, 6, 8, 9, and rh10 (Table 1, Figure 2). In contrast to the small and medium sized airways, transduction of the large airways (500–1000 μm) representing segmental bronchi was inefficient. Only AAV8 and AAVrh10 demonstrated definitive transduction of the large airways on immunohistochemical analysis.

Real-time quantitative PCR was performed to confirm the presence of and quantify the GFP transgene in the histologic specimens. We used the cycle threshold (C_T) to determine the amount of transgene (GFP) DNA expressed in each specimen. As more efficiently transduced epithelium expresses higher levels of transgene product, the cycle threshold allows us to quantify cell transduction with each adeno-associated viral vector serotype. We compared the C_T values of beta actin, a gene found in all ovine lung tissue, to the C_T values of GFP in our samples (Table 2). Relative quantity (RQ) was established to a positive reference control. AAV9 and AAV8 showed the best relative quantity of GFP DNA compared to the control. While AAV6 and AAV rh10 demonstrated less GFP expression, and therefore poorer transduction.

DISCUSSION

In our study we examined the transduction efficacies of AAV vector serotypes in lung epithelium following intratracheal injection in neonatal sheep. This is the first study to analyze the efficiency of epithelial cell transduction in the neonatal ovine model using various AAV serotypes. We demonstrated that five of the six serotypes examined (AAV1, AAV6, AAV8, AAV9, and AAVrh10) effectively transduced lung epithelial cells at one-week post injection. Transduction was limited to the area of injection (the RUL) and was not present in the heart, liver, or additional ipsilateral or contralateral lung parenchyma suggesting a localized effect of our delivery technique and viral serotypes. AAV5 showed no GFP expression when examined using stereomicroscopy and was not sectioned for histology or further quantified. Furthermore, we demonstrated that GFP expression varied depending on the size of the intrapulmonary airway examined. All five serotypes that effectively transduced lung epithelial cells did so in the small and medium sized airways. Conversely, expression was low in large airways in which only AAV8 and AAVrh10 resulted in effective transduction. The percentage of GFP expressing cells in the lung epithelium (for all airway sizes combined) ranged from 22% to 69%. Quantitative PCR confirmed variable levels of transduction of pulmonary epithelial cells with AAV serotypes containing the GFP

transgene. Ultimately, AAV8 transduced epithelial cells of all airways (small, medium and large) and demonstrated high levels of GFP DNA amplicons suggesting that it is the most efficient AAV serotype from our study group. Interestingly, we were unable to identify GFP by qPCR following injection of the AAV1 serotype despite appreciating transduction by stereomicroscopy and immunohistochemistry. This may indicate that AAV1 was the least successful at lung epithelial transduction and therefore had very low levels of GFP DNA present. Different AAV serotypes are known to have different cell tropism related to variations in serotype capsid protein structure. These variations likely resulted in the different transduction efficiencies of pulmonary epithelial cells with the different AAV serotypes seen in the current study.

Previous studies indicate that transduction of 5–10% of airway epithelia may be sufficient to achieve therapeutic correction of the defective chloride channel function in CF [13]. We were able to achieve levels of transduction in the RUL equivalent if not greater than those believed to be therapeutic. Our study, however, is limited in this analysis because we isolated a single lobe of the lung after delivering a high concentration of viral vector to that localized area. Although we did see uniform delivery throughout the right upper lobe, high viral titers over a small surface area may effectively overload the mechanical and immunologic barriers of the airway epithelium resulting in elevated levels of transduction. Additionally, our studies provide only short-term analysis of transduction following intratracheal delivery. Previous studies in humans and large animal models suggest that repeated AAV administration will be required to maintain long-term high, therapeutically relevant levels of pulmonary transgene expression [14–16]. Future studies in our model investigating the entire lung epithelium as well as the long-term transduction efficiency are required to evaluate the clinical relevance of our findings.

In the current study a high vector titer in a small lung volume was used to overcome some barriers to transduction. Future studies targeting the entire lung in sheep may require enhancing the transduction potential of AAV vectors. Earlier studies in rodent models have attempted to bypass the barriers to lung epithelial cell transduction by using agents to open cell tight junctions and circumvent natural resistance mechanisms to viruses. In vitro studies using Na-caprate, which reversibly opens the epithelial tight-junctions for a short period of time, resulted in widespread staining of the trachea and main bronchi and increased virus-mediated transgene expression in the lungs by more than 90-fold. The combination of virus/DEAE dextran and Na-caprate resulted in significantly enhanced transgene expression, and gene transfer to the trachea and main bronchi as well as the large, small and medium airways was demonstrated [17]. Also, reagents that help AAV vectors bypass the natural resistance of the apical surface to infection can augment AAV transduction 10- to 100-fold [18] [19]. Similarly dosage, proliferation rates, and target cell access are factors involved in efficient transduction by AAV vectors in lung epithelia [20].

One advantage of AAV vectors over LV vectors is the presence of viral capsid receptors on the luminal surface of the epithelial cell. That being said, each AAV serotype exhibits a specific binding domain for the cell receptor resulting in variable cell tropism. Pseudotyping the capsid protein of AAV5, AAV6, and AAV9 to AAV2 have also been employed to

improve airway epithelial transduction in murine models of gene therapy [21] and may be useful in future studies in the ovine model.

Previous studies in rodent models have evaluated AAV transduction of the lung epithelium. These studies demonstrated long-term persistence of gene expression after transduction with AAV serotypes 1–9 and many recombinant and novel vectors [22] [20] [21]. Although results from these murine studies are valuable, the large animal ovine model has many benefits compared to the murine model for studying lung gene transfer. [23]. The sheep lung has a number of characteristics that support its use as a model for studies of human pulmonary physiology and disease in preference to rodents [24]. These include greater anatomical similarity with human lungs and closer similarity in the distribution of specialized airway epithelial cells [25]. In addition, the similar size of sheep and human lungs facilitates analysis of pulmonary function, *in vivo* imaging procedures, and better replicates the ratio of the dose of viral vector to size of target organ that will be seen clinically. An additional benefit of the sheep model is the capacity to direct regional instillation of study drugs or vectors and tissue sampling using bronchoscopy [26].

Although we have demonstrated good short-term transduction with multiple AAV serotypes additional barriers to the clinical use of gene therapy for pulmonary diseases exist. Specifically, most pulmonary diseases, like CF, will require repeat doses of corrective gene therapy to maintain appropriate levels of the therapeutic gene. This is particularly true for safer, nonintegrating viral vector systems such as AAV. A known barrier to repeat viral vector administration is an immune response to the viral vector and transgene through neutralizing antibodies [27]. In the future we hope to use the results of the current study in a combined approach of in utero gene therapy to induce immune tolerance and postnatal intratracheal viral vector administration to maintain therapeutic levels of transgene expression.

ABBREVIATIONS

LGT	Lung gene therapy
AAV	Adeno-associated viral
GFP	Green fluorescent protein
RT	real-time
qPCR	quantitative polymerase chain reaction
RUL	right upper lobe

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Figure 1.

Whole organ stereomicroscopy using GFP band pass filter and red anti-auto fluorescence filter. Clusters of cells expressing GFP are bright green (AAV1, 6, 8, 9, rh10) and are identified throughout the RUL. Bright clusters are absent in the RUL of the animal injected with AAV5.

Figure 2.

Light microscopy with immunoperoxidase staining. Sections were stained with rabbit anti-GFP IgG (primary antibody) then Super-picture polymer HRP followed by Chromogen DAB. Slides were counterstained with hematoxylin. Using this method cells expressing GFP appear brown while all other epithelial cells stain blue. For the positive control we stained for surfactant B protein which is expressed in all lung epithelial cells using an identical

method as discussed above. Negative controls were created by staining the samples without a primary antibody.

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Table 1

A radial grid was used to quantify the percentage of airway epithelial cells demonstrating GFP expression after injecting AAV serotypes. This was performed in triplicate for each airway size and values are represented as the mean $\pm$ one standard deviation. Of note, the large airways were too large to quantify on the radial grid system and thus a “+” or “-” is used to indicate if GFP staining was present or not.

Serotype	% epithelium small (80–100 μ m)	% epithelium medium (200–400 μ m)	expression large (500–1000 μ m)
AAV1	40.3 $\pm$ 9.62	33.3 $\pm$ 18.16	–
AAV6	22.2 $\pm$ 6.37	27.8 $\pm$ 23.69	–
AAV8	33.3 $\pm$ 4.17	47.2 $\pm$ 6.36	+
AAV9	34.7 $\pm$ 6.37	69.4 $\pm$ 24.41	–
AAVrh10	43.0 $\pm$ 12.73	40.3 $\pm$ 13.39	+

Table 2

RT qPCR was conducted to further quantify transduction after AAV serotype injection. C_T (difference in threshold cycles between GFP and β -actin reference control) was calculated for each AAV serotype tested and compared to a positive reference control containing all samples tested (C_T). If many genome copies exist it takes fewer cycles to reach the threshold, therefore, smaller C_T values indicate more genome copies of the transgene (GFP). When the C_T value is negative this indicates that the sample tested has more genome copies of the transgene (GFP) relative to the endogenous control. When the C_T value is positive this indicates that the sample contains less genome copies of the transgene (GFP) relative to the endogenous control. The C_T further compares each serotype sample to a standardized positive control. Serotype samples with lower C_T are comparatively closer to the positive control and therefore have more genome copies of the transgene (GFP). Relative quantity (RQ) was then calculated using 2^{-C_T} . RQ is a commonly reported value for qPCR and allows the reader to compare our data with other papers using qPCR.

	Avg GFP C_T	Avg β -actin C_T	C_T (avg GFP C_T - β -actin C_T)	C_T (Serotype C_T -Control C_T)	RQ(2^{-C_T})
AAV1	Undetermined	33.27	Undetermined	Undetermined	Undetermined
AAV6	37.17	34.10	3.07	14.26	.0000509
AAV8	21.92	26.97	-5.05	6.14	0.0142
AAV9	20.23	27.97	-7.75	3.44	0.09214
AAVrh10	35.16	28.81	6.35	17.54	.00000525
Control	22.93	34.12	-11.19	0	1