Progress with Recombinant Adeno-Associated Virus Vectors for Gene Therapy of Alpha-1 Antitrypsin Deficiency

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Abstract

The pathway to a clinical gene therapy product often involves many changes of course and strategy before obtaining successful results. Here we outline the methodologies, both clinical and preclinical, that went into developing a gene therapy approach to the treatment of alpha-1 antitrypsin deficiency lung disease using muscle-targeted recombinant adeno-associated virus. From initial gene construct development in mouse models through multiple rounds of safety and biodistribution studies in rodents, rabbits, and nonhuman primates to ultimate human trials, this review seeks to provide insight into what clinical translation entails and could thereby inform the process for future investigators.

Introduction

HERE WE WILL DISCUSS the methodology that went into designing the preclinical and clinical investigations for the adeno-associated virus (AAV) clinical gene therapy vector for alpha-1 antitrypsin (AAT) deficiency lung disease, how the therapy has evolved as a result of this work, and the potential course for future research and clinical trials. This overview is intended to provide insight to investigators who are working to develop a translation therapeutic plan for their disease of interest.

AAT, also termed *SERPINA1* or M-AAT, is a serine protease inhibitor largely produced in the liver. AAT is secreted from hepatocytes and carries out the majority of its function to inhibit neutrophil elastase and other neutrophil-derived proteases and defensins within the lung. Mutations in AAT that decrease serum levels lead to an increased risk of emphysema, with shortened life expectancy, largely the result of unopposed neutrophil elastase in the lung parenchyma. Life expectancy is further decreased in smokers and ex-smokers.¹ Mutations in AAT can also result in sporadic liver disease as the result of accumulations of mutant AAT polymers, specifically the PiZ mutant protein (glu342lys, E342K), within the hepatic endoplasmic reticulum.^{2–5} In the case of the PiZ protein, the mutation causes a portion of the protein to form a loop structure that inserts itself into the active sight of other PiZ protein molecules. It is this action that creates the protein aggregates in the hepatic endoplasmic reticulum and dramatically decreases serum AAT levels in these patients (because of inability to secrete the protein).

AAT deficiency is perfectly suited for a gene therapy approach to treatment for several reasons (see Table 1). It is a

monogenetic disorder, meaning that the gene therapy vector will need only to deliver the single AAT gene itself in order to correct the phenotype. Another positive feature as a target for gene therapy is that there is a wide therapeutic range for serum AAT levels, eliminating the need for tight transcriptional control. Previous work has shown that serum levels of AAT of 11 μ M (571 μ g/ml) are necessary to prevent lung disease, and protein replacement studies have established that levels greater than 80 mg/ml have no adverse clinical effect. Protein replacement in AAT-deficient individuals has also established that human AAT can be delivered without immune responses. In terms of gene therapy vector production, AAT has the advantage of a coding sequence small enough to be easily packaged within an AAV. Gene therapy delivery options are widened because AAT is a secreted protein, allowing for production of AAT from sites other than the lung or liver, such as the muscle, while still reaching the intended target, the lung via the blood. Finally, the precisely defined therapeutic threshold of 11 μ M on a simple blood test provides an ideal endpoint for clinical trials and eventual licensure of an rAAV-AAT gene therapy product.

While other gene delivery methods (recombinant adenoviral vectors, cationic liposome vector delivery, retroviral vectors, and naked DNA injections) were initially tried, subsequent gene therapy attempts have focused on correction of the lung phenotype via an AAV vector encoding wild-type human AAT delivered to skeletal muscle.^{6–13} In this review we will focus on the development of gene therapy for AAT-associated lung disease through elevation of human AAT serum levels (hAAT=M-AAT). In the final section we will discuss potential options for treating AAT-associated liver disease in addition to the lung disease.

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TABLE 1. POTENTIAL ADVANTAGES OF ALPHA-1 ANTITRYPSIN DEFICIENCY AS A TARGET FOR GENE THERAPY

Single gene disorder
Short length of coding sequence relative to adeno-associated virus packaging size
Secreted protein allowing for multiple choices for target cells
Wide therapeutic window
Well-established, readily assayed endpoint for clinical trials

Preclinical Development of RAAV2-AAT Vectors

Selection of route and construct design

While hepatic delivery of a therapeutic AAT encoding vector would be the most physiologic source for expression, there has long been a concern that driving increased AAT expression in hepatocytes already burdened with subclinical disease (as might be found in patients with Z-AAT accumulation or latent virus infection) could precipitate hepatic injury or failure.¹⁴ Early work demonstrated that sustained AAT expression and secretion could be obtained from skeletal myofibers alleviating the concern of precipitating liver disease.¹⁵ Several promoters were tested to optimize expression of AAT. Ultimately, a hybrid cytomegalovirus (CMV) enhancer with a chicken beta-actin promoter and a hybrid rabbit beta-globin intron was found to have the most robust AAT expression from liver and was subsequently confirmed to be effective in intramuscular baboon studies.^{16,17}

Mouse and primate studies

Initial work in the lab demonstrated that human AAT could be expressed long-term from C57BL/6 mice following intramuscular injection with an rAAV2-AAT vector with minimal immune response.¹⁵ This allowed for toxicology and biodistribution studies to be performed in C57BL/6 mice using the planned clinical human gene therapy DNA construct.¹⁸ Toxicology/biodistribution was performed following intramuscular and intravenous (to mimic inadvertent intravascular delivery in a patient) delivery. Widespread distribution was less following intramuscular injection, and no vector DNA was detected in the gonads of the mice. Similar results were obtained when vector was delivered to New Zealand white rabbits, including a lack (or very low copy number) of vector genomes present in the gonads.¹⁸

A safety study performed in a baboon model showed no significant adverse events.¹⁷ However, when the baboons were administered vector encoding the human AAT (hAAT) gene, circulating anti-hAAT antibodies were generated. This result is similar to that seen previously in Balb-C mice. No anti-AAT antibodies were generated when the baboons were administered a vector encoding the baboon AAT gene. Mild muscle inflammation around the injection site was seen in both vector and non-vector-injected animals. Serum creatine kinase levels, a marker of myofiber injury, were elevated in only four samples, with three of those samples collected either before vector delivery or in a saline control animal. Germline transmission was also assessed by assaying for vector DNA in gonadal tissue 4 months after vector delivery via quantitative real-time PCR. At that time point, vector DNA copy numbers were at or below the limit of detection

in the gonads examined (≤ 40 –400 vg copies/10,000 cell genomes). This study was one of the final steps in the pre-clinical vector development and safety testing before proceeding to clinical trials.

Phase 1 Clinical Trial: AAV2 (Published 2006)

The first human clinical trial of an AAV encoding hAAT protein was published in 2006.¹⁹ Protein replacement therapy was discontinued 28 days before dosing in patients receiving protein replacement. The dose of recombinant AAV2 encoding hAAT was given at a dose range of 2.1×10^{12} to 6.9×10^{13} vector genomes (vg) per subject delivered intramuscularly into the upper arm (deltoid muscle) of the non-dominant arm. The construct was driven by a chicken beta-actin promoter with a CMV enhancer. A CT scan of the upper arm was performed before injection to confirm normal muscle morphology. Doppler ultrasonography during the injection confirmed that major arteries and veins were avoided. No serious adverse events were recorded in this study, including a lack of muscle toxicity as evidenced by normal physical examination and serum creatine kinase levels (a marker of muscle damage). No muscle biopsies were performed in this clinical trial. Preexisting anti-AAV antibodies were recorded in all patients (and were not an exclusion criteria) before dosing, with levels increasing following dosing with the clinical vector. Anti-AAT antibodies were low in patients before dosing with no increase measured following vector administration. Residual hAAT levels were measured in patients receiving protein replacement therapy, because of an apparently inadequate washout period before vector dosing. These residual levels likely made detection of vector-derived hAAT impossible as one patient that was not on protein replacement therapy before dosing had vector-derived levels within the range of the residual amounts seen in the replacement therapy patients. Based on the prior mouse studies, one might have predicted that a dosage of 1×10^{12} vg/kg would be required to achieve detectable levels in patient serum. Therefore, the fact that serum levels were low or undetectable in this trial was not surprising. At the time, it was anticipated that rAAV2 might have expressed higher levels in humans on a vg/kg basis than it had in mice, but this was not observed with rAAV2-AAT.

Alternatives to AAV2 and Preclinical Toxicology/Biodistribution of AAV1 (Published 2006, 2007)

In parallel with the AAV2 clinical trial discussed above, investigation began looking at whether there were AAV serotypes that would result in more efficient muscle AAT expression. Previous work in other labs had shown an advantage of other rAAV serotypes over AAV2 for Factor IX expression.^{20,21} In 2006 Lu et al. determined that, of rAAV1–5, rAAV1 had the most efficient murine muscle transduction, resulting in hAAT serum levels 100-fold higher than with rAAV2 delivery.²² They also determined that the resultant hAAT was able to interact with neutrophil elastase and had the same level of elastase inhibition as human liver-derived AAT.²²

With the subtherapeutic serum AAT levels in the AAV2 clinical trial discussed above and this data showing a marked advantage of AAV1, the decision was made to move

to AAV1 for subsequent clinical trials. In order to progress to the clinics, a toxicity/biodistribution study was performed in 170 C57Bl/6 mice and 26 New Zealand white rabbits.²³ The vector genome construct was identical to that used in the rAAV2 study. Mouse toxicology (three dose groups) included histology on all major organs as well as isolation of genomic DNA from these organs, as well as blood, to assay for vector genomes following intramuscular injections. The murine biodistribution study (three dose groups) was performed following either intramuscular or intravenous delivery (to mimic an inadvertent intravascular delivery in a patient). Vector genome analysis was performed in all major organs, with particular importance placed on vector genomes in the gonadal DNA. An identical study, with the addition of the GFP group (GFP within an identical backbone) and analysis of serial semen collection to assess the possibility of germline transmission, was performed in rabbits. At higher doses (1.2×10^{13} vg/kg), muscle inflammation at the injection site, anti-AAT antibody responses, and the presence of vector DNA in both the blood as well as peripheral organs (including the gonads) could be seen in mice. At a dose of 3×10^{12} vg/kg, vector DNA was also detected in the semen and gonads of rabbits. In both mouse and rabbits, vector copy number was highest at earlier time points with concentration decreasing over time. It should be noted that the cell type within the semen or gonads in which the vector DNA was present was not determined; therefore, this data does not indicate that germline transmission was possible.

Phase 1 Clinical Trial: AAV1 (Published 2009)

As discussed above, transgene-derived hAAT levels in the first clinical trial were only above background in 1 of the 12 subjects dosed, and a strategy to improve expression was sought that involved switching to an AAV1 capsid.^{19,24} For this phase 1 trial, the same hAAT gene cassette used in the first trial was packaged in the AAV1 capsid and 9 AAT-deficient ($<11 \mu\text{M}$) patients were dosed by intramuscular injection. Vector doses were similar to the first trial at 6.9×10^{12} , 2.2×10^{13} , and 6.0×10^{13} vg per subject. Mild injection-site reactions were seen in 7 of 9 subjects following dosing, and 1 subject developed an *Escherichia coli* epididymitis (deemed unrelated to vector injection). No abnormalities in serum chemistry, including creatine kinase levels, or hematology were noted. Blood samples were collected for vector biodistribution analysis on days 1, 3, 14, and 90. Vector DNA was detected in the blood of 2 of 3 patients in the lower dose cohorts and 3 of 3 patients in highest dose cohort, with maximal vector concentration at day 1. Vector DNA was undetectable in all subjects but one by day 14 or 90. Unlike the rabbits, the patient semen had no detectable vector DNA. Patients in the highest dose cohort maintained subtherapeutic levels for at least 90 days and up to 1 year in the face of an effector T-cell response to AAV capsid. No immune response was detected against hAAT.

Phase 2 Clinical Trial: AAV1 (Interim Results Published 2011, Final Results 2013)

Because the phase 1 trial with AAV1 encoding the hAAT showed evidence of dose-dependent expression, but at subtherapeutic levels, phase 2 employed a dose escalation in

order to increase transgene expression to therapeutic levels ($11 \mu\text{M}$ [$571 \mu\text{g/ml}$]).²⁵ The previous two clinical trials of AAV encoding hAAT used a plasmid transfection method to produce the clinical vector.^{19,24} However, previous work has shown that the use of a recombinant herpes simplex virus (HSV) complementation system for rAAV vector production can result in greater vector yields (and modestly increased infectivity of vector on a per vg basis), allowing for dose escalation.^{26,27} The HSV complementation system for vector production was validated in a mouse toxicology study comparing it to the previously used traditional transfection method and found that the HSV vector system had increased packaging efficiency as well as increased *in vivo* vector expression and a similar safety profile as traditionally produced vector.²⁸

The phase 2 clinical trial consisted of 3 dose cohorts of rAAV1-CB-hAAT at 6×10^{11} , 1.9×10^{12} , or 6×10^{12} vg/kg, with 3 patients per cohort. The total dose in this study ranged from 3.3×10^{14} to 4.3×10^{14} vg per subject. Because of the limits of vector concentration without causing viral aggregation and precipitation, it was necessary to deliver the highest dose cohort's vector in 100 intramuscular injections. Significantly, this trial demonstrated a linear serum hAAT dose-response relationship.²⁵ However, the serum AAT levels were still well below thresholds set for a therapeutic effect. Interestingly, despite the presence of CD8-positive T-lymphocytes being present in the injected muscle at day 90, serum hAAT levels persisted in patients at stable levels for over 1 year without the need for immunosuppressive therapy.^{25,29} Further characterization of the inflammatory infiltrate surrounding the myofibers expressing hAAT determined that a population of regulatory T-cells was present that was activated in response to AAV capsid.²⁹ This regulatory response is likely what allowed the long-term hAAT expression in the face of the inflammatory infiltration.

Future Directions

While the preceding narrative demonstrates that significant advances in expression level can be obtained through alterations in vector design (promoter choice, for example) and serotype selection (rAAV2 $\rightarrow$ rAAV1), there is still a need to increase serum AAT levels to the minimum effective levels used in protein replacement therapy ($11 \mu\text{M}$ [$571 \mu\text{g/ml}$]) in order to obtain clinical relevance. There are many strategies that can be employed either alone or in combination that could increase expression levels of AAT. One way to increase expression is to increase the number of myocytes that are transduced by the vector. One way that has been shown to effectively target the entire limb is to employ a regional limb delivery method, whereby the vector is delivered into the vasculature of a limb that has been isolated from the systemic circulation using a tourniquet.^{30–38} These methods have long been used in human patients to treat limb neoplasia and proof-of-concept gene therapy delivery studies have been performed in patients with muscular dystrophy.^{31,33} There is also evidence that limb infusion delivery has a lower level of immune stimulation compared with intramuscular delivery, which may increase transgene expression.³⁸

Other methods to decrease the immune response could be employed, including systemic immunosuppression, either in response to clinical evidence of inflammation (rise in serum

creatine kinase, indicating myocyte damage) or prophylactically for a short period of time beginning at or before vector delivery to prevent an immune response from being initiated. Another strategy to decrease the immune response might be to select or create a capsid type with lower pre-existing immunity or immune profile in humans.^{39,40,41} Expression could be potentially increased through codon optimization of the AAT gene or through the use of self-complimentary AAV vectors.

Another approach to therapy for AAT deficiency would be to safely target hepatocytes by using RNAi, zinc finger, or CRISPR/Cas9 technologies to stop expression of mutant PiZ in the liver, allowing safe expression of wild-type hAAT from hepatocytes, either providing a “liver-sparing” lung disease gene therapy or potentially a mode to treat both liver and lung disease. Our laboratory has demonstrated that RNAi technology can be used to decrease PiZ expression either alone or in the form of a dual-function vector that allows simultaneous knockdown of mutant AAT and expression of wild-type hAAT.^{42,43} Zinc finger technology delivered via AAVs has been used to target the liver in a hemophilia model.⁴⁴ Proof-of-concept studies have also demonstrated that CRISPR/Cas9 technology can be used to target the liver to treat a genetic disease.⁴⁵ With all of the new technologies available to address AAT gene therapy, the future prospects for licensed gene therapy products for this disease seem very bright.

Acknowledgments

The work described here was funded by the NHLBI (P01HL59412, R01HL69877), the NHLBI-GTRP, the NGVL Toxicology Center (RR16586), the NIDDK (P01DK58327), and the Alpha One Foundation.

Author Disclosure Statement

T.R.F. is a paid consultant for Dimension Therapeutics and for Editas Medicine.

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Received for publication June 2, 2015;
accepted after revision June 11, 2015.

Published online: June 11, 2015.