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Brief Reports

Phase 2 Clinical Trial of a Recombinant Adeno-Associated Viral Vector Expressing α_1-Antitrypsin: Interim Results

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Abstract

Recombinant adeno-associated virus (rAAV) vectors offer promise for the gene therapy of α_1-antitrypsin (AAT) deficiency. In our prior trial, an rAAV vector expressing human AAT (rAAV1-CB-hAAT) provided sustained, vector-derived AAT expression for >1 year. In the current phase 2 clinical trial, this same vector, produced by a herpes simplex virus complementation method, was administered to nine AAT-deficient individuals by intramuscular injection at doses of 6.0×10^{11}, 1.9×10^{12}, and 6.0×10^{12} vector genomes/kg ($n=3$ subjects/dose). Vector-derived expression of normal (M-type) AAT in serum was dose dependent, peaked on day 30, and persisted for at least 90 days. Vector administration was well tolerated, with only mild injection site reactions and no serious adverse events. Serum creatine kinase was transiently elevated on day 30 in five of six subjects in the two higher dose groups and normalized by day 45. As expected, all subjects developed anti-AAV antibodies and interferon-γ enzyme-linked immunospot responses to AAV peptides, and no subjects developed antibodies to AAT. One subject in the mid-dose group developed T cell responses to a single AAT peptide unassociated with any clinical effects. Muscle biopsies obtained on day 90 showed strong immunostaining for AAT and moderate to marked inflammatory cell infiltrates composed primarily of CD3-reactive T lymphocytes that were primarily of the $CD8^+$ subtype. These results support the feasibility and safety of AAV gene therapy for AAT deficiency, and indicate that serum levels of vector-derived normal human AAT >20 μg/ml can be achieved. However, further improvements in the design or delivery of rAAV-AAT vectors will be required to achieve therapeutic target serum AAT concentrations.

Introduction

Individuals with α_1-antitrypsin (AAT) deficiency have mutations in the *SERPINA1* gene that cause reduced secretion of AAT from the liver and consequent impaired antiprotease activity in the lung, resulting in early-onset pulmonary emphysema (Silverman and Sandhaus, 2009). More than 95% of AAT-deficient individuals have the Z-type of AAT instead of the normal M-type AAT (Brantly *et al.*, 1991). Gene therapy approaches to treatment of AAT deficiency, using recombinant adeno-associated viral (rAAV) vectors expressing AAT, have been evaluated in preclinical and clinical studies (Song *et al.*, 1998, 2002; Poirier *et al.*, 2004; Brantly *et al.*, 2006, 2009; De *et al.*, 2006; Liqun Wang *et al.*, 2009; Halbert *et al.*, 2010; Chulay *et al.*, 2011). We previously conducted a phase 1 clinical trial with an rAAV vector expressing human AAT (rAAV1-CB-hAAT), produced by a plasmid transfection method, in which sustained expression of AAT was achieved, but serum levels were substantially below the levels considered to be therapeutic (Brantly *et al.*,

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Registration: This study has been registered at Clinical Trials.gov (NCT01054339).

2009). Production of rAAV1-CB-hAAT using a recombinant herpes simplex virus (HSV) complementation system (Kang *et al.*, 2009; Thomas *et al.*, 2009) can generate much higher yields, enabling a substantial increase in dose in clinical studies, and the HSV-produced vector is more potent when given by intramuscular injection in mice (Chulay *et al.*, 2011). We report here preliminary results from a phase 2 clinical trial of rAAV1-CB-hAAT produced by the HSV-based method.

Research Design and Methods

Participants

Individuals of either gender were eligible for study inclusion if they had a diagnosis of AAT deficiency, a serum AAT level $<11\,\mu M$, a forced expiratory volume in 1 sec (FEV_1) >25% of predicted, and had not received AAT augmentation therapy in the 3 months before enrollment and planned not to receive it for 12 months after enrollment.

Vector production and characterization

The rAAV1-CB-hAAT vector was identical to the vector used in a phase 1 clinical trial (Brantly *et al.*, 2009) except that it was made using a recombinant HSV complementation system in suspension baby hamster kidney (BHK) cells (Kang *et al.*, 2009; Thomas *et al.*, 2009), based on the method of Conway and colleagues (1999) and purified by Convective Interaction Media (CIM) QA Monolith anion-exchange chromatography (BIA Separations, Villach, Austria) followed by AVB Sepharose affinity chromatography (GE Healthcare Life Sciences, Piscataway, NJ). It was produced in compliance with current Good Manufacturing Practice at SAFC Pharma (Carlsbad, CA) and characterized in compendial assays or product-specific assays as described previously (Chulay *et al.*, 2011).

Study design and conduct

This is a nonrandomized, open-label, multicenter, sequential, three-arm, phase 2 clinical trial evaluating the safety and efficacy of administration of rAAV1-CB-hAAT conducted under an investigational new drug (IND) application with approval by institutional review boards and in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained before any study procedures were performed. Three cohorts of three subjects each received rAAV1-CB-hAAT at dose levels of 6×10^{11}, 1.9×10^{12}, or 6×10^{12} vector genomes (VG)/kg body weight by intramuscular injection on a single occasion. Subjects in cohort 1 received 10 intramuscular injections distributed across a single muscle site, subjects in cohort 2 received 32 intramuscular injections distributed across three muscle sites, and subjects in cohort 3 received 100 intramuscular injections distributed across 10 muscle sites. Each injection was given in a volume of 1.35 ml, at the appropriate vector concentration to achieve the desired total vector dose. The clinical protocol specified that subjects could choose to have injections administered using topical anesthetic cream or conscious sedation with intravenous midazolam.

A data and safety monitoring board reviewed safety data for the first two dose level cohorts before the next higher dose cohort was enrolled. Safety is being monitored by evaluation of adverse events, hematology (complete blood count with white cell differential) and clinical chemistry parameters (electrolytes, glucose, albumin, globulin, blood urea nitrogen, creatinine, creatine kinase, bilirubin, and hepatic enzymes), histological examination of muscle biopsies at 3 and 12 months, and measurement of serum antibodies to AAT. Efficacy is being evaluated by measurement of serum concentrations of M-type AAT and total AAT. Additional information being collected includes changes in antibody responses to AAV and T cell responses to AAV and AAT.

Laboratory assessments

Antibodies to AAT were measured by ELISA, using a modification of a previously described method (Brantly *et al.*, 2006) in which serum from a cynomolgus macaque immunized against human AAT by injection with rAAV1-CB-hAAT was used as the reference standard. All other assays were performed as previously described (Brantly *et al.*, 2009), including measurement of antibodies to AAV1 using a neutralization assay, T cell responses by *ex vivo* interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay and polychromatic flow cytometry of peripheral blood mononuclear cells (PBMCs) after stimulation with AAV or AAT peptides, and M-specific AAT by ELISA. Epitope mapping of the ELISPOT response to an AAT peptide pool was performed with a matrix of subpools to identify the single peptide that was reactive.

Muscle biopsies were performed with a disposable Price muscle biopsy clamp (V. Mueller catalog #SU20910; Cardinal Health Medical Products, McGaw Park, IL) to obtain two approximately $2\times1\times0.5$ cm specimens. Half of each specimen was immediately frozen and the other half was prepared for histopathology by fixation in 10% neutral buffered formalin for 4 to 24 hr and then transferred to 70% ethanol until processing. Paraffin serial sections (4 μm) were stained with hematoxylin and eosin (H&E) and by immunohistochemistry for human AAT as previously described (Poirier *et al.*, 2004). Inflammatory cell immunophenotype was determined with monoclonal antibodies that recognize CD3, CD20, CD68 (Dako, Carpinteria, CA), and CD4 (Cellmarque, Rocklin, CA) following standard clinical validation on an autostainer (Dako).

Results

Characterization of rAAV1-CB-hAAT

The four 25-liter batches of crude cell lysate yielded a total of 1.0×10^{16} VG, with an overall product recovery during purification of 23%. The study drug met all release criteria, including sterility, endotoxin (<0.3 EU/ml), and absence of detectable mycoplasma, adventitious viruses, replication-competent AAV, and replication-competent HSV. Residual BHK DNA and protein were 4.1 and 15.9 ng/ml, respectively, and residual HSV DNA and protein were 76 and 834 ng/ml, respectively. Vector concentration measured by quantitative PCR was 5.0×10^{12} VG/ml, vector infectivity measured as median tissue culture infective dose ($TCID_{50}$) was 4.7×10^{10} IU/ml, and hAAT expression measured by ELISA in infected HEK 293 cell supernatant was 2.2 μg/ml.

Preliminary clinical trial results

Nine subjects were enrolled and received intramuscular injections of rAAV1-CB-hAAT between June 2010 and October 2010. All were white and seven were female, with a

mean age of 51.1 (range, 20–68) years and mean weight of 71.2 (range, 55–90) kg. The AAT phenotype was ZZ for eight subjects and SZ for one.

Administration of rAAV1-CB-hAAT by multiple intramuscular injections was well tolerated in all subjects. On the basis of subject preferences, injections were performed with topical anesthetic cream for the three subjects in cohort 1 and for two subjects in each of cohorts 2 and 3, and under conscious sedation using intravenous midazolam for one subject in each of cohorts 2 and 3.

All subjects reported at least one adverse event of mild to moderate intensity. The most frequently reported adverse events were injection site reactions (discomfort, erythema, bruising, or pain) of mild intensity, which occurred in eight of nine subjects. A list of all adverse events is provided in Table 1. No serious adverse events were reported.

Table 1. Summary of Adverse Events

	Mild	*Moderate*	*Severe*	*Total*
Events related to study agent or its administration				
General disorders and administration site conditions				
Influenza-like illness	1			1
Injection site discomfort	6			6
Injection site erythema	2			2
Injection site hemorrhage	7			7
Injection site pain	1	1		2
Malaise	1			1
Investigations				
Blood creatine phosphokinase increased	2	1		3
Musculoskeletal and connective tissue disorders				
Muscle strain	1			1
Vascular disorders				
Phlebitis, superficial	1			1
Events not related to study agent or its administration				
Gastrointestinal disorders				
Nausea	1			1
General disorders and administration site conditions				
Pyrexia	1			1
Infections and infestations				
Gastroenteritis, viral		1		1
Oral candidiasis	1			1
Urinary tract infection	1			1
Injury, poisoning, and procedural complications				
Arthropod bite	1			1
Postprocedural discomfort	3			3
Procedural pain	2	2		4
Metabolism and nutrition disorders				
Dehydration		1		1
Musculoskeletal and connective tissue disorders				
Back pain	1			1
Joint sprain	1			1
Limb discomfort	1			1
Muscle spasms	1			1
Muscle strain	1			1
Myalgias	2			2
Pain in extremity		1		1
Nervous system disorders				
Headache	2			2
Respiratory, thoracic and mediastinal disorders				
Sinus headache	1			1
Throat irritation	1			1
Upper respiratory tract infection	1			1
Skin and subcutaneous tissue disorder				
Furuncle	1			1
Rash, erythematous	1			1
Skin hemorrhage	1			1
Vascular disorders				
Orthostatic hypotension		1		1
Phlebitis, superficial	2			2
Postmastectomy lymphedema syndrome	1			1

Data represent the number of subjects who reported the listed adverse event on one or more occasions.

Measurement of serum M-specific AAT levels demonstrated a dose-dependent increase after injection of rAAV1-CB-hAAT, which peaked on day 30 (mean value of 572 n*M* in the highest dose cohort) and then declined on day 45 with little change thereafter (mean value of 240 n*M* on day 90 in the highest dose cohort). The average peak serum M-AAT level in the lowest dose cohort in this study was more than 2-fold higher than the average peak serum M-AAT level in the highest dose cohort in the previous phase 1 study with transfection-produced vector (Fig. 1). Serum M-AAT levels for individual subjects are shown in Fig. 2.

Serum creatine kinase (CK) was transiently elevated on day 30 in two of three subjects in cohort 2 and in three of three subjects in cohort 3, and had normalized by day 45 in four subjects and by day 60 in one subject (Fig. 2). There were no clinically significant changes in any other clinical chemistry or hematology parameter.

As expected on the basis of previous preclinical and clinical testing, all subjects in all three dose level cohorts developed neutralizing antibodies against AAV (Table 2) and IFN-γ ELISPOT responses to AAV peptides (Fig. 3). There was no apparent relationship between the dose of vector administered and the magnitude of the IFN-γ ELISPOT responses to AAV peptides. Both AAV1-specfic $CD8^+$ and $CD4^+$ T cell responses were detected and had a cytokine profile similar to that seen in the previous clinical trial with this vector (Brantly *et al.*, 2009).

None of the subjects has developed antibodies to AAT, but one subject in the mid-dose cohort (subject 401) developed IFN-γ ELISPOT responses to a pool of AAT peptides at month 1 that persisted at months 2 and 3 (baseline and screening samples were negative). Epitope mapping identified a single peptide (peptide 46, DTEEEDFHVDQVTTV) distant from the site of the *PI*Z* mutation that was the target of the ELISPOT response. Cytokine flow cytometry after stimulation with the AAT peptide pool showed that this subject had a response to AAT mediated by $CD4^+$ T cells expressing IFN-γ but not tumor necrosis factor (TNF)-α and by $CD8^+$ T cells expressing both IFN-γ and TNF-α. On the basis of review of history and physical examination findings at each visit and hematology and clinical chemistry data, there was no evidence that the T cell response to this AAT peptide was associated with any untoward clinical effects.

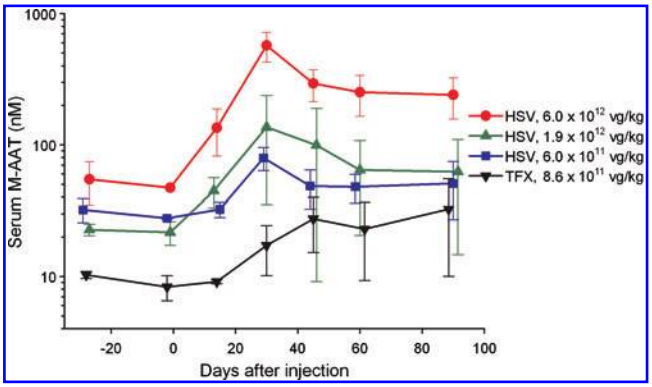

FIG. 1. Serum M-specific α_1-antitrypsin (AAT) concentration after injection of rAAV1-CB-hAAT produced by plasmid transfection (TFX) or the herpes simplex virus (HSV) method. Values shown represent means ± SD. The dose of vector administered to subjects is indicated in the figure legend. Values for the TFX group are from a previous study (Brantly *et al.*, 2009). Values for the 6×10^{11} VG/kg HSV group do not include results for subject 303, who had an AAT phenotype of SZ; the monoclonal antibody used to determine serum M-specific AAT concentrations has little cross-reactivity with Z-type AAT but cross-reacts strongly with S-type AAT, causing results for this assay in this subject to be spuriously high. Color images available online at www.liebertonline.com/hum

Histological examination of muscle biopsies, obtained on day 90 from eight subjects, showed moderate to marked mononuclear endomysial and perivascular inflammatory infiltrates composed primarily of mature lymphocytes and smaller populations of monocytes and plasma cells (Fig. 4). There was also prominent myofiber regeneration, as evidenced by numerous basophilic myofibers with large vesicular nuclei containing prominent nucleoli. Occasional myofibers undergoing active necrosis with phagocytosis by macrophage-like cells were identified. No significant endomysial fibrosis was seen. Immunohistochemical staining indicated that CD3-immunoreactive T lymphocytes comprised the most abundant single subset of mononuclear cells, with $CD8^+$ cells accounting for slightly more of the total inflammatory cell population than CD4-reactive cells. Scattered CD20-immunoreactive B lymphocytes and CD68-reactive macrophages were also seen. There was strong immunostaining of AAT within endomysial and perimysial blood vessels, not associated with cell components, and in perimysial and endomysial connective tissue. Focal individual myofibers showed weaker AAT immunoreactivity of the sarcoplasm, with some myofibers having a dispersed granular pattern of reactivity.

Discussion

The most significant finding in this study was a clear demonstration of a linear dose–response relationship. This is the first time, to the authors' knowledge, that such a linear relationship between physical dose of a gene therapy product and expression of a therapeutic protein has been shown in humans. Previous clinical trials with the rAAV2-CB-hAAT vector (Brantly *et al.*, 2006, 2009) and with an AAV2 vector expressing clotting factor IX (Manno *et al.*, 2003, 2006) did not have sufficient numbers of subjects with sustained expression at various doses to enable demonstration of a linear dose response, and in clinical trials with an AAV1 vector expressing lipoprotein lipase (Stroes *et al.*, 2008) the end point was indirect, and thus cannot be compared directly with our results. Our observation indicates that human rAAV gene therapy for AAT deficiency behaves in a predictable fashion and is an important step in the ability to design and conduct appropriate safety and efficacy studies in support of product licensure.

However, AAT is one of the most abundant serum proteins (normal concentration, 20 to 50 μM or about 1,000 to 2,500 μg/ml), and the peak serum AAT levels achieved after delivery of 6×10^{12} VG/kg by multiple intramuscular injections (between 412 and 694 n*M*, equivalent to 21 to 36 μg/ml) were below the target therapeutic concentration (> 11 μM, equivalent to 572 μg/ml) required to reduce the risk of emphysema. Further improvements in product delivery or product design will therefore be required to achieve

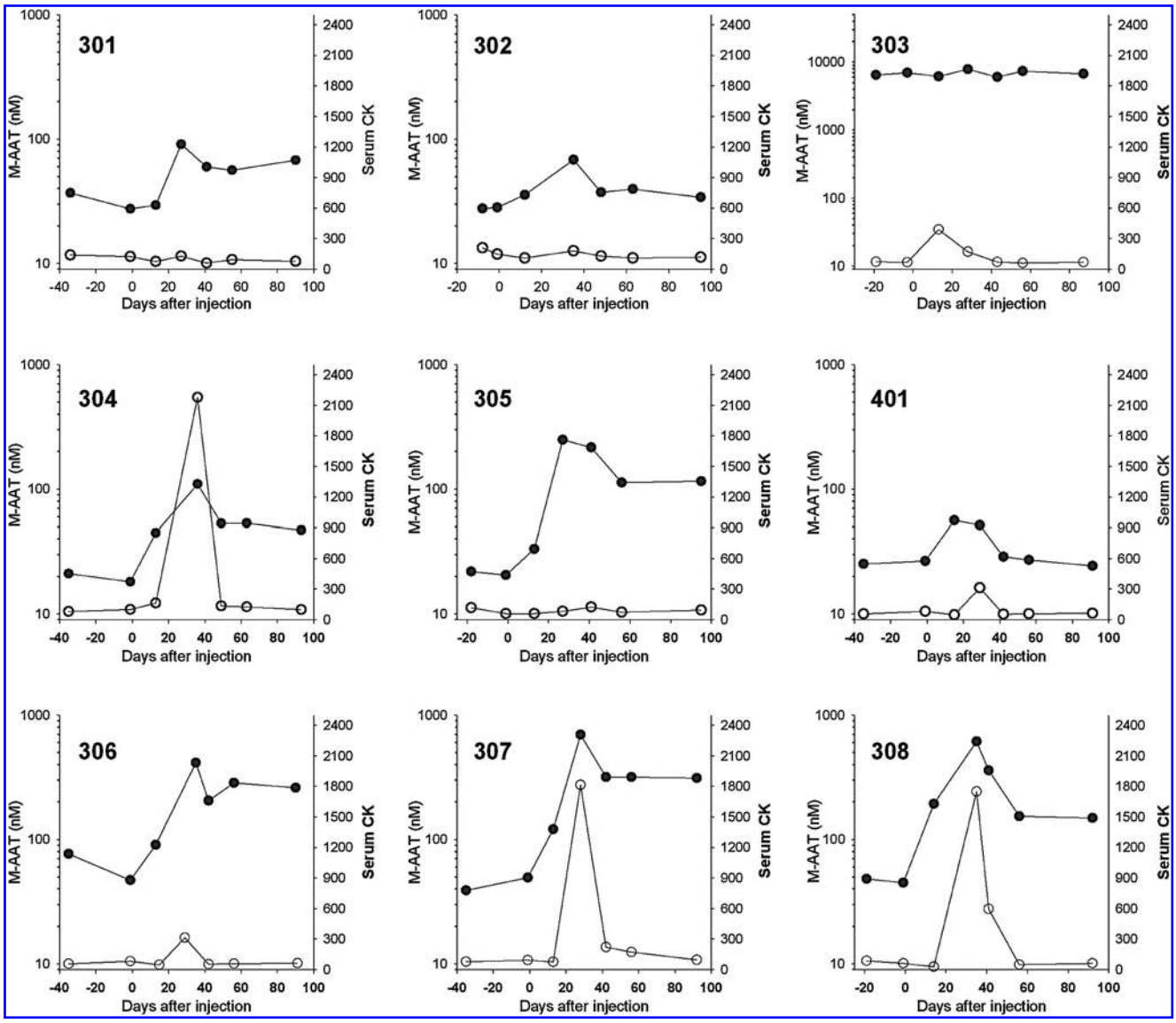

FIG. 2. Serum M-specific α_1-antitrypsin (AAT) concentration (solid symbols) and serum creatine kinase (CK) levels (open symbols) after injection of rAAV1-CB-hAAT in individual subjects. Subject 303 had an AAT phenotype of SZ, and results for the M-specific AAT ELISA in this subject are spuriously high. Subjects 305 and 307 were the two male subjects.

Table 2. Neutralizing Antibody Responses to AAV

		Low dose			*Middle dose*			*High dose*		
Serotype	*Visit*	*Subject 301*	*Subject 302*	*Subject 303*	*Subject 304*	*Subject 305*	*Subject 401*	*Subject 306*	*Subject 307*	*Subject 308*
AAV1	Screen	<5	<5	80	<5	<5	<5	<5	<5	160
	Day 14	5,120	5,120	40,960	10,240	5,120	5,120	20,480	5,120	20,480
	Month 1	5,120	2,560	20,480	10,240	10,240	10,240	10,240	2,560	20,480
	Month 3	20,480	40,960	20,480	40,960	20,480	40,960	81,920	40,960	81,920
AAV2	Screen	<5	<5	80	<5	<5	<5	<5	<5	80
	Month 3	160	<5	10,240	160	80	320	640	320	81,920
AAV8	Screen	<5	<5	80	<5	<5	<5	<5	<5	80
	Month 3	80	20	5,120	1,280	80	2,560	1,280	80	10,240

rAAV-lacZ vectors mixed with serial dilutions of serum were used to infect Huh7 cells. Results are expressed as the reciprocal of the highest serum dilution that inhibited β-galactosidase expression by 50%.

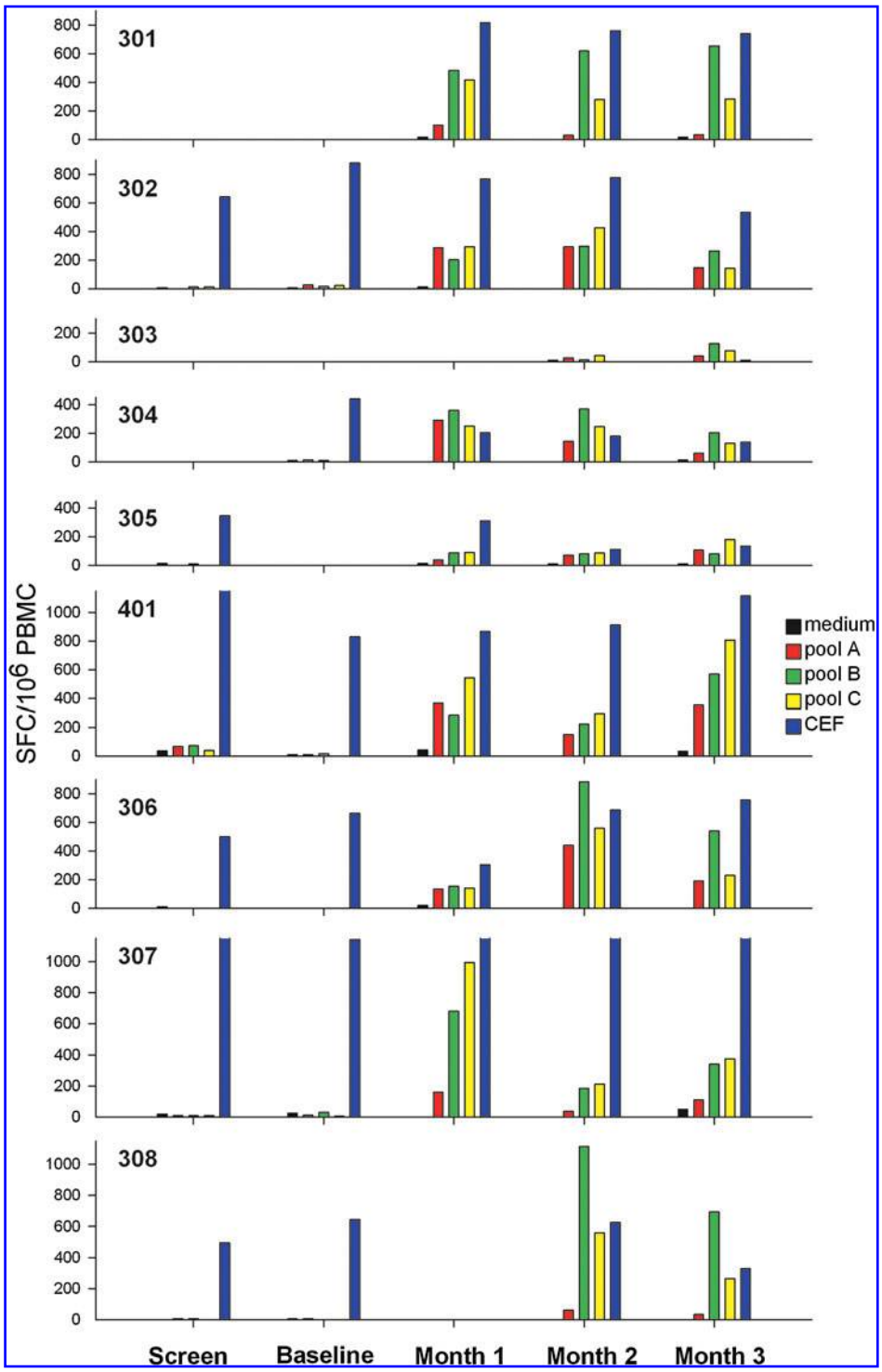

FIG. 3. Time course of IFN-γ ELISPOT responses to pools of AAV1 capsid peptides or controls. PBMCs were obtained at screening, baseline, and 1, 2, and 3 months after vector administration and were stimulated with each of three pools (A, B, and C) of AAV1 capsid peptides (15-mers overlapping by 10 amino acids) or with a positive control peptide pool (CEF). SFC, spot-forming cells.

therapeutic target serum AAT concentrations. For example, administration of an rAAV1 vector expressing a CTLA4Ig transgene by a regional intravenous method achieved serum concentrations 5- to 8-fold higher than multiple intramuscular injections of the same vector in cynomolgus macaques (Toromanoff *et al.*, 2008), and suggests that regional vascular delivery of rAAV1-CB-hAAT might result in higher serum AAT concentrations. It is also possible that regional vascular delivery may elevate expression levels by reducing anti-AAV immune responses (Toromanoff *et al.*, 2010), or that a similar result could be achieved by short-term administration of immunosuppressive drugs. The use of alternative AAV serotypes should also be considered. AAV1 was selected for use in the current clinical trial on the basis of evidence of improved transduction efficiency with AAV1 compared with AAV2 after intramuscular injection in mice (Xiao *et al.*, 1999; Chao *et al.*, 2000; Gao *et al.*, 2002; Rabinowitz *et al.*, 2002; Lu *et al.*, 2006). However, more recent data

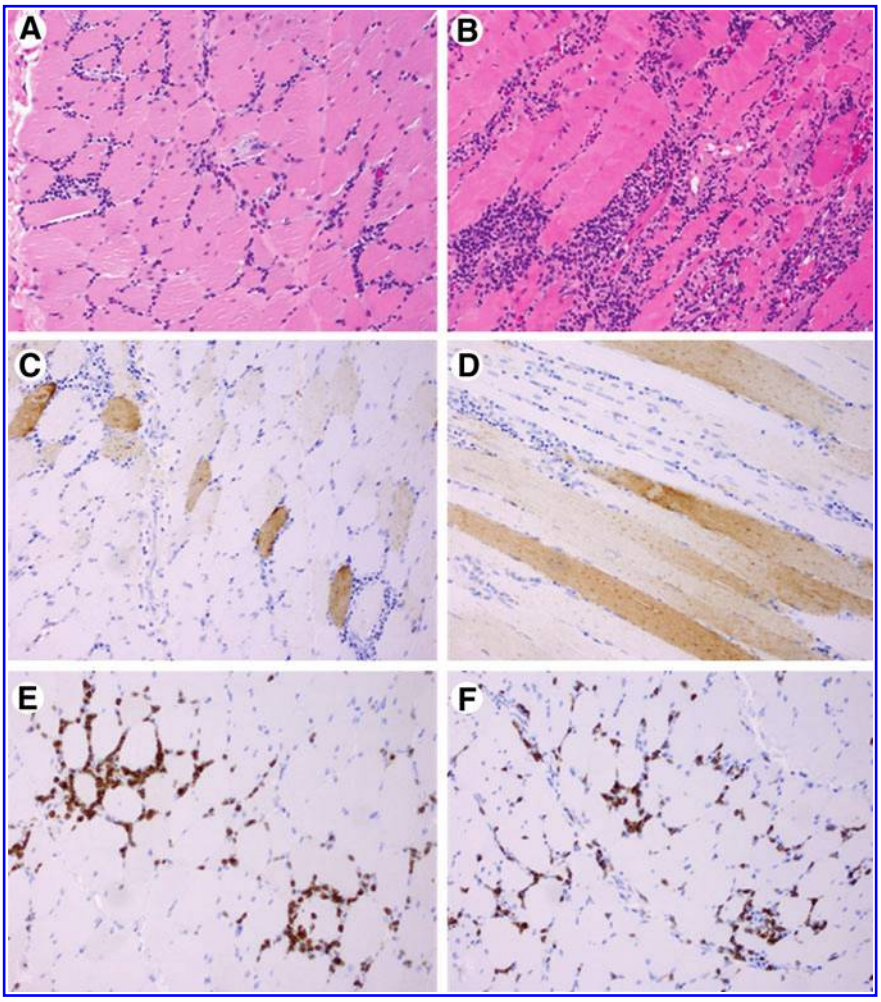

FIG. 4. Histology and immunohistochemical study of skeletal muscle. (**A**) H&E-stained section showing a moderate endomysial inflammatory reaction composed primarily of mononuclear cells. (**B**) H&E-stained section showing a marked endomysial inflammatory reaction. (**C**) Immunohistochemistry for AAT, showing individual weak to moderate granular reactivity in individual myofibers on cross-section. (**D**) Immunohistochemistry for AAT, showing individual weak to moderate granular reactivity in individual myofibers cut longitudinally. (**E**) Immunohistochemistry for CD3, showing a high proportion of T lymphocytes comprising the inflammatory infiltrate. (**F**) CD8-immunoreactive T cells comprise a significant subset of the total lymphocytic infiltrate.

indicate that other serotypes, including recombinant serotypes and other nonnaturally occurring serotypes, may transduce muscle cells more efficiently than AAV1 (Rodino-Klapac *et al.*, 2007; Asokan *et al.*, 2010; Qiao *et al.*, 2010; Pulicherla *et al.*, 2011). A combination of these approaches may be necessary in order to ultimately achieve the goal of effective gene therapy for AAT deficiency. Results of the current study provide a foundation for designing rational therapeutic protocols.

Results of this study provide additional evidence of the safety of AAV gene therapy. In the highest dose cohort (6×10^{12} VG/kg), subjects received a total of between 3.3×10^{14} and 4.3×10^{14} VG, administered in a total of 135 ml distributed over 100 intramuscular injections, with only mild and transient discomfort at the injection sites.

As expected, all subjects developed anti-AAV antibodies and IFN-γ ELISPOT responses to AAV peptides. In a previous clinical trial with the same vector, anti-AAV immune responses were not associated with any significant decline in peak AAT expression; expression rose irregularly during the first 30 to 180 days and was then sustained at similar levels through day 365. In the present study, serum CK levels were elevated in most subjects in the higher two dose level cohorts on day 30 after injection, which corresponded to the time of peak serum AAT expression, and there was histological evidence of inflammatory cells in muscle biopsy samples 3 months after injections, but no clinical symptoms suggestive of ongoing myositis. It is not known if the T cells seen in muscle biopsies are AAV specific, or if antivector immune responses are responsible for the observed decline in AAT expression after day 30.

We documented T cell response to a single AAT peptide in one subject but found no evidence of untoward clinical effects (no symptoms, no antibody response to AAT, no abnormal liver function tests, and no change in total AAT concentration). The fact that the epitope eliciting this T cell response was distant from the site of the missense mutation is puzzling. Although there is no evidence that the glycosylation pattern of AAT expressed from muscle is different from that of AAT expressed from liver, it is possible that altered glycosylation of AAT may break tolerance and trigger adaptive immune responses in some individuals under certain circumstances. Alternatively, this subject may have had low levels of preexisting reactive T cells that were not detected in the peripheral blood before vector administration. It is reassuring that the three subjects in the highest dose cohort did not mount any detectable T cell responses to AAT peptides.

In summary, results from this clinical trial support the feasibility and safety of AAV gene therapy of AAT

deficiency, although further improvements in the design or delivery of rAAV-AAT vectors will be required to achieve therapeutic target serum AAT concentrations.

Acknowledgments

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Author Disclosure Statement

D.R.K., G.Y., and J.D.C. are employees of and hold share options in Applied Genetic Technologies Corporation, and have a conflict of interest to the extent that this work potentially increases their personal financial interests. J.M.W. is a consultant to ReGenX Holdings, and is a founder of, holds equity in, and receives a grant from affiliates of ReGenX Holdings; in addition, he is an inventor on patents licensed to various biopharmaceutical companies, including affiliates of ReGenX Holdings. None of the other authors has a competing financial interest.

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