Preclinical Evaluation of a Recombinant Adeno-Associated Virus Vector Expressing Human Alpha-1 Antitrypsin Made Using a Recombinant Herpes Simplex Virus Production Method

Jeffrey D. Chulay,¹ Guo-Jie Ye,¹ Darby L. Thomas,¹ David R. Knop,¹ Janet M. Benson,²
Julie A. Hutt,² Gensheng Wang,² Margaret Humphries,³ and Terence R. Flotte³

Abstract

Recombinant adeno-associated virus (rAAV) vectors offer promise for gene therapy of alpha-1 antitrypsin (AAT) deficiency. A toxicology study in mice evaluated intramuscular injection of an rAAV vector expressing human AAT (rAAV-CB-hAAT) produced using a herpes simplex virus (HSV) complementation system or a plasmid transfection (TFX) method at doses of 3×10^{11} vg (1.2×10^{13} vg/kg) for both vectors and 2×10^{12} vg (8×10^{13} vg/kg) for the HSV-produced vector. The HSV-produced vector had favorable *in vitro* characteristics in terms of purity, efficiency of transduction, and hAAT expression. There were no significant differences in clinical findings or hematology and clinical chemistry values between test article and control groups and no gross pathology findings. Histopathological examination demonstrated minimal to mild changes in skeletal muscle at the injection site, consisting of focal chronic interstitial inflammation and muscle degeneration, regeneration, and vacuolization, in vector-injected animals. At the 3×10^{11} vg dose, serum hAAT levels were higher with the HSV-produced vector than with the TFX-produced vector. With the higher dose of HSV-produced vector, the increase in serum hAAT levels was dose-proportional in females and greater than dose-proportional in males. Vector copy numbers in blood were highest 24 hr after dosing and declined thereafter, with no detectable copies present 90 days after dosing. Antibodies to hAAT were detected in almost all vector-treated animals, and antibodies to HSV were detected in most animals that received the highest vector dose. These results support continued development of rAAV-CB-hAAT for treatment of AAT deficiency.

Introduction

ALPHA-1 ANTITRYPSIN DEFICIENCY is caused by mutations in the SERPIN1A gene that typically generate a protein with impaired secretion from the liver, resulting in low serum concentrations of alpha-1 antitrypsin (AAT) and impaired anti-protease activity in the lung, leading to early-onset pulmonary emphysema (Silverman and Sandhaus, 2009). The potential for using a recombinant adeno-associated virus (rAAV) vector for delivery of the wild-type (M) AAT gene has been explored. Initial efforts examined delivery of rAAV-AAT vectors to muscle, lung, or liver (Song *et al.*, 1998, 2001; Virella-Lowell *et al.*, 2005; Liqun Wang *et al.*, 2009). Although robust expression could be achieved with each delivery method, the

use of muscle injection for development of a clinical approach has several advantages, such as a more favorable profile of anti-capsid effector T-cell responses (Manno *et al.*, 2006; Brantly *et al.*, 2009) and a lower level of dissemination to distant sites (Manno *et al.*, 2003, 2006). The pursuit of an intramuscular (IM) approach was first focused on the use of an rAAV-AAT vector delivered within an adeno-associated virus (AAV) serotype 2 capsid (Brantly *et al.*, 2006), but levels of gene expression were very low and generally undetectable. Studies from a number of groups showed that the use of the AAV1 capsid in muscle was substantially more efficient than AAV2 (Xiao *et al.*, 1999; Chao *et al.*, 2001; Gao *et al.*, 2002; Rabinowitz *et al.*, 2002; Hauck and Xiao, 2003), and work from members of our group demonstrated that human AAT

¹Applied Genetic Technologies Corporation, Alachua, FL 32615.

²Lovelace Biomedical and Environmental Research Institute, Albuquerque, NM 87108.

³University of Massachusetts Medical School, Worcester, MA 01655.

(hAAT) produced from the muscle was functional (Lu *et al.*, 2006).

In a Phase 1 clinical trial, an rAAV virus vector expressing hAAT when delivered within the AAV1 capsid (rAAV1-CB-hAAT) achieved sustained expression of AAT, but serum levels were substantially below the levels considered to be therapeutic (Brantly *et al.*, 2009). The rAAV1-CB-hAAT vector used in that study was made using a plasmid transfection (TFX) production method; it was evaluated in toxicology and biodistribution studies that demonstrated dose-dependent inflammatory reactions detected histologically at the injection site, and vector DNA was detected in most organs in a dose- and time-dependent manner (Flotte *et al.*, 2007).

Production of rAAV1-CB-hAAT using a recombinant herpes simplex virus (HSV) complementation system (Kang *et al.*, 2009; Thomas *et al.*, 2009) has achieved much higher yields, enabling a substantial increase in dosage in clinical studies. In preparation for a Phase 2 clinical trial of rAAV1-CB-hAAT, we performed a bridging toxicology study comparing the vector made by either a plasmid TFX method or the HSV complementation method.

Research Design and Methods

Vector production

The rAAV1-CB-hAAT vector consists of an AAV serotype 1 capsid structure containing a single-stranded DNA molecule with AAV2 inverted terminal repeats flanking a gene cassette comprised of a cytomegalovirus (CMV) immediate-early enhancer/chicken β -actin promoter, a hybrid chicken β -actin/rabbit β -globin intron, cDNA encoding hAAT, and an SV40 polyadenylation signal. This vector was produced by two methods.

The TFX-produced vector was made by TFX of human embryonic kidney (HEK) 293 cells with two plasmids, one containing the AAV-CB-hAAT expression cassette and the other an AAV/adenovirus hybrid plasmid containing AAV serotype 2 *rep* and AAV serotype 1 *cap* genes and all adenovirus packaging and helper functions required for production of rAAV (Grimm *et al.*, 1998). The cells were lysed with 0.5% (v/v) Polysorbate 80, treated with Benzonase, disrupted by passage through a microfluidizer, and clarified by centrifugation. The vector was purified by a three-column chromatography process (CHT Hydroxyapatite followed by Q Sepharose HP and then Sephacryl S-300) and then concentrated by tangential flow filtration, buffer exchanged with lactated Ringer's solution, and filtered through a 0.2- μ m filter. This is the same process that was used to produce the vector used in a Phase 1 clinical trial (Brantly *et al.*, 2009).

The HSV-produced vector was made using a recombinant HSV (rHSV) complementation system in suspension baby hamster kidney (sBHK) cells (Kang *et al.*, 2009; Thomas *et al.*, 2009). The sBHK cells were coinfecting with two rHSV helper viruses. One contained AAV serotype 2 *rep* and AAV serotype 1 *cap* genes. The other contained the AAV-CB-hAAT expression cassette inserted into the thymidine kinase locus of an HSV-1 virus (d27.1) that has a deletion in the UL54 gene, and thus does not express the immediate-early protein ICP27 and is thereby replication-incompetent in normal cells but can be propagated in V27 cells (Vero cells stably transformed with the UL54 gene). The cells were harvested by lysis with Triton X-100 detergent, treated with

Benzonase, clarified by filtration, concentrated, and buffer exchanged; the resulting intermediate was purified by column chromatography using CIM Q Monolith anion-exchange chromatography followed by AVB Sepharose affinity chromatography. The product was then concentrated by tangential flow filtration, buffer exchanged with lactated Ringer's solution, and filtered through a 0.2- μ m filter.

Vector characterization

Vector concentration was determined by a quantitative real-time PCR assay as previously described (Kang *et al.*, 2009), using primers and probe that target the SV40 polyadenylation sequence in the vector.

Vector infectivity was determined by a TCID₅₀ assay. HeLaRC32 cells (which express AAV *rep* and *cap* genes) were coinfecting with serial dilutions of vector and a saturating amount of human adenovirus type 5 (Ad5). After 3 days, cell lysates were analyzed for the presence of vector sequences by PCR, using primers and probe specific for the SV40 sequence present in the vector. Samples from each well that achieve a value above the limit of quantification of the PCR assay were scored as positive, and the TCID₅₀ was calculated using the Spearman-Kärber method (Miller and Ulrich, 2001).

Expression of hAAT was determined by coinfection of HEK 293 cells cultured in six-well plates with vector (1,000, 10,000, or 100,000 vg/cell) and Ad5 (10 IU/cell). Controls included mock-infected cells and cells infected with only Ad5. Five hours after infection, infectious medium was replaced with 2 ml of fresh medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum) and the plates incubated at 37°C, 5% CO₂ for a total of 58 hr. Cultures were harvested, cell debris was removed by centrifugation, and AAT expressed by the transduced cells and secreted into the culture medium was quantified by hAAT-specific ELISA.

Vector purity was determined by silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis using 1×10^{10} vg per lane. Vectors were resuspended in 4 $\times$ loading buffer, subjected to electrophoresis on a NuPAGE Novex 4–12% Bis-Tris denaturing polyacrylamide gel (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction, and silver-stained using the SilverXpress Silver Staining Kit (Invitrogen) for total proteins. The density of silver-stained bands was determined using a Molecular Imager ChemiDoc XRS System with Quantity One software (Bio-Rad Laboratories, Hercules, CA). Purity was estimated by comparing the sum of the densities of the three AAV viral capsid proteins (VP1, VP2, and VP3) with the sum of the densities for all proteins in the lane. The density of the VP3 band was also compared between the HSV-produced and TFX-produced vector and the HSV-produced vector after purification by CsCl gradient centrifugation.

Residual HSV protein was determined by ELISA. Serial dilutions of test serum or affinity column-purified HSV protein standards were added to wells of microtiter plates precoated with rabbit polyclonal anti-HSV antibody, incubated overnight, washed, reacted with horseradish peroxidase (HRP)-labeled rabbit polyclonal anti-HSV antibody, incubated 2 hr, rinsed, and reacted with Supersignal ELISA

Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA); the relative luminescence units (RLU) were determined in a plate reader. The HSV protein concentration was calculated based on the RLU of a series of dilutions of test samples and the standard curve generated from the HSV protein reference standard curve. The limit of quantification in this assay is 1 ng/ml.

Endotoxin was determined by Limulus Amebocyte Lysate Endosafe assay (Charles River Laboratories, Charleston, SC) according to the manufacturer's instructions. Standard procedures were used to test for bacterial, fungal, and mycoplasma contamination.

For electron microscopy, samples of purified vector were applied to carbon-coated electron microscopy grids, rinsed twice with ultrapure water, negatively stained with 2% uranyl acetate for 1 min, and examined by transmission electron microscopy at 100,000-fold magnification. Empty capsids can be readily distinguished from full capsids as doughnut-shaped particles, resulting from the uptake of uranyl acetate in the core of the capsid or from collapse and differential staining of the particle. Total particles and empty particles were counted on each of three images from a sample of rAAV1-CB-hAAT vector produced by the HSV method (total of 1,268 particles counted) or TFX method (total of 1,251 particles counted).

Toxicology study design

Four groups of 42 adult C57BL/6 mice (21 males, 21 females) each were injected by the IM route with one of four test articles: the TFX group received rAAV1-CB-hAAT produced using a plasmid TFX method at a dose of 3×10^{11} vg per mouse (1.2×10^{13} vg/kg); the HSV groups received rAAV1-CB-hAAT produced using an rHSV helper virus method at two different dosage levels [3×10^{11} vg per mouse (1.2×10^{13} vg/kg) and 2×10^{12} vg per mouse (8×10^{13} vg/kg)]; and the control group received placebo (lactated Ringer's solution). IM injections were given in a volume of 180 μ l in each hind limb (90 μ l in each quadriceps and 90 μ l in each gastrocnemius) on a single day. Ten animals (five males, five females) from each group were sacrificed at 21, 60, or 90 days after injection. The remaining 12 animals (six males, six females) from each group were used to provide blood samples at study days 1, 21, 60, and 90 for measurement of vector DNA concentration.

Blood for hematology (hemoglobin, hematocrit, red cell count, platelet count, and white cell count with differential) and clinical chemistry (creatinine kinase, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, creatinine, and blood urea nitrogen) was obtained on the day of sacrifice. Blood for quantitative PCR analysis of vector DNA was obtained 1, 21, 60, and 90 days after injection. At necropsy, blood was obtained for measurement of serum hAAT concentration; the brain, heart, liver, lungs, kidneys, spleen, adrenal glands, thymus, and uterus were weighed; and samples of skeletal muscle, liver, spleen, heart, kidney, lung, lymph nodes, diaphragm, jejunum, pancreas, gonads, and brain were obtained for histopathology.

Quantitation of vector in blood

Blood was collected in ethylenediaminetetraacetic acid by submandibular puncture and snap-frozen in liquid nitrogen,

followed by storage at -80°C until analysis. DNA was extracted from 50–190 μ l of blood using a QiaAmp DNA Blood Mini kit (Qiagen, Valencia, CA), with one positive control (blood spiked with rAAV1-CB-hAAT at 1×10^8 vg/ml) and one negative control (normal mouse blood) included in each batch of DNA extraction. DNA concentration was determined by measuring UV absorbance at a wavelength of 260 nm (A260) using a Beckman Coulter DU 640 Spectrophotometer. Vector concentration was determined by a quantitative real-time PCR assay as previously described (Kang *et al.*, 2009), and was expressed as copy numbers per microgram of DNA.

Quantitation of hAAT expression

Expression of hAAT was quantified by ELISA. Microtiter plates were coated with 100 μ l of goat anti-hAAT antibody (MP Biomedical, Solon, CA) diluted 1:500 in 0.2 M sodium carbonate-bicarbonate buffer (pH 9.4) overnight at 4°C , washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), and then blocked with blocking buffer (1% nonfat milk in PBS-T) for 1 hr at room temperature. Serial dilutions of hAAT standards (Athens Research & Technology, Athens, GA), test samples, and a positive control (pooled normal human serum; Innovative Research, Plymouth, MN) were added to the plate and incubated at room temperature for 2 hr. Plates were washed with PBS-T, and HRP-labeled goat anti-hAAT (Abcam, Cambridge, MA) diluted 1:5,000 in blocking buffer was added and plates incubated for 1 hr at room temperature. Plates were washed with PBS-T, and 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma, St. Louis, MO) was added followed by stop solution after 5 min. Absorbance (OD_{450}) was measured using a μ Quant plate reader (BioTek, Winooski, VT) and data analyzed using the Gen5 data analysis software. The assay has a lower limit of quantitation of 1 ng/ml.

Detection of antibodies to HSV and hAAT

Antibodies to HSV were detected by ELISA as above, except that microtiter plates were coated with 0.5 μ g of total protein from an extract of HSV-infected Vero cells diluted in blocking buffer. The anti-HSV reference standard was a pool of five monoclonal antibodies specific for HSV-1 proteins ICP0, ICP5, ICP35, gD, and gG (Virusys, Taneytown, MD), and the secondary antibody was HRP-labeled goat anti-mouse IgG (Abcam) diluted 1:5,000 in blocking buffer. A pool of sera from a group of mice injected with rAAV1-CB-hAAT was used as an internal positive control. Results were expressed relative to the internal positive control, which was assigned an anti-HSV titer of 1 U/ml.

Antibodies to hAAT were detected by ELISA as above, except that microtiter plates were coated with 1 μ g of hAAT (Athens Research & Technology) in 100 μ l, the anti-hAAT reference standard was serum from a cynomolgus macaque injected with rAAV1-CB-hAAT, and HRP-labeled goat anti-monkey IgG (Fitzgerald Industries International, North Acton, MA) and HRP-labeled goat anti-mouse IgG (Abcam) were used as a secondary antibody for the reference standard and the test samples, respectively. Results were expressed relative to an internal positive control, which was assigned an anti-hAAT titer of 1 U/ml.

Statistical analyses

Differences in the proportion of empty particles on electron micrograph images were compared using the χ^2 test. Group mean body weight (by sex and dose group) and group mean organ weight measurements (by sex, dose group, and sacrifice time [subgroup]) were analyzed using statistics programs in the PathTox database (PathTox Version 4.2.2, Xybion, Cedar Knolls, NJ). After testing for an overall trend among test groups by an analysis of variance, Bartlett's test was used to establish the homogeneity of the data. If the data were homogeneous, group differences were evaluated using a modified Dunnett's test (Dunnett, 1955, 1980). If data were nonhomogeneous, group differences were assessed using a modified *t* test. Statistical significance was assessed at $P \leq 0.05$.

For hematology, clinical chemistry, serum hAAT concentrations, and vector distribution in blood, one-way analysis of variance (ANOVA) was used to evaluate differences in mean values among placebo and treatment groups. For hematology and clinical chemistry parameters, Dunnett's multiple comparison test (Dunnett, 1955, 1980) was performed to assess differences between treated and control groups. For vector distribution and hAAT data, *F*-test contrasts based on pooled variance estimates from the ANOVA were performed, with no adjustment for multiple pairwise comparisons; the pairwise comparisons of interest were between (1) individual treated groups and placebo controls, (2) low-dose HSV and TFX groups, and (3) high-dose HSV and low-dose HSV groups. Pairwise comparisons were made at each time point, and for hAAT data, the magnitudes of differences between groups were compared across time points.

Both the vector distribution and hAAT data exhibited variability that scaled with mean values. Logarithmic transformation of these data using an estimated offset constant (Rocke and Durbin, 2003) was used prior to analysis to stabilize variances and satisfy the homogeneity of variance assumption underlying the ANOVA regression model. Logarithmic transformations were also performed for several of the hematology and clinical chemistry data prior to analysis.

Statistical calculations were performed using the SAS software system, Version 9.1 (SAS Software Corp., Cary, NC). All reported *P* values are two-sided, and statistical significance was assessed at $P = 0.05$ and $P = 0.01$.

TABLE 1. CHARACTERIZATION OF rAAV1-CB-hAAT VECTORS

Parameter	Results	
	TFX-produced vector	HSV-produced vector
Vector concentration (vg/ml)	6.4×10^{12}	6.0×10^{12}
Infectious titer (IU/ml)	2.0×10^{10}	1.3×10^{11}
hAAT expression ($\mu\text{g/ml}$)	6.0	13.2
Purity	93.6%	97.6%
Endotoxin (EU/ml)	0.22	0.13
HSV protein (ng/ml)	Not applicable	60

EU, endotoxin units; HSV, herpes simplex virus; IU, infectious units; TFX, transfection; vg, vector genomes.

Results

Characterization of rAAV1-CB-hAAT

Characteristics of the rAAV1-CB-hAAT vector stocks produced by the TFX and HSV methods are summarized in Table 1. They had similar concentrations as determined by quantitative PCR, but vector infectivity, determined by a TCID₅₀ assay in HeLaRC32 cells, was approximately sixfold higher and hAAT expression, determined in HEK 293 cells, was approximately twofold higher with the HSV-produced vector (Fig. 1). Both vectors had very low levels of endotoxin, and tests for sterility and mycoplasma contamination were negative. The HSV-produced vector had low levels of residual HSV protein (60 ng/ml). Silver-stained SDS-PAGE analysis indicated that both vectors had purity >90%, although small amounts of extra bands in addition to AAV VP1, VP2, and VP3 were visible in the TFX-produced vector (Fig. 2). At an equivalent loading of 1×10^{10} vg per lane, the density of VP3 was 34% higher with the TFX-produced vector and 24% higher with the HSV-produced vectors than with an HSV-produced vector that underwent subsequent CsCl gradient purification, suggesting there were empty viral particles in the non-CsCl-purified vector preparations. Electron micrographs of purified vectors showed a higher proportion of empty capsids in the TFX-produced vector than in the HSV-produced vector. Examples of empty par-

FIG. 1. Expression of hAAT in HEK 293 cells transduced with rAAV1-CB-hAAT produced by a recombinant herpes simplex virus method and purified by CIM Q and AVB columns (HSV), or produced by a transfection method and purified by hydroxyapatite, Q Sepharose, and Sephacryl S-300 column chromatography (TFX). Mock, mock-infected cells; Ad5, cells infected with type 5 adenovirus only; rAAV 1K, 10K, or 100K, cells transfected with Ad5 and rAAV1-CB-hAAT at a multiplicity of infection of 1,000, 10,000, or 100,000, respectively. Values represent the mean $\pm$ SEM concentration of hAAT in the culture supernatant. Serum hAAT concentrations were significantly higher in animals receiving the HSV-produced vector than in mice receiving the TFX-produced vector (*F*-value 65.16, *df*=1,29, $P < 0.0001$).

FIG. 2. Silver-stained SDS-PAGE analysis of rAAV1-CB-hAAT. Lane 1, molecular weight markers; lane 2, HSV-produced vector purified by CIM Q and AVB column chromatography; lane 3, TFX-produced vector purified by hydroxyapatite, Q Sepharose, and Sephacryl S-300 column chromatography; lane 4, HSV-produced vector purified by CIM Q and AVB columns followed by CsCl gradient centrifugation. A total of 1×10^{10} vg was added to lanes 2, 3, and 4.

ticles with attenuated central electron density are indicated by the arrows in Fig. 3. The mean $\pm$ SD proportion of empty capsids was $2.45\% \pm 0.24\%$ for the HSV-produced vector and $12.18\% \pm 2.16\%$ for the TFX-produced vector ($\chi^2 = 87.19$, 1 *df*, $P < 0.0001$).

Toxicology study results

IM injections of both vectors were well tolerated in all animals, with no apparent local reactions observed clinically. Two male and two female mice died or were euthanized during the study due to non-test article-related causes (severe dermatitis unresponsive to treatment in two females and trauma from submandibular bleeds or found dead the morning after injection in one each). There were no test article-related clinical findings. Skin lesions (dermatitis with alopecia) occurred in all groups including the control animals (no dose-response effect was seen) and were more common in females than in males.

There were no significant differences in mean body weights between test article and control groups at any time point for males. For females, group mean body weights were lower than those of the control group at week 8 in the TFX group, at weeks 7 and 9 in the lower-dose HSV group, and at weeks 5–8 in the high-dose HSV group (Fig. 4). In all instances where group mean body weights were lower, values were between 92.4% and 95.3% of the corresponding control value.

Vector administration had no adverse effect on hematology or serum chemistry parameters, and there were no gross pathology findings. For the nine organs that were weighed at necropsy, there were several instances of statistically significant differences in organ weight, organ-to-body weight or organ-to-brain weight ratios in one or more treatment groups compared with the control group, but none of these weight differences was associated with histological changes in the relevant organs.

FIG. 3. Electron micrographs of HSV-produced rAAV1-CB-hAAT purified by CIM Q and AVB columns (*left*) and TFX-produced rAAV1-CB-hAAT purified by hydroxyapatite, Q Sepharose, and Sephacryl S-300 column chromatography (*right*). The arrows point to examples of capsids with electron-dense centers (empty capsids), which are more common with the TFX-produced vector than with the HSV-produced vector.

FIG. 4. Group mean body weights of C57BL/6 mice after administration of rAAV1-CB-hAAT vector or placebo. TFX, transfection-produced vector; HSV, recombinant herpes simplex virus-produced vector; LD, 3×10^{11} vg/mouse; HD, 2×10^{12} vg/mouse.

Histopathological findings

Microscopic findings associated with exposure to vector were limited to the gastrocnemius and quadriceps femoris muscles injected with vector and the draining lymph nodes. No other test article-related effects were detected in any of the other tissues examined.

Histological lesions related to test article exposure were identified in animals from all vector-treated groups at all three sacrifice time points, and consisted primarily of mononuclear cell inflammation and muscle cell degeneration, regeneration, and vacuolization (Fig. 5). Comparison of lesion scores for the inflammatory and degenerative response in the skeletal muscles of the mice injected with low or high doses of the HSV-produced vector demonstrated: (1) a dose response to the amount of vector injected; (2) a diminishment in the inflammatory and degenerative response over time; and (3) a more pronounced inflammatory and degenerative response in the female mice compared with male mice at 21 days post injection (Table 2).

At 21 days post injection, the inflammatory and degenerative responses in male and female mice injected with the

lower dose of HSV-produced vector were slightly greater than in mice injected with the TFX-produced vector, but difference between these two groups in the amount of inflammation and degeneration were no longer apparent by 60 days post injection.

The myofiber vacuolization in mice exposed to HSV-produced vector also showed a dose response at day 21 post injection, was greater in females than males at 21 days post injection, and diminished over time. In contrast, myofiber vacuolization was not apparent in mice exposed to TFX-produced vector at 21 days post injection but was apparent at day 60 and increased in intensity at day 90.

Serum hAAT concentrations

Dose- and sex-dependent expression of hAAT was observed in mice administered the HSV-produced vector (Table 3). The vector dose was 6.7-fold higher in the high-dose HSV group than the low-dose HSV group. In females, serum hAAT levels appeared to increase proportional to the increased dose (serum hAAT levels were 4.8- to 8.5-fold higher in the high-dose HSV group compared with the

FIG. 5. Histological appearance of muscles after injection of rAAV1-CB-hAAT. The tissue is from the day 21 sacrifice of a female injected with the lower dose of HSV-produced vector. (A) Segmental myofiber degeneration and necrosis with regeneration. The large arrow points to an individual myofiber with segmental degeneration and necrosis and associated lymphocytic, histiocytic, and neutrophilic inflammation. The small arrow points to a focus of myofiber regeneration. (B) Myofiber vacuolization. Myofibers below the line contain peripheral vacuoles (small arrow).

TABLE 2. MEAN SEVERITY SCORES^a OF INJECTION SITE LESIONS IN C57BL/6 MICE AFTER INJECTION OF rAAV1-CB-hAAT

Group	Placebo				TFX ^b 1.2×10 ¹³ vg/kg				HSV ^b 1.2×10 ¹³ vg/kg				HSV ^b 8×10 ¹³ vg/kg			
	R	M	D	V	R	M	D	V	R	M	D	V	R	M	D	V
Males																
Day 21	0	0	0	0	0.4	0.5	0.2	0	0.7	1.5	1.0	0.9	1.5	1.9	1.8	2.2
Day 60	0	0.1	0	0	0.6	1.2	0.4	1.1	1.0	1.2	0.2	0.9	1.5	1.9	0.7	0.7
Day 90	0	0	0	0	0.9	1.0	0.4	1.2	0.7	0.7	0.2	1.0	1.2	1.4	0.9	0.6
Females																
Day 21	0	0.1	0.1	0	0.2	0.7	0.3	0	1.2	1.6	1.5	1.3	1.7	2.0	2.0	2.5
Day 60	0	0	0	0	0.3	1.0	0.2	0.5	0.9	1.3	0.2	1.2	1.3	2.2	0.8	0.8
Day 90	0	0.1	0.1	0	0.9	1.1	0.8	1.4	0.9	1.0	0.5	0.7	1.2	1.3	0.4	0.3

^aSeverity score scale: 0, none; 1, minimal; 2, mild; 3, moderate; 4, severe.

^bTFX, transfection-produced rAAV1-CB-hAAT; HSV, recombinant herpes simplex-produced vector.

^cR, regeneration; M, mononuclear cell inflammation; D, degeneration; V, vacuolization.

low-dose HSV group at the three time points tested). In males, the increase in serum hAAT levels was more than dose-proportional (serum hAAT levels were 15- to 33-fold higher in the high-dose HSV group compared with the low-dose HSV group at the three time points tested). There was also a modest sex-dependent effect on hAAT expression at the 1.2×10¹³ vg/kg dose of TFX-produced or HSV-produced vector; at the three time points tested, serum hAAT levels were 1.2- to 2.2-fold higher in males than in females in the TFX group and 1.0- to 1.9-fold higher in males than in females in the low-dose HSV group.

At equivalent dosage levels of 3×10¹¹ vg (1.2×10¹³ vg/kg), hAAT expression was higher in animals receiving HSV-produced vector than in animals receiving TFX-produced vector. At the three time points tested, serum hAAT levels were 1.2- to 2.9-fold higher in the low-dose HSV group than in the TFX group in males and 1.4- to 2.7-fold higher in the low-dose HSV group than in the TFX group in females. These differences were statistically significant at days 21 and 60 in females and day 60 in males. Serum hAAT levels were also significantly higher in the high-dose HSV groups compared with the low-dose HSV groups at all time points.

TABLE 3. SERUM hAAT CONCENTRATIONS AFTER INJECTION OF rAAV1-CB-hAAT IN C57BL/6 MICE

Sex	Day	Serum hAAT concentration (μM, mean ± SD) Dosage level (vg/kg)		
		TFX 1.2×10 ¹³	HSV 1.2×10 ¹³	HSV 8×10 ¹³
Male	21	1.08 ± 1.57	1.08 ± 0.86	35.95 ± 9.18 ^b
	60	0.92 ± 0.44	2.65 ± 1.71 ^a	39.88 ± 8.95 ^b
	90	0.70 ± 0.25	1.51 ± 0.37	27.77 ± 5.60 ^b
Female	21	0.40 ± 0.25	1.08 ± 0.21 ^a	9.24 ± 2.34 ^b
	60	0.59 ± 0.14	1.40 ± 0.34 ^a	6.76 ± 0.52 ^b
	90	0.56 ± 0.03	0.80 ± 0.21	4.40 ± 1.27 ^b

^aSignificantly different from TFX group, *p* < 0.01.

^bSignificantly different from low-dose HSV group, *p* < 0.01.

hAAT, human alpha-1 anti-trypsin; HSV, recombinant herpes simplex-produced vector; SD, standard deviation; TFX, transfection-produced vector; vg, vector genomes.

Vector concentrations in blood

In all vector-treated groups, vector copy number in blood was highest 24 hr after dosing and declined thereafter, with no detectable copies present in any vector-treated group 90 days after dosing (Fig. 6). Vector copy numbers were generally higher in females than in males, and were significantly higher in low-dose HSV females compared with TFX females on days 1 and 21, in high-dose HSV females compared with low-dose HSV females at days 1, 21, and 60, and in high-dose HSV males compared with low-dose HSV males at days 21 and 60.

Antibodies to hAAT and HSV

Antibodies to hAAT were detected in all three vector-treated groups (Table 4). In the TFX and low-dose HSV groups, the average anti-hAAT antibody titer was highest at day 21 and decreased at day 60 and day 90. The average anti-hAAT antibody titer was lower in the high-dose HSV group than in the other vector-treated groups and was similar at all three time points.

Antibodies to HSV were detected almost exclusively in the high-dose HSV group (Table 4). In the groups that received placebo or vector made by transfection, no anti-HSV antibodies were detected except for a low level in one placebo animal at day 60 and one TFX animal at day 90. In the low-dose HSV group, anti-HSV antibodies were detected in one female at day 60, two females at day 90, and none of the males at any time point. In the high-dose HSV group, anti-HSV antibodies were detected in none of the animals at day 21, in three of five males and four of four females at day 60, and in three of five males and five of five females at day 90. The average anti-HSV antibody concentration in the high-dose HSV group did not increase between day 60 and day 90.

Discussion

AAT deficiency is caused by mutations in the SERPIN1A gene. The mutation phenotype is designated by its protease inhibitor (PI*) type, with normal AAT designated PI*M. More than 95% of clinical AAT deficiency is due to homozygosity for the PI*Z allele (Glu342Lys) (Crystal *et al.*, 1989).

FIG. 6. Biodistribution of rAAV1-CB-hAAT DNA in blood of C57BL/6 mice. TFX, transfection-produced vector; HSV, recombinant herpes simplex virus-produced vector; LD, 3×10^{11} vg/mouse; HD, 2×10^{12} vg/mouse; LOQ, lower limit of quantitation. Each symbol represents the value for an individual mouse.

The range of serum AAT concentrations is 20 to $53 \mu\text{M}$ in normal individuals, 3 to $7 \mu\text{M}$ in patients with the ZZ phenotype, and 10 to $23 \mu\text{M}$ in patients with the SZ phenotype (Brantly *et al.*, 1991). Epidemiological data indicate that patients with the ZZ phenotype but not the SZ phenotype are at increased risk of developing pulmonary emphysema; achieving trough serum AAT concentrations $>11 \mu\text{M}$ ($572 \mu\text{g/ml}$) with weekly intravenous infusions of AAT protein purified from normal plasma donors has been the basis for approval of several products used for augmentation therapy in AAT-deficient patients.

The high serum AAT concentration required to achieve a therapeutic benefit is a major challenge in the development of a gene therapy product for treatment of AAT deficiency. To address this challenge, we have evaluated an rAAV-hAAT vector produced using a recombinant HSV complementation system and an improved column chromatography purification process that achieves much higher yields than previous TFX methods. When evaluated by *in vitro* assays and compared with TFX-produced vector, the HSV-produced vector had favorable characteristics in terms of purity assessed by silver-stained SDS-PAGE, efficiency of transduction and hAAT expression, and the ratio of full to empty AAV particles in electron micrographs. Compared

with the TFX-produced vector, the proportion of empty particles in the HSV-produced vector was lower as assessed by either electron microscopy (2.45% vs. 12.18%) or densitometry of silver-stained bands in SDS-PAGE (24% vs. 34% more VP3 compared with CsCl-purified vector). Both of these methods provide an imprecise estimate of the proportion of empty particles, and neither can fully explain the $>100\%$ increase in transgene expression in animals injected with the HSV-produced vector, which suggests that the HSV-produced vector is more infectious or less susceptible to degradation after infection than the TFX-produced vector. Although results in rodents do not necessarily translate to results in humans, if a similar twofold increase in transgene expression levels with the HSV-produced vector compared with the TFX-produced vector were to occur in humans, this might allow for a reduction in the vector dosage required to achieve a therapeutic effect. In addition, the marked increase in vector yield with the HSV-based production method will enable administration of much higher total dosages to patients with AAT deficiency.

Although improved overall yields of rAAV production using the HSV complementation system have been reported previously, it was important to ask if there are any differences in the safety profile of HSV-produced vector compared

TABLE 4. ANTIBODIES TO hAAT AND HSV IN C57BL/6 MICE AFTER INJECTION OF rAAV1-CB-hAAT

Antigen	Number positive/number tested (mean value $\pm$ SD)			
	Placebo	TFX	Low HSV	High HSV
hAAT				
Day 21	0/10	8/10 (16.6 ± 12.6)	10/10 (15.1 ± 10.1)	8/10 (3.0 ± 2.2)
Day 60	0/10	9/10 (5.3 ± 3.5)	10/10 (3.6 ± 3.3)	6/9 (4.0 ± 2.8)
Day 90	0/9	10/10 (2.7 ± 1.8)	10/10 (1.7 ± 1.1)	7/10 (2.5 ± 2.1)
HSV				
Day 21	0/10	0/10	0/10	0/10
Day 60	1/10 (1.0)	0/10	1/10 (0.13)	7/9 (29.6 ± 40.0)
Day 90	0/9	1/10 (1.7)	2/10 (5.2 ± 3.5)	8/10 (23.1 ± 28.2)

hAAT, human alpha-1 antitrypsin; HSV, herpes simplex virus; TFX, transfection-produced vector at 1.2×10^{13} vg/kg; Low HSV, HSV-produced vector at 1.2×10^{13} vg/kg; High HSV, HSV-produced vector at 8×10^{13} vg/kg.

Antibody values are in arbitrary units per milliliter compared with a reference standard assigned a value of 1 U/ml.

with TFX-produced vector. Results from the current bridging toxicology study support the safety of rAAV1-CB-hAAT produced using the HSV complementation method when injected IM in mice. There were no local or systemic clinically apparent adverse effects, no adverse effects on hematology or clinical chemistry parameters, and no gross pathology findings. Histological findings at the injection site were minimal to moderate, decreased over time, and their frequency was proportional to hAAT expression as measured by serum hAAT levels. Similar dose-dependent histological changes at the injection site were seen in a previous study in mice injected IM with TFX-produced rAAV1-CB-hAAT (Flotte *et al.*, 2007).

At a dose of 1.2×10^{13} vg/kg, serum hAAT concentrations were higher in mice injected with the HSV-produced vector than in mice injected with the TFX-produced vector. These results are consistent with the higher efficiency of transduction and hAAT expression observed in the *in vitro* assays. Higher serum hAAT concentrations were also observed in male mice compared with female mice, especially with the high-dose HSV-produced vector. Sex differences were not observed in a previous toxicology study of rAAV1-CB-hAAT administered by IM injection (Flotte *et al.*, 2007), but a male sex advantage has been noted after portal vein delivery of rAAV2 and rAAV5 vectors in murine studies of both factor IX and phenylalanine hydroxylase gene transfer (Davidoff *et al.*, 2003; Mochizuki *et al.*, 2004). It is possible that the higher serum hAAT levels in male mice in the present study are due to leakage of the vector to the systemic circulation and liver-derived expression due to the use of the CB promoter.

Initial studies with an rAAV vector expressing hAAT packaged in serotype 2 capsid and injected IM in mice indicated that high levels of antibody to hAAT developed in BALB/c mice and appeared to abrogate hAAT expression, whereas immunocompromised nude mice and C57BL/6 mice did not appear to develop antibodies to hAAT, and sustained expression of hAAT was detected in serum from these latter mice (Song *et al.*, 1998). Subsequent studies in C57BL/6 mice reported that IM injection of an rAAV-hAAT vector packaged in serotype 1 capsid resulted in a marked increase in serum hAAT concentrations compared with the same vector packaged in serotype 2 capsid, and was associated with low but detectable levels of anti-hAAT antibodies (Lu *et al.*, 2006). In the present study, serum hAAT concentrations tended to decrease with time after dosing, concurrent with development of antibodies to hAAT. Preliminary studies have identified AAT:anti-AAT immune complexes in the serum of C57BL/6 mice injected with rAAV1-CB-hAAT, and dissociation of these immune complexes *in vitro* during measurement of serum hAAT concentration by ELISA results in a higher measured AAT concentration (G. Ye, unpublished observations). In contrast, antibodies to hAAT have not been reported among the thousands of patients with AAT deficiency who have received weekly intravenous infusions of purified AAT protein. Thus, results in mice injected with rAAV1-CB-hAAT may underestimate the serum AAT concentrations that will be achieved in patients injected with this vector.

The process used for purification of HSV-produced rAAV1-CB-hAAT is highly effective in removing HSV proteins, and a low-level residual HSV protein (20 ng/ml at the

dilution injected) was present in the batch of vector used in the toxicology study. The residual HSV protein is not detectable by immunoblotting (G. Ye, unpublished observations) and is assumed to consist of very small amounts of many different HSV proteins present in HSV-infected cells. This low level of HSV protein appeared to be sufficient to induce low levels of anti-HSV antibodies in mice that received high doses of the HSV-produced vector. It is not known if the lower doses of HSV-produced vector that will be used in clinical trials will result in either an increase in anti-HSV antibodies in the approximately 80% of persons >40 years of age who have preexisting antibodies to HSV (Xu *et al.*, 2006) or development of anti-HSV antibodies in those who have not been previously infected with HSV. It is unlikely that immune responses to small amounts of HSV protein will have any deleterious effect, because in HSV vaccine studies much larger amounts of HSV proteins combined with strong adjuvants have been administered by IM injection to large numbers of healthy volunteers with no important adverse effects (Corey *et al.*, 1999; Stanberry *et al.*, 2002; Bernstein *et al.*, 2005).

In summary, results of the preclinical evaluation of rAAV1-CB-hAAT produced using an rHSV complementation system support the planned clinical evaluation of this product.

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Author Disclosure Statement

J.D.C., G.Y., D.L.T., and D.R.K. are employees of Applied Genetic Technologies Corporation, which has a proprietary interest in the product described in this article. None of the other authors has a competing financial interest.

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Address correspondence to:

Dr. Jeffrey D. Chulay
Applied Genetic Technologies Corporation
11801 Research Drive
Alachua, FL 32615

E-mail: jchulay@agtc.com

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