

Sustained Alpha-Sarcoglycan Gene Expression after Gene Transfer in Limb-Girdle Muscular Dystrophy, Type 2D

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Objective: The aim of this study was to attain long-lasting alpha-sarcoglycan gene expression in limb-girdle muscular dystrophy, type 2D (LGMD2D) subjects mediated by adeno-associated virus (AAV) gene transfer under control of a muscle specific promoter (tMCK).

Methods: rAAV1.tMCK.hSGCA (3.25×10^{11} vector genomes) was delivered to the extensor digitorum brevis muscle of 3 subjects with documented SGCA mutations via a double-blind, randomized, placebo controlled trial. Control sides received saline. The blind was not broken until the study was completed at 6 months and all results were reported to the oversight committee.

Results: Persistent alpha-sarcoglycan gene expression was achieved for 6 months in 2 of 3 LGMD2D subjects. Markers for muscle fiber transduction other than alpha-sarcoglycan included expression of major histocompatibility complex I, increase in muscle fiber size, and restoration of the full sarcoglycan complex. Mononuclear inflammatory cells recruited to the site of gene transfer appeared to undergo programmed cell death, demonstrated by terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick-end labeling and caspase-3 staining. A patient failing gene transfer demonstrated an early rise in neutralizing antibody titers and T-cell immunity to AAV, validated by enzyme-linked immunospot on the second day after gene injection. This was in clear distinction to other participants with satisfactory gene expression.

Interpretation: The findings of this gene replacement study in LGMD2D subjects have important implications not previously demonstrated in muscular dystrophy. Long-term, sustainable gene expression of alpha-sarcoglycan was observed following gene transfer mediated by AAV. The merit of a muscle-specific tMCK promoter, not previously used in a clinical trial, was evident, and the potential for reversal of disease was displayed.

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Successful gene therapy for muscle disease will require sustained gene expression using tissue specific regulatory elements resulting in therapeutic proteins expressed at sufficient levels to improve function.¹ In this early

phase in the evolution of gene therapy for muscle disease, it would be advantageous to assess gene expression at the site of gene transfer in a single patient at sequential time points. This would require consecutive biopsies of the

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This study has been registered at Clinicaltrials.gov as NCT00494195.

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same muscle over a designated time period. This multiple biopsy regimen is clinically impractical and subject to reduced expression levels as vector genomes are lost from the site of gene transfer by prior removal of transduced muscle fibers. In lieu of this approach and to maximize interpretation, we have examined gene expression levels at different time points in 6 different limb-girdle muscular dystrophy, type 2D (LGMD2D), alpha-sarcoglycan (α SG)-deficient patients receiving intramuscular gene transfer of the full length α SG cDNA under control of a truncated muscle creatine kinase promoter (tMCK).^{2,3} In 3 patients previously reported,⁴ post gene transfer muscle biopsies were examined between 6 weeks and 3 months. This report extends the period of observation after gene transfer for 6 months in 3 additional patients and tests the capacity of the tMCK promoter to sustain long-term gene expression. There is currently no effective treatment of LGMD2D, a disease that varies in severity depending on levels of α SG protein expression; at 1 end of the spectrum patients suffer from ambulatory loss between 12 and 15 years of age, whereas others maintain walking until the third to fourth decade.^{5,6}

It would be misleading to present the gene transfer findings of the first 3 of 6 LGMD2D patients as new information in this report, because the results of these cases have been published.⁷ However, summarizing the data from these patients does provide an overall perspective by which to appreciate the findings of the entire cohort. In the first 3 LGMD2D cases, the full-length human α -SG gene (hSGCA) was transferred to the extensor digitorum brevis (EDB) muscle using ultrasound guidance and electromyographic monitoring. The hSGCA dose was 3.25×10^{11} vector genomes (vg) delivered in 1.5ml. Two patients (Cases 1 and 3) had follow-up muscle biopsies at 6 weeks, and the third underwent muscle biopsy at 3 months. Analysis (performed blinded to side of gene transfer) revealed transduction of 57% of fibers in Subject 1, 69% in Subject 2, and 62% in Subject 3. Sections from these same blocks taken for Western blots showed a 4- to 5-fold increase in all 3 cases. Two signs of muscle repair included restoration of the full sarcoglycan complex on the side of gene transfer in all cases and an increase in muscle fiber size compared to the control side receiving only vector diluent in 1 case (Subject 3). A potentially important finding in all 3 cases was the expression of major histocompatibility complex (MHC) class I molecules on the sarcolemma of virtually every muscle fiber on the side of upregulated gene expression, contrasting sharply with the lack of expression on the control side. MHC class II expression was not observed. CD4- and CD8-positive infiltrates tended to be focal (so total numbers were not significantly increased), but sug-

gested recruitment to the side of gene transfer. Interferon (IFN)- γ enzyme-linked immunospots (ELISpots) showed a minimal but definite response to a specific adeno-associated virus (AAV) capsid pool in 1 of 3 cases at days 14 and 43, suggesting a transient T-cell-mediated immune response to capsid pool 2 of AAV1. There was no evidence of an immune response to newly expressed α -SG peptides.

The results of the first 3 cases provided a template on which to move forward. Although dose escalation had been originally proposed in the IND, the findings in the first cohort precluded any need to change the regimen, and the final 3 cases received the same amount of vector, to the same muscle, providing an unequivocal basis for comparison at a later time point. The fundamental question to be addressed in Cohort 2 was whether gene expression could be maintained at a robust level for an additional 3 months (total of 6 months) using the tMCK promoter. Additionally, we sought to ascertain the potential consequences of universally expressed MHC class I on virtually every muscle fiber on the side of gene transfer and whether a focal cellular inflammatory infiltrate was poised to attack transduced muscle fibers. Perhaps a somewhat more difficult question to address, but a question of interest would be the increase in muscle fiber size at the 3-month time point and whether this was a chance occurrence or a result of sustained gene expression.

Subjects and Methods

Study Subjects

Subject eligibility included proof of *SGCA* mutations of both alleles, ability to cooperate for testing, willingness to practice contraception during the study (if appropriate), negative pregnancy test (for females), and no evidence of cardiomyopathy, diabetes, or organ system abnormalities of bone marrow, liver, or kidney. Human immunodeficiency virus infection, hepatitis A, B, or C, and known autoimmune diseases were exclusion criteria. Institutional review board (IRB)-approved consent forms were obtained by the principal investigator (J.R.M.) and signed by subjects. Taking immunosuppressive drugs or glucocorticoids during the trial was prohibited, and patients were required to be off treatment for 3 months prior to enrollment.

Novel *SGCA* Mutations

Variations within the *SGCA* coding sequence were detected in all 6 patients by direct sequencing analysis of the entire coding region. In 2 of the patients, previously unreported variants were described. The results of our studies suggest probable pathogenicity of these mutations.

SUBJECT 6. The c.434C>A mutation results in an amino acid substitution at a highly conserved residue (p.A145E), and ablates an AciI restriction endonuclease site. Pathogenicity was assessed by reducing the possibility that this was a rare

polymorphism rather than a disease-causing allele. DNA from the patient and from 96 wild-type controls (CEPH parent sets) were polymerase chain reaction (PCR)-amplified using a forward primer in exon 5 (5'-CCCTGCTGCCATACCAAG-3') and a reverse primer at the junction of exon 5 and intron 5 (5'-GCACACCTACCCTTCTTTTCG-3'), resulting in a 206bp fragment. Restriction digest was performed with *AclI* (New England Biolabs, Beverly, MA) under the following conditions: 6.5 μ L H₂O, 1.5 μ L 10 \times NEBuffer 3, 1 μ L restriction enzyme, 6 μ L PCR product. Samples were incubated at 37°C for 1 hour and heat inactivated at 65°C for 20 minutes. Expected fragment sizes were 129 and 77 bp for the patient allele and 129, 45, and 32 bp for the wild-type allele. Digestion fragments were analyzed via gel electrophoresis on a 4% GenePure Sieve agarose (ISC Bioexpress, Kaysville, UT) in 1 \times tris-boric acid-ethylenediaminetetraacetic acid stained with ethidium bromide. Consistent with pathogenicity, the c.434C>A allele was detected only in the patient and not in the 192 control chromosomes.

SUBJECT 5. Sequencing from lymphocyte-derived genomic DNA samples in each parent of Patient 5 revealed that each carried 1 of the putative mutations. Using an archived muscle biopsy specimen from the patient, mRNA was extracted from forty 10 μ m sections using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's recommended protocol. To assess the splicing implications of the c.956+2_956+19del mutation, gene-specific reverse transcriptase (RT) PCR was performed using the SuperScript One-step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen), with a forward primer within exon 6 (5'-GCACCCCACTTCCGCGTTGAC-3') and a reverse primer within exon 9 (5'-GAATGAGGGGCACCTGGGCG-3'). The 5' untranslated region (UTR) deletion detected in genomic DNA was confirmed by gene-specific RT-PCR using a forward primer 5' of the canonical UTR (5'-AGGTGACTGGAAGGTTGCTG-3') and a reverse primer within the coding region of exon 1 (5'-CAGAAGAGGGATCTGGGTTG-3'). In each case, amplicons were gel-purified and TA cloned into the pCR 2.1TOPO vector; clones were subsequently sequenced using ABI dye terminator sequencing technologies on an ABI 3130xl sequencer.

With amplification of exons 6 through 9, several species of mRNA were detected that confirm the pathogenicity of the c.956+2_956+19del mutation. Of the 2 most abundant, 1 contained a deletion of exon 7, resulting in an out-of-frame transcript (r.748_956del), whereas the second contained the last 79 nucleotides from intron 6 and the first 46 nucleotides from exon 7 as a pseudoexon between exons 6 and 8 (r.747_957 ins748-79_793). The c.-205_-37del, in contrast, ablates most of the SGCA promoter (including the putative transcriptional start site at c.-175) as predicted by neural network algorithms⁸ and is thus predicted to result in a null allele.

Immune Responses

Subjects were prescreened for serum neutralizing antibodies to AAV1 according to a previously published protocol,⁹ with follow-up titers on post gene transfer days 2, 7, 14, 28, 42, 60,

90, 120, 150, and 180. In addition, an AAV particle binding enzyme-linked immunoassay (ELISA) assay, similar to that previously published by our group,⁹ used serum obtained pretreatment coinciding with neutralizing antibody titers. For this assay, the AAV1 antigen coating protocol was modified to use 2 \times 10⁹ vg particles per 96 wells (100 μ l added per well of 2 \times 10¹⁰ AAV1 particles/ml in carbonate coating buffer). Prescreening and follow-up studies to identify potential T-cell immunity were done on these same days, with continued follow-up for 2 years. This was assessed using an IFN- γ ELISpot assay according to previously described methods.¹⁰⁻¹² Antigens for the ELISpot assay included 3 AAV1 capsid peptide pools and the full α -SG protein.^{7,11,12}

Vector Production

rAAV1.tMCK.hSGCA was produced at the Harvard Gene Therapy Initiative according to current good manufacturing practices. Vector production followed previously published methods using plasmid DNA tritransfection of HEK293 cells, followed by iodixanol and anion exchange column chromatography purification.¹³ The vector was formulated in sterile phosphate-buffered saline, and passed all quality control acceptance criteria established by the US Food and Drug Administration (FDA) for strength, identity, and purity.

Efficacy Evaluation

Efficacy was evaluated by blinded analyses of muscle tissue assessing α -SG gene expression by immune stains of muscle sections and Western blot analysis, with quantification assessed by densitometry with comparisons between sides. This included 4 of 6 blocks in each case, with limitations imposed by spread of vector related to connective tissue barriers in the muscle and by the directional planes of injection. Bioquant image analysis software (Bioquant Image Analysis Corporation, Nashville, TN) was used for quantitative image analysis. Muscle cross sections (12 μ m) were subdivided into series of 4 random \times 10 images. To ensure reliable measurements, staining of the sections and recording of all images were performed during 1 session with the use of fixed exposure settings and the avoidance of pixel saturation. The lower-intensity threshold was set at background using a LGMD2D section stained with Alexa Fluor 594 goat antimouse antibody only, and positive fluorescence was quantified for each section (area percentage). The total α -SG fluorescent signal (area percentage) was reported as a ratio to the normal control section, which was set at 100.

The results of the gene expression findings were presented to our oversight Data Safety Monitoring Board at the National Institutes of Health in a written report before the blind was broken. MHC I and II antigens (Dako, Carpinteria, CA) were assessed on muscle sections. CD4+ and CD8+ (BD Biosciences, San Jose, CA) mononuclear cells were reported as number/mm² area. Muscle morphometrics included fiber size histograms.

Statistical analyses were based on differences between the sides in the total number of cells per square millimeter of area expressing CD4+ and CD8+ mononuclear cells, MHC I and II antigens, and muscle fiber size using a paired *t* test (*p* < 0.05).

TABLE 1: Mutations in the SGCA Gene in 6 Subjects

| Patient # | Age/Onset | Gender | cDNA | Protein | Days between Gene Transfer and Biopsy |
|-----------|--------------------|--------|---|---|---------------------------------------|
| 1 | 14/9 ^a | M | c.229C>T (homozygous) | p.Arg R77Cys | 43 |
| 2 | 11/3 ^a | M | c.371T>C (homozygous) | p.Ile124Thr | 92 |
| 3 | 14/8 ^a | M | (i) c.371T>C | (i) p.Ile124Thr | 50 |
| | | | (ii) 409G>A | (ii) p.Glu137Lys | |
| 4 | 43/10 ^b | F | (i) c.229C>T | (i) p.Arg77Cys | 183 |
| | | | (ii) c.739G>A | (ii) p.Val246Met | |
| 5 | 34/10 ^a | F | (i) c.956+2_956+19del ^c <i>r.[748_956del,747_957 ins748-79_793]</i> | (i) p.[Val250AlafsX50]+ [Val250SerfsX22] | 183 |
| | | | (ii) c.-205_-37del ^c <i>r.0?</i> | (ii) p.0? | |
| 6 | 23/10 ^a | M | (i) c.100C>T | (i) p.R34C | 176 |
| | | | (ii) c.434C>A ^c | (ii) p.A145E | |

Patients 1 to 3 were previously reported⁷ (referred to as Cohort 1 in text). Patients 4 to 6 have not previously been reported and are referred to as Cohort 2. Three previously unreported mutations were detected as indicated. In Patient 5, 2 previously unreported mutations were detected; the effect of the first 1 listed on mRNA splicing was determined by mRNA transcript analysis from skeletal muscle, and is listed in italics. For further discussion, see text.

Age refers to age at time of gene transfer. Onset refers to age of onset of symptoms

^aCurrently wheelchair dependent.

^bCurrently ambulatory.

^cPreviously undetected mutation.

Results

Study Design

This was a, double-blind, randomized controlled trial of rAAV1 containing the full length human SGCA under control of the tMCK promoter (rAAV1.tMCK.hSGCA) injected into the EDB muscle of LGMD2D patients.⁷ Table 1 summarizes the mutations, age, gender, and disease severity of the current 3 patients enrolled in Cohort 2 (Patients 4, 5, and 6) and provides comparative information to Cohort 1 (Patients 1, 2, and 3, previously reported⁷). The older age of patients in Cohort 2 was incidental to those fulfilling IRB approved criteria for enrollment. The study was approved by the Recombinant DNA Advisory Committee (#0610-815; October 31, 2006) and the FDA (IND# is BB-IND 13434).

Gene transfer was performed in the intensive care unit at Nationwide Children's Hospital. Approximately 4 hours before gene transfer, subjects received a dose of intravenous methylprednisolone, 2 mg/kg (not to exceed 1g total). At the time of the procedure, investigators received labeled syringes (left and right) on ice containing either viral vector or phosphate-buffered saline from the pharmacy. Injection sides were determined by a computer-generated random numbers sequence. Unblinding envelopes were held in the pharmacy. Before needle insertion into the muscle, the skin was anes-

thetized with 1% lidocaine. Gene delivery was guided by ultrasound and electromyographic recordings (37 mm Teca Myoject injection recording needle) to ensure that muscle was the destination of the delivered product. The picture of the gene delivery to EDB is shown in the report of Cohort 1.⁷ After injection, the empty syringes were resealed and stored at -20°C . Repeat doses of methylprednisolone were given again at 24 and 48 hours postinjection as an anti-inflammatory agent. We have unpublished data that support the use of glucocorticoids to enhance gene expression both in mice and nonhuman primates.

EDB muscles were removed bilaterally from patients at 6 months (Table 1 shows exact times of gene transfer and muscle biopsy) and processed for transgene expression and immune reaction to vector and newly expressed protein.¹²

Safety and Efficacy

Patients were monitored in the hospital for the first 24 hours after gene transfer, including hourly vital signs for 4 hours and then every 4 hours until discharge. Outpatient evaluations continued and included days 1, 2, 7, 14, 30, 60, 120, and 180, and at the end of years 1 and 2 (after the first month, outpatient visits could vary by up to 7 days). Photographs of the injection sites were

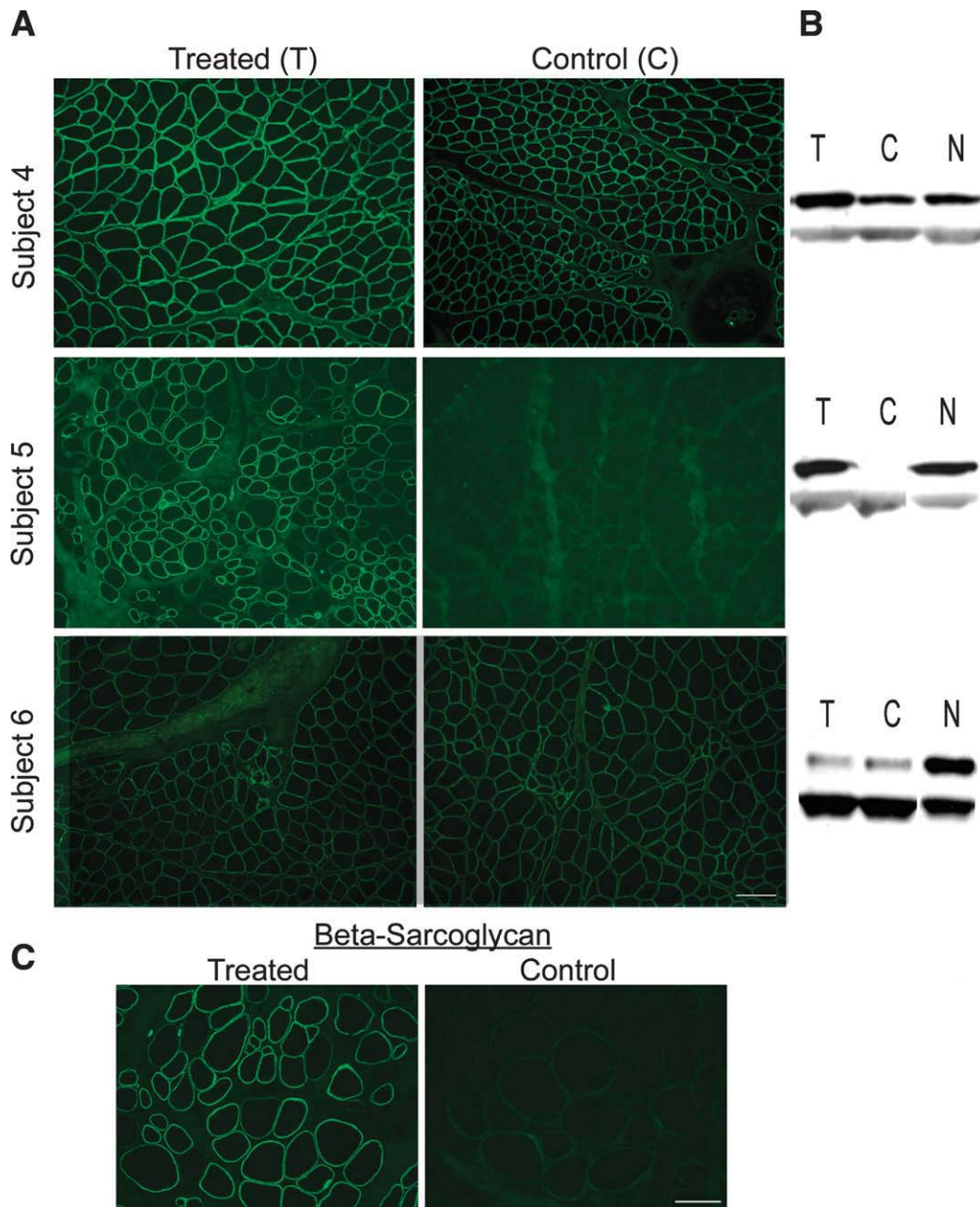


FIGURE 1: (A) Subjects 4 to 6: post gene transfer tissue sections from EDB muscles were stained with antibody to alpha-sarcoglycan (α SG). Subjects 4 and 5 showed increased staining on the treated side (T) compared to the control side (C). Subject 6 showed no difference in α -SG staining intensity between before and after gene transfer (findings verified by Bioquant Image Analysis) (scale bar = $150\mu\text{m}$). (B) Subjects 4 to 6: Western blots (WBs) show increased α -SG gene expression on the side of gene transfer compared to the contralateral side (treated on left, control on right) for Subjects 4 and 5; residual gene expression from mutant protein was prominently exhibited in Subject 4. Subject 6 showed no increase in α -SG expression between gels from before and after transfer. WBs normalized to actin (lower band) and each is compared with normal (N) muscle for comparison. (C) Subject 5: α -SG staining (Subject 5) demonstrates restoration on the side of gene transfer, with absent staining on the control side (scale bar = $100\mu\text{m}$). Other sarcoglycans (delta and gamma) were also restored (not shown).

taken immediately and 8 to 12 hours after gene transfer and at each follow-up visit. Patients complained of varying degrees of pain after EDB muscle biopsies that required narcotic analgesics for the first night after muscle biopsy. The degree of pain described by the 3 patients in Cohort 2 was unanticipated, based on experience in

the prior cohort, but should be noted by others performing the procedure.

No serious adverse events were encountered in any subject. Two had sore throats (3 weeks and 9 months after gene transfer); 1 was positive for streptococcal A and was treated with azithromycin by a primary care

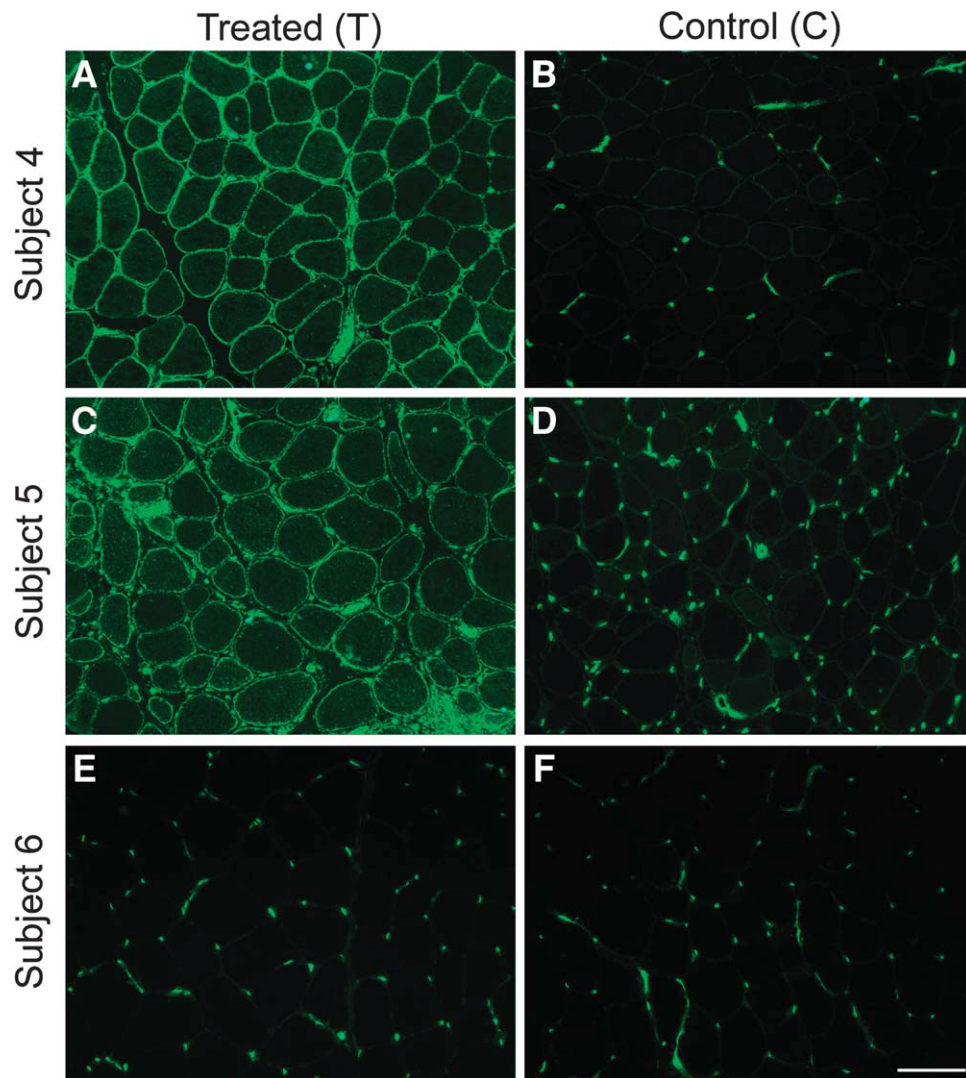


FIGURE 2: Major histocompatibility complex (MHC) I staining of sarcolemmal membrane of muscle sections on treated (T) and control (C) sides. Subjects 4 and 5 show staining on the treated (A, C) but not the control (B, D) sides. Subject 6 shows no MHC I staining on the treated side (E) or the control side (F). (scale bar = 100 μ m). Microvascular circulation is positive for MHC I on both sides in all subjects.

physician. A nagging concern in Subject 6 was an elevated white blood cell (WBC) count with normal differential that reached 21,700/mm³ on day 2 after gene transfer, 19,200/mm³ on day 7, and 20,100/mm³ on day 13. It then remained in normal range (<12,500/mm³) and suddenly increased transiently on day 200 (22,400/mm³). The patient was repeatedly checked for respiratory and urinary tract infections, lymphadenopathy, and organomegaly, and no cause was ever found. The AAV capsid T-cell response seen by the IFN- γ ELISpot assay (presented below) was the only finding that seemed to correlate with the elevated WBCs.

Gene Expression

Figure 1 shows findings of post gene transfer muscle tissue by immune stains and Western blots. Patient 4 (Subject 1

in Cohort 2) had the mildest phenotype of all 6 patients receiving gene transfer. α -SG expression was seen in the EDB on both sides, but gene expression increased by 2-fold on the side of gene transfer (right) as assessed by 2 separate methods, Bioquant Image Analysis and Western blots. Gene expression reached wild-type levels on the side of gene transfer. We also found an increase in mean muscle fiber diameter when comparing the control to the gene transfer side (untreated, 28.2 \pm 11.1 μ m; treated, 52.2 \pm 13.1 μ m). The side of gene transfer was validated by quantitative PCR using vector-specific primer probes that amplified a unique 5' untranslated leader sequence of the α -SG cassette on the right side only. hSGCA transgene copies increased by an average of 150-fold over baseline (representing 0.06 copies per nucleus) in the side of increased gene expression compared to the contralateral

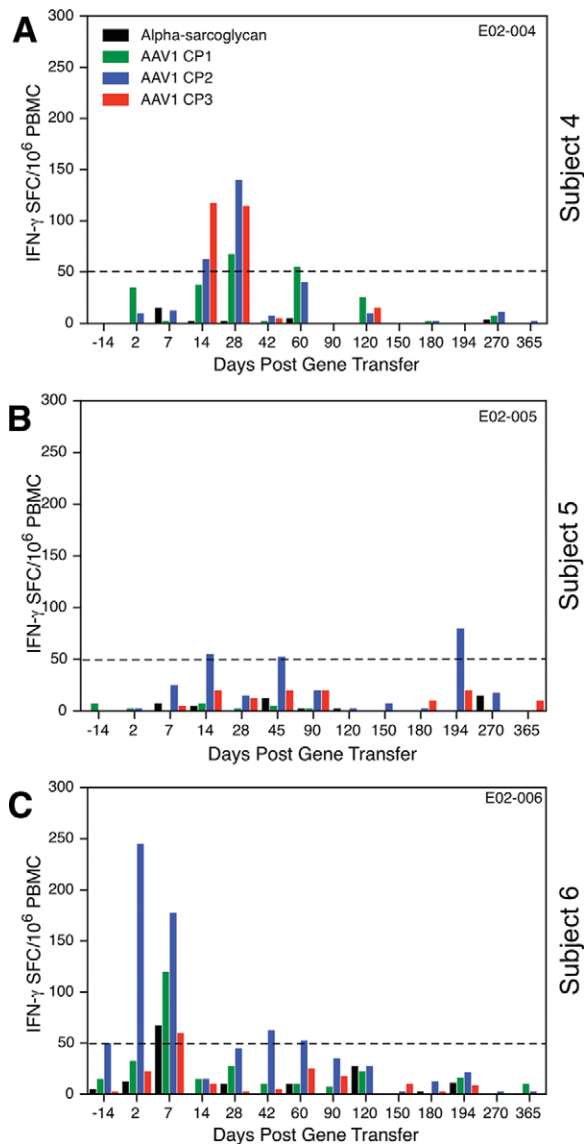


FIGURE 3: (A) Subject 4 (E02-004): α -SG stimulated interferon (IFN)- γ ELISpot assays showed no increase in spot forming colonies (SFC) per million peripheral blood mononuclear cells (PBMCs) at any time point (from 14 days before gene transfer to day 365). Adeno-associated virus (AAV)1 capsid peptide pool stimulation (CP2 blue and CP3 red) were positive for T-cell response (exceeded confidence limits of >50 spot forming cells/million PBMCs) at days 14 and 28. The response to AAV1 capsid pool 1 (CP1 green) was minimally positive at day 14. (B) Subject 5 (E02-005): α -SG and AAV1 capsid IFN- γ ELISpot assays were negative preceding and throughout the course of gene transfer up to day 365 with the exception of a slight reaction at day 194 to AAV1 capsid pool 2. (C) Subject 6 (E02-006): the α -SG-stimulated IFN- γ ELISpot assays (black) were consistently negative before and after gene transfer. However, this patient showed a prolific AAV1 capsid response as early as day 2 that was also present on day 7. This was distinctly earlier than other patients in Cohort 2 or in Cohort 1 (previously published⁷).

side. The RNase P gene was used as an internal control to normalize for genomic input and confirm the absence of PCR inhibitors in the sample DNA.

Patient 5 was wheelchair-dependent because of severe generalized weakness. There was little residual skeletal muscle α -SG providing clear differentiation between the control side treated with saline and the side of gene transfer (left). At 6 months after gene transfer, α -SG levels reached wild-type levels accompanied by full restoration of the sarcoglycan complex (see Fig 1). Muscle fibers ranged in size from 10 μ m to 50 μ m, without a clear difference between the sides. Quantitative PCR using an α -SG-specific primer set validated gene transfer to the left EDB. hSGCA transgene copies increased by an average of 1,000-fold over baseline (representing 0.64 copies per nucleus) in the side of increased gene expression compared to the contralateral side.

Patient 6 was the exception compared to all previous 5 patients participating in this clinical gene therapy trial. In this case, the 6-month EDB muscle biopsies showed low level gene expression on both sides, and the side of gene transfer (left) could not be differentiated by Bioquant Image analysis or Western blots (see Fig 1). There was also a very striking paucity of transgene copy numbers per nucleus that was 30-fold lower compared to Patient 5 and >3-fold lower compared to Patient 4 (0.02 copies per nucleus). A 1-time muscle biopsy makes it difficult to differentiate between loss of gene expression versus poor muscle transduction at the time of gene transfer. One distinctive feature in this muscle biopsy is the lack of expression of MHC I antigen on any muscle fiber in the biopsy. This is in direct contrast to the findings in Patients 4 and 5 of this cohort (Fig 2), as well as MHC I expression on the sarcolemma from patients in the previous cohort.⁷ The patient was found to have both early humoral and T-cell responses to AAV1 capsid. The IFN- γ ELISpot assay demonstrated T-cell activation to AAV1 capsid as early as day 2 after gene transfer (also present on day 7) (Fig 3). This is in clear contradistinction to every other patient undergoing gene transfer in this trial, where responses if present were not seen until day 14, as seen in Patient 4 of this cohort and in the first 3 patients in Cohort 1.⁷ α -SG antigen-specific T-cell responses as measured in IFN- γ ELISpot assay were not observed in peripheral blood mononuclear cells (see Fig 3), in contrast to our experience in transferring rAAV.mini-dystrophin.⁸ Accompanying the AAV capsid-induced T-cell response in Patient 6, we found a very rapid rise in AAV-neutralizing antibody titers reaching levels >30 \times greater than seen in other cases (Fig 4, Table 2). Collectively, these findings favor an amnesic response related to pre-existing immunity to AAV. Also of interest, the very rapid immune response in both humoral and T-cell immunity was temporally related to the enigmatic elevations of the peripheral blood WBCs, which were increased in

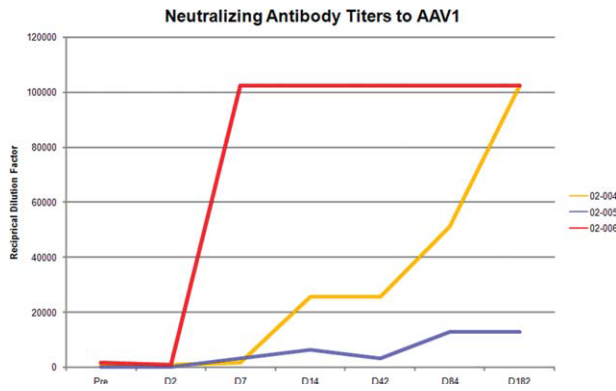


FIGURE 4: Neutralizing antibody titers to adeno-associated virus (AAV)1 in Subjects 4 to 6 starting 14 days before through day 182 after (D182) gene transfer. In all 3 subjects, serum neutralizing antibody titers to AAV1 were very low (see Table 2 for pretreatment titers). Follow-up studies showed a slow rise in serum AAV1 titers in Subjects 4 and 5. This contrasted with the rapid rise and peak elevation by week 1 in Subject 6. Plateau in elevation for Subject 6 (red) represents limit of assay.

the first week after gene transfer and in other weeks that followed.

Recognizing that the pre-existing AAV immunity uncovered in Patient 6 can present challenges, it was surprising that the neutralizing antibody titer (1:1,600) pretreatment was only 2-fold higher compared to Patient 4 (see Table 2). AAV-binding ELISA¹⁴ was utilized in an attempt to corroborate the cell-based AAV1 neutralization data, and revealed a robust particle binding titer of 1:200 in only Patient 6 (Table 3), whereas titers of Patients 4 and 5 were at baseline (<1:50).

A final point of interest is that inflammatory cells were found in the post gene transfer muscle biopsies in Patients 4, 5, and 6, with differences between cases. CD4+ cells were increased on the side of gene transfer in Patients 4 and 5 and decreased in Patient 6 (Patient 4,

24.8 ± 8.6 vs 9.5 ± 4.6 cells/mm²; Patient 5, 50.8 ± 29.5 vs 13.2 ± 5.9 cells/mm²; Patient 6, 7 ± 3.1 vs 15.5 ± 5.1 cells/mm² [*p* < 0.05]), whereas CD8+ cell counts only reached significance on the side of gene transfer in Patient 4 (20.4 ± 9.6 vs 0.7 ± 0.2 cells/mm² [*p* < 0.05]). Most of the inflammatory infiltrate was focal, perivascular, and in the perimysial connective tissue, at sites distant from individual muscle fibers (Fig 5). We did not encounter invasion of transduced muscle fibers, as seen in T-cell-mediated inflammatory myopathies,¹⁵ suggesting that recruited cells were ineffectual. In the report of the first 3 subjects undergoing gene transfer with rAAV1.tMCK.hSGCA, we showed that focal cell infiltrates expressed terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) and interpreted this to imply that recruited inflammatory cells were undergoing programmed cell death.⁷ In this second cohort, many of the infiltrating cells in Subjects 4 and 5 were positive for both TUNEL and caspase-3 (see Fig 5), supporting a similar apoptotic fate.¹⁶

Discussion

Gene transfer of rAAV1.tMCK demonstrated persistent gene expression in 2 of 3 patients for as long as 6 months. The findings continue to demonstrate the potential merit of AAV1 for protocols employing direct muscle injection.^{17,18} The cumulative observations from 2 successive gene therapy trials using identical conditions of transfer of the hSGCA gene demonstrated robust gene expression in subjects undergoing muscle biopsy at 6 weeks (n = 2), 3 months (n = 1), and 6 months (n = 2). These findings appear to address the fundamental question of whether the vector (rAAV1), transgene (hSGCA), and promoter (tMCK) are adequate for further study and can provide the necessary elements

TABLE 2: Enzyme-Linked Immunoassay for Neutralizing Antibody Titers against Adeno-Associated Virus 1 before and after Gene Transfer

| Time after Injection | Patient 4 (E02-004) | Patient 5 (E02-005) | Patient 6 (E02-006) |
|----------------------|---------------------|---------------------|---------------------|
| Pretreatment | 1:800 | <1:50 | 1:1,600 |
| Day 7 | 1:1,600 | 1:3,200 | >1:102,400 |
| 2 weeks | 1:25,600 | 1:6,400 | >1:102,400 |
| 6 weeks | 1:25,600 | 1:3,200 | >1:102,400 |
| 12 weeks | 1:51,200 | 1:12,800 | >1:102,400 |
| 26 weeks (6 months) | >1:102,400 | 1:12,800 | >1:102,400 |

The results for Subject 6 are 1 dilution higher before treatment compared to Subject 4. However, by day 7 the neutralizing antibody titers rose >30-fold compared to other subjects.

TABLE 3: Binding Antibody Ratios against Adeno-Associated Virus 1 before Gene Transfer

| Dilution | Patient 4 (E02-004) | Patient 5 (E02-005) | Patient 6 (E02-006) |
|----------|------------------------|------------------------|------------------------|
| 1:50 | 0.016 | 0.006 | 10.178 |
| 1:100 | 0.032 | -0.045 | 6.059 |
| 1:200 | 0.003 | 0.013 | 3.335 |
| 1:400 | 0.014 | -0.001 | 1.666 |
| 1:800 | 0.029 | 0.003 | 0.812 |
| 1:1,600 | 0.014 | 0.000 | 0.397 |

Binding antibody ratios against adeno-associated virus 1 shows dramatic differences before treatment for Subject 6 compared to Subjects 4 and 5. Although neutralizing antibody titers (Table 2) failed to correlate with gene expression, the unequivocally higher (>1,000-fold higher) binding antibody ratio in Patient 6 predicted an amnestic response with early onset humoral and T-cell immunity and poor gene transfer.

to maintain long-term gene expression. The results from this study are encouraging in this regard. Two patients with long-term gene expression showed definite increase in muscle fiber size accompanying persistent gene expression for 3 months (Cohort 1)⁷ and 6 months (Cohort 2). The other patient with very good gene expression for 6 months (Cohort 2, Subject 5) was severely affected, and we could not discern differences between the side receiving vector and the placebo side. The severity of the dystrophic muscle damage may have impeded change. Additional experience will be required to address potential restoration in severe phenotypes.

The overall experience from this trial is different from our immunological findings related to 2 scenarios observed in the Duchenne muscular dystrophy (DMD) gene therapy trial.¹³ In 1 DMD case, we detected a T-cell response in the ELISpot assay directed against an amino acid sequence present in the mini-dystrophin gene but absent from a deleted region of the patient's endogenous dystrophin gene. This situation is unlikely to be encountered in LGMD2D, an autosomal recessive disease requiring mutations on both alleles to produce a clinical phenotype. Because missense mutations predominate in LGMD, even a heterozygous deletion mutation at 1 allele, as seen in Patient 5, is not likely to predispose to an immune response from transgene expression. In the DMD trial, an immune reaction was also induced by a novel epitope expressed on revertant muscle fibers that are usually not encountered in LGMD2D patients. On the other hand, we did find that pre-existing immunity to AAV precluded gene expression in this current LGMD2D clinical gene therapy trial. In this case, a rapid amnestic response to AAV was

documented (both B and T cells) and predicted an immune-mediated response that correlated with a lack of significant α -SG expression in the muscle biopsy taken at the 6-month time point. Absent MHC I expression on muscle fibers and the extremely low vector genome levels in the muscle in the post gene transfer biopsy support this argument. It is difficult to discern, however, if the loss of transduced muscle fibers was caused exclusively through cell-mediated immunity versus a possible role for neutralizing or blocking antibodies hindering initial muscle fiber transduction. The importance of the finding of pre-existing immunity to AAV is that it demonstrates the potential need for cautious selection of patients enrolled in gene transfer trials, especially those not using immunomodulatory therapies. T-cell responses directed against AAV disrupting gene expression have previously been reported in a clinical trial gene transfer of Factor IX.¹⁴ The observation in the current trial that AAV-binding ELISA appeared to identify the patient with pre-existing

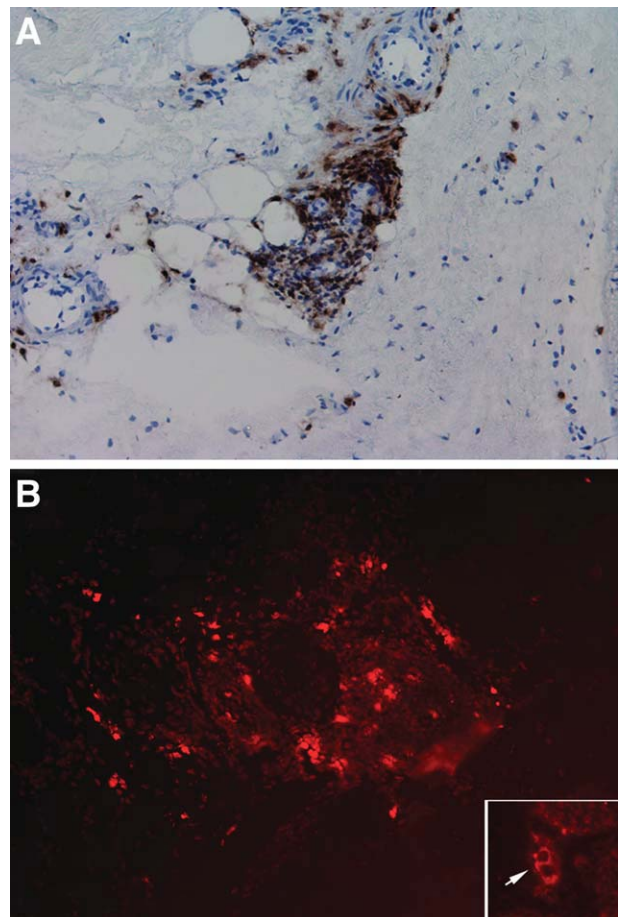


FIGURE 5: (A) Subject 4. Focal collection of CD8+ T cells shown in perimysial connective tissue of muscle biopsy after gene transfer. (B) Subject 4. TUNEL staining demonstrates inflammatory cells undergoing apoptosis confirmed by anti-caspase 3 antibody (inset).

immunity to rAAV may indicate a potential role for this laboratory assay in patient selection for gene therapy trials.

The overall favorable findings in this clinical trial lay the foundation for further gene therapy steps that can be taken for LGMD2D patients, a patient group with few treatment options. Persistent gene expression without adverse effects speaks to the safety of gene transfer to muscle and the application of the tMCK promoter that has not previously been used in skeletal muscle gene therapy protocol. The study also opens the door for potential safe and effective delivery of the sarcoglycan gene through the circulation using a protocol similar to that which we have employed in nonhuman primates, permitting vascular delivery to specific muscle groups in the lower limbs.¹⁹

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Potential Conflicts of Interest

Nothing to report.

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