Sustained Whole-Body Functional Rescue in Congestive Heart Failure and Muscular Dystrophy Hamsters by Systemic Gene Transfer

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Background—The success of muscular dystrophy gene therapy requires widespread and stable gene delivery with minimal invasiveness. Here, we investigated the therapeutic effect of systemic delivery of adeno-associated virus (AAV) vectors carrying human δ-sarcoglycan (δ-SG) gene in TO-2 hamsters, a congestive heart failure and muscular dystrophy model with a δ-SG gene mutation.

Methods and Results—A single injection of double-stranded AAV serotype 8 vector carrying human δ-SG gene without the need of any physical or pharmaceutical interventions achieved nearly complete gene transfer and tissue-specific expression in the heart and skeletal muscles of the diseased hamsters. Broad and sustained (>12 months) restoration of the missing δ-SG gene in the TO-2 hamsters corrected muscle cell membrane leakiness throughout the body and normalized serum creatine kinase levels (a 50- to 100-fold drop). Histological examination revealed minimal or the absence of central nucleation, fibrosis, and calcification in the skeletal muscle and heart. Whole-body functional analysis such as treadmill running showed dramatic improvement, similar to the wild-type F1B hamsters. Furthermore, cardiac functional studies with echocardiography revealed significantly increased percent fractional shortening and decreased left ventricular end-diastolic and end-systolic dimensions in the treated TO-2 hamsters. The survival time of the animals was also dramatically extended.

Conclusions—Systemic gene transfer of δ-SG by the AAV serotype 8 vector could effectively ameliorate cardiac and skeletal muscle pathology, profoundly improve cardiac and whole-body functions, and significantly prolong the lifespan of the treated TO-2 hamsters. (Circulation. 2005;112:2650-2659.)

Key Words: viruses ■ cardiomyopathy ■ gene therapy

Muscular dystrophies are common and debilitating genetic diseases that afflict a significant population of children and adults worldwide. No effective treatment is currently available. Besides the loss of limb functions, progressive muscle deterioration in the patients eventually leads to respiratory and heart failure. These diseases affect the striated muscles throughout the body, both skeletal and cardiac. Efficient whole-body delivery of the therapeutic genes or stem cells to correct the genetic defects has been sought.1-5 Gene vectors derived from the nonpathogenic and replication-defective adeno-associated viruses (AAV) are among the most promising systems for muscle and heart gene delivery.6-11 Local intramuscular injection has demonstrated the effectiveness of AAV vector-mediated gene transfer.2,6,12-16 The blood circulation system could serve as a natural conduit for systemic gene delivery, but the capillary vasculature in the muscle tissues imposes a major physical and biological barrier limiting gene vector dissemination. As a result, both physical17,18 and pharmaceutical methods6,10,19,20 have been developed to overcome the barrier. These methods, however, are either limited to limb muscles or associated with severe invasiveness and/or pharmaceutical side effects.21 The discovery of novel serotypes of AAV22-27 has prompted us to explore the utility of these new serotypes for systemic gene delivery to muscle without invasive inter-
Gene Therapy for Dilated Cardiomyopathy

Zhu et al

AAV Vector Construction and Production

Recombinant double-stranded AAV vectors containing human SG cDNA driven by the cytomegalovirus (CMV) promoter or by the synthetic muscle-specific promoter17 (SP) C5–27 were constructed by the standard cloning protocols. The vector DNA was packaged into AAV2 or AAV8 viral particles with the commonly used triple-plasmid transfection method18 and purified twice by the standard CsCl density ultracentrifugation technique. The titers of the purified viral stocks were determined by DNA dot blot method in standard CsCl density ultracentrifugation technique. The titers of the purified viral stocks were determined by DNA dot blot method in standard CsCl density ultracentrifugation technique.

Animals and Vector Administration

All experiments involving wild-type F1B and dystrophic TO-2 hamsters (Bio Breeders, Fitchburg, Mass) were approved by the University of Pittsburgh Animal Care and Use Committee. For vector administration, the neonatal TO-2 hamsters (age, 10 days) were injected intraperitoneally with AAV2 or AAV8 CMV-δSG vectors (1×10^{12} vector genomes), whereas the adult TO-2 hamsters (age, 1.5 months) were injected intravenously with the AAV8 SP-δSG vector (1×10^{12} vector genomes).

Histology and Serum Creatine Kinase Enzyme Assay

Muscle or other tissues were collected at the indicated time points after vector injection. For detection of SGs, tissue samples were processed by cryo-thin-sections at 6- to 8-μm thickness. Detection of the δ-, β-, γ- and δ-SG by immunofluorescent staining or by Western blotting was also described previously.14 To detect fibrosis and calcification, Masson’s trichrome staining and Von Kossa staining, respectively, were performed according to histology handbooks. For in vivo tests of muscle cell membrane integrity, Evans blue dye (10 mg/mL PBS) was injected into the jugular vein of hamsters at a dose of 0.1 mg/g body weight. The hamsters were euthanized 3 hours later, and muscles were collected and cryosectioned. Evans blue dye–positive myofibers, which show strong red fluorescence, were observed under the fluorescent microscope.

Echocardiography Analysis

Cardiac functions were analyzed by transthoracic echocardiography as described9 with a 13-MHz probe and a SEQUOIA 512 echocardiography machine (Acuson). The F1B hamsters were anesthetized with 60 mg/kg and TO-2 hamsters with 30 mg/kg sodium pentobarbital by intraperitoneal injection for consistent heart rates. Two-dimensional targeted M-mode imaging was obtained from the short axis immediately below the level of the mitral valve. M-mode tracings were recorded and measurements (left ventricular end-systolic dimension, left ventricular end-diastolic dimension, heart rate, left ventricle posterior wall thickness, and percentage fractional shortening) were made as previously reported,9 with the operator blinded to hamster genotype and treatment.

Results

Systemic and Long-Term SG Gene Transfer in Heart and Muscle by AAV8 in Neonatal Hamsters

First, we investigated side by side the gene transfer efficiencies of the most commonly used AAV2 and the newly discovered AAV8 carrying a therapeutic gene, the human δ-SG, in the hamsters of TO-2 strain, a naturally occurring congestive heart failure and LGMD 2F animal model. Our results showed that a single injection of the AAV8-CMV-δSG vector (1×10^{12} vector genomes) by the intraperitoneal route in the 10-day-old neonatal TO-2 hamsters achieved nearly complete gene transfer in the heart and all the skeletal muscles we examined in hamsters at 3 months of age (2.5 months after gene transfer). In contrast, the AAV2 vector achieved only very limited gene transfer in the heart and various muscles (Figure 1a), except in the diaphragm and...
abdominal muscles, which were proximal to the peritoneal cavity and therefore readily infected. In addition, the AAV8-mediated systemic human δ-SG gene transfer and expression were both widespread and persistent from 3 months (Figure 1a) to 6 and 12 months (Figure 1b) during the studies. Careful examination of cross sections of the entire hind-leg tibialis anterior (TA) muscle at 6 and 12 months after gene transfer revealed δ-SG expression in nearly 100% of the myofibers (Figure 1b). Overexpression of δ-SG was observed in a fraction of the heart and diaphragm muscle cells (Figure 1a), in which both the plasma membrane and the cell bodies were stained positive by an antibody specific to the human δ-SG. It was also noticeable that overexpression of human δ-SG in hamster heart persisted for at least 6 months, which contributed to the recruitment of the missing components of SG complex such as α-SG onto the cardiomyocyte membrane (Figure 1b). The cardiac structure and morphology were normal and free of cell infiltration. Similar phenomena were also seen after local delivery in the heart and muscle.9,14 Throughout the duration of the experiments, no immune rejection and overt toxicity were observed by monitoring the general health, physical activities, and tissue histology of the treated hamsters. These results indicate that AAV8 is capable of systemic and long-term gene delivery in neonatal hamsters with high efficiency.

Systemic and Long-Term SG Gene Transfer and Muscle-Specific Expression by AAV8 in Adult Hamsters

We next investigated whether comparable efficiency of δ-SG gene transfer could be achieved in the adult TO-2 hamsters. Here, we chose to use a muscle-specific, synthetic promoter SP37 instead of the CMV promoter to drive the δ-SG gene, because AAV8 is also capable of long-term gene transfer to a number of non-muscle tissues, especially the adult liver.3,22,40 The AAV8 δ-SG vector driven by the synthetic promoter SP was injected intravenously (1×10^{12} vector genomes per animal) via the jugular vein in the 1.5-month-old

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Figure 1. Efficient and sustained whole-body δ-SG gene transfer by AAV8 in heart and skeletal muscle in neonatal hamsters. Neonatal TO-2 hamsters (age, 10 days; weight, ~10 g) were injected intraperitoneally with 1×10^{12} vector genomes of AAV2- or AAV8-CMV-δ-SG (n=4 per group). Expression of human δ-SG was examined by immunofluorescent staining of tissue sections. a, Heart and muscle at 2.5 months after AAV2 or AAV8 intraperitoneal injection in neonates. Microscopic photos were taken with a ×10 lens. Insets show representative areas with higher magnification to show the ringlike cell membrane staining of δ-SG on the myofiber cross sections. b, TA muscles at 6 or 12 months and heart at 6 months after AAV8 intraperitoneal injection in neonates.
adult TO-2 hamsters, which already manifested pathological signs of muscular dystrophy. Similar to the neonatal hamsters treated with AAV8-CMV-\(\delta\)-SG, the adult-treated hamsters also exhibited nearly complete and long-term \(\delta\)-SG expression in the heart (Figure 2a, top) and all of the skeletal muscles examined (see below), including TA muscle (Figure 2a, bottom).

As expected, \(\delta\)-SG expression was muscle specific and could not be detected in nonmuscle tissues by either immunofluorescent staining or Western blotting (Figure 2b and 2c), despite the fact that the AAV \(\delta\)-SG vector DNA was present in the liver at a very high copy number and variably in other nonmuscle tissues (Figure 2d). It is noteworthy that unlike the shortened form of muscle-specific CK promoters, the SP promoter was found highly active in both the heart and diaphragm. These results demonstrated that efficient and stable systemic \(\delta\)-SG gene delivery in the heart and muscle of adult TO-2 hamsters could also be achieved and that striated muscle-specific gene expression could be obtained by use of the synthetic muscle promoter.

Restoration of Muscle Cell Membrane Integrity and Amelioration of Muscle Pathology by Systemic Gene Transfer

We next investigated whether systemic \(\delta\)-SG in the muscle could render therapeutic effects at the cellular and histological levels. Immunofluorescent staining of \(\alpha\)-, \(\beta\)-, \(\gamma\)-, and \(\delta\)-SGs on the cross-thin-sections of the heart and muscle samples revealed that expression of the missing \(\delta\)-SG gene restored the missing SG complex onto the plasma membrane of TO-2 hamsters, which were treated as either neonates (Figure 3a) or adults (see below). The recovery of the SG complex on the striated muscle cell membrane has corrected the underlying biochemical deficiency and consequently restored the integrity of the dystrophic muscle membrane, which otherwise would be very leaky. In vivo examination with Evans blue, a commonly used small-molecule dye for cell membrane integrity test, showed no leakage in the myofibers of AAV8-treated TO-2 hamsters but substantial leakage in those of untreated TO-2 hamsters (Figure 3b).

Additional evidence of systemic restoration of the muscle cell membrane integrity was the dramatic diminution of a
hallmark sign of the muscular dystrophy, thus the elevated serum levels of CK, which came from the leaky dystrophic muscle cells. When examined at 2, 5, and 8 months after AAV8 vector injection, the serum CK activities of the TO-2 hamsters treated at the neonatal age were found to be completely normalized, indistinguishable from the wild-type F1B hamsters. In contrast, the serum CK activities in the untreated, age-matched TO-2 hamsters were up to 100-fold higher (Figure 3c, left). More compellingly, the serum CK activities in the TO-2 hamsters, which were treated at the adult age, also declined >100-fold to normal levels and remained normal when examined at 1 and 5 months after vector injection (Figure 3c, right). Complete normalization of the serum CK activities further substantiated the remarkable gene transfer efficiency revealed earlier by immunofluorescent staining of δ-SG in the muscle (Figure 1). Thus, the histological and biochemical assays strongly suggest that AAV8 has efficiently and stably transferred the therapeutic δ-SG gene into the vast majority, if not all, of the striated muscle cells in the neonatal and adult hamsters.

Systemic gene delivery in both neonatal and adult TO-2 hamsters was also found to systemically ameliorate muscle pathology such as muscle degeneration and fibrosis. In the hamsters treated at the neonatal age, muscle degeneration was completely prevented as a result of gene transfer in all examined muscles (Figure 4a [top] and 4c). The percentages of myofibers containing centrally localized nuclei (CN), a classic sign of muscle degeneration and regeneration, was very low (<1%) in hamsters examined at 3.5 and 9 months of age, identical to those of age-matched wild-type F1B hamsters (Figure 4b). In contrast, the untreated age-matched TO-2 hamsters exhibited CN rates >70% (Figure 4b). Furthermore, in the TO-2 hamsters treated as adults, highly efficient gene transfer of δ-SG also improved muscle morphology (Figure 4a [bottom] and 4c). The CN rates were reversed from >70% before the treatment to <5% after the treatment in hamsters examined at 6 months of age (Figure 4b). Similar reversal was also previously observed after local intramuscular injection of an AAV δ-SG vector in the hind-leg muscles of the adult Bio14.6 hamsters, the parental line of the TO-2 hamsters. The above data demonstrated that in both neonatal and adult TO-2 hamsters, AAV8 vectors could achieve significant therapeutic efficacy in the skeletal muscle by systemic gene transfer.
Amelioration of Cardiomyopathy and Improvement in Cardiac Functions by Systemic Gene Transfer

Because congestive heart failure is the major cause of premature death of the TO-2 hamsters and a population of LGMD patients, we next investigated whether robust gene transfer and expression in the hearts could result in efficient therapeutic effects on the cardiomyopathy. Indeed, the treated hamsters, when examined at 8.5 months after neonatal gene therapy, were found to have normal gross heart morphology (Figure 5a, top). The hearts also exhibited normal ventricle structures and lacked any pathological signs of cardiomyopathy, as revealed by hematoxylin and eosin staining of cross sections of the entire heart (Figure 5a, bottom).

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ogy and histology were indistinguishable from the age-matched wild-type F1B controls. In contrast, autopsy of the age-matched untreated TO-2 hamsters showed markedly dilated heart, prominent calcification plaques on the heart surface (Figure 5a, top), atrium thrombosis, and secondary pathology of heart failure such as renal dysfunctions, enlarged liver, swollen lungs, and severe ascites in both the chest and peritoneal cavities (data not shown). Microscopic findings indicated cardiomyocyte degeneration and necrosis, pronounced fibrosis, and calcification in the untreated hamsters (Figure 5b). However, those pathological signs were essentially absent in the AAV-treated hamsters (Figure 5b).

In addition to the histopathology, we also examined the major cardiac functional indices of the hamsters using non-invasive echocardiography. The data were collected in hamsters 4 and 8 months of age for the groups treated as neonates and in hamsters 5 months of age for those treated as adults. Significant improvement (Figure 6a and 6b) was observed in percent fractional shortening, an index of systolic function; left ventricle end-systolic and end-diastolic dimensions; indices of chamber dilation; and left ventricle posterior wall thickness, an index of cardiac hypertrophy. In all of these parameters, the treated TO-2 hamsters were statistically equivalent to the wild-type F1B hamsters but significantly

Figure 6. Serial echocardiographic changes after i-SG gene delivery. a, Echocardiography of wild-type F1B, TO-2 control, and TO-2 treated with AAV8 as neonates (n=4) or as adults. b, Representative transthoracic left ventricular M-mode echocardiograms of 8-month-old TO-2 treated with AAV8 as neonates. *Statistically significant difference from untreated TO-2 (P<0.01).
Gene Therapy for Dilated Cardiomyopathy

Discussion

In the present study, we have for the first time achieved profound and global therapeutic efficacies in a heart failure and muscular dystrophy animal model by means of AAV8-mediated whole-body striated muscle gene delivery without any physical or pharmaceutical interventions. Importantly, the therapeutic efficacies were obtained in both neonatal and adult animals as a result of widespread and extensive transfection and expression. To treat the hereditary muscle diseases, it is vital to deliver the therapeutic genes into as many target cells as possible because the gene products are often cellular proteins with functions that are “cell autologous,” unlike circulating proteins such as the clotting factors that need only a small percentage of normal levels to obtain significant therapeutic benefits. In this respect, gene therapy and cell therapy for muscular dystrophies are particularly challenging. In our study, the nearly complete gene transfer and long-term, muscle-specific expression of the human δ-SG gene in both heart and muscle were key to the success of global rescue of the disease phenotypes.

Recently, we found that AAV8 vectors could cross the capillary blood vessel barriers in the skeletal muscle and heart more efficiently than other AAV serotype vectors tested. In the present study, we have extensively investigated the therapeutic effects after AAV8-mediated δ-SG gene delivery in a severe LGMD animal model, the TO-2 hamsters, which show both muscle pathology and dilated congestive heart failure. The TO-2 animal model is believed to have a more severe phenotype than the Bio14.6 hamster model, which is described as a mouse model for the muscular dystrophy and heart failure combined disease. The TO-2 hamsters develop progressive and severe muscle weakness and dilated congestive heart failure as early as 4 months of age. Thus, the TO-2 animal model is valuable for the evaluation of the therapeutic potential of local and systemic gene transfer and expression. The TO-2 hamsters are considered to be a suitable model for the study of new therapeutic approaches for muscular dystrophies and heart failure.

Improvement in Whole-Body Endurance and Prolongation of Lifespan by Systemic Gene Transfer

Finally, we examined whether the efficient systemic δ-SG gene transfer could render whole-body functional rescue and ultimately prolongation of lifespan of the diseased animals. Treadmill running was used to test the whole-body endurance as an indicator of both skeletal and cardiac muscle functions at the age of 4 and 9 months. The running distances (in meters) of the AAV-treated TO-2 hamsters were found to be equivalent to that of normal F1B hamsters but markedly longer than that of untreated TO-2 hamsters in both age groups (AAV8-treated versus untreated TO-2, 765±127 versus 220±46 m at 4 months and 474±113 versus 78±11 m at 9 months) (Figure 7a and video material in the Data Supplement, which is available online at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.105.565598/DC1). Previously, we have observed in an in vitro test full contractile force recovery as a result of local injection of an AAV-δSG vector into the TA muscles of the Bio14.6 hamsters, the parental line of the TO-2 hamsters. In this study, we also attempted to measure the TA muscle contractile force improvement in the TO-2 hamsters after systemic gene delivery. Our preliminary results comparing the TA muscles of wild-type F1B and the untreated TO-2 hamsters, however, showed no statistical difference in the generation of specific force (data not shown). This made it difficult to obtain any meaningful evaluation of this parameter in the TO-2 hamsters. A possible reason may be the lack of pathological hypertrophy of the TA muscles in the TO-2 hamsters.

Furthermore, long-term δ-SG expression in muscles throughout the body also led to an improvement in the general health and prolongation of the lifespan of the TO-2 hamsters. Without the gene therapy treatment, all of the animals died of heart failure and muscular dystrophy at 32 to 43 weeks of age (median survival time, 37 weeks). The much-shortened lifespan was a major reason for us to choose the TO-2 hamsters in this study to examine the whole-body therapeutic efficacies of gene therapy. As expected, all of the AAV8-treated TO-2 hamsters survived beyond 48 weeks (the duration of the study) (Figure 7b). These data demonstrated the overall efficiency of systemic gene transfer and its global therapeutic effects in the heart failure and muscular dystrophy animal model.
failure with a much-shortened lifespan. Previously, gene therapy approaches have been used to treat LGMD hamsters and mice but only with local gene delivery in either the skeletal muscle or the heart. Until now, technical hurdles and poor efficiency in systemic gene delivery in muscle and heart have prevented achievement of any meaningful whole-body therapeutic benefits such as an increase in treadmill running endurance and the prolongation of lifespan. A recent report described the use of AAV6 and high doses of vascular endothelial growth factor (VEGF) to achieve systemic gene delivery in a Duchenne muscular dystrophy animal model, the mdx mice. Those mice do not manifest severe phenotypes of muscular dystrophy such as shortened lifespan seen clinically in Duchenne muscular dystrophy patients, however, so it was difficult to evaluate the lifesaving effects of systemic gene delivery. In addition, although vascular endothelial growth factor is a potent blood vessel–permeable reagent that facilitated the dissemination of the AAV6 viral particles into the muscle tissues, the short-term and long-term safety of high dose administration of vascular endothelial growth factor remains to be investigated. It is apparently advantageous to achieve systemic gene delivery by AAV8 into the striated muscles without the use of additional pharmacological reagents.

AAV8-mediated systemic gene transfer delivers the genes not only to the muscle and heart but also to other unintended tissues, particularly the adult liver, which has the highest copy numbers of the vector DNA. Nonspecific gene transfer and expression may result in unwanted side effects. Because targeted gene transfer with selective infectivity to the muscle and heart is currently unrealistic, a practical measure we took in this study was the use of an SP that conferred tissue-specific δ-SG gene in both heart and skeletal muscles. Previously, the most commonly used muscle-specific promoter in the gene therapy studies was the muscle CK promoter and its derivatives. However, the shortened muscle CK promoters were not sufficiently active in the heart and diaphragm and may show preference to certain muscle fiber types. However, the universal gene expression in the striated muscles by the SP promoter observed in our study, particularly in the heart and diaphragm, is very meaningful because cardiac and respiratory failures are the main causes of premature death in dystrophic patients. Muscle-specific expression should also lower the potential immune response that could result from transgene expression in the antigen-presenting cells. Indeed, we did not observe any discernable immune response by the TO-2 hamsters to the human δ-SG protein that was encoded by the AAV vector under the control of the SP promoter. That may partially contribute to the long-term gene expression after gene delivery in the adult TO-2 hamsters, which had mature immune systems at the time of gene delivery.

In summary, the unprecedented gene delivery efficiency and therapeutic efficacy for both cardiomyopathy and muscular dystrophy demonstrated here in an animal model should pave the way for further preclinical studies in large animal models and eventually in clinical trials for this and other genetic diseases.

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References

Clinical Perspective

Muscular dystrophies commonly arise from single gene mutations. In humans and hamsters (TO-2), mutations in the δ-SG gene cause LGMD with dilated cardiomyopathy. As a potential treatment, gene therapy would introduce functional genes to replace the defective genes. However, a major challenge has been to achieve body-wide gene delivery with prolonged expression in skeletal and cardiac muscle. This study used AAV-8 to deliver human δ-SG into TO-2 hamsters. Derived from a small non-pathogenic virus, recombinant AAV vectors are highly efficient for in vivo gene delivery and long-term expression. Dosens of AAV serotypes have now been identified with differential organ tropism. In the present report, intravenous delivery of high doses of AAV-8 encoding δ-SG produced ~100% gene transfer in skeletal and cardiac muscle, marked improvement in muscle and cardiac functions, and prolonged lifespan. The efficacy and simplicity of this approach (compared with invasive surgical or chemical interventions) make the potential application to human diseases very alluring. However, many questions must be answered before human therapeutic interventions are attempted. Scaled for differences in body mass, the treatment of a 3- to 70-kg human requires an enormous scale-up of virus production. Will AAV-8 retain a similar effectiveness in humans? Will preexisting anti-AAV antibodies block AAV therapy or portend adverse immune responses? Will unregulated expression of δ-SG prove toxic in humans? Thus, the great promise of AAV8 observed in hamsters requires more painstaking studies to uncover the potential utility in humans.
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