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Virus-Delivered Small RNA Silencing Sustains Strength in Amyotrophic Lateral Sclerosis

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Mutations in superoxide dismutase cause a subset of familial amyotrophic lateral sclerosis and provoke progressive paralysis when expressed in mice. After retrograde transport to the spinal cord following injection into muscles, an adeno-associated virus carrying a gene that encodes a small interfering RNA was shown to target superoxide dismutase messenger RNA for degradation. The corresponding decrease in mutant superoxide dismutase in spinal motor neurons preserved grip strength. This finding provides proof of principle for the selective reduction of any neuronal protein and supports intramuscular injections of a small interfering RNA–encoding virus as a viable therapy for this type of familial amyotrophic lateral sclerosis.

Ann Neurol 2005;57:773–776

Small interfering RNA (siRNA) can promote degradation of specific messenger RNA (mRNA), and thus protein species,[1] and it may be useful as a therapy in neurodegenerative diseases where accumulation of a toxic protein drives pathogenesis. Putting these molecules to use in treating human disease involves overcoming two substantial hurdles. The first hurdle is achieving sustained synthesis of small RNA species, because the half-life of these molecules is short. This problem has been largely solved by the recent design of genes that reliably produce effective small RNA species by transcription.[1] The second challenge is delivering the siRNA to the desired target cells.

Delivery is especially a problem for a disease such as amyotrophic lateral sclerosis (ALS), in which the lower motor neurons at risk are distributed throughout the spinal cord. ALS causes weakness, stiffness, difficulty speaking and swallowing, and death secondary to failure of respiratory muscles, usually within 3 to 5 years after diagnosis. Current therapies are marginally effective. Mouse models of ALS are based on expression of high levels of mutations in superoxide dismutase (SOD1) that cause dominantly inherited ALS in humans.[2] Mutant SOD1 causes disease by an acquired toxicity of the mutant proteins, not through loss of dismutase activity.[3] Complete absence of SOD1 does not cause disease.[4] We now demonstrate that siRNA delivered to spinal motor neurons through retrograde transport of adeno-associated virus (AAV-2) injected into muscles substantially decreases an abundant target protein (SOD1) and produces a functional impact, delaying loss of grip strength in a mouse model of ALS. Thus, decreasing SOD1 through methods such as virus-delivered siRNA may represent a viable therapy for SOD1-mediated familial ALS.

Materials and Methods

Design of Small Interfering RNA

siRNA directed against SOD1 was constructed as described[5] with the pSuper vector using the sequences 5′-GGCCTGCATGGATTCCATG-3′ for the siRNA and 5′-GGC**T**TGCATGGATT**A**CATG-3′ for the siRNA mismatch. All constructs were verified by automated sequencing.

Transfection and Immunoblotting

Human T98G cells were grown in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum and 50μg/ml pen/strep (Invitrogen, La Jolla, CA). Transfections with pSuper vectors were done by using Effectene (Qiagen, Valencia, CA). Cells were harvested 72 hours after transfection, lysed in 1X Laemmli buffer, resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis,

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Received Feb 14, 2005. Accepted for publication Feb 28, 2005.

Published online Apr 25, 2005, in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.20453

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and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Membranes were incubated with 1:2,000 dilution of SOD1 antibody[6] or 1:3,000 dilution of α-tubulin antibody (Sigma, St. Louis, MO) in 5% nonfat skim milk/0.1% Tween 20 in phosphate-buffered saline, followed by incubation with horseradish peroxidase–conjugated anti–rabbit or anti–mouse IgG (Amersham Pharmacia Biosciences, Piscataway, NJ), and visualized with enzyme chemiluminescence (Amersham Pharmacia Biosciences).

Immunocytochemistry

Mice were perfused transcardially with saline and 4% paraformaldehyde in 0.1M phosphate buffer. The spinal cord was removed and embedded in Tissue-Tek O.C.T. Compound (Sakura, Torrance, CA), and the lumbar cord was sectioned by cryostat (30μm sections). Spinal cord sections were incubated with 1:100 anti-SOD1 (SC-8637; Santa Cruz Biotechnology, Santa Cruz, CA) and 1:100 anti-green fluorescent protein (GFP; Molecular Probes, Eugene, OR) in Tris-buffered saline, 3% donkey serum (Sigma), and 0.25% Triton X-100 (Sigma). Secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Microscopy was performed using a Bio-Rad confocal microscope using Lasersharp (Bio-Rad, Richmond, CA).

Design of Viral Vectors

Recombinant AAV-2 carrying enhanced green fluorescent protein (EGFP; ClonTech, Palo Alto, CA) driven by the cytomegalovirus promoter and the siRNA construct driven by the H1RNA promoter was produced in HEK293 cells (ATCC) by calcium phosphate transient transfection of vector plasmid, pAAV/Ad8, and pXX6 helper plasmid, as described previously.[7] Virus was purified by CsCl density gradient dialysis and heparin affinity column chromatography (Amersham Biotech) using vector services from Virapur, LLC (San Diego, CA). Titers were determined by quantitative polymerase chain reaction (PCR) methods, as described previously, and were diluted to 1×10^{12} DNAse-resistant particles per milliliter.[8]

Green Fluorescent Protein Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated using Trizol reagent (Invitrogen). Reverse transcription (RT) was performed with Superscript kit (Invitrogen) using oligo dT primers. For amplification, primers were designed for the *EGFP* gene using 5′ primer ACTTCAAGATCCGCCACAAC and 3′ primer GAACTCCAGCAGGACCATGT. Thirty-five cycles of PCR were performed on samples including control samples with no RT. A positive band of 178bp was present in the RT+ samples, whereas no band appeared in the control samples without RT, indicating that EGFP was expressed in AAV-injected animals.

Injection of Mice and Behavioral Testing

All surgical procedures were performed in accordance with National Institutes of Health guidelines and with institutional approval. At aged 45 days, 20 randomly assigned transgenic mice with the G93A human SOD1 mutation (B6SJL-TgN[SOD1-G93A]1Gur; Jackson Laboratories, Bar Harbor, ME) were injected with 25μl AAV-siRNA-GFP or AAV-missense-GFP into the right lower hind limb using a Hamilton syringe. Motor function was tested weekly with a Grip Strength Meter (Columbus Instruments, Columbus, OH). Grip strength meter testing was performed by allowing the animals to grasp a platform with one hind limb (left or right), followed by pulling the animal until it released the platform; the force measurement was recorded in four separate trials.

Results

A transcribable siRNA[5] against human SOD1 mRNA was designed to test the principle of targeted reduction of mRNA and accumulated protein as a possible therapy for SOD1 mutant–mediated ALS. Similar vectors against SOD1 have been used in vitro.[9,10] To test the efficacy in vivo, we constructed AAV-2 viral vectors with an siRNA gene, which also encodes GFP, so that transduced cells could be identified (Fig 1A). In cultured human cells, the siRNA vector decreased SOD1 protein levels, whereas a 2bp missense control did not (see Fig 1B).

As an in vivo test, we injected the lower part of the hind limb on one side of a cohort of 7-week-old SOD1^{G93A} mutant mice with AAV-2 carrying either siRNA against SOD1 or the missense control. As expected,[11] GFP mRNA was present in the spinal cord (Fig 2A) of animals injected with either AAV-2-GFP-siRNA or the missense construct. This demonstrates retrograde transport of virus from the muscle to the spinal cord. Motor neurons expressing GFP showed lower levels of SOD1 (see Fig 2B). To determine the functional effect of reduced SOD1 in motor neurons after gene delivery, we measured hind-limb grip strength on each side of the animal weekly for 9 weeks. Because only one hind limb was injected, each animal's noninjected side served as a negative injection control. The side injected with AAV-2-siRNA against SOD1 showed remarkable preservation of grip strength,

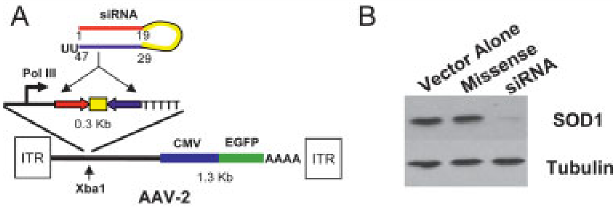

Fig 1. Small interfering RNA (siRNA) against superoxide dismutase (SOD1) decreases SOD1 protein levels. (A) A gene encoding an siRNA structure that targets human SOD1 messenger RNA was constructed and cloned into adeno-associated virus 2 (AAV-2), which also contains a green fluorescent protein gene. (B) Cultured human (T98G) cells were transfected with a gene encoding an siRNA directed against human SOD1. Cells were harvested after 72 hours, and SOD1 levels were measured by immunoblotting.

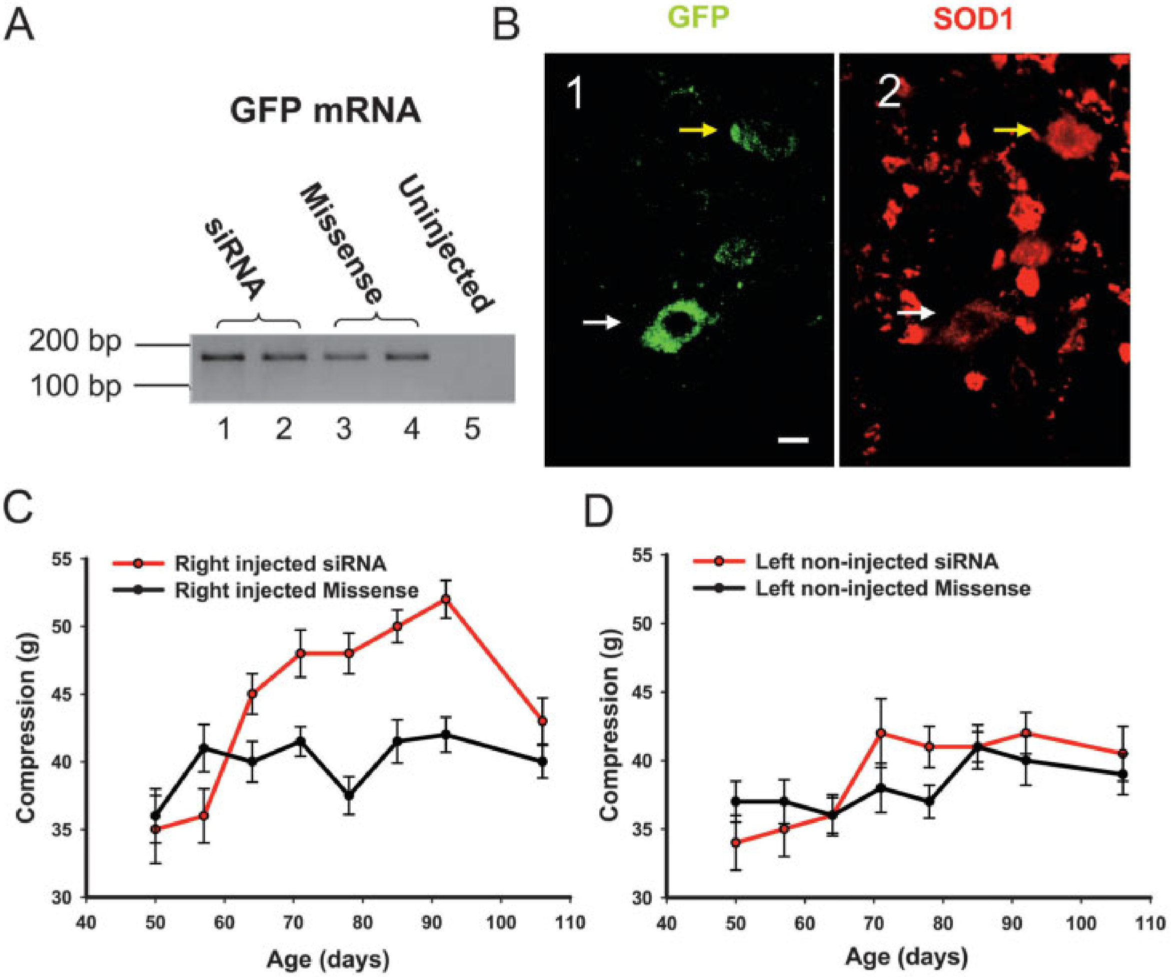

Fig 2. Virus-delivered small interfering RNA (siRNA) against superoxide dismutase (SOD1) delays loss of grip strength in an $SOD1^{G93A}$ *amyotrophic lateral sclerosis (ALS) mouse model.* $SOD1^{G93A}$ *mice were injected in the lower leg with* 25×10^{10} *adeno-associated virus 2 (AAV-2)–siRNA-green fluorescent protein (GFP) viral particles at 45 days. Spinal cords were harvested at 106 days. Spinal neurons containing the viral genome delivered by retrograde transport were identified by reverse transcription polymerase chain reaction for GFP messenger RNA (A) or by indirect immunofluorescence (B). A strongly GFP-positive neuron* (1, white arrow) *shows decreased levels of SOD1* (2, white arrow), *whereas a weakly GFP-negative neuron* (1, yellow arrow) *shows relatively high levels of SOD1 expression* (2, yellow arrow). *(C) Lower right leg muscles of ALS* ($SOD1^{G93A}$) *mice were injected with AAV-2-siRNA-GFP (n = 10) or the missense construct (n = 10). The left side was not injected (D). Hind-limb grip strength was measured weekly in the AAV-2-siRNA-GFP– and AAV-2-missense-GFP–injected animals. Grip strength was preserved in the mice treated with AAV-2-siRNA-GFP.*

whereas both the noninjected side and animals injected with the control virus did not (see Fig 2C, D).

Discussion

Although long-lived preservation of grip strength late in disease would not be possible as the disease progressed into the noninjected upper part of the limb, where we did not target the innervating motor neurons, these results demonstrate regional rescue of motor neurons that received the AAV-2 siRNA construct and provide the proof of principle for use of RNA interference in treating ALS in vivo in adulthood. Although AAV or other viral vectors can be directly injected into nervous system tissue and may be effective in mouse models of neurodegeneration,[12] such approaches in humans may be fraught with significant morbidity from the trauma of injections, especially if the target is the spinal cord at multiple levels, such as would be anticipated in ALS. In contrast, we anticipate that reduction in mutant SOD1 by AAV-2–mediated delivery of siRNA after peripheral injection may be an effective therapy for SOD1 familial ALS patients. The safety of a similar approach in humans has already been demonstrated by injection of AAV-2 into muscle as a means of generating clotting factor replacement.[13] Indeed, a clinical trial using AAV-2 to deliver a growth

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factor (insulin-like growth factor-1) is to be initiated in ALS patients. It is likely that knockdown of other proteins by virus-delivered siRNA also will be effective for future therapeutic targets both in sporadic ALS and other neurodegenerative diseases.

Toxicity of SOD1 mutants to motor neurons is noncell autonomous; that is, it does not derive solely from mutant damage directly within motor neurons.[14] Indeed, expression of SOD1 mutants only in motor neurons has not produced disease or pathology,[15,16] and individual motor neurons surrounded by nonmutant neighbors survive indefinitely despite expressing mutant SOD1 at levels sufficient to generate early-onset disease when expressed ubiquitously.[14] Regarding this, our evidence indicates that within the spinal cord, selective reduction of mutant SOD1 in motor neurons is sufficient to significantly slow motor neuron killing, demonstrating, as expected, that mutant damage developed directly within motor neurons contributes in an important way to toxicity. Although no evidence currently is available to support direct damage by mutant SOD1 within muscle cells, we cannot exclude that the viral-delivered siRNA used here might also reduce mutant SOD1 expression in muscle, and that a proportion of the benefit we have documented may arise from decreased mutant expression in muscle. Indeed, disconnection at the neuromuscular synapse is among the earliest abnormalities in mutant SOD1 mice,[17] and increased synthesis of insulin-like growth factor-1 by muscles[18] can slow toxicity from SOD1 mutants; albeit, it is unclear whether this latter benefit derives from insulin-like growth factor-1 action on the neuron or the muscle. Future efforts using viruses that are not retrogradely transported are necessary to determine whether reducing SOD1 mutant synthesis exclusively within muscles will be beneficial.

This study was supported by grants from the Amyotrophic Lateral Sclerosis (ALS) Association, Lou Gehrig Challenge Grant (D.W.C., T.M.M.), Project ALS (B.K.K., F.H.G.), and the NIH (National Institute of Neurological Disorders and Stroke, NS 27036, D.W.C., National Institute on Aging, AG000975-04, T.M.M.).

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