Retrograde Viral Delivery of IGF-1 Prolongs Survival in a Mouse ALS Model
Brian K. Kaspar, Jerónia Lladó, Nushin Sherkat, Jeffrey D. Rothstein, Fred H. Gage

Amyotrophic lateral sclerosis (ALS) is a progressive, lethal neuromuscular disease that is associated with the degeneration of spinal and brainstem motor neurons, leading to atrophy of limb, axial, and respiratory muscles. The cause of ALS is unknown, and there is no effective therapy. Neurontrophic factors are candidates for therapeutic evaluation in ALS. Although chronic delivery of molecules to the central nervous system has proven difficult, we recently discovered that adenovirus can be retrogradely transported efficiently from muscle to motor neurons of the spinal cord. We report that insulin-like growth factor 1 prolongs life and delays disease progression, even when delivered at the time of overt disease symptoms.

Overexpression of superoxide dismutase-1 (SOD1) gene mutations in mice and rats recapitulates the clinical and pathological characteristics of amyotrophic lateral sclerosis (ALS) in humans, in which motor neurons (MNs) degenerate and animals die shortly after onset of symptoms (1–9). Compounds active in retarding symptoms in this model have been shown to be predictive for clinical efficacy in patients with ALS (10). Typically, compounds that show any level of efficacy must be continuously delivered well before symptom onset in order to delay disease or increase survival times in the mouse model. Given the lack of preclinical diagnostic tools, this approach is unpredictable in the clinic (2–7). Although investigation of these molecules continues, the targeted delivery of a therapy to MNs remains problematic.

We recently discovered that adenovirus-associated virus (AAV) is retrogradely transported from presynaptic terminals of projecting neurons through the entire length of the axon and can enter the projecting cell nucleus, providing sustained gene delivery (11–13). We used the retrograde transport ability of AAV in the ALS animal model by injecting AAV into respiratory and motor limb muscles to directly target the affected MNs and test the efficacy of two neurontrophic factors (NTFs), insulin growth factor 1 (IGF-1) and glial cell line–derived neurontrophic factor (GDNF) (14–16). Our results reveal that IGF-1 delays the onset of behavioral symptoms and sustains life in the SOD1 mutant to a greater degree than GDNF, even when administered at the onset of overt clinical symptoms. The importance of retrograde delivery was accentuated by the finding of the significantly reduced effects of IGF-1 delivered by lentivirus, a virus that is not transported efficiently (17), when compared to the effects of IGF-1 delivered by AAV. The marked effects of IGF-1 on onset and survival are accompanied by robust survival and preserved morphology of MNs and decreased gliosis. The actions of IGF-1 occur at least in part through an antiapoptotic mechanism, as evidenced by the inhibition of caspase-3 and -9 cleavage and DNA fragmentation.

Retrograde transport from MNs that innervate muscles requires the virus to bind to viral receptors on the axon terminal, with subsequent transport over a long distance to the MN nucleus, allowing sustained gene expression (Fig. 1A). We investigated the ability of AAV to target specific subsets of MNs that project to defined muscles in 90-day-old transgenic mice that express the G93A SOD1 transgene, the high-expressing SOD1 mutant mouse that displays disease onset at 90 days and dies ~30 days later (8). AAV carrying green fluorescent protein (AAV-GFP) injected into the hindlimb quadriceps and intercostal muscles showed GFP expression in choline acetyltransferase (CHAT)–positive MNs within the ventral horn of the lumbar and thoracic spinal cord, respectively, with no evidence of glial cell transduction (Fig. 1B and C). Quantification revealed that more than 400 MNs were positive for GFP in the spinal cord, and transcription of GFP was confirmed by reverse transcription–polymerase chain reaction (PCR) (18). We found a dose response for retrograde transport to the spinal cord by injecting virus at titers between 4 × 107 and 3 × 1010 viral particles into the quadriceps muscles and performing PCR for vector sequences on the lumbar spinal cord. A dose of 1 × 1010 viral particles was required to achieve transport to the lumbar spinal cord; however, in one of three experiments, vector sequence was found at 4 × 109 viral particles (Fig. 1D). Up to 1.1% of the virus injected at a dose of 1 × 1010 viral particles was transported to the lumbar region of the spinal cord, as assessed by quantitative PCR (18).

To explore two NTFs that have been reported to have potent effects on MN survival (14–16), AAV vectors expressing GDNF or IGF-1 were injected bilaterally into the hindlimb quadriceps and intercostal muscles of G93A SOD1 animals before disease onset at 60 days of age, with a dosage of 1 × 1010 particles per injection. AAV-GFP was used as control vector. At the age of 91 days, disease onset was observed in GFP-treated animals (n = 25 mice), as assessed by a decline in hindlimb function in the rotarod test. IGF-1 treatment (n = 25 mice) delayed the onset by 31 days compared to a 16-day delay of onset in GDNF-treated animals (n = 15 mice) (χ² = 34.14, P < 0.0001) (Fig. 1E). GDNF-treated animals showed a smaller, 11-day increase in median survival compared to GFP-treated controls (134 days versus 123 days, χ² = 29.05, P < 0.0001). IGF-1–treated animals showed a larger, significant improvement in life-span, with a 37-day increase in median survival compared to controls (160 days versus 123 days, χ² = 19.40, P < 0.0001). The maximal life-span of IGF-1–treated animals was 265 days, compared to 140 days in the control group (Fig. 1F). Thus, it appears that injections of IGF-1 not only delayed the onset but also slowed the rate of disease progression. In contrast, GDNF appears only to have delayed the onset of symptoms (19).

Next, we tested the therapeutic potential of the two NTFs at the time of disease onset, by giving injections into the hindlimb quadriceps and intercostal muscles at 90 days of age. GDNF treatment (n = 20 mice) had minimal effects, with a 7-day median increase in survival compared to control GFP animals (n = 20 mice) (130 days versus 123 days, respectively; χ² = 18.92, P < 0.0001). In contrast, IGF-1 treatment (n = 30 mice) extended median life-span by 22 days compared to the GFP group (n = 30 mice) (146 days versus 124 days, χ² = 29.40, P < 0.0001) (Fig. 2A). Assessment of neuromuscular function was performed on IGF-1–treated animals by quantitative grip strength and rotarod per-
formance (Fig. 2, B to D). Between 100 and 110 days of age, GFP-treated animals showed a marked decrease in performance, whereas the IGF-1–treated animals displayed the greatest deficits ~20 days later. Additionally, IGF-1–treated animals maintained their weights over a longer period of time, compared to a 15% weight loss in GFP-treated animals (Fig. 2E). IGF-1 treatment had hypertrophic and protective effects against muscle atrophy, resulting in a 20% higher muscle mass in IGF-1–treated animals compared to GFP-treated animals at 115 days of age (18). These combined results suggest that addition of IGF-1 after the onset of overt motor dysfunction results not only in an extension of life but also in a delay in the functional decline associated with the disease.

Histological evaluation of the lumbar spinal cord revealed that IGF-1 treatment prevented the pathological changes typical of the transgenic disease model. Neurotrophic factor treatment produced a qualitative reduction in neuropil and cellular vacuolization in animals at 110 days of age (Fig. 3, A and B). Average MN counts per section in the lumbar spinal cord in IGF-1–treated animals injected at 90 days of age (n = 3 mice) were similar to counts in control wild-type animals (n = 3 mice) (26.58 ± 1.10 versus 25.5 ± 1.04), whereas GFP-treated animals (n = 3 mice) showed a substantial loss of MNs (14.99 ± 0.85). There were no significant differences in MN counts between GFP and IGF-1 treatments when animals were classified as end-stage (Fig. 3E). An estimate of the total number of MNs in the lumbar spinal cord showed that IGF-1 promoted a 78% increase in MN survival when compared to the GFP group. The most vulnerable MNs in ALS, which are the large MNs, were also significantly preserved in the IGF-1–treated animals, as analyzed by morphometric measurements, with a 66% increase in survival of this subgroup of neurons when compared to the GFP group (Fig. 3F). At the end-stage, animals treated with IGF-1 continued to have 34% more large neurons (>250 μm²) as compared to untreated G93A SOD1 mice (18). Staining for nonphosphorylated neurofilament by SMI-32 revealed higher numbers of neurons in IGF-1–treated animals than in GFP-treated animals (Fig. 3, C and D).

To assess the requirement for retrograde transport of AAV–IGF-1 to the spinal cord to achieve therapeutic effects, we used a vector that would maintain long-term expression in the muscle only, i.e., that would not be retrogradely transported to the spinal cord (20). Vescicular stomatitis virus glycoprotein– pseudotyped lentiviral vector (LV) expressing IGF-1 (n = 10 mice) or GFP (n = 6 mice) was injected in a manner similar to that used in the AAV experiments, without transport to the spinal cord, and survival was evaluated. Muscle production of IGF-1 was similar between LV- and AAV-injected animals 3 weeks post-injection, on the basis of transcript and protein measurements from muscle biopsies (18, 19). LV–IGF-1 increased median survival by 9 days over GFP controls (132 days versus 123 days, χ² = 6.863, P = 0.0088), a period significantly shorter than the 22-day increase seen with AAV–IGF-1, suggesting that delivery to both the muscle and spinal cord is the most efficacious delivery method (Fig. 2F).

The beneficial effects of IGF-1 treatment were not solely restricted to neurons. There was also a significantly reduced amount of astrogliosis, as assessed by glial fibrillary acidic protein (GFAP) staining, suggesting a delayed activation of astrocytes in the IGF-1–treated animals (Fig. 3, C and D). Overexpression of G93A SOD1 has been shown to be associated with neuropil, neuronal, and astroglial accumulations of ubiquitin–positive aggregated protein (21). GFP-treated G93A SOD1 animals exhibited large, ubiquitin–positive aggregates in the spinal cord. However, IGF-1 treatment resulted in smaller, focalized inclusions, suggesting a delay in the pathological course of aggregate formation or enhanced degradation of aggregates. Importantly, there were no significant changes found in the levels of G93A SOD1 protein in the spinal cord between IGF-1– and GFP-treated animals (18).

Consistent with the finding that apoptosis is involved in ALS (22, 23), GFP-treated animals exhibited large numbers of cells that were positive for terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) at 110 days of age, compared to little to no TUNEL reactivity in IGF-1–treated ani-
mals (Fig. 4A). One reported mechanism of action of IGF-1 is to increase the phospho-
rylated state of Akt, a protein kinase acti-
vated by insulin and various growth factors
that is involved in blocking proapoptotic
pathways through receptor-mediated phos-
phatidylinositol 3-kinase signaling (24).

We found that AAV–IGF-1-treated animals
had 38% higher levels of phosphorylated
Akt when compared to GFP controls (Fig.
4D). Phosphorylated Akt has been shown to
prevent cleavage of caspase-9, thereby in-
hibiting apoptosis. Signaling within the ap-
opoptotic pathway, including the cleavage of
caspase-3 and -9, is a target for disease
intervention in ALS (23).

IGF-1 significantly reduced the amount
of caspase-9 cleavage. At 110 days of age,
IGF-1 decreased the cleaved 37- and 39-kD
subunits by more than 63% compared to the
GFP group, indicating that IGF-1 can block
caspase activation involved in the apoptotic
pathway (Fig. 4C). In addition, cleaved
caspase-3 immunohistochemistry was less
evident in IGF-1–treated animals compared
to the GFP group (16 ± 5 cells versus
117 ± 7 cells, respectively; n = 5 mice
each, P < 0.001) (Fig. 4B). Furthermore,
IGF-1–treated animals showed a 59%
decrease in the levels of tumor necrosis
factor-α within the lumbar spinal cord
compared to GFP controls (n = 5 mice),
indicating that IGF-1 also works to delay
the associated glial (microglial and astro-
gial) response seen in ALS.

We describe AAV-NTF delivery in vivo
to the hindlimb and intercostal muscles in a
mouse model of ALS, which results in a
significant delay in the decline of motor func-
tion, a prolongation of MN survival, a de-
crease in parenchymal gliosis, and most
importantly, a prolongation in survival. Fur-
thermore, these effects were evident even
with late delivery of therapy—at the time of
symptom onset—that is comparable to the
method and time of treatment that needs to be
used for the human disease. The mechanism
of increased survival may be multifactorial,
both clinically and biochemically. Death of
the transgenic mice likely reflects both loss of
respiratory function and wasting and weight
loss due to muscle weakness, leading to star-
vation and dehydration. Thus, at the clinical
level, delivery of the agent to both limb and
respiratory MNs is appropriate. At the cellu-
lar and biochemical level, gliosis is believed
to contribute to disease progression, with re-
sultant insults including excitotoxicity, oxi-
dative stress, and initiation of apoptotic cas-
cades (25, 26). Previous in vitro data have
demonstrated that IGF-1 can also prevent
excitotoxic MN degeneration (27). AAV–
IGF-1 delivery clearly delayed loss of MNs and also increased muscle mass. Those two effects alone could be responsible for the increased survival in mice. However, lentiviral delivery of IGF-1 to muscle, without retrograde transport, only produced a modest effect in survival. This finding suggests that the retrograde transport and MN soma expression of IGF-1 were responsible for the majority of the neuroprotection. Our studies do not allow us to determine if the protection is due to effects of the intraneuronal expression of IGF-1 and/or its release to surrounding neuropil. The decrease in gliosis seen with IGF-1 by delivery—either through paracrine effects of the secreted IGF-1 or through limited neuronal injury and associated cellular responses—may also be protective. Recent studies using anti-inflammatory agents have also demonstrated increased survival associated with limited gliosis (28, 29).

Importantly, past studies have suggested that many different trophic factors are MN-protective. NTFs such as ciliary neurotrophic factor, GDNF, and brain-derived neurotrophic factor have been unsuccessful in human trials. However, subcutaneous delivery of a recombinant growth factor, IGF-1, has had marginal success in one of two human trials (14). Recent studies have demonstrated that viral muscle and intra parenchymal delivery of GDNF can increase survival in G93A mice (15, 16, 30–34). Our direct comparison of these two factors clearly demonstrates a superior effect of IGF-1.

The marginal efficacy in these past human trials may be due, in part, to the limited delivery of the protein to the target neurons and glia in the spinal cord. AAV vectors will provide for long-term muscular secretion of IGF-1, thereby avoiding the half-life and stability issues seen with protein therapeutics (14, 35). Furthermore, AAV vectors have the useful property of retrograde transport, such that spinal MNs can be selectively targeted, permitting local secretion of IGF-1 to have a broader effect on surrounding cells and not be confined only to the MNs that transported the virus.

Our results demonstrate substantial behavioral, functional, and pathological improvements in a clinically relevant model of MN disease after intramuscular AAV–IGF-1 delivery. A clinical trial testing this approach is being designed.

**References and Notes**

9. About 5 to 10% of the ALS cases are familial, with many different trophic factors being responsible. NTFs such as ciliary neurotrophic factor, GDNF, and brain-derived neurotrophic factor have been unsuccessful in human trials. However, subcutaneous delivery of a recombinant growth factor, IGF-1, has had marginal success in one of two human trials (14). Recent studies have demonstrated that viral muscle and intra parenchymal delivery of GDNF can increase survival in G93A mice (15, 16, 30–34). Our direct comparison of these two factors clearly demonstrates a superior effect of IGF-1.

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