Therapeutic benefits of cardiotrophin-1 gene transfer in a mouse model of spinal muscular atrophy

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Spinal muscular atrophy (SMA) is a recessive autosomal disorder characterized by degeneration of lower motor neurons caused by mutations of the survival motor neuron gene (SMN1). No curative treatment is known so far. Mutant mice carrying homozygous deletion of Smn exon 7 directed to neurons display skeletal muscle denervation, moderate loss of motor neuron cell bodies and severe axonal degeneration. These features, similar to those found in human SMA, strongly suggest the involvement of a dying back process of motor neurons and led us to test whether neurotrophic factors might have a protective role in SMA. We report here the therapeutic benefits of systemic delivery of cardiotrophin-1 (CT-1), a neurotrophic factor belonging to the IL-6 cytokine family. Intra-muscular injection of adenoviral vector expressing CT-1, even at very low dose, improves median survival, delays motor defect of mutant mice and exerts protective effect against loss of proximal motor axons and aberrant cytoskeletal organization of motor synaptic terminals. In spite of the severity of SMA phenotype in mutant mice, CT-1 is able to slow down disease progression. Neuroprotection could be regarded as valuable therapeutic approach in SMA.

INTRODUCTION

Spinal muscular atrophy (SMA) is one of the most frequent genetic causes of death in childhood. SMA is an autosomal-recessive disorder with heterozygosity frequency of one in 35 persons (1). SMA is characterized by degeneration of lower motor neurons leading to muscle paralysis and atrophy. Homozygous deletion or conversion events of SMN1 exon 7 are the most frequent mutations found in SMA patients (reviewed in 2). SMN1 encodes a protein involved in several processes including pre-mRNA splicing, transcription and metabolism of ribosomal RNA (reviewed in 3). However, the molecular pathway linking SMN1 defect to SMA phenotype remains unclear.

The absence of Smn gene duplication in mouse and the embryonic lethality of mice carrying homozygous deletion of Smn in all cell types have led us to adopt the Cre–loxP system to direct deletion of Smn exon 7 in a given tissue (2). This system allowed circumvention of embryonic lethality and reproduction of the most frequent mutation found in patients. Mutant mice in which Smn mutation has been directed to neurons (neuronal model) exhibit the functional and morphological criteria of spinal muscular atrophy (4–8) without mimicking the complex genetic situation found in human patients. Mutant mice display progressive motor defects leading to complete paralysis and death with median survival of 33 days. Characterization of the neuromuscular system of mutant mice has revealed skeletal muscle denervation, severe motor axonal degenerative process and moderate loss of motor neuron cell bodies, features similar to those found in human SMA (4–8).

Neurotrophic factors have been regarded as therapeutic agents able to slow down motor neuron cell death and axonal degeneration (reviewed in 9). Therapeutic benefits have...
been reported in models of motor neuron disease including wobbler and pmn mice (10–14). Cardiotrophin-1 (CT-1), a cytokine belonging to the IL-6 family (15), has been shown to be able to prevent or delay motor axonal degeneration in pmn and in transgenic mice overexpressing SOD1 G93A, a mouse model of amyotrophic lateral sclerosis (ALS) (16,17).

Here, we have investigated the therapeutic potential of CT-1 in a neuronal mouse model of SMA. Intramuscular injection of adenoviral vectors represents an efficient means of achieving gene transfer and systemic delivery of CT-1 in vivo (16). In this study, we treated newborn SMA mutant mice and observed a therapeutic benefit in both functional and morphological parameters.

RESULTS

Therapeutic benefits of high dose of AdCT-1 in mouse model of SMA

We first determined the bioactivity of adenoviral vector constructs, both in vitro and in vivo. After delivery of adenoviral vectors expressing CT-1 (AdCT-1) (16) or LacZ (AdLacZ) (18), supernatants of HeLa cultured cells or homogenates of gastrocnemius from 30-day-old mutant mice were tested in a CT-1 ELISA assay. Significant production of CT-1 was detected in HeLa cells or gastrocnemius after AdCT-1 delivery (Fig. 1).

Neonatal intra-muscular injections of AdCT-1 or AdLacZ were performed in 14 and 12 mutant mice (NSE-Cre, SmnF7/F7), respectively, while 16 mutant mice did not receive any treatment. We delivered $10^8$ p.f.u. per animal, a dose named ‘high’, reported to display therapeutic benefit in two other mouse models of neurodegeneration (16,17). Seven AdCT-1- and eight AdLacZ-injected mutant mice were saved for survival and rotarod tests, while the others were sacrificed at day 30 for molecular or morphological analysis. AdCT-1 injection resulted in significant improvement in the median survival of mutant mice (44.4 days ± 1.3 SE, $n = 7$) compared with that of AdLacZ (33.7 days ± 3, $n = 8$, $P = 0.0005$) or untreated mutant mice (33.7 days ± 1.7 SE, $n = 16$, $P = 0.009$; Fig. 2A). Induced motor activity was evaluated by rotarod test in AdCT-1, AdLacZ or untreated mutant mice. AdCT-1-treated mutant mice had significantly better performance at 5 r.p.m. from 15 to 30 days of age than that of AdLacZ or untreated mutant mice ($P < 0.05$; Fig. 2B). At 10 r.p.m., AdCT-1-treated mice were able to maintain their balance for more than 3 min, except at day 30, a performance out of the AdLacZ-treated animal range (Fig. 2B).

To know whether AdCT-1 has any effect on terminal motor axons, labeling of neuromuscular junctions (NMJ) was performed on hindlimb muscles contralateral to intra-muscular injection of AdCT-1 or AdLacZ from 30-day-old mutant mice. Whole-mount preparations of muscle fibers from tibialis anterior or extensor digitorum longus were stained with rhodamine-conjugated α-bungarotoxin and with a monoclonal antibody directed against the 160 kDa subunit of neurofilament (NF) to label the acetylcholine receptor (AChR) and NF middle subunit, respectively (Fig. 3). A marked reduction in number of abnormal neuromuscular junctions filled with NF was observed in 30-day-old AdCT-1 mutant mice (41 out of 161 NMJ, 25.5%) when compared with AdLacZ mutant mice of the same litter [51 out of 128 NMJ, 40%, $P(x^2) = 0.01$, data not shown].

Semi-quantitative RT–PCR analysis of Smn transcripts extracted from spinal cord of AdCT-1 or non-injected mutant mice did not reveal any difference in the Smn amount or spliced products, indicating that therapeutic benefits of CT-1 were not caused by modification of Cre recombinase activity nor Smn transcript regulation (data not shown).

Low dose of AdCT-1 results in therapeutic benefits without side effects

Despite the marked therapeutic benefit of AdCT-1 on SMA disease progression, AdCT-1 induces toxic side effect as shown
by loss of weight of AdCT-1 treated mutant mice when compared with those treated with AdLacZ (Fig. 4). To determine if a reduction in CT-1 amount could lower its toxicity while preserving its therapeutic effect, 20 other mutants were injected with a 2.5 × 10⁶ p.f.u. dose (referred to as 'low dose'). Median survival was significantly enhanced with this treatment, reaching a value of 39.6 days ± 1 SE (P = 0.04, n = 13; Fig. 2A). Motor performances at both 5 and 10 r.p.m. were improved as determined by the rotarod test (Fig. 2B). This low dose of AdCT-1 was also able to prevent disorganization of neuromuscular junctions, with 45% reduction in number of abnormal NMJ (11 out of 69 NMJ, 16%) when compared with AdLacZ-treated mutant mice of the same litter [33 out of 54 NMJ, 61%, P(\chi²) < 0.0001, Fig. 3].

CT-1 was shown to be able to attenuate axonal degeneration of phrenic nerves in pmn and transgenic mice overexpressing SOD1G93A, a mouse model of ALS (16,17,19). To determine whether CT-1 has a similar effect in the SMA mouse model, semi-thin transverse sections of phrenic nerve were examined in AdCT-1, untreated mutant mice and control littermates. Light microscopic examination of cross-sections stained with...
toluidine blue revealed significant loss of myelinated axon number in phrenic nerves of 30-day-old untreated mutant mice (200 ± 3, n = 10, 14% reduction) compared with that of control littermates (232 ± 3 SE, n = 5, P < 0.001; Fig. 5). At 4 weeks of age, a significant, although moderate, preservation of phrenic axon number was observed in AdCT-1-treated mutant mice (216 ± 2, n = 3, P = 0.02) when compared with that of untreated mutants (200 ± 3, n = 10, 8% increase, Fig. 5).

**DISCUSSION**

Mutant mice carrying homozygous deletion of Smn exon 7 directed to neurons fulfill the criteria of spinal muscular atrophy including skeletal muscle denervation, abnormal morphology of motor neurons, moderate loss of motor neurons and severe loss of proximal motor axons. Muscle denervation of mutant mice was determined by defect of motor behavior, and the presence of group of atrophic muscle fibers associated with re-expression of the AChR outside the neuromuscular junctions (4,5). Loss of terminal motor axons was confirmed using immunolabeling and transmission electron microscopic examination of neuromuscular junctions from mutant mice (4,5). In addition, analysis of mutant mice revealed abnormal accumulation of neurofilaments in terminal motor axons while in human SMA such detailed analysis has not yet been performed. Abnormal morphology of motor neurons including deep invagination of the nuclear envelope was identified in both the SMA mouse model and human fetuses predicted to have SMA type I, suggesting that these changes are early signs of motor neuron degeneration (4,5,7). Moderate loss of motor neurons was observed in both our mouse model (5) and human SMA type I fetuses or neonate SMA patients who did not display any significant changes in the number of lower motor neuron cell bodies (7,8). These data suggest that loss of motor neurons is a moderate and late manifestation in both human and mouse SMA. Finally, severe loss of proximal motor axons of lumbar ventral roots was detected in both our mouse model (78% reduction) (5) and in SMA type I patients (65% reduction, six patients analyzed) (6). Moderate loss of motor neuron cell bodies associated with severe loss of proximal motor axons was found in mutant mice of the same age, which strongly suggests the involvement of a dying back process of motor neurons (5). Quantitative study of both motor neuron cell bodies and proximal motor axons was not performed in

**Figure 3.** Analysis of neuromuscular junctions of control, AdCT-1 and AdLacZ mutant mice. In toto immunostaining of NMJ from teased muscle fibers was performed in 30-day-old control and mutant mice treated with AdLacZ or AdCT-1 (low dose). AChR (red) and NF-M (green) labeling reveals thin terminal axons in controls (arrow), while in AdLacZ mutant mice synaptic gutters are filled with NF (arrowhead). Note the reduction in number of terminal axons filled with NF in mutant mice treated with low dose of AdCT-1. An example of normal NMJ in an AdCT-1 treated mouse is indicated by the arrow. Scale bar: 30 μm.
SMA patients or fetuses so far. Although the Smn defect was directed to neurons and not to the other cell types, neuronal mutant mice provide thus a relevant model of human SMA and a valuable system for in vivo selection of compounds having neuronal input.

Intramuscular injection of AdCT-1 was shown to be an appropriate mean for systemic delivery of CT-1 and skeletal muscle represents an efficient reservoir from which CT-1 is secreted (16). No axonal retrograde transport of CT-1 from skeletal muscle to motor neurons was demonstrated using this approach (14). We report here that cardiotrophin-1 has beneficial effects on main pathological hallmarks found in SMA. AdCT-1 treatment at high dose improves median survival, delays motor defect of mutant mice as determined by rotarod test and protects both proximal and distal motor axons from degeneration. Side effects including low body weight and cardiotoxicity were previously described after administration of high doses of CT-1 (16). Interestingly, at very low dose (100-fold less), CT-1 still has definite therapeutic benefits, in a dose-dependent manner, without side effects on body weight. Further investigation is required to determine whether low dose of CT-1 may attenuate or prevent CT-1-mediated cardiotoxicity. Our results show that CT-1 is able to attenuate the SMA phenotype despite the severity of disease progression of mutant mice. These data indicate that neurotrophic factors are able to delay the neurodegenerative process in SMA.

Although CT-1 has been identified as a neurotrophic factor for the survival of subgroups of motor neurons during the embryonic period (20), we report here that CT-1 delivery in the postnatal period leads to definite protection against degenerative process of motor axons in SMA, suggesting that CT-1 can also play an important neuroprotective role during the postnatal period. This role could be of great interest considering that protecting subgroups of motor neurons against degeneration could be sufficient to increase the number of functional motor units, leading to motor and functional improvement of SMA. Progressive paralysis of SMA mutant mice leading to death has, however, been observed, feature also observed in pnm and ALS mouse models (16,17), suggesting a limited action of CT-1. Associating CT-1 with other neuroprotective drugs might amplify the therapeutic benefit of this molecule. Although elucidating SMA pathogenesis should help in identifying key molecular intermediates for targeted therapeutics, the therapeutic benefit of neuroprotection may warrant further investigation as potential therapeutic strategy in SMA.

SMA, ALS and pnm mouse models are caused by mutations of genes encoding proteins involved in distinct pathways (21–23). CT-1 exerts, however, therapeutic benefits in all mutants, indicating that systemic delivery of neurotrophic factors from skeletal muscle is a valuable therapeutic strategy of motor neuron diseases including SMA. Several neurotrophic factors (CNTF, BDNF, IGF-1) have been suggested for the treatment of motor neuron diseases. In ALS patients, however, the repeated subcutaneous injection of these factors as recombinant proteins was complicated by their toxicity or poor bioavailability (24). Improving delivery through continuous systemic liberation of growth factors was able to prevent side effects in patients and should allow accurate evaluation of the therapeutic benefit of growth factors (25). Gene transfer in skeletal muscle, an efficient mean for systemic delivery of neurotrophic factors in vivo, might represent an alternative approach.

In the scope of future clinical trials, the use of adenoviral vectors rising important safety concerns (26) could be circumvented by non-viral methods. Electrottransfer of plasmid encoding CT-1 into skeletal muscle has been proved to be almost as efficient as adenovirus-mediated gene transfer (27), representing therefore an attractive and safe alternative for therapeutic interventions.

**MATERIAL AND METHODS**

**Mice**

Mutant mice carrying a deletion of Smn exon 7 directed to neurons (NSE-Cre<sup>+</sup>, Smn<sup>F7/F7</sup>) have been generated by crossing (Smn<sup>F7/F7</sup>) mice to those carrying the (Smn<sup>F7/+</sup>, NSE-Cre<sup>+</sup>) genotype. (NSE-Cre<sup>+</sup>, Smn<sup>F7/F7</sup>) mutant mice were selected by PCR amplification analysis of DNA obtained from tail biopsy as previously described (4). (Smn<sup>F7/+</sup>) mice of the same litter were used as controls. Mice were seronegative for common mouse and bacterial pathogens. All animal procedures were performed in accordance with institutional guidelines (agreements A91-228-2 and 3429).

**Cell culture and CT-1 ELISA**

HeLa cells were grown to a density of 3 × 10<sup>6</sup> cells per 25 cm<sup>2</sup> dish. Adenoviral infection was conducted during 1 h at different multiplicities of infection (from 10 to 160 p.f.u./cell). Conditioned media (2 ml) were sampled from 24 to 48 h after infection. CT-1 ELISA has been described in detail elsewhere.

Figure 4. Weight evolution of mutant mice as a function of treatment. High doses of AdCT-1 prevented treated animals from gaining weight, revealing the toxic effect of CT-1, in contrast to low doses of CT-1.
This ELISA allows determination of CT-1 level in both conditioned media and muscle homogenates. After neonatal intra-muscular injection of AdCT-1 or AdLacZ, skeletal muscles have been dissected out from 30-day-old mutant mice and homogenized as previously described (24).

**Adenovirus CT-1 and LacZ and intra-muscular injection of adenoviral vectors**

Construction of AdCT-1 and AdLacZ has been described previously (16,18). Newborn mice (5–7-day-old) were briefly anesthetized by hypothermia on ice. Vectors were diluted in 0.9% NaCl to 10⁶ p.f.u./100 μl or 2.5 × 10⁶ p.f.u./100 μl and injected unilaterally into three muscle groups—gastrocnemius (50 μl), triceps brachii (30 μl) and along the muscles of the dorsal trunk (20 μl)—as previously described (17).

**Survival and rotarod test**

Age of death was recorded by daily observation of mice. Mice were killed using CO₂, as recommended by institutional guidelines, when they were no longer able to move or to bear their weight. Rotarod test was used to assess motor performance as previously described (5). Results are given as the time that mice were able to maintain their balance on the rod.

**Histologic analysis of phrenic nerves**

In 30-day-old mice, phrenic nerves were dissected at the thoracic inlet and immersed in 2.5% glutaraldehyde, 0.5% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), rinsed in PB, and postfixed in 2% osmium tetroxide. After three washes with PB, each sample was dehydrated in a graded series of ethanol and embedded in Epon. Semi-thin transverse sections of phrenic nerve (1 μm) were stained with toluidine blue and examined under a Zeiss Axiophot microscope.

**Immunofluorescence staining of NMJ**

In toto immunostaining of NMJ was performed as previously reported (5). In brief, skeletal muscles were directly fixed for 1 h in 2% formaldehyde, then incubated for 1 h in PBS (pH 7.4) containing 0.1 M glycine. Presynaptic motor nerve terminals were stained with monoclonal antibody directed against the 160 kDa isoform of neurofilament (NN18, Chemicon Inc., CA, USA) and AChR with rhodamine-conjugated α-bungarotoxin (α-BgTx, Molecular Probes, Eugene, OR, USA). Motor end plates were labeled on whole mount preparations of muscle fibers and the teased fibers were mounted in Mowiol-glycerol. Analysis of NMJ was performed in 30-day-old control and mutant mice of the same litter. In control NMJ, the terminal axon labeled with NF displays a very thin caliber within the synaptic gutter labeled with α-BgTx. NMJ was selected as abnormal when synaptic gutters were filled with NF. The number of abnormal neuromuscular junction of mutant mice injected with a high dose of CT-1 was compared with that of mutant mice injected with AdLacZ and belonging to the same litter. The same procedure was applied to evaluate the effect of low dose of AdCT-1. Confocal analysis was performed as previously described (28). Doubly-labeled NMJs were observed with a confocal multiphoton microscope (Leica SP-2; argon laser at 488 nm for FITC and He–Ne laser at 543 nm for TRITC) through an oil immersion objective (X40; NA 1.25).
Statistical analysis

Statistical comparisons were performed using Student’s t-test for the rotarod test and number of myelinated axons. Median survival was determined using the Kaplan–Meier method, followed by the log rank test. The chi-squared test was used for statistical comparison of neuromuscular junction morphology.

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