Adenoviral-mediated mouse model of motor impairment in distal spinal muscular atrophy type V

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Distal spinal muscular atrophy type V (dSMA-V) is a hereditary neurodegenerative axonal neuropathy and a glycyl-tRNA synthetase (GARS)-associated neuropathy that is caused by a mutation in GARS. Herein, we report a new GARS-associated neuropathy mouse model using an adenovirus vector system equipped with a neuron-specific promoter. In this model, we showed an increased number of activated microglia around the L129P mutant-expressing motor neuron cell bodies and the increased nerve injury signal in L129P mutant-expressing motor neuron cell bodies. In addition, we found that the mouse model exhibited impaired motor function caused by the L129P mutant. We propose that the neuroinflammation may be associated with motor neuron impairment in GARS-associated neuropathies.

Keywords: recombinant adenovirus; glycyl-tRNA synthetase; motor impairment; ATF3; microglia

Introduction

Glycyl-tRNA synthetase (GARS), a member of the aminoacyl-tRNA synthetase family, is expressed ubiquitously in both neuronal and nonneuronal cells. Mutations in the GARS gene were found in Charcot-Marie-Tooth disease (CMT) type 2D, which is a neurodegenerative axonal neuropathy characterized by impaired motor function and sensory loss in the extremities (Antonellis et al. 2003). CMT2D is a neuromuscular disorder with a phenotype similar to that of distal spinal muscular atrophy type V (dSMA-V), which can be distinguished from CMT2D only by the absence of sensory loss (Barisic et al. 2008). The GARS L129P mutation is reportedly linked to dSMA-V (Christodoulou et al. 1995; Antonellis et al. 2003).

Although CMT2D and dSMA-V are associated with a mutation in GARS, the underlying molecular mechanisms for the motor impairment in GARS-associated neuropathies remain unclear. Among CMT disease pathophysiology, neuroinflammation may explain motor impairment in GARS-associated neuropathies. Understanding the pathophysiology of amyotrophic lateral sclerosis (ALS), an inherited neurodegenerative disease characterized by the progressive loss of upper and lower motor neurons, may help identify the underlying mechanisms of motor impairment in GARS-associated neuropathies. The most prominent known causes of familial ALS are mutations in the ubiquitously expressed protein superoxide dismutase 1 (SOD1), similar to the GARS protein mutation that induces GARS-associated neuropathies (Rosen et al. 1993). Several studies have suggested that microglia-associated motor impairment is associated with neuronal degeneration in ALS (Boillée et al. 2006; Dibaj et al. 2012). These studies suggest that neuroinflammation induced by a GARS mutation is a mechanism for GARS-associated neuropathies.

These possible GARS-associated neuropathy mechanisms were supported by our in vivo adenovirus mouse models. To confirm the effect of the L129P mutant GARS in dSMA-V, we generated in vivo mouse models using neuron-specific adenovirus vectors and investigated the mechanism for motor neuron impairment in GARS-associated neuropathies. We employed an adenovirus vector system equipped with a neuron-specific promoter to achieve strong transgene expression restricted entirely to neurons in vivo. Our data indicate that the neuroinflammation is potential mechanisms underlying dSMA-V phenotypes.

Materials and methods

Animals

All experiments were performed in six-week-old male C57BL/6 mice. Mice were housed in a temperature- and humidity-controlled environment on a 12-h light/12-h dark cycle and were provided food and water ad libitum. Cages were changed weekly. All animal procedures were
performed in accordance with the guidelines of the US National Institute of Health and approved by the Kyung Hee University Committee on Animal Research. Every effort was made to minimize the number of animals used and the amount of animal suffering.

**Neuron-specific recombinant adenoviruses**

Adenovirus vectors were constructed as previously reported (Seo et al. 2014). Briefly, as shown in Figure 1A, we used that of the mouse choline acetyltransferase (ChAT) gene (GenBank accession number NC_000080) for the promoter (Misawa et al. 1992; Lönnerberg et al. 1996). Recombinant pAdEasy-Chat-hGARS<sup>wt</sup>,-CMV/green fluorescent protein (GFP) (AdhGARS<sup>wt</sup>/Chat) and pAdEasy-Chat-hGARS<sup>L129P</sup>,-CMV/GFP (AdhGARS<sup>L129P</sup>/Chat) were transfected into AD293 cells (Invitrogen, Carlsbad, CA, USA), specifically, the E1-expressing packaging cell line, for further propagation and amplification. Amplified viruses in AD293 cells were concentrated and purified by density gradient separation using cesium chloride (CsCl) and ultracentrifugation. Finally, the CsCl-concentrated viruses were replaced with optimal buffer (10 mM Tris-Cl pH 8.0, 2 mM MgCl<sub>2</sub>, 5% sucrose) using membrane dialysis.

![Diagram](image_url)

**Figure 1.** Neuron-specific expression of hGARS proteins in peripheral nerves. (A) Schematic representation of promoter and expression units in each adenovirus. (B) mRNA expression of GAP-43. Seven days after adenovirus infection, mRNA expression of GAP-43 in DRG samples was determined by RT-PCR. (C) Semi-quantification of the relative intensity of bands derived by RT-PCR. GAPDH was used as an internal control (Con, n = 4; WT, n = 5; L129P, n = 5). (D) Co-localization of GFP (green) and ChAT (red), a marker of motor neurons, indicated adenovirus-infected motor neuronal cell bodies in spinal ventral horn cross sections (scale bar = 200 μm).
Motor performance was studied in mice injected with the recombinant adenoviruses using the cytotoxic effect method (Nyberg-Hoffman et al. 1997). The titers of AdhGARS\(^{wt}\)/ChAT and AdhGARS\(^{L129P}\)/ChAT used in these experiments were 5.1 \(\times\) 10\(^9\) (pfu/mL) and 2.6 \(\times\) 10\(^9\) (pfu/mL), respectively.

**Viral administration procedures**

Recombinant adenoviruses were injected into the sciatic nerve for anterograde and retrograde transduction. Mice were anesthetized with Zoletil (Virbac, Carros Sedecs, France; 30 mg/kg) and Rompun (Bayer, Leverkusen, Germany; 10 mg/kg), and the sciatic nerve was surgically exposed by incision of the musculus gluteus superficialis. The nerve was gently placed using forceps, and virus diluent of AdhGARS\(^{wt}\)/ChAT (1.5 \(\mu\) L) or AdhGARS\(^{L129P}\)/ChAT (3 \(\mu\) L) was injected with Fast Green (Sigma, St. Louis, MO, USA) to visualize virus infusion. The injections were performed with a fine capillary microinjection needle pulled using the NARISHIGE puller (PC-10) attached via rubber tubing to a 25-\(\mu\)L Hamilton syringe to minimize nerve insult. The skin incision was closed with sutures (B/Braun, Melsungen, Germany; Silkam 4.0). In all sciatic nerve-associated experiments, we used the upper part of the site where the adenovirus vectors were injected.

**Behavioral tests**

Motor performance was studied in mice injected with the recombinant virus (AdhGARS\(^{wt}\)/ChAT or AdhGARS\(^{L129P}\)/ChAT) on both sides of the sciatic nerve. All behavioral tests were performed seven days after the sciatic nerves were infected with adenovirus vector.

The fixed bar consisted of a wooden bar (2 cm in diameter, 40 cm long, and 40 cm above the ground) held horizontally on both ends. To accomplish the fixed bar test, each mouse was placed onto the bar, and only the mice that stayed on the rod for more than 3 min were used for the tests. Groups of mice (Con, \(n = 4\); Sham, \(n = 4\); WT, \(n = 6\); L129P, \(n = 5\)) were placed onto the bar and tested for their ability to remain on the bar for 3 min.

In the modified tail suspension test (MTST), each mouse was suspended by the tail end 50 cm above a table and remained hanging for 10 min. We scored the duration of the hind limbs’ full extension within a 10-min session. This procedure was repeated three times for each mouse. Full extension of the hind limbs was defined as complete opening of the hind limbs and extension of their phalanges.

**Immunofluorescence labeling**

Tissues collected from the L4/L5 spinal cord at seven days of virus administration were dissected after perfusion with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The samples were immersed in the same solution overnight at 4°C and then in 30% sucrose in 0.1 M PB for three days. The samples were then embedded in optimum cutting temperature compound and frozen immediately in dry ice. Frozen sections (16 \(\mu\)m) were cut from tissues and placed onto slides (Fisher Scientific, Houston, TX, USA). For staining, slides were fixed in 4% PFA for 10 min. After three washes with phosphate-buffered saline (PBS), the samples were permeabilized in PBS containing 0.3% Triton X-100 and blocked with 10% bovine serum albumin overnight at 4°C. The primary antibodies, rabbit anti-Flag (1:500; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-ATF3 (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-ChAT (1:1000; Millipore, Billerica, MA, USA), and rabbit anti-Iba-1 (1:1000; Wako Chemicals, Richmond, VA, USA) were placed on the slide, and the slides were incubated for 1 h at room temperature. After three 10-min washes in PBS, samples were incubated for 2 h at room temperature in Alexa Fluor 594 donkey anti-mouse secondary antibodies. The slides were then washed with PBS and mounted. The immunofluorescence was detected and analyzed by a laser scanning confocal microscope (LSM700, Carl Zeiss, Oberkochen, Germany).

**Reverse transcription polymerase chain reaction (RT-PCR)**

After seven days of virus administration, the L4/L5 dorsal root ganglia (DRG) were collected and stored appropriately for RT-PCR. Isolated total RNA (5 \(\mu\)g) from the DRG was reverse-transcribed into first-strand complementary DNA (cDNA) using a reverse-transcription kit (Invitrogen). RT-PCR reactions were performed on the cDNA template with Taq polymerase PCR master mix (Cosmogenetech, Seoul, South Korea) in a total volume of 25 \(\mu\)L. GAPDH amplification with 1 \(\mu\)L cDNA was performed first to quantify the cDNA. Quantified cDNA was used for the reactions with the other primer sets. The designed primer sets were as follows: GAPDH-Forward, 5'-CACGACTGTCATCG-3', GAPDH-Reverse, 5'-GGTTTGGCTTCGAGGCCAAGGAG-3'; and GAP43-Forward, 5'-AGGCCAAGGAGAGGATGAT-3'; GAP43-Reverse, 5'-GGTTTGGCTTCGCTACAGC-3'. Thermal cycling conditions were 5 min at 94°C, 28–33 amplification cycles for 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and then another 7 min at 72°C. Each reaction was performed in triplicate. The results were visualized using 2% agarose gel electrophoresis stained with GelRed (Biotium, Hayward, CA, USA).

**Statistical analysis**

Differences in the means among groups were statistically analyzed using analysis of variance followed by a
Bonferroni post-hoc test. Differences were considered statistically significant at the \( p < 0.01 \) level after analysis of three independent experiments.

Results

Growth-associated protein 43 (GAP-43) was induced by peripheral nerve injury and was highly expressed in the growth cone during regeneration (Cafferty et al. 2004). To investigate the difference in adenoviral-induced damage to DRG cell bodies due to the WT and L129P mutant proteins, we assessed the relative levels of GAP-43 mRNA as a marker of peripheral nerve injury. The RT-PCR result showed that the L129P mutant did not cause an increase of GAP-43 in DRG cell bodies, and both the WT and L129P mutant proteins caused the same level of adenoviral-induced damages (Figure 1B and C). Thus, these findings suggest the WT and L129P mutant hGARS proteins are expressed at the same level in peripheral nerves, and the damage they cause to peripheral nerves is similar.

Next, to understand the underlying mechanisms of dSMA-V motor impairment, we evaluated several molecular markers, ChAT, activating transcription factor 3 (ATF3), and Iba-1, to identify their relationship with dSMA-V motor impairment. First, to assess whether adenovirus infection in the spinal ventral horn is specific to motor neurons, we immunostained adenovirus-infected spinal cord samples with the antibody against ChAT, a motor neuron marker. Figure 1D shows GFP/ChAT double-positive signals, indicating adenovirus-infected motor neurons in the spinal ventral horn.

ATF3 is considered an injury marker in the nervous system (Tsujino et al. 2000; Ohba et al. 2003) and is expressed in degenerating motor neurons (Vlug et al. 2005). To assess whether the L129P mutant hGARS protein affected ATF3 expression in motor neurons, we performed ATF3 immunostaining in adenovirus-infected spinal cords seven days after adenovirus infection. Image analysis showed that ATF3 was expressed in GFP-positive motor neuronal cell bodies of the L129P mutant hGARS-expressing ventral horn (Figure 2A and B). However, Figure 2A also shows that ATF3 was rarely expressed in GFP-positive motor neuronal cell bodies of the WT hGARS-expressing ventral horn compared with the L129P mutant hGARS-expressing ventral horn. The quantitative data indicated a significant increase of ATF3 expression in the L129P mutant hGARS-expressing ventral horn compared with the WT GARS-expressing ventral horn (Figure 2B). Thus, these findings suggest that L129P mutant hGARS-induced ATF3 expression is involved in motor impairment and is correlated with motor impairment in GFP-positive motor neurons.

Microglial activation contributes substantially to motor neuron death and degeneration (Beers et al. 2006; Boillée et al. 2006). To confirm the influence of L129P mutant hGARS on microglial activation in this dSMA-V adenovirus model, we performed immunostaining to quantify microglia activation using Iba-1 as a marker of microglia. Seven days after adenovirus infection, a significant increase in Iba-1 immunostaining was observed around GFP-positive motor neurons in the L129P mutant hGARS ventral regions compared with WT hGARS regions, indicating that L129P mutant hGARS motor neurons are involved in microglia-mediated inflammation (Figure 3A and B). Thus, these findings indicate microglia-induced inflammation plays an important role in motor neuron dysfunction in this dSMA-V mouse model.

Due to a GARS mutation, dSMA-V is a peripheral neuropathy characterized by progressive weakness and atrophy. Seven days after the sciatic nerves were infected with the adenovirus, we performed different types of motor impairment tests, the fixed bar test and MTST, to assess motor neuron impairment. In the fixed bar test, WT GARS-expressing mice moved efficiently along the bar without falling off (Figure 4A). By contrast, mutant GARS-expressing mice showed reduced mobility along the bar and were unable to remain on it (Figure 4A). In the MTST, WT GARS-expressing mice were able to fully extend, with a complete opening of the adenovirus-infected hind limbs,

![Figure 2. Increase of motor neuron-damaging signals in L129P mutant GARS-expressing spinal ventral horn. (A)](image)
for longer periods of time, compared with a low degree of opening and shrinkage of mutant GARS-expressing mice (Figure 4B). Thus, our data suggest that expression of the L129P mutant GARS protein may be involved in coordinated motor activity in peripheral nerves. Collectively, in this model, behavioral measures of peripheral nerve dysfunction are useful for detecting motor function in the dSMA-V phenotype.

**Discussion**

Muscular weakness and atrophy are among the general clinical features of CMT, including dSMA-V. In this dSMA-V animal model, we found motor impairment in L129P mutant GARS-expressing mice compared with WT hGARS-expressing mice using behavioral tests (Figure 4). However, the functional, morphological, and molecular knowledge of motor impairment in dSMA-V is lacking. Herein, we discuss two possibilities (ATF3 and microglia activation) that explain the motor-dysfunctional factors in dSMA-V.

Motor neurons in the spinal ventral horn respond to injurious stimuli by ATF3 activation (Tsujino et al. 2000; Hai & Hartman 2001). In previous studies, ATF3 activation was associated with deterioration of motor neurons (Vlug et al. 2005; de Waard et al. 2010). In addition, ATF3 was linked to cell death programs (Hai & Hartman 2001; Nakagomi et al. 2003; Raivich et al. 2004) and, thus, can serve as a marker of degenerating motor neurons. However, in dSMA-V or CMT2D, the relationship between ATF3 expression and motor neurons is poorly understood. The establishment of ATF3 as a contributing factor to motor neuron vulnerability in vivo is important for understanding motor neuronal degenerative diseases. Our data showed that the number of ATF3-positive cells increased in L129P mutant hGARS-expressing motor neurons compared with WT hGARS-expressing neurons (Figure 2A and B). These results suggest that in dSMA-V, the expression of L129P mutant hGARS induces ATF3 activation in ventral motor neurons, and ATF3 activation triggers cell death programs similar to the effect of ATF3 seen in ALS (Vlug et al. 2005).

Previous studies have emphasized that microglia contribute to motor neuronal death and neurodegenerative disease progression in diseases such as ALS (Beers et al. 2006; Boillée et al. 2006; Yamanaka et al. 2008). Additionally, ALS familial type 8, spinal muscular atrophy (SMA), and adult-onset proximal SMA are attributed to a mutation in the vesicle-associated membrane-associated protein, protein B (Nishimura et al. 2004). Thus, understanding ALS mechanisms is likely to help identify the

**Figure 3.** Increase of microglial activation in L129P mutant GARS-expressing spinal ventral horn. (A) GFP expression (green) and immunofluorescent staining against Iba-1 (a macroglial marker) in the ventral horn. Iba-1-positive cells localized around GFP-positive L129P mutant hGARS-expressing neuronal cell bodies more so than around WT hGARS-expressing cell bodies. (B) Number of Iba-1-positive cells surrounding a GFP-positive cell body from the spinal cord sections in a microscopic field (300 μm × 300 μm). Error bars indicate the SD (statistically significant differences using the paired t-test; WT, n = 5; L129P, n = 7; *p < 0.01).

**Figure 4.** Motor impairment in the dSMA mouse model. (A) Coordinated motor activity analysis using the fixed bar test. The percentage of mice indicates the relative number of animals that remained on the bar compared with controls. **p < 0.01. (B) MTST of control, Sham-operated, GARS WT- and L129P mutant-expressing mice. The duration of full hind limb extension indicated the time (seconds) taken to completely open the hind limbs. Con, n = 4; Sham, n = 4; WT, n = 6; L129P, n = 5. **p < 0.01.
underlying pathophysiology of motor impairment in GARS-associated neuropathies. However, the contribution of microglia-mediated damage to the onset and progression of motor neuronal impairment in dSMA-V has not been established. Our data showed that regions around GFP-positive motor neurons in the L129P-expressing ventral horn contain more Iba-1-positive microglial cells compared with the WT hGARS-expressing ventral horn (Figure 3A and B). These results suggest the possibility that L129P mutant hGARS proteins in motor neurons induce microglial activation and the subsequent release of pro-inflammatory cytokines that drive disease progression. Thus, it seems likely that neuroinflammation induced by microglia is one of causes of motor impairment in GARS-associated neuropathies, similar to that of found in ALS model which is induced by SOD1 protein mutation (Boillée et al. 2006; Dibaj et al. 2012). Furthermore, these results have important implications for the development of successful therapies for dSMA-V by targeting microglia.

Sensory impairment does not have the same prevalence as motor impairment in CMT2D and dSMA-V (Barisic et al. 2008). dSMA-V has similar phenotypes as CMT2D, such as impairment of peripheral axons and absence of defects associated with myelin sheaths in Schwann cells (Antonellis et al. 2003). However, dSMA-V is distinguished from CMT2D only by the absence of sensory loss (Barisic et al. 2008). GAP-43 is a neuron-specific growth-associated protein expressed in DRG neurons (Bergman et al. 1999). GAP-43 expression is increased in DRG neurons after peripheral nerve injury (Kato et al. 2010) and is a frequently used marker for nerve degeneration after nerve injury. After adenovirus infection of mouse sciatic nerves, both WT and L129P mutant hGARS-expressing DRG cell bodies showed the same level of GAP-43 mRNA expression (Figures 1B and 3C). These results indicate that WT and L129P mutant-expressing DRG neurons were affected equally by the adenovirus vectors or by the physical damages associated with the technique used to infect the cells. In addition, if we interpret these results differently, sensory neurons are not affected by L129P mutant hGARS expression in this mouse model. However, further investigation is necessary to propose a reasonable mechanism for the absence of sensory impairment in dSMA-V using this in vivo model.

In conclusion, motor neuron cell death by ATF3 activation and neuroinflammation by microglial activation in the ventral horn may be involved in disease onset and progression as well as other disease-causing factors; for example, a noncanonical role of GARS in axons or a toxic effect of mutant GARS may exist. Previous studies suggested that the partial innervation in neuromuscular junctions of GARS neuropathy animal models using transgenic mice is involved in motor impairment (Seburn et al. 2006; Stum et al. 2011). In this adenovirus animal model, the abnormal innervation in neuromuscular junctions could not be excluded as a cause of motor impairment. Further evaluation is necessary to identify the relationship between the abnormal innervations and motor impairment in the animal model. Therefore, this overexpressing animal model can help explain the underlying mechanism of motor impairment in dSMA-V and help identify new therapeutic targets for dSMA and CMT2D.

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