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Title

Histopathological Changes of the Spinal Cord and Motor Neuron Dynamics in SOD1 Tg Mice

Running title: Dynamics of Motor Neurons in the Spinal Cord of SOD1 Tg Mice

Authors

Masaharu Tanaka, Kengo Homma, Aki Soejima

Affiliations

Research Unit/Neuroscience, Sohyaku. Innovation Research Division, Mitsubishi Tanabe Pharma Corporation, 1000, Kamoshida-cho, Aoba-ku, Yokohama-shi 227-0033, Japan

Address for correspondence:

Masaharu Tanaka, PhD,

Research Unit/Neuroscience, Sohyaku. Innovation Research Division, Mitsubishi Tanabe Pharma Corporation, 1000, Kamoshida-cho, Aoba-ku, Yokohama-shi 227-0033, Japan

Tel: 070-2448-3643, email: tanaka.masaharu@mb.mt-pharma.co.jp

Nonstandard abbreviations used in this paper:

ALS, amyotrophic lateral sclerosis; SOD1^{G93A} Tg, Cu/Zn superoxide dismutase transgenic; MN, motor neuron

Abstract

We analyzed the histopathological changes and the number of motor neurons (MNs) in the lumbar spinal cord of Cu/Zn superoxide dismutase transgenic (SOD1^{G93A}Tg) mice, which are frequently used as a disease model of amyotrophic lateral sclerosis (ALS). In SOD1^{G93A}Tg mice, hyaline inclusions and foamy vacuoles in the neuronal cell body were observed at 7 weeks of age before neurologic symptoms, and large vacuoles, spheroid formation, and nerve cell aggregation became prominent after 13 weeks of age. The number of healthy MNs was 28.7 to 37.1 cells/animal in wild-type mice and 9.3 to 13.6 cells/animal in transgenic (Tg) mice. Furthermore, the number of MNs, including degenerative neurons, in Tg mice was 27.3–36.1 cells/animal at 18 weeks of age and 17.8–19.6 cells/animal at 21 weeks of age. The present results provide useful information for the development of drugs in ALS treatment.

(141 words)

Keywords

Amyotrophic lateral sclerosis, degenerated neuron, histopathology, hyaline inclusion, motor neuron, SOD1^{G93A}Tg mice

Amyotrophic lateral sclerosis (ALS) is an unexplained progressive neurodegenerative disease in which motor neurons (MNs) are selectively damaged. Generally, the MN system is broadly divided into upper and lower MNs, which connect the brain to the spinal cord and connect the spinal cord to the periphery, respectively. ALS is characterized by the impairment of both the upper and lower MNs. Although more than 20 different mutant genes are known to cause ALS^{1,2}, including Cu/Zn superoxide dismutase (SOD1)³, TAR DNA-binding 43-kDa protein⁴, sarcoma fusion⁵, and chromosome 9 open reading frame 72⁶, the exact cause remains unknown, and research is still ongoing. Transgenic (Tg) mice overexpressing mutated SOD1 show progressive paralysis and muscle atrophy of the lower limbs due to cell death in the MN. These types of mice reproduce well the symptoms and pathological changes of ALS; therefore, they are frequently used as an animal model of ALS⁷. In addition, mutated SOD1 Tg mice have a long history, and many research results have been reported⁸⁻¹². Pathological lesions in this disease model were mainly limited to the spinal cord (particularly lumbar), brainstem, descending spinal tracts, and neuromuscular junctions. Moreover, various pathological features have been reported in the spinal cord before the onset of neurological symptoms, including mitochondrial vacuolation^{8,9}, Golgi fragmentation¹⁰, and neurofilament-rich inclusions¹¹. Spinal MNs are said to expire later, and the number of lumbar spinal MNs decreases by approximately 50% by the end stage¹². However, no detailed report has been provided on the time-course analysis of histopathological changes and the dynamics of MN quantity in the spinal cord of this animal model.

In this study, we collected the basic information of SOD1^{G93A}Tg mice and aimed to contribute to the development of drugs for ALS treatment by analyzing in detail the histopathological changes and the number of MNs in the spinal cord of SOD1^{G93A}Tg mice.

Six-week-old male and female B6Cg-Tg (SOD1)¹Gur/J mice (C57/B6) were purchased from Charles River (Tsukuba, Ibaraki, Japan). The animals were housed in the animal facility of Mitsubishi Tanabe Pharma Corporation (MTPC) and were acclimated for one week. The rearing conditions were as follows: 12 h light/dark cycle (lights on at 7:00 a.m.), 20 °C–26 °C temperature, and 26%–70% humidity. The animals had access to food and water *ad libitum*. The experiments were approved by the Institutional Animal Care and Use Committee of the Research Laboratories of MTPC.

A total of 80 mice were used in this study. Tg (n = 6 per sex) and wild-type (WT) (n = 4 per sex) mice were sacrificed at 7, 13, 18, and 21 weeks of age. Mice were anesthetized by inhalation of sevoflurane (Mylan Inc., Tokyo, Japan), and transcardiac blood sampling was performed. The animals were then sacrificed by exsanguination from the abdominal aorta.

The whole spinal cord was removed and fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for histopathology. Cross-sections of the lumbar spinal cord were embedded in paraffin and then sectioned (5 µm). Five hematoxylin and eosin (HE)–stained sections were prepared for each animal, and the section with the strongest change was evaluated for histopathological observations. Six and four mice in the Tg and WT groups were evaluated, respectively.

The number of MNs was counted using the HE-stained sections. The number of large neurons $\geq 25 \mu\text{m}$ in the anterior horn of the spinal gray matter was counted using five HE sections for each animal, and the mean value was defined as the number of MNs per animal. In addition, the number of large neurons $\geq 25 \mu\text{m}$, including degenerating cells, was similarly counted. The MN quantity obtained from three mice in the Tg and WT groups were used for analysis.

Statistical significance was assessed using Student's *t*-test. The values given in the text and figures are mean $\pm$ standard deviation of the mean.

Genotyping results from Charles River confirmed that the animals used in this study were Tg mice.

In this study, neurological symptoms, such as hind limb paresis, were observed at 13 weeks of age, and obvious atrophy of the thigh muscle was observed at 21 weeks of age. There were no sex-related differences in these changes.

No significant changes were observed in WT mice throughout the evaluation period (Fig. 1A). On the other hand, in Tg mice, hyaline inclusions and foamy vacuoles were observed in MN cell bodies at seven weeks of age, and the incidence and degree of change were stronger in the hyaline inclusion than in the foamy vacuole (Fig. 1B). The following changes were mainly observed at 13 weeks of age: Large vacuoles were observed in the spinal gray matter and white matter. Neuronal cell aggregation with glial cells and spheroid formation were observed in the gray matter, but no apparent neuronal necrosis was observed (Figs. 1C and 1D). Most lesions were observed in the anterior horn of the gray matter, but large vacuolation was

observed not only in the gray matter but also in the anterior or lateral funiculus of the white matter. In addition, the hyaline inclusions in neuronal cells observed at 7 weeks of age were not prominent after 13 weeks of age. No apparent increase in glial cells was observed in Tg mice at any age. No apparent sex differences were noted in these histopathological changes (Table 1).

The number of healthy MNs $\geq 25 \mu\text{m}$ was constant over time in male and female WT mice; however, it significantly decreased in Tg mice compared with WT mice at 7 weeks of age in both sexes. The numbers of healthy MNs $\geq 25 \mu\text{m}$ in male and female WT mice ranged from 31.2–37.1 cells/animal and 28.7–31.9 cells/animal throughout the evaluation period, respectively. For male and female Tg mice, the numbers of healthy MNs $\geq 25 \mu\text{m}$ ranged from 13.6–9.9 cells/animal and 13.6–9.3 cells/animal throughout the evaluation period, respectively (Fig. 2). The number of large neurons $\geq 25 \mu\text{m}$, including cells with degenerative changes (e.g., hyaline inclusion and foamy vacuole), was significantly reduced in Tg mice compared with WT mice only at 21 weeks of age. No degenerative neurons were observed in WT mice. Thus, 31.2–37.1 cells/animal and 28.7–31.9 cells/animal were noted in male and female WT mice throughout the evaluation period, respectively. By contrast, 31.2–32.9 cells/animal and 19.6 cells/animal were noted in male Tg mice at 7–18 weeks of age and at 21 weeks of age, respectively. Similarly, 27.3–36.1 cells/animal and 17.8 cells/animal were noted in female Tg mice at 7–18 weeks of age and at 21 weeks of age, respectively (Fig. 3).

ALS is a progressive nervous system disease that affects the nerve cells in the brain and spinal cord and

impairs the control of the muscles that are needed for movement, speaking, eating, and breathing, thus leading to death. Edaravone and riluzole are the only drugs available for the treatment of this fatal disease.

Several animal models of ALS have been developed in recent years¹⁻⁶. In particular, SOD1 Tg mice are the most prominent ALS model and has been used for drug efficacy assessments¹³⁻¹⁸. Various analyses, including analyses on neurological symptoms, various proteins, RNA, muscle mass, survival rate, and presence/absence of sex difference using SOD1^{G93A}Tg mice (C57/B6) with a homogeneous genetic background among SOD1^{G93A}Tg mice, are currently being conducted. In the present study, we focused on the histopathological changes and the changes in the number of MNs in the lumbar spinal cord of SOD1^{G93A}Tg mice.

Chiu et al.¹² investigated the pathological analysis of the spinal cord in SOD1 Tg mice. They reported that vacuoles are the initial pathological changes in spinal MNs. Moreover, a vacuole has been reported as a swelling of the mitochondrial membrane on the basis of electron microscopy^{8,9}. However, according to the observations of the present study, the initial pathological changes in spinal MNs were foamy vacuoles and hyaline inclusions, and the degree was high and strong for hyaline inclusion. Although the relationship between hyaline inclusion and foamy vacuole remains unclear, the initial histological change observed in the spinal cord of SOD1^{G93A}Tg mice was revealed to be hyaline inclusion. These changes were more pronounced in younger mice but were less pronounced in mice older than 13 weeks. Moreover, although hyaline inclusions and foamy vacuoles were observed at seven weeks of age, neurological symptoms (e.g.,

hind limb paresis) were observed at nine weeks of age (data not shown).

Regarding the MN count in the spinal cord, Ferrucci et al.¹⁹ described that the classical immunostaining for antigens that were routinely thought to be specific to MNs (e.g., the nonphosphorylated epitope of neurofilament proteins and choline acetyltransferase [ChAT]) may also occur in other neuronal types of the spinal cord. In addition, they mentioned that the HE stain is the most appropriate tissue stain for MN identification. Therefore, in the current study, we performed MN counting by using HE staining rather than immunostaining. In general, when applied to rodents, an MN diameter $\geq 20 \mu\text{m}$ represents the most common cutoff for selectively counting alpha-MNs and simultaneously ruling out a large part of the gamma-MN population^{20,21}. Other authors brought this limit up to $30 \mu\text{m}$ in mouse spinal cord^{22,23}. In the current study, we used a cutoff of $25 \mu\text{m}$ for MN counting on the basis of this information.

Chiu et al.¹² evaluated the number of spinal MNs in SOD1 Tg mice by using ChAT immunostaining and reported that MNs were reduced by up to 50% in end-stage disease (136 days old). However, the results in the HE-stained specimens in the present study showed that the number of healthy MNs in Tg mice had already been halved at seven weeks. Moreover, the number of MNs, including degenerating neurons, decreased by half at 21 weeks of age, which is close to the end stage of Tg mice; these results are similar to that of Chiu et al.¹². Considering that ChAT immunostaining showed positive reactions even in MNs with degeneration, Chiu et al.¹² may have counted ChAT-positive cells, including degenerative MNs (data not shown).

In conclusion, the initial histopathological changes observed in SOD1^{G93A}Tg mice were not only foamy vacuoles but also hyaline inclusions in spinal cord MNs. Furthermore, the number of healthy MNs had already been halved before neurological symptoms were observed in Tg mice. This is important information to consider in a drug intervention study using SOD1^{G93A}Tg mice and is beneficial for the future development of therapeutic drugs for ALS.

Disclosure of potential conflicts of interest: The authors declare that they have no conflicts of interest.

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References

1. Ferraiuolo LK, Grierson J, Sendtner AJ, and Shaw PJ. Molecular pathways of motor neuron injury in amyotrophic lateral sclerosis. *Nat Rev Neurol* 7: 616-630. 2011.
2. Rademakers R, van Blitterswijk M. Motor neuron disease in 2012: novel causal genes and disease modifiers. *Nat Rev Neurol* 9: 63-63. 2013.
3. Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng H-X, Rahmani Z, Krizus A, McKenna-Yasek D, Cayabyab A, Gaston SM, Berger R, Tanzi

- RE, Halperin JJ, Herzfeldt B, Van den Bergh R, Hung W-Y, Bird T, Deng G, Mulder DW, Smyth C, Laing NG, Soriano E, Pericak-Vance MA, Haines J, Rouleau GA, Gusella JS, Horvitz HR and Brown Jr RH. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **362**: 59-62. 1993.
4. Kabashi E, Valdmanis PN, Dion P, Spiegelman D, McConkey BJ, Velde CV, Bouchard J-P, Lacomblez L, Pochigaeva K, Salachas F, Pradat P-F, Camu W, Meininger V, Dupre N and Rouleau GA. TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nat Genet.* **40**: 572-574. 2008.
 5. Kwiatkowski TJ, Bosco DA, Leclerc AL, Tamrazian E, Vanderburg CR, Russ C, Davis A, Gilchrist J, Kasarskis EJ, Munsat T, Valdmanis P, Rouleau GA, Hosler BA, Cortelli P, de Jong PJ, Yoshinaga Y, Haines JL, Pericak-Vance MA, Yan J, Ticozzi N, Siddique T, McKenna-Yasek D, Sapp PC, Horvitz HR, Landers JE and, Brown Jr RH. Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science* **323**: 1205-1208. 2009.
 6. DeJesus-Hernandez M, Mackenzie IR, Boeve BF, Boxer AL, Baker M, Rutherford NJ, Nicholson AM, Finch NA, Flynn H, Adamson J, Kouri N, Wojtas A, Sengdy P, Hsiung G-Y R, Karydas A, Seeley WW, Josephs KA, Coppola G, Geschwind DH, Wszolek ZK, Feldman H, Knopman DS, Petersen RC, Miller BL, Dickson DW, Boylan KB, Graff-Radford NR and Rademakers R. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9orf72 causes chromosome 9p-linked FTD and ALS.

- Neuron **72**: 245-256. 2011.
7. Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliendo J, Hentati A, Kwon YW, Deng H-X, Chen W, Zhai P, Sufit RL, Siddique T. Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. *Science* **264**: 1772-1775. 1994.
 8. Wong PC, Pardo CA, Borchelt DR, Lee MK, Copeland NG, Jenkins NA, Sisodia SS, Cleveland DW, and Price DL. An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* **14**: 1105-1116. 1995.
 9. Dal Canto MC and Gurney ME. Neuropathological changes in two lines of mice carrying a transgene for mutant human Cu, Zn SOD, and in mice overexpressing wild type human SOD: A model of familial amyotrophic lateral sclerosis (FALS). *Brain Res* **676**: 25-40. 1995.
 10. Mourelatos Z, Gonatas NK, Stieber A, Gurney ME, and Dal Cant MC. The Golgi apparatus of spinal cord motor neurons in transgenic mice expressing mutant Cu, Zn superoxide dismutase becomes fragmented in early, preclinical stages of the disease. *Proc Natl Acad Sci* **93**: 5472-5477. 1996.
 11. Tu P-A, Raju P, Robinson KA, Gurney ME, Trojanowski JQ, and Lee VM-Y. Transgenic mice carrying a human mutant superoxide dismutase transgene develop neuronal cytoskeletal pathology resembling human amyotrophic lateral sclerosis lesions. *Proc Natl Acad Sci* **93**: 3155-3160. 1996.
 12. Chiu AY, Zhai P, Dal Cant MC, Peter TM, Kwon YW, Prattis SM, and Gurney ME. Age-dependent penetrance of disease in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Mol Cell*

- Neurosci **6**: 349-362. 1995.
13. Waibel S, Reuter A, Malessa S, Blaugrund E andC Ludolph A. Rasagiline alone and combination with riluzole prolongs survival in an ALS mouse model. *J Neurol* **251**: 1080-1084. 2004.
 14. Smith RA, Miller TM, Yamanaka K, Monia BP, Condon TP, Hung G, Lobsiger CS, Ward CM, McAlonis-Downes M, Wei H, Wancewicz EV, Bennett CF and Cleveland DW., Antisense oligonucleotide therapy for neuro degenerative disease. *J Clin Invest* **116**: 2290-2296. 2006.
 15. Shin JH, Cho SI, Lim HR, Lee JK, Lee YA, Noh JS, Joo IS, Kim K-W and Gwag BJ. Concurrent administration of Neu2000 and lithium produces marked improvement of motor neuron survival, motor function, and mortality in a mouse model of amyotrophic lateral sclerosis. *Mol Pharmacol* **71**: 965-975. 2007.
 16. Lepore AC, O'Donnell J, Kim AS, Williams T, Tuteja A, Rao MS, Kelley LL, Campanelli JT and Maragakis NJ. Human glial-restricted progenitor transplantation into cervical spinal cord of the SOD1 mouse model of ALS. *PLoSOne* **6**: e25968. 2011.
 17. Takata M, Tanaka H, Kimura M, NagaharanY, Tanaka K, Kawasaki K, Seto M, Tsuruma K, Shimazawa M and Hara H. Fasudil, a rho kinase inhibitor, limits motor neuron loss in experimental model of amyotrophic lateral sclerosis. *Br J Pharmacol* **170**: 341-351. 2013.
 18. Bucchia M, Ramirez A, Parente V, Simone C, Nizzardo M, Magri F, Dametti S, and Corti S. Therapeutic Development in Amyotrophic Lateral Sclerosis. *Clinical Therapeutics* **37**: 668-680. 2015.

19. Ferrucci M, Lazzeri G, Flaibani M, Biagioni F, Cantini F, Madonna M, Bucci D, Limanaqi F, Soldani P and Fornai F. In search for a gold-standard procedure to count motor neurons in the spinal cord. *Histol Histopathol* **33**: 1021-1046. 2018.
20. Carriedo S.G., Yin H.Z. and Weiss J.H. Motor neurons are selectively vulnerable to AMPA/Kainate receptor-mediated injury in vitro. *J. Neurosci.* **16**: 4069-4079. 1996.
21. Avossa D., Grandolfo M., Mazzarol F., Zatta M. and Ballerini L. Early signs of motoneuron vulnerability in a disease model system: characterization of transverse slice cultures of spinal cord isolated from embryonic ALS mice. *Neuroscience* **138**: 1179-1194. 2006.
22. Martin, L.J., Liu Z., Chen K., Price A.C., Pan Y., Swaby J.A. and Golden W.C. Motor neuron degeneration in amyotrophic lateral sclerosis mutant superoxide dismutase-1 transgenic mice: Mechanisms of mitochondriopathy and cell death. *J. Comp. Neurol.* **500**: 20-46. 2007.
23. Fornai F., Longone P., Ferrucci M., Lenzi P., Isidoro C., Ruggieri S. and Paparelli A. Autophagy and amyotrophic lateral sclerosis: The multiple roles of lithium. *Autophagy* **4**: 527-530. 2008.

Figure legends

Fig. 1

Photographs showing histopathology (HE staining) of the lumbar spinal cord. (A) Seven-week-old male WT mice: no significant changes were observed. (B) Seven-week-old male Tg mice: hyaline inclusions (solid circles) and foamy vacuoles (dotted circles) were observed in the cell bodies of MNs in the gray matter. (C) Thirteen-week-old male Tg mice: large vacuole and nerve cell aggregation with glial cells (solid circles) were observed in the gray matter. (D) Twenty-one-week-old female Tg mice: large vacuoles and spheroid formation (arrowhead) were observed in the gray matter.

Scale bar = 50 μ m; (A, C), 100 μ m; (B, D)

Fig. 2

Number of healthy MNs ≥ 25 μ m in both sexes at each age. Values are expressed as mean $\pm$ SD.

Significantly different from WT ($P < 0.01$). *Significantly different from WT ($P < 0.001$).

Fig. 3

Number of MNs ≥ 25 μ m, including degenerative neurons, in both sexes at each age. Values are expressed as mean $\pm$ SD. **Significantly different from WT ($P < 0.01$).

Table 1 Histopathological changes in SOD1Tg mice lumbar spinal cord

Week age	Sex	Hyaline inclusion				Foamy vacuole				Large vacuole				Nerve cell aggregation				Spheroid formation			
		–	±	+	2+	–	±	+	2+	–	±	+	2+	–	±	+	2+	–	±	+	2+
7W	Male	0	0	3	3	1	4	1	0	6	0	0	0	4	0	2	0	6	0	0	0
	Female	0	1	2	3	4	1	1	0	6	0	0	0	6	0	0	0	6	0	0	0
13W	Male	4	1	1	0	0	2	3	1	0	0	6	0	3	2	1	0	2	4	0	0
	Female	0	6	0	0	0	1	4	1	2	3	1	0	3	2	1	0	4	1	1	0
18W	Male	6	0	0	0	1	4	0	1	0	0	3	3	0	2	4	0	3	1	2	0
	Female	6	0	0	0	2	1	3	0	0	0	4	2	2	2	2	0	5	1	0	0
21W	Male	4	1	1	0	1	1	4	0	0	0	1	5	1	0	5	0	3	1	2	0
	Female	6	0	0	0	5	0	1	0	0	0	1	5	3	0	3	0	3	0	3	0

N=6 per group, –: Non remarkable change, ±: Weak, +: Slight, 2+: Moderate changes

Figure 1

Figure 2

Figure 3