

Short Communication

Histopathological changes of the spinal cord and motor neuron dynamics in SOD1 Tg mice

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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. (DOI: 10.1293/tox.2021-0056; J Toxicol Pathol 2022; 35: 129–133)

Key words: 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 protein*⁴, *sarcoma fusion*⁵, and *chromosome 9 open reading frame 72*⁶, 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 reported^{8–12}. Pathological lesions in this disease model were mainly limited to the spinal cord (particularly lumbar), brainstem, descending spinal tracts, and neuromuscular junctions. Moreover, various pathologi-

cal 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.), 20–26 °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%

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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 μ m). 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 ≥ 25 μ 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 ≥ 25 μ 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 (Fig. 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 ob-

served in Tg mice at any age. No apparent sex differences were noted in these histopathological changes (Table 1).

The number of healthy MNs ≥ 25 μ 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 ≥ 25 μ 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 ≥ 25 μ 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 ≥ 25 μ 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 years^{1–6}. In particular, SOD1 Tg mice are the most prominent ALS model and has been used for drug efficacy assessments^{13–18}. 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

Table 1. Histopathological Changes in the Lumbar Spinal Cord of SOD1 Tg Mice

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.

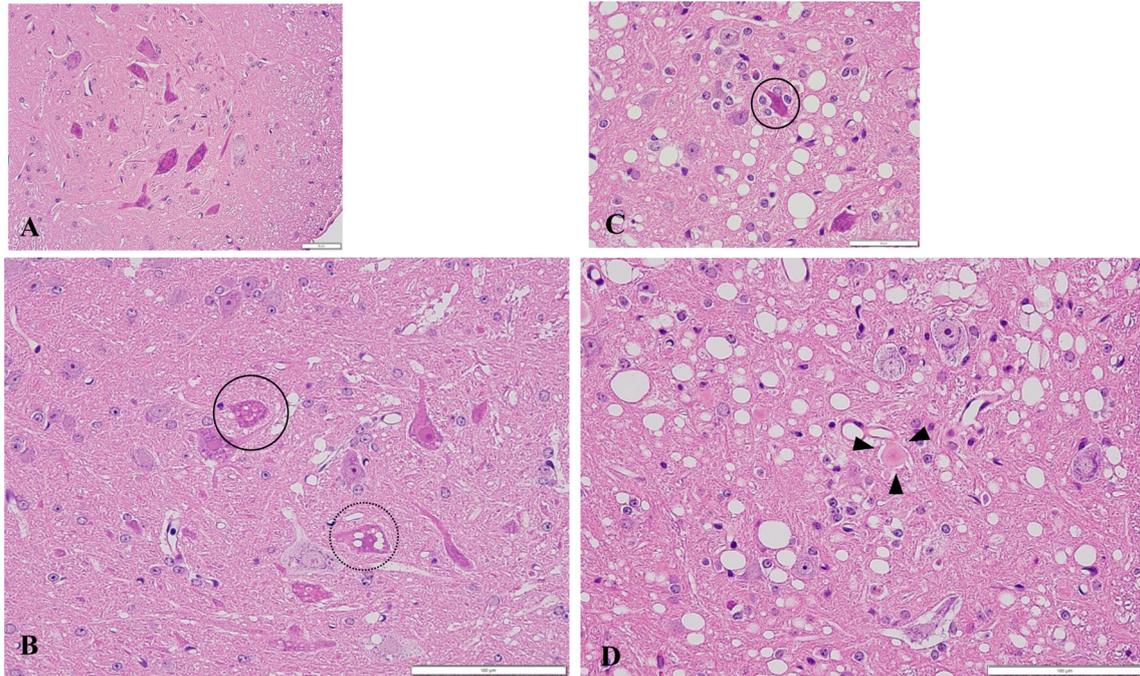


Fig. 1. Photographs showing histopathology (HE staining) of the lumbar spinal cord. (A) Seven-week-old male WT mice: no significant changes were observed. (B) Seven-week-old male Tg mice: hyaline inclusions (solid circles) and foamy vacuoles (dotted circles) were observed in the cell bodies of MNs in 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 $\geq 25 \mu$ 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 ($p < 0.001$).

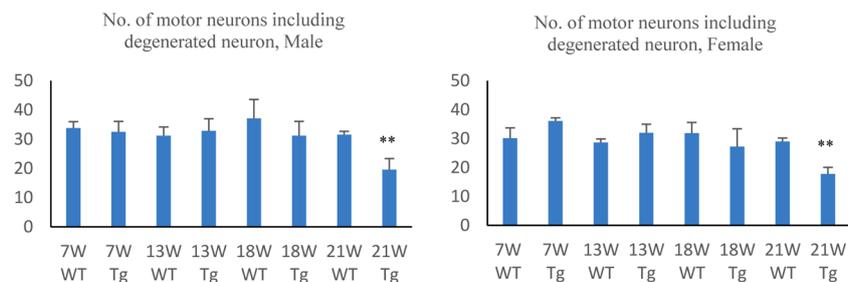


Fig. 3. Number of MNs $\geq 25 \mu$ m, including degenerative neurons, in both sexes at each age. Values are expressed as mean \pm SD. **Significantly different from WT ($p < 0.01$).

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 ≥ 20 μm 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 μm in mouse spinal cord^{22, 23}. In the current study, we used a cutoff of 25 μm 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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