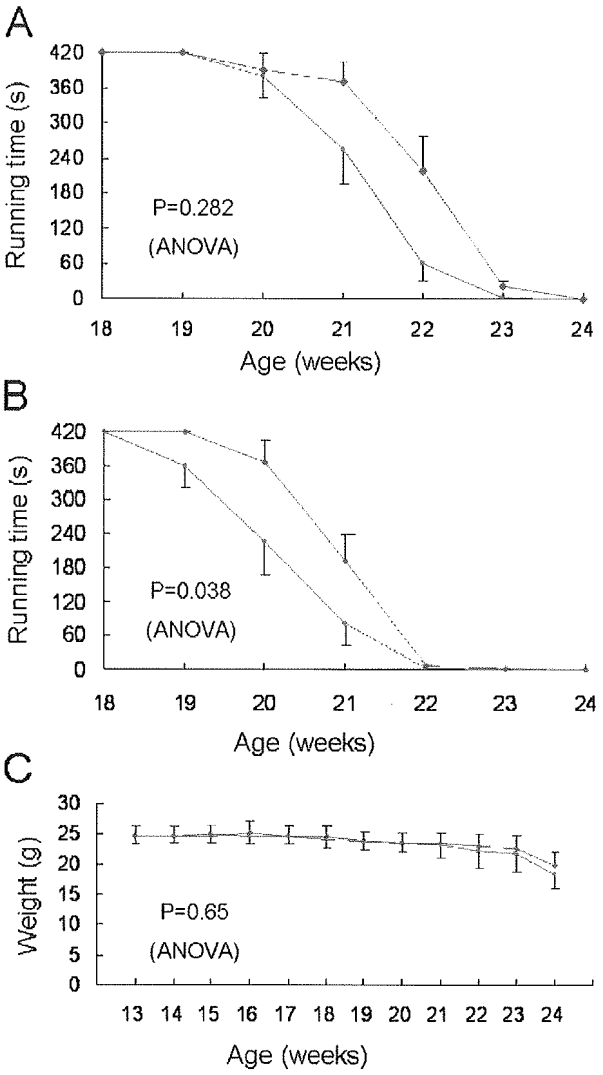

Fig. 4. Motor function of Tg mice (mutant H46R SOD1) tested with the Rotarod. For the 5 rpm task (A), there was no significant difference between the two groups. However, the assessment with the Rotarod task at 20 rpm was much more improved in the gal-1-treated group than in the control group ($P = 0.038$) (B). Red line, gal-1 group ($n = 10$); blue line, control group ($n = 9$). Body weight measurements of the transgenic mice treated with rhGAL-1/ox or physiological saline (C). Red line, gal-1 group ($n = 14$); blue line, control group ($n = 14$). Error bars represent SD.

with those in the gal-1-treated group (18.4 ± 2.4 versus 19.7 ± 2.3 g) (Fig. 4C); however, there was no statistical significance of the body weights between the two groups (ANOVA; $P = 0.65$).

Histopathological evaluation of spinal cords with 147-day-old mice: effect of rhGAL-1/ox on motor neuron survival

In H&E-stained sections, several pathological features were seen in both gal-1-treated group and control group. Neurite swellings, eosinophilic inclusion bodies similar to Lewy body-like hyaline inclusions in human ALS, and astrocytic proliferations were detectable in the anterior horns of the spinal cord. Large anterior horn cells were decreased in number in both groups, however, histological evaluation using Nissl-stained spinal cord sections of the 147-day-old mice suggested a neuroprotective effect of rhGAL-1/ox on spinal motor neuron survival.

In Nissl-stained sections, more anterior horn cells of L_{4-5} segments were preserved in the gal-1-treated group (Fig. 5A) than in the control group (Fig. 5B) ($P = 0.007$, Table 2). Furthermore, we compared the number of remaining large anterior horn cells at the cervical level (C_{5-6}) between the gal-1-treated group and the control group. At the cervical level, gal-1-treated Tg mice also had a greater number of large anterior horn cells than the control group ($P = 0.039$, Table 2). In both the cervical and lumbar spinal cords, there was no significant difference in the number of anterior horn cells between the

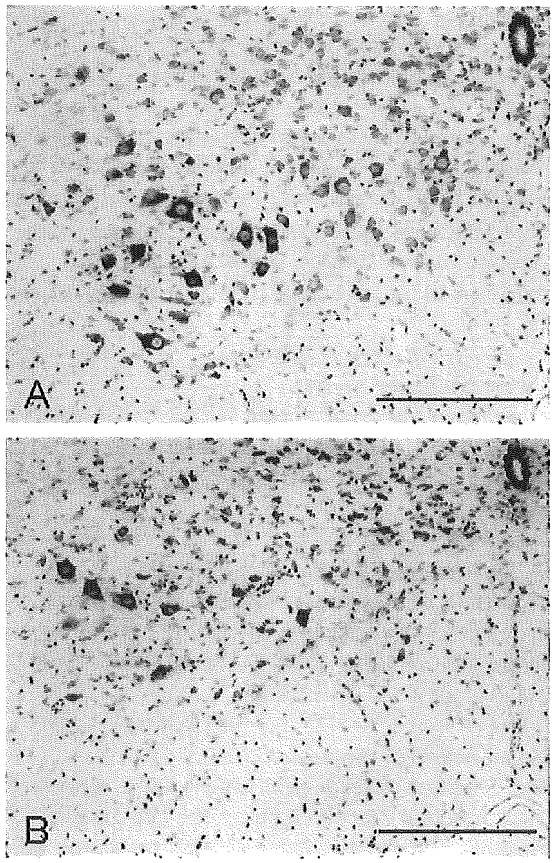

Fig. 5. Histological evaluation of the lumbar cord in 147-day-old mice. (A) rhGAL-1/ox-treated mice, (B) physiological saline-treated mice. In Nissl-stained sections, neuronal cells were well preserved in the anterior horn of the lumbar cord in the gal-1-treated group. Scale bars = 200 μm.

Table 2
The number of large anterior horn neurons/section of spinal cord L_{4-5} and C_{5-6} at postnatal day 147

	n	Lumbar cord		Total	Cervical cord		Total
		L	R		L	R	
Control group	5	3.8 ± 0.3	3.7 ± 0.3	7.5 ± 0.6*	5.7 ± 1.0	5.7 ± 1.1	11.4 ± 2.1*
Gal-1 group	6	6.9 ± 0.8	6.5 ± 0.7	13.5 ± 1.5*	10.0 ± 1.3	10.1 ± 1.6	20.1 ± 2.8*

Values tabulated are mean ± SEM. Statistical comparisons were with a two-tailed Student's *t* test.
n: number of mice examined; R: right side of the spinal cord; L: left side of the spinal cord.
* Gal-1 vs. Control, $P < 0.05$.

injected side (left) and the non-injected side (right) ($P > 0.05$, Table 2).

Discussion

The results of the present study showed the therapeutic effect of rhGAL-1/ox for H46R SOD1 Tg mice, an animal model of FALS. The administration of rhGAL-1/ox prevented the Tg mice from losing spinal anterior horn neurons. In contrast to the control group, rhGAL-1/ox-treated mice showed better behavioral performance and a prolonged life span, consistent with the preservation of spinal motor neurons. In the present study, rhGAL-1/ox was injected into the left gastrocnemius muscle. However, anterior horn cells were well preserved not only in the left side but also in the right side of the anterior horn of the lumbar cord. Moreover, the number of anterior horn cells was well preserved even in the cervical cord. Therefore, it seems that the effect of rhGAL-1/ox on the anterior horn cells is not through retrograde axonal transport.

Galectin-1, a member of the family of β-galactoside-binding lectins, is isolated as a homodimer of the 14.5 kDa subunit. Galectin-1 is present in various tissues and organs, including the lung, heart, skeletal muscle, skin, placenta, thymus, lymph node, brain, spinal cord, and peripheral nerve (Kasai and Hirabayashi, 1996). Several functions for galectin-1 have been proposed in those tissues: cell growth, cell differentiation, apoptosis, cell–cell interaction, and cell–matrix interaction (Perillo et al., 1998).

The galectin-1 molecule has six cysteine residues and, when it is oxidized, three disulfide bonds are formed (Inagaki et al., 2000). An oxidized form of galectin-1 showed axonal regeneration-enhancing activity; however, it lacked a property of lectin to bind to lactose (Inagaki et al., 2000). On the other hand, a reduce form of galectin-1 possessed lectin properties but showed no axonal regeneration-enhancing activity. Indeed, a galectin-1 mutant, in which all six cysteine residues were replaced by serine, induced lectin activity but lacked axonal regeneration-promoting activity (Inagaki et al., 2000).

These three intramolecular disulfide bonds appear to represent a stable conformation of oxidized galectin-1. As these strong covalent linkages are not broken down easily, injected rhGAL-1/ox probably acted as an oxidized form of galectin-1, showing axonal regeneration-enhancing activity. Indeed, rhGAL-1/ox confirmed that the protein promotes axonal regeneration in both in vitro experiments (Horie et al., 2004) and the in vivo acellular nerve regeneration model (Fukaya et al., 2003).

On the other hand, because direct application of oxidized galectin-1 to isolated primary sensory neurons does not alter their morphology, it is hypothesized that galectin-1 may stimulate non-neuronal cells to produce a factor that promotes Schwann cell migration while enhancing axonal regeneration (Horie et al., 1999, 2004). To date, the following issues have been addressed: (1) identification of target cells of galectin-1 among non-neuronal cells surrounding axons and/or neurons; (2) understanding of the mechanism whereby oxidized galectin-1 promotes axonal regeneration.

Recent reports have given possible answers to these questions. The macrophage is one target cell for oxidized galectin-1, and an axonal regeneration-promoting factor is secreted from macrophages stimulated by oxidized galectin-1 in vitro (Horie et al., 2004). Recently, Horie et al. have shown the following results: (1) macrophages bear specific receptors to rhGAL-1/ox on their cell membranes; (2) rhGAL-1 stimulates tyrosine phosphorylation of proteins in macrophages, suggesting that rhGAL-1/ox specifically binds to macrophages to activate their signal transduction pathway; (3) rhGAL-1/ox induces macrophages to secrete a factor(s) to promote axonal regeneration; (4) rhGAL-1/ox stimulates macrophages to enhance Schwann cell migration. Surprisingly, the axonal promoting activity of the conditioned medium secreted from galectin-1-activated macrophages is distinctively stronger than various trophic factors, such as nerve growth factor (NGF), insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), and ciliary derived neurotrophic factor (CNTF) in vitro (Horie et al., 2004). Further experiments need to be conducted to identify the factor released from rhGAL-1/ox-stimulated macrophages.

To date, the mechanism of motor neuron degeneration in ALS remains unknown; however, several neurotrophic factors (NTFs) or other therapeutic agents have been studied because of their potential ability to protect against motor neuron degeneration. Indeed, these factors have been extensively studied in animal models of ALS. Several agents have shown delay of disease onset and/or survival prolongation, and these agents have been viewed as a new therapeutic strategy for ALS. As for these therapeutic agents,

the mechanisms of action have been considered to be as follows: (1) free radical scavengers (Barneoud and Curet, 1999; Dugan et al., 1997; Gurney et al., 1996); (2) glutamate inhibitors (Gurney et al., 1996); (3) copper chelator (Hottinger et al., 1997); (4) stabilizers of mitochondria (Klivenyi et al., 1999); (5) caspase inhibitors (Li et al., 2000); (6) microglial activation inhibitors (Kriz et al., 2002); and (7) NTFs. At present, riluzole, a glutamate receptor antagonist, is commercially available for patients with ALS (Rowland and Schneider, 2001). As for NTFs, some trials have been performed on patients with ALS; the subcutaneous delivery of IGF-I had marginal success in one of two human trials (Kaspar et al., 2003); however, other NTFs such as the CNTF, the glial cell line-derived neurotrophic factor (GDNF), and the brain-derived neurotrophic factor (BDNF) have been unsuccessful in human trials (Dawbarn and Allen, 2003).

Several investigations have revealed that the impairment of axonal transport is the early event of spinal motor neurons in ALS; disturbance of axonal transport may occur initially and subsequently cause accumulation of neurofilaments in the perikarya and the proximal portion of axons (Collard et al., 1995; Williamson and Cleveland, 1999; Zhang et al., 1997). Impairment of the axonal transport may trap galectin-1 in the perikarya and the proximal portion of the axons of the anterior horn cells in ALS (Kato et al., 2001). It has recently been reported that the axotomy of facial nerve induced transient upregulation of galectin-1 mRNA, suggesting that facial nerve injury can trigger the synthesis of galectin-1 in neuronal cell bodies (Akazawa et al., 2004). Several studies have also shown that galectin-1 is likely to be released from muscle cells and subsequently act as a factor for myogenesis in vivo (Goldring et al., 2002a,b; Gu et al., 1994).

If motor neuron axons and skeletal muscles truly need galectin-1 for their maintenance or survival, depletion of this protein may cause degeneration of the motor neurons and skeletal muscles. Although the mode of action of galectin-1 on spinal motor neurons remains unclear, the results of the present study show a potential therapeutic effect of galectin-1 for patients with ALS.

Acknowledgments

This work was in part supported by The Nakabayashi Trust for ALS Research and a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

References

Akazawa, C., Nakamura, Y., Sango, K., Horie, H., Kohsaka, S., 2004. Distribution of the galectin-1 mRNA in the rat nervous system: its transient upregulation in rat facial motor neurons after facial nerve axotomy. Neuroscience 125, 171–178.

Barneoud, P., Curet, O., 1999. Beneficial effects of lysine acetylsalicylate, a soluble salt of aspirin, on motor performance in a transgenic model of amyotrophic lateral sclerosis. Exp. Neurol. 155, 243–251.

Bruijn, L.I., Houseweart, M.K., Kato, S., Anderson, K.L., Anderson, S.D., Ohama, E., Reaume, A.G., Scott, R.W., Cleveland, D.W., 1998. Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. Science 281, 1851–1854.

Cleveland, D.W., 1999. From Charcot to SOD1: mechanisms of selective motor neuron death in ALS. Neuron 24, 515–520.

Collard, J.F., Cote, F., Julien, J.P., 1995. Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis. Nature 375, 61–64.

Dawbarn, D., Allen, S.J., 2003. Neurotrophins and neurodegeneration. Neuropathol. Appl. Neurobiol. 29, 211–230.

Dugan, L.L., Turetsky, D.M., Du, C., Lobner, D., Wheeler, M., Almli, C.R., Shen, C.K., Luh, T.Y., Choi, D.W., Lin, T.S., 1997. Carboxyfullerenes as neuroprotective agents. Proc. Natl. Acad. Sci. U. S. A. 94, 9434–9439.

Elroy-Stein, O., Bernstein, Y., Groner, Y., 1986. Overproduction of human Cu/Zn superoxide dismutase in transfected cells: extenuation of paraquat-mediated cytotoxicity and enhancement of lipid peroxidation. EMBO. J. 5, 615–622.

Fukaya, K., Hasegawa, M., Mashitani, T., et al., 2003. Oxidized galectin-1 stimulates the migration of Schwann cells from both proximal and distal stumps of transected nerves and promotes axonal regeneration after peripheral nerve injury. J. Neuropathol. Exp. Neurol. 62, 162–172.

Goldring, K., Jones, G.E., Thiagarajah, R., Watt, D.J., 2002a. The effect of galectin-1 on the differentiation of fibroblasts and myoblasts in vitro. J. Cell Sci. 115, 355–366.

Goldring, K., Jones, G.E., Sewry, C.A., Watt, D.J., 2002b. The muscle-specific marker desmin is expressed in a proportion of human dermal fibroblasts after their exposure to galectin-1. Neuromuscul. Disord. 12, 183–186.

Gu, M., Wang, W., Song, W.K., Cooper, D.N., Kaufman, S.J., 1994. Selective modulation of the interaction of alpha 7 beta 1 integrin with fibronectin and laminin by L-14 lectin during skeletal muscle differentiation. J. Cell Sci. 107, 175–181.

Gurney, M.E., Pu, H., Chiu, A.Y., Dal Canto, M.C., Polchow, C.Y., Alexander, D.D., Caliendo, J., Hentati, A., Kwon, Y.W., Deng, H.X., Chen, W., Zhai, P., Sufit, R.L., Siddique, T., 1994. Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. Science 264, 1772–1775.

Gurney, M.E., Cutting, F.B., Zhai, P., Doble, A., Taylor, C.P., Andrus, P.K., Hall, E.D., 1996. Benefit of vitamin E, riluzole, and gabapentin in a transgenic model of familial amyotrophic lateral sclerosis. Ann. Neurol. 39, 147–157.

Hashimoto-Gotoh, T., Mizuno, T., Ogasahara, Y., Nakagawa, M., 1995. An oligodeoxyribonucleotide-directed dual amber method for site-directed mutagenesis. Gene 152, 271–275.

Hirano, A., Nakano, I., Kurland, L.T., Mulder, D.W., Holley, P.W., Saccomanno, G., 1984. Fine structural study of neurofibrillary changes in a family with amyotrophic lateral sclerosis. J. Neuropathol. Exp. Neurol. 43, 471–480.

Horie, H., Kadoya, T., 2000. Identification of oxidized galectin-1 as an initial repair regulatory factor after axotomy in peripheral nerves. Neurosci. Res. 38, 131–137.

Horie, H., Inagaki, Y., Sohma, Y., Nozawa, R., Okawa, K., Hasegawa, M., Muramatsu, N., Kawano, H., Horie, M., Koyama, H., Sakai, I., Takeshita, K., Kowada, Y., Takano, M., Kadoya, T., 1999. Galectin-1 regulates initial axonal growth in peripheral nerves after axotomy. J. Neurosci. 19, 9964–9974.

Horie, H., Kadoya, T., Hikawa, N., Sango, K., Inoue, H., Takeshita, K., Asawa, R., Hiroi, T., Sato, M., Yoshioka, T., Ishikawa, Y., 2004. Oxidized galectin-1 stimulates macrophages to promote axonal regeneration in peripheral nerves after axotomy. J. Neurosci. 24, 1873–1880.

Hottinger, A.F., Fine, E.G., Gurney, M.E., Zurn, A.D., Aebischer, P., 1997. The copper chelator D-penicillamine delays onset of disease and

extends survival in a transgenic mouse model of familial amyotrophic lateral sclerosis. Eur. J. Neurosci. 9, 1548–1551.

Inagaki, Y., Sohma, Y., Horie, H., Nozawa, R., Kadoya, T., 2000. Oxidized galectin-1 promotes axonal regeneration in peripheral nerves but does not possess lectin properties. Eur. J. Biochem. 267, 2955–2964.

Ioannou, P.A., Amemiya, C.T., Garnes, J., Kroisel, P.M., Shizuya, H., Chen, C., Batzer, M.A., de Jong, P.J., 1994. A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. Nat. Genet. 6, 84–89.

Kadoya, T., Oyanagi, K., Kawakami, E., Hasegawa, M., Inagaki, Y., Sohma, Y., Horie, H., in press. Oxidized galectin-1 advances the functional recovery after peripheral nerve injury. Neurosci. Lett. (Available online in ScienceDirect).

Kasai, K., Hirabayashi, J., 1996. Galectins: a family of animal lectins that decipher glycocodes. J. Biochem. (Tokyo) 119, 1–8.

Kaspar, B.K., Llado, J., Sherkat, N., Rothstein, J.D., Gage, F.H., 2003. Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. Science 301, 839–842.

Kato, T., Kurita, K., Seino, T., Kadoya, T., Horie, H., Wada, M., Kawanami, T., Daimon, M., Hirano, A., 2001. Galectin-1 is a component of neurofilamentous lesions in sporadic and familial amyotrophic lateral sclerosis. Biochem. Biophys. Res. Commun. 282, 166–172.

Klivenyi, P., Ferrante, R.J., Matthews, R.T., Bogdanov, M.B., Klein, A.M., Andreassen, O.A., Mueller, G., Wermer, M., Kaddurah-Daouk, R., Beal, M.F., 1999. Neuroprotective effects of creatine in a transgenic animal model of amyotrophic lateral sclerosis. Nat. Med. 5, 347–350.

Kriz, J., Nguyen, M.D., Julien, J.P., 2002. Minocycline slows disease progression in a mouse model of amyotrophic lateral sclerosis. Neurobiol. Dis. 10, 268–278.

Levanon, D., Lieman-Hurwitz, J., Dafni, N., Wigderson, M., Sherman, L., Bernstein, Y., Laver-Rudich, Z., Danciger, E., Stein, O., Groner, Y., 1985. Architecture and anatomy of the chromosomal locus in human chromosome 21 encoding the Cu/Zn superoxide dismutase. EMBO J. 4, 77–84.

Li, M., Ona, V.O., Guegan, C., Chen, M., Jackson-Lewis, V., Andrews, L.J., Olszewski, A.J., Stieg, P.E., Lee, J.P., Przedborski, S., Friedlander, R.M., 2000. Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. Science 288, 335–339.

Manabe, Y., Nagano, I., Gazi, M.S., Murakami, T., Shiote, M., Shoji, M., Kitagawa, H., Abe, K., 2003. Glial cell line-derived neurotrophic factor protein prevents motor neuron loss of transgenic model mice for amyotrophic lateral sclerosis. Neurol. Res. 25, 195–200.

Perillo, N.L., Marcus, M.E., Baum, L.G., 1998. Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death. J. Mol. Med. 76, 402–412.

Ripps, M.E., Huntley, G.W., Hof, P.R., Morrison, J.H., Gordon, J.W., 1995. Transgenic mice expressing an altered murine superoxide dismutase gene provide an animal model of amyotrophic lateral sclerosis. Proc Natl. Acad. Sci. U. S. A. 92, 689–693.

Rowland, L.P., Schneider, N.A., 2001. Amyotrophic lateral sclerosis. N. Engl. J. Med. 344, 1688–1700.

Shefner, J.M., Brown Jr., R.H., Cole, D., Chaturvedi, P., Schoenfeld, D., Pastuszak, K., Matthews, R., Upton-Rice, M., Cudkowicz, M.E., 2001. Effect of neurophilin ligands on motor units in mice with SOD1 ALS mutation. Neurology 57, 1857–1861.

Tu, P.H., Raju, P., Robinson, K.A., Gurney, M.E., Trojanowski, J.Q., Lee, V.M., 1996. Transgenic mice carrying a human mutant superoxide dismutase transgene develop neuronal cytoskeletal pathology resembling human amyotrophic lateral sclerosis lesions. Proc. Natl. Acad. Sci. U. S. A. 93, 3155–3160.

Wang, L.J., Lu, Y.Y., Muramatsu, S., Ikeguchi, K., Fujimoto, K., Okada, T., Mizukami, H., Matsushita, T., Hanazono, Y., Kume, A., Nagatsu, T., Ozawa, K., Nakano, I., 2002. Neuroprotective effects of glial cell line-derived neurotrophic factor mediated by an adeno-associated virus vector in a transgenic animal model of amyotrophic lateral sclerosis. J. Neurosci. 22, 6920–6928.

Warita, H., Itoyama, Y., Abe, K., 1999. Selective impairment of fast anterograde axonal transport in the peripheral nerves of asymptomatic transgenic mice with a G93A mutant SOD1 gene. Brain Res. 819, 120–131.

Williamson, T.L., Cleveland, D.W., 1999. Slowing of axonal transport is a very early event in the toxicity of ALS-linked SOD1 mutants to motor neurons. Nat. Neurosci. 2, 50–56.

Wong, P.C., Pardo, C.A., Borchelt, D.R., Lee, M.K., Copeland, N.G., Jenkins, N.A., Sisodia, S.S., Cleveland, D.W., Price, D.L., 1995. An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. Neuron 14, 1105–1116.

Zhang, B., Tu, P., Abtahian, F., Trojanowski, J.Q., Lee, V.M., 1997. Neurofilaments and orthograde transport are reduced in ventral root axons of transgenic mice that express human SOD1 with a G93A mutation. J. Cell Biol. 139, 1307–1315.