position 93 (G93A) and histidine to arginine at position 46 (H46R) (Nagai et al. 2001). Similar to its murine counterpart, the transgenic rats that express human SOD1 transgene ALS-associated mutations develop striking motor neuron degeneration and paralysis.

Although the mechanism of ALS is still unclear, there are many hypotheses concerning its cause of ALS, including loss of neurotrophic support to motor neurons (Rowland and Shneider 2001). The insulin-like growth factors (IGF-I and IGF-II) are neurotrophic factors expressed in the central nervous system that promote the survival and differentiation of neuronal cells including motor neurons. They could be of therapeutic value in human neurodegenerative disorders, including ALS (Adem et al. 1994; Hawkes and Kar 2003; Narai et al. 2005). Evidence that IGF-I rescues motor neurons in vitro and in animals. (Kaspar et al. 2003) has led to therapeutic trials of human recombinant IGF-I in patients with ALS (Nagano et al. 2005).

The biological actions of the IGFs are mediated through specific cell membrane receptors designated as the IGF-I and IGF-II receptors (Sepp-Lorenzino 1998; Hawkes and Kar 2003; Kim et al. 2004). Alterations of the IGF-I and IGF-II binding sites in the spinal cord of the patients with ALS would support their involvement in the pathology of ALS (Dore et al. 1996; Chung et al. 2003; Kar et al. 2006).

In the present study, we used the SOD1 (H46R) mutant Tg rat as an *in vivo* model of ALS and performed immunohistochemical studies to investigate the changes of the IGF-II receptor in the spinal cord.

Materials and Methods

Animals and clinical assessment

In this study we used nine Tg male Spargue Dawley rats as well as nine non-Tg rats (Japan SLC, Inc., Hamamatsu). The Tg rats expressing H46R mutant human copper-zinc superoxide dismutase (SOD1) were genotyped by polymerase chain reaction (PCR) assay using DNA obtained from the tail as described previously (Nagai et al. 2001). H46R Tg rats were divided into 3 groups: pre-symptomatic (aged 23 weeks, $n = 3$), onset (aged 26 weeks, just after onset, $n = 3$) and end-stage (aged 29 weeks, $n = 3$); and compared with age-matched non-transgenic littermate controls. In each Tg rat, we carefully observed the development of the symptoms of ALS. When the rats developed distinct muscle weakness in their unilateral hindlimb, they were included in the second group (onset) of the rats. All experimental protocols and procedures were approved by the Animal Committee of the Tohoku University Graduate School of Medicine, Japan.

Histopathological analysis

Nine Tg and nine control rats were anesthetized and killed by transcardial perfusion with saline and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The lumbar (L4-5) spinal cords were rapidly removed and post-fixed in the same fixative at 4°C overnight, then embedded in paraffin according to the standard protocol. Transverse sections (5 μm thick) were cut and submitted for histopathology and immunohistochemistry. A set of the sections was stained with hematoxylin and eosin (H & E).

Immunohistochemistry

For double immunohistochemistry of the spinal cord, every fifth section per animal was mounted on silanized glass slides (Dako Cytomation Co. Ltd., Gopenhagen, Denmark) and deparaffinized. The sections were quenched with 0.3% hydrogen peroxide in 10% methanol for 20 min at room temperature (RT), and rinsed in phosphate buffer saline (PBS, pH 7.4). After blocking with 5% normal serum was performed for 20 min at RT to avoid the non-specific binding of antibodies, we used the following primary antibodies: mouse anti-IGF-II receptor monoclonal antibody (1:50, overnight, 4°C; BD Transduction Laboratories, CA, USA), rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:10,000, overnight, 4°C; Dako Cytomation Co. Ltd.), and rabbit anti-ionized calcium-binding adapter molecule-1 (Iba-1) polyclonal antibody (1:3,000, overnight, 4°C; Wako Pure Chemicals, Osaka). After incubation with a mixture of biotinylated anti-mouse and anti-rabbit IgG secondary antibodies (1:400, 1 hr, RT; Vector Laboratories, Burlingame, CA, USA), the immunoreactivity was enhanced with avidin-biotin peroxidase complex (ABC) kit (Vector Laboratories). We used two kinds of color substrates as a chromogen. For the visualization of the IGF-II receptor we used 3,3-diaminobenzidine and nickel (blue-gray color), which was the first staining of the double immunostain-

ing (Vector Laboratories). For the staining of GFAP and Iba1 we used Nova Red (light-red color), which was the second staining of the double immunostaining (Vector Laboratories). To prevent cross-reaction between the first and the second immunoreactivities, we performed the double immunohistochemistry sequentially, and used an avidin-biotin blocking kit (Vector Laboratories) between the two sets of immunohistochemistry.

Quantitative analysis

Sections were examined and microphotographed under a light microscope (at 200 × magnification, Olympus BX50). We evaluated a total of five transverse sections from three different rats at each stage. The estimated motor neuron counts were performed on HE-stained sections in the lumbar spinal cord. Cells were selected as motor neurons if they were > 30 μm in diameter, multipolar with neuronal morphology, and located in the anterior horn of the spinal cord. The resulting data provided the estimated number of motor neurons per unilateral anterior horn. For semi-quantification of the double immunohistochemistry, we counted the double-immunoreactive areas in the photographed digital images of the anterior horns (60 × 45 μm for 2,048 × 1,536 pixels) using Image J software (National Institutes of Health, Bethesda, MD, USA). The average area (pixels/μm) double immunoreactive for IGF-II receptor and GFAP in the unilateral anterior horn in each rat was used for statistical analysis.

Statistical analysis

Values are expressed as the means ± S.D. The statistical analysis was performed using GraphPad PRISM (San Diego, CA, USA). Differences among the experimental groups were examined for significance using one way analysis of variance (ANOVA) among means of value with the group of rats as the independent factor. We tested multiple pair-wise comparisons between means by Bonferroni-Dunn post hoc test.

Results

The clinical course of transgenic rats

The Tg rats expressing the human SOD1 mutant (H46R) developed motor neuron disease with the onset of this clinical weakness at a mean age of around 180 days. Clinically apparent weakness, denoted by dragging of one hindlimb without limb tremor, was evident somewhat later. Simultaneously with the onset of clinical weakness, the affected rats showed prominent weight loss. While the initial clinical manifestation of weakness was unilateral leg paralysis, this progressed and became bilateral in the H46R Tg rats. In the early stages of the illness, another distinctive abnormality was increased tone in the tail musculature, resulting in an elevated, segmentally spastic tail posture. As the disease progressed, the rats exhibited marked muscle wasting in the hind limbs, typically dragging themselves about the cage using the forelimbs. Thereafter, the forelimbs also became weak, in association with further weight loss. At the end-stage, the affected rats could not drink water and died. The mean age of death was around 200 days.

We estimated the numbers of motor neurons in each anterior horn of the control littermates and

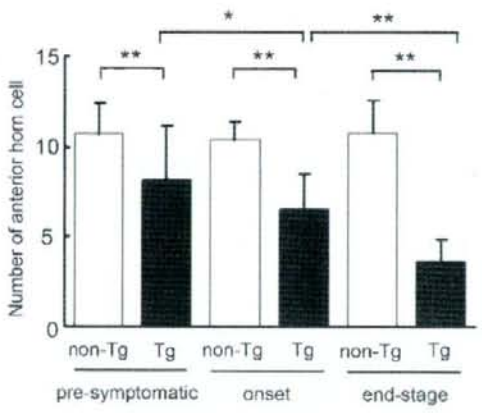

Fig. 1. Numbers of anterior horn cells: Numbers of anterior horn cells in the lumbar spinal cord at the pre-symptomatic, onset and end-stage. Mean number of the anterior horn cells from non-Tg rats (*open bar*) and H46R Tg rats (*solid bar*) are presented. The estimated numbers of motor neurons in the anterior horn of the spinal cord were almost the same in the control rats, while those in the Tg rats were gradually decreased: pre-symptomatic stage non-Tg, 10.7 ± 1.7; Tg, 8.3 ± 3.0 anterior horn cells/slice; onset stage non-Tg, 10.4 ± 1.0; Tg, 6.5 ± 1.9 anterior horn cells/slice; end-stage non-Tg, 10.8 ± 1.8; Tg, 3.6 ± 1.3 anterior horn cells/slice. Bonferroni-test $^{**}p < 0.01$; $^{*}p < 0.05$. The error bars denote the S.D.

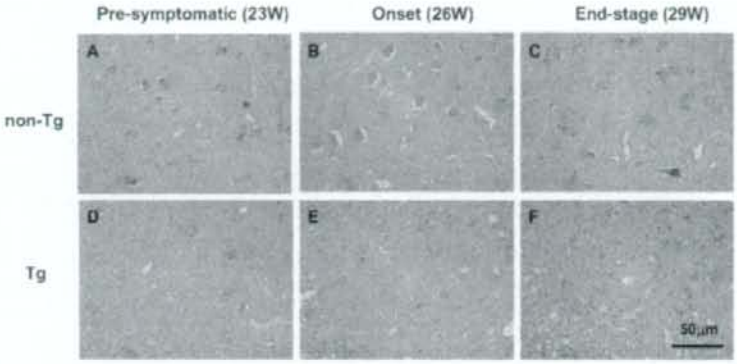

Fig. 2. Anterior horn cells with hematoxylin and eosin staining: Decreased number of anterior horn cells in the anterior horn of the lumbar spinal cord in non-Tg rats and H46R Tg rats at pre-symptomatic, onset and end-stage. Sections were stained with hematoxylin and eosin. Scale bar: 50 μm. Note that a decrease in the number of anterior horn cells in the lumbar spinal cord is evident at the pre-symptomatic stage in H46R Tg rats.

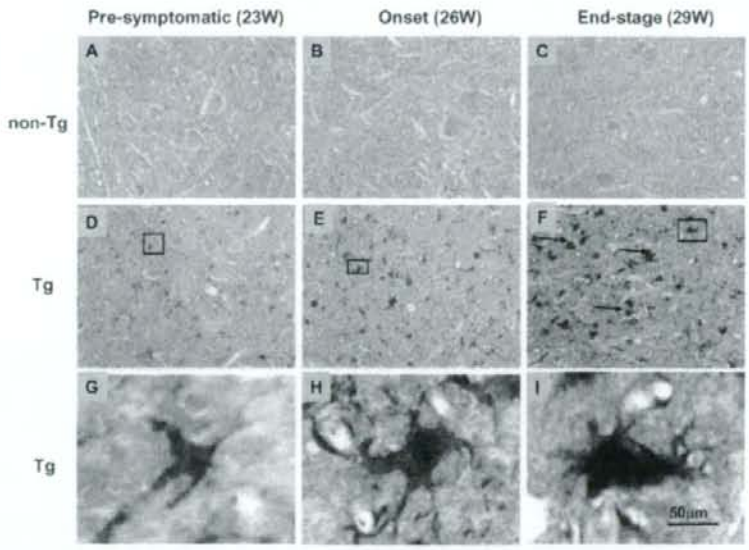

Fig. 3. Double-immunostaining for IGF-II receptor and GFAP: Double-immunostaining for Insulin-like growth factor-II (IGF-II) receptor and glial fibrillary acidic protein (GFAP) in non-Tg rats and H46R Tg rats at pre-symptomatic, onset and end-stage. Scale bar: 50 μm. IGF-II receptor and GFAP double-positive cells were found in the anterior horns of the lumbar spinal cord, and were co-stained with GFAP in the end-stage (F, I) of H46R Tg rats (arrows). The co-localization of IGF-II receptor and GFAP were visualized as a dark-red using Nova Red and DAB+Nickel chromogen (I). While there was evidence of GFAP-immunoreactive astrocytes in the pre-symptomatic stage (D, G) and at the onset (E, H), these reactive astrocytes were colored only light-red (G, H), indicating that the cells visualized were GFAP positive only.

Tg rats as a function of age. As indicated in Fig. 1, the estimated numbers declined abruptly in parallel with the development of clinical paralysis. In the spinal cords of the Tg rats, the drop-off in estimated motor neuron numbers preceded the onset of clinical weakness.

Histopathological studies in the spinal cords

The H46R Tg rats exhibited neuropathological abnormalities associated with the degeneration of motor neurons in the anterior horns of the spinal cord (Fig. 2). They also showed evidence of proliferation of small non-neuronal cells with the morphological characteristics of astroglia and microglia. In the pre-symptomatic stage at 23 weeks of age, the numbers of large, multipolar neurons in the anterior horn (motor neurons) were decreased (Fig. 2D) as compared to non-Tg littermates (Fig. 2A) , while the numbers of hypertrophic astrocytes were increased (Fig. 2D). By 26 weeks of age, when clinical weakness became apparent, there was a marked loss of large, multipolar neurons (Fig. 2E) as compared to non-Tg littermates (Fig. 2B). At that time, numerous hypertrophic astrocytes and microglia were evident in all stages of transgenic rats, as were sites of swelling in axons in the anterior horn (Fig. 2E). Many inclusions were characterized by a dense core and clear peripheral halo, strongly resembling the Lewy body-like hyaline inclusions seen in the spinal cords of human ALS patients. These were detected in the neuropil, motor neurons, and astrocytes (data not shown). At 29 weeks of age, corresponding to the end-stage when the H46R Tg rats clinically displayed quadriplegia or a moribund state, the rats of this end-stage showed severe loss of the anterior horn cells with gliosis of the spinal cords (Fig. 2E).

Immunohistochemical analyses for IGF-II receptor

In the anterior horn of the spinal cord of the H46R Tg rats, immunohistochemistry using the antibody against the IGF-II receptor showed intensely stained IGF-II receptor-positive glial cells with the appearance of astrocytes, but few IGF-II receptor-positive glial cells were observed in the spinal cord of the non-Tg littermates (Fig. 3), which were evident at the end-stage in H46R Tg rats (Fig. 3F). However, there were not evident at the pre-symptomatic stage (Fig. 3D) or at the onset of the disease (Fig. 3E). The IGF-II receptor-positive cells showing the morphology of astrocytes were confirmed as astrocytes by double-stained immunohistochemistry using the antibody against GFAP, which is a specific marker for astrocytes. There was a 125-fold increase in IGF-II receptor/GFAP double positive areas in the anterior horn of the spinal cord of the H46R Tg rats as compared to non-Tg littermates using ImageJ software on images captured electronically (Fig. 4). This increase was statistically significant ($p < 0.001$). IGF-II receptor-positive cells were not stained simultaneously with Iba1, which is a specific marker for microglia, in both Tg (Fig. 5D-I) and non-Tg (Fig. 5A-C) rats. In the spinal cord of non-Tg rats, few IGF-II receptor-positive astrocytes were detected although some IGF-II receptor-positive neurons were observed in the anterior horn.

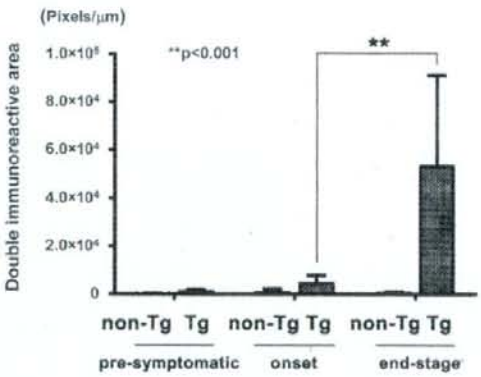

Fig. 4. Volume of double-positive areas of IGF-II receptor and GFAP: Volume of double-positive areas (pixels/μm) of insulin-like growth factor-II (IGF-II) receptor and glial fibrillary acidic protein (GFAP) in the anterior horn of the lumbar spinal cord. IGF-II receptor and GFAP double-positive area was significantly (**$p < 0.001$) increased in the anterior horn of the lumbar spinal cord in the end-stage of ALS transgenic rats.

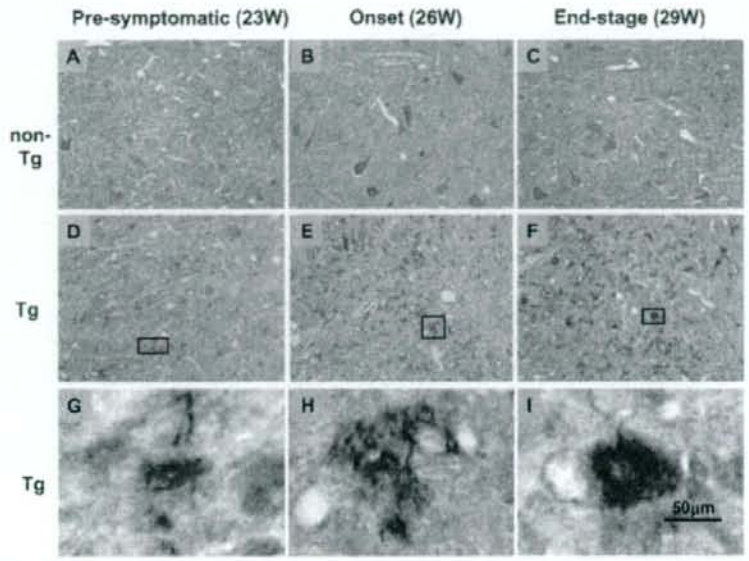

Fig. 5. Double-immunostaining for IGF-II receptor and Iba1: Double-immunostaining for insulin-like growth factor-II (IGF-II) receptor and ionized calcium-binding adapter molecule-1 (Iba1) in the anterior horn of the lumbar spinal cord from non-Tg rats and H46R Tg rats at pre-symptomatic, onset and end-stage. Scale bar: 50 μm. Iba1-immunoreactive microglia were visualized as light-red due to Nova RED chromogen in Tg rats at pre-symptomatic (D, G), symptomatic (E, H) and end-stage (F, I). However, there were no IGF-II receptor and Iba1 double positive cells, which would be colored by both Nova Red and DAB + Nickel color substrates.

Discussion

Our results showed the apparent loss of motor neurons in the anterior horn of the lumbar spinal cord in H46R Tg rats as described in our previous report (Nagai et al. 2001). We estimated the numbers of motor neurons in each anterior horn of the Tg rats as well as in control littermates and confirmed that the estimated number declined abruptly in parallel with the development of clinical paralysis. The H46R Tg rats also showed evidence of proliferation of small non-neuronal cells with the morphological characteristics of astroglia and microglia.

Various growth factors and their receptors are expressed differentially in ALS. The insulin-like growth factors are neurotrophic factors expressed in the central nervous system that promote the survival and differentiation of neuronal cells including motor neurons. The ability of IGFs to enhance the outgrowth of spinal motor neurons makes it a potential therapeutic agent for patients with ALS (Kaspar et al. 2003). Several studies have reported positive effects of IGF-I in reducing motor neuron death, delaying the onset of motor performance decline and the increasing life span in SOD1 mouse models of ALS and in one clinical trial. The IGF-1 studies in humans have reported that the progression of functional impairment in patients receiving high doses of IGF-1 was reduced by 26% vs patients receiving placebo (Lai et al. 1997). However, a second clinical trial produced no positive results (Borasio et al. 1998), and there currently is a phase III randomized, double-blind, placebo-controlled clinical IGF-1 trial underway. Therefore, we examined further the expression of the receptors of IGFs in the model of ALS.

A number of studies suggested that the level of expression of IGF receptors in reactive astro-

cytes was increased with the disease progression in human ALS, SOD1 Tg rats and mice (Adem et al. 1994; Chung et al. 2003). During development, astrocytes have been recognized as a source of cytokines that are involved in the growth and differentiation of neuronal cells and glial cell populations (Raff et al. 1985; Du and Dreyfus 2002). Recently, using immunohistochemistry, apparent increases of IGF-I receptors in reactive astrocytes in the anterior horns of the spinal cord in SOD1 G93A Tg mice were observed (Chung et al. 2003). The IGF-II receptor is a multifunctional single transmembrane glycoprotein that, along with the cation-dependent M6P (CD-M6P) receptor, mediates the trafficking of M6P-containing lysosomal enzymes from the trans-Golgi network to lysosomes. In the present study, immunohistochemistry using the antibody against the IGF-II receptor showed intensely stained IGF-II receptor-positive glial cells with the appearance of astrocytes in the anterior horn of the spinal cord of the H46R Tg rats. This was evident at the end-stage, however, but not evident at the pre-symptomatic stage or at the onset of the disease. The IGF-II receptor- positive glial cells showing the morphology of astrocytes were confirmed to be astrocytes by double-stained immunohistochemistry using the antibody against GFAP. This result was compatible with the expression of IGF-I receptors in Tg mice (Chung et al. 2003). On the other hand, we observed Iba1 reactive hypertrophic microglia in the pre-symptomatic, onset and end-stage of the Tg rats. However, IGF-II receptor-positive reactive microglia were not observed in the H46R Tg rats, although Kihira T and co-workers (Kihira et al. 2007) reported that some microglia expressing IGF-II have neuroprotective effects on the motor neurons in patients with ALS.

The apparent increase in IGFI and II receptors in the anterior horn in ALS spinal cords may be due to the loss of IGF-related trophic factors leading to receptor upregulation in an attempt to maintain neuronal homeostasis and insure neuronal survival. This study suggests that the expression of IGF receptors may play a key role in the pathogenesis, and that IGFs may have therapeutic applications in ALS.

Acknowledgments

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