Zinc Transporters (ZnT3 and 6) Are Downregulated in the Spinal Cords of Patients with Sporadic Amyotrophic Lateral Sclerosis

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Running Title: Downregulation of ZnTs in ALS

Grant information:

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Basic Research (C) 24590664)(to I.H.), the Collaborative Research Projects of the Brain Research Institute, Niigata University, (2214 (to I.H.) and 2521 (to M.K.)) and the Japan Amyotrophic Lateral Sclerosis Association (to M.K).

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(**Abstract)**

The loss of homeostasis of essential metals is associated with various diseases, including neurodegenerative diseases. Previous studies showed that the levels of zinc (Zn) were significantly higher in the cerebrospinal fluid of patients with amyotrophic lateral sclerosis (ALS). Zn transporters and metallothioneins tightly control intracellular and extracellular Zn levels. We here investigated the protein levels of ZnT, a Zn transporter family, in ALS patients and model mice. The mRNA expression of ZnT1, 3, 4, 5, 6, 7, and 10 was assessed in the spinal cords of human control subjects. ZnT3 and 6 protein levels were significantly diminished in the spinal cords of sporadic ALS patients compared with controls. Furthermore, immunohistochemical staining demonstrated decreased ZnT3 and 6 immunoreactivity in the ventral horn of the spinal cords in ALS patients. Moreover, immunohistochemical analysis revealed that all ZnTs expressed in the spinal cords were localized in a distinct subset of motor neurons. In addition, ZnT3 and 6 protein levels were not altered in SOD1 (G93A) mutant transgenic mice before and after the onset of ALS symptoms compared with controls. These results suggest that ZnT3 and 6 protein levels were decreased in the spinal cords of sporadic ALS patients; however, this did not occur merely via loss of motor neurons.

Key words: biometal; ER stress; Immunohistochemistry

(Introduction)

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that exhibits selective loss of cortical and spinal motor neurons. The majority of ALS cases are sporadic, while approximately 5%–10% of ALS cases are familial (Pasinelli and Brown 2006). The genetic cause of most of the familial (20%) and part of the sporadic (2%) ALS cases (Akimoto et al. 2011) is mutation in the Cu/Zn superoxide dismutase (*SOD1*) gene, in which more than 150 different mutations have been identified in ALS patients (Renton et al. 2014). *SOD1* (G93A) mutant transgenic (Tg) mice are widely studied as an ALS model (Valentine et al. 2005). Although several hypotheses have been proposed to explain motor neuron death, the underlying mechanism remains unclear.

The disruption of homeostasis of essential metals [e.g., copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn)] is associated with various diseases; neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and ALS are well-known examples (Barnham and Bush 2008; Vonk and Klomp 2008). However, the mechanism underlying the involvement of such metals in neurodegenerative diseases remains unclear. We recently reported increased Cu, Fe, Mg, and Zn levels in the cerebrospinal fluid (CSF) of ALS patients. Moreover, increased levels of such metals were observed in CSF of patients with AD, PD, and idiopathic basal ganglia calcification (IBGC) (Hozumi et al. 2011; Hozumi et al. 2010). Conversely, the levels of metals (e.g., Cu) were decreased in the hair of IBGC patients (Takagi et al. 2013). This suggests that metabolic disorders of biometals are involved in the pathological processes of IBGC. Mutations in a gene for a phosphate transporter, *PiT2* (*SLC20A2*), were found in some familial IBGC cases (Wang et al. 2012; Yamada et al. 2014). Accumulating evidence suggests that the aberrant metabolism of some biometals is closely linked to the pathogenesis of several neurodegenerative diseases.

Zn plays important roles in the functions of various proteins because approximately 3−10% of human genes encode Zn-containing proteins (Andreini et al. 2006; Maret 2013). Therefore,

Zn deficiency causes many human disorders, including growth retardation, dermatological lesions, and neuronal dysfunctions (Fukada and Kambe 2011). Zn transporters and metallothioneins (MTs) play a pivotal role in the regulation of intracellular and extracellular Zn homeostasis. We have reported that immunopositivity for both MT-I/II and MT-III was diminished in the spinal cords of sporadic ALS patients compared with controls. In particular, MT-III immunopositivity in astrocytes in the gray matter of the lumbar spinal cord was diminished as ALS advanced (Hozumi et al. 2008). In addition, we have shown that the administration of an adenovirus encoding MT-III prevents neuronal death and prolongs life span in *SOD1* (G93A) mutant Tg mice (Hashimoto et al. 2011). However, we have not examined Zn transporters in the spinal cords of ALS patients.

Zn transporters are classified into two families: ZnTs (Zn transporters; SLC30A family) and Zips (Zrt/Irt-like protein; SLC39A family). ZnTs transport Zn from the cytoplasm to the extracellular space or to intracellular compartments, whereas Zips transport Zn from the extracellular space or intracellular compartments into the cytoplasm. Thus, the two Zn transporters are critical for the maintenance of Zn homeostasis in the cytoplasm and the intracellular compartments of the secretory pathway (Fukada and Kambe 2011; Kambe et al. 2004).

MT and Zn transporters (ZnTs and Zips) seem to play a pivotal role in ALS pathophysiology. We scrutinized ZnT (ZnT1–10) levels in the spinal cords of sporadic ALS patients and in *SOD1* (G93A) mutant Tg mice, at the protein level and immunohistochemically, because ZnT3 levels are reportedly decreased in the cerebral cortex of AD patients.

MATERIALS AND METHODS

Antibodies

Antibodies were purchased from the following manufacturers: anti-β-actin (C4; Santa Cruz Biotechnology, Santa Cruz, CA); anti-β-tubulin III (2G10; Sigma-Aldrich, St. Louis, MO);

anti-choline acetyltransferase (ChAT; 1.B3.9B3; Millipore Corporation, Temecula, CA); anti-CNPase (11-5B; Sigma-Aldrich); anti-GAPDH (6C5; Santa Cruz Biotechnology); anti-glial fibrillary acidic protein (GFAP; GA5; Millipore Corporation); anti-SLC30A1 (C-term; Abgent, Inc., San Diego, CA); SLC30A3 (17363-1-AP; Proteintech Group Inc., Chicago, IL); anti-SLC30A5 (HPA035373; Atlas Antibodies AB, Stockholm, Sweden); anti-SLC30A6 (13526-1-AP; Proteintech Group Inc.); anti-SLC30A7 (13966-1-AP; Proteintech Group Inc.); and Alexa Fluor® 555 goat anti-mouse IgG (H+L), highly cross-adsorbed, and Alexa Fluor® 488 goat anti-rabbit IgG (H+L), highly cross-adsorbed (Invitrogen Corp., Carlsbad, CA). We confirmed the immunospecificity of ZnT antibodies using at least two distinct antibodies.

Subjects

Clinical procedures were performed in accordance with the latest version of the Helsinki Declaration. All participants involved in this study provided written informed consent. The study was approved by the Ethics Committee of the Gifu University Graduate School of Medicine and Gifu Pharmaceutical University. The spinal cords were taken from 10 ALS sporadic patients who had no other patient with ALS in the family and from 14 controls who had diseases other than neurodegenerative disorders and no spinal lesions.

Animals

All animal experiments were performed in accordance with NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Committee for Animal Research of Gifu Pharmaceutical University. Six-week-old male C57BL/6N mice (20–25 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The mice were maintained in a room at 23 °C under a constant day–night rhythm and given food and water *ad libitum*. Tg mice overexpressing mutated human *SOD1* (G93A) [B6SJL-Tg (SOD1-G93A) 1Gur/J] were purchased from the Jackson Laboratory (Bar Harbor, ME). The onset of disease was determined by the motor

function deficit observed in the rotarod performance test; the endpoint of ALS mice was defined as when mice are unable to eat a food by itself, which is from 18 to 20 weeks, as described previously (Tanaka et al. 2012).

Analysis of mRNA Levels in Human Tissues

Human tissue total RNAs (Premium Total RNA) were purchased from Takara Bio Inc. (Otsu, Japan). The total RNA sources were pooled from 1–10 persons. Reverse transcription was performed using the SuperScript® VILO cDNA Synthesis Kit (Invitrogen Corp.). mRNA expression was measured in duplicate using the TaqMan-based real-time PCR assay on a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA), as described previously (Kaneko et al. 2007).

Analysis of Protein Levels in Human and Mouse Spinal Cords

The human spinal cords were homogenized with 10 volumes of a buffer consisting of 10 mM Tris-HCl (pH 7.6), 420 mM NaCl, 1 mM EDTA, 1% NP-40, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride, as described previously (Kaneko et al. 2010). The lysates were centrifuged at 20,000 ×*g* for 30 min. The mouse spinal cords were lysed using RIPA buffer with protease (P8340; Sigma-Aldrich) and phosphatase inhibitor cocktails (P2850 and P5726; Sigma-Aldrich), described previously (Tanaka et al. 2012). Protein equivalent samples were subjected to western blotting on the same gel and membrane; the detection was performed with goat anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP antibodies and the ECL Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) using a LAS-3000 mini luminescent image analyzer (Fujifilm, Tokyo, Japan). Quantitative analysis was performed using the Multi Gauge software (Fujifilm). ZnT levels are normalized by the amount of GAPDH. We confirmed that the other internal controls, β-actin and α-tubulin, exhibited similar tendencies in the ZnT levels when normalized by them.

Immunohistochemistry

Coronal sections of the human and mouse spinal cords, which were fixed in formaldehyde and paraformaldehyde, respectively, followed by paraffin embedding, were subjected to immunohistochemistry, as described previously (Omura et al. 2006). The tissues were sectioned into 4-μm-thick slices and then subjected to heat treatment in 10 mM sodium citrate buffer (pH 6.0) for 10 min using a microwave oven. Immunofluorescence staining was performed with anti-ZnT polyclonal antibodies and anti-neural cell-specific marker monoclonal antibodies, followed by Alexa Fluor® 555 goat anti-mouse IgG (H+L), highly cross-adsorbed, and Alexa Fluor® 488 goat anti-rabbit IgG (H+L), highly cross-adsorbed. Fluorescence images were acquired using an LSM 700 confocal microscope (Carl Zeiss AG, Gottingen, Germany). Diaminobenzidine (DAB) immunostaining was performed using anti-ZnT3 and anti-ZnT6 polyclonal antibodies (1:100 dilution), as described previously (Inden et al. 2013). The slices were treated with biotinylated goat anti-rabbit secondary antibodies (Histofine SAB-PO kit; Nichirei Co., Tokyo, Japan). Products of the streptavidin–biotin peroxidase complex were visualized using DAB (Nichirei Co.).

Statistics

All data are expressed as mean ± SEM. Statistical evaluation was performed using two-tailed Student's *t*-tests.

RESULTS

We determined the tissue distribution of ZnTs and the presence of ZnTs in the spinal cord. qPCR analysis using normal human tissues showed that ZnT1, 3, 4, 5, 6, 7, and 10 were expressed in central nervous system (CNS) tissues, including the spinal cord (Fig. 1). Conversely, ZnT2 and 8 were almost completely absent in CNS tissues (Fig. 1). It is noteworthy that ZnT3 specifically existed in CNS but not in peripheral tissues. Therefore, we selected ZnT1,

3, 4, 5, 6, 7, and 10 for quantitative analysis in ALS patients.

Western blot analysis showed that ZnT3 and 6 protein levels were significantly reduced in sporadic ALS patients compared with non-ALS controls, whereas ZnT5 protein levels showed a decreasing tendency but no statistical significance (Fig. 2). ZnT1 and 7 exhibited almost the same level between control and ALS samples (Fig. 2). ZnT4 and 10 were not evaluated because we were unfortunately unable to find an antibody that effectively and reliably detects these proteins in the spinal cord.

We further compared ZnT3 and 6 expression in the spinal cord between ALS patients and controls. Immunohistochemical staining demonstrated the decreased numbers of ZnT3 and ZnT6-immunopositive neurons in the ventral horn of the gray matter of the spinal cord of the patients with ALS, compared to those of controls, respectively. The immunopositivity of ZnT3 and ZnT6 in the residual motor neurons in ALS seemed to be reduced although immunohistochemical assessment is not appropriate for quantitative analysis. (Fig. 3A and B, and Supplementary Fig. 1A and B).

We immunohistochemically examined ZnTs expression in the spinal cord. ZnT1, 3, 5, 6, and 7-immunopositive cells correspond to class III β-tubulin/Tuj-1-positve cells in the gray matter (Fig. 4A; Supplementary Fig. 2A), whereas they were not detected with GFAP (Fig. 4B) or CNPase in the gray matter (Fig. 4C). Furthermore, we examined ZnT expression in spinal motor neurons using anti-ChAT antibody that recognizes cholinergic neurons. Immunohistochemical analysis showed that all ZnTs expressed in the spinal cord were partially, but not entirely, colocalized with ChAT-immunoreactive neurons (Fig. 4D; Supplementary Fig. 2B).

We examined ZnT3 and ZnT6 expression levels in *SOD1* (G93A) mutant Tg mice at ages before (10 weeks) and after (14 weeks) the onset of ALS symptoms with degeneration of motor neurons, as well as at the endpoint (18–20 weeks). ZnT3 and ZnT6 levels, which are normalized to GAPDH levels, were not altered in the *SOD1* Tg mice at each time point compared with controls (Fig. 5).

DISCUSSION

In the present study, we demonstrated that the majority of ZnTs (ZnT1, 3, 4, 5, 6, 7, and 10), with the exception of ZnT2 and 8, were expressed in the spinal cord, as the expression and distribution of ZnTs in the spinal cord had not been determined previously. In addition, we showed that ZnTs expressed in the spinal cord were selectively localized in neurons, and not in glia. Furthermore, we found that ZnT3 and 6 levels in particular were significantly decreased in the spinal cords of ALS patients, whereas ZnT1 and 7 levels were almost unchanged. Conversely, we demonstrated that *SOD1* mutation-induced neurodegeneration observed in Tg mice did not affect ZnT3 and 6 protein levels. These results seem to contradict the notion that the decrease in ZnT3 and 6 protein levels in ALS patients is caused only by the loss of motor neurons. If the reduction of ZnT3 and ZnT6 resulted from neuronal loss, the neurodegeneration in the SOD1 mice also induced decreased levels of ZnT3 and ZnT6. However, our result demonstrated that SOD1 mutation-induced neuronal loss failed to reduce the ZnTs expression. Therefore, the findings support our notion that impaired neuronal Zn homeostasis is an early event in the pathogenesis of sporadic patients with ALS.

In this study, we focused on ZnT, and not on the Zip family, as dysfunction of ZnT3 and 10 is reportedly associated with AD (Adlard et al. 2010; Beyer et al. 2012; Beyer et al. 2009; Bosomworth et al. 2013) and PD (Quadri et al. 2012; Tuschl et al. 2012). Moreover, dysfunction of some ZnTs causes endoplasmic reticulum (ER) stress (Ellis et al. 2004; Ishihara et al. 2006), which results in neuronal death in ALS. The ZnT family is mainly expressed in the secretory pathway, which comprises the ER and the Golgi apparatus, and secretory vesicles, in which one-third of all proteins, including secretory, membrane-bound, and vesicle-resident proteins, undergo folding, assembly, and modification processes. Zn is required for these secretory processes as an essential component of their structure and function. In addition, Zn has been reported to be stored in and released from the ER in a manner similar to that reported for calcium (Ca) (Maret 2013; Yamasaki et al. 2012). Thus, the disruption of Zn and Ca

homeostasis leads to ER stress and disease (Ellis et al. 2004; Ishihara et al. 2006).

The involvement of ER stress in ALS has been reported (Atkin et al. 2008; Ito et al. 2009). A series of SOD1 mutations associate directly and specifically with the cytosolic region of Derlin-1, which is an ER membrane-anchored protein and a component of the ER-associated degradation (ERAD) machinery (Fujisawa et al. 2012; Lilley and Ploegh 2004; Nishitoh et al. 2008; Ye et al. 2004). This interaction inhibits Derlin-1 function and leads to the disruption of the ERAD system, resulting in ER stress, which is linked to motor neuronal death (Fujisawa et al. 2012; Nishitoh et al. 2008). Recent studies have demonstrated that Zn depletion induces SOD1 conformational changes that allow the interaction between SOD1 and Derlin-1, which leads to ER stress (Fujisawa et al. 2012; Homma et al. 2013). Interestingly, this study showed that treatment with TPEN (a specific Zn chelator) or tunicamycin (an ER stress inducer) induced the expression of five ZIPs and three ZnTs (ZIP3, 7, 9, 13, and 14 and ZnT3, 6, and 7). It is noteworthy that ZnT3 was most strongly induced by the ER stressor (Homma et al. 2013). Taken together, these results show that an intracellular turbulence of Zn can induce ER stress and cause motor neuronal death in ALS pathogenesis.

Moreover, we previously demonstrated using immunohistochemical techniques that MT-I/II and MT-III expression were downregulated in the spinal cords of sporadic ALS patients (Hozumi et al. 2008). MTs are small cysteine-rich metal (Cu/Zn)-binding proteins that have multiple functions, such as regulating metal homeostasis (Hozumi 2013). Those results suggest that the decrease in MT proteins in ALS patients is also involved in the disruption of Zn homeostasis and ALS pathogenesis. Furthermore, Zip10 and 12 are expressed predominantly in CNS (Chowanadisai et al. 2013; Lemaire et al. 2012; Schmitt-Ulms et al. 2009), in which their roles remain poorly understood. To elucidate the mechanism of ALS pathogenesis, the study of Zip protein levels in ALS patients will be indispensable.

In this study, we demonstrated that ZnT3 and 6 protein levels were markedly and significantly reduced in the spinal cords of ALS patients. Furthermore, ZnT5 levels showed a

tendency to be decreased, albeit not significantly. As these ZnTs were expressed in neurons, including motor neurons, these results suggest that the downregulation of these proteins in the spinal cords of ALS patients is due only to the loss of motor neurons. To address this issue, we immunohistochemically analyzed ZnT3 and 6 expression in the spinal cords of ALS patients, which indicated that ZnT3 and 6-immunoreactive neurons were decreased in the ventral horn of the spinal cords of ALS patients. Conversely, the analysis of *SOD1* mutant Tg mice revealed that SOD1 mutation-induced motor neuronal loss did not affect ZnT3 and 6 protein ZnTs, including ZnT3 and 6, were observed to be widely expressed in the neurons of the whole spinal cord, in addition to the motor neurons of the ventral horn. Although ZnT5 was most abundantly and selectively expressed in motor neurons, the expression levels of this protein were not significantly downregulated. These results provide evidence that the decrease in the levels of ZnTs, including ZnT3 and 6, precedes the degeneration of motor neurons in sporadic ALS patients. However, to further support the hypothesis that impaired neuronal Zn homeostasis is an early event in ALS pathogenesis, it is necessary to determine whether the decrease in ZnT3 and 6 protein levels occurs prior to the loss of expression of neuronal markers at each clinical stage, as demonstrated in AD (Beyer et al. 2009).

ACKNOWLEDGMENTS

We wish to thank Professor Shu-ichi Ikeda, Department of Neurology & Rheumatology Shinshu University School of Medicine for kindly approving to provide samples and helpful advice. We are also grateful to Dr. M. Shimazawa and Dr. K. Tsuruma, Laboratory of Molecular Pharmacology, Department of Biofunctional Evaluation, Gifu Pharmaceutical University for technical assistance.

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FIGURE LEGENDS

Fig. 1. Expression patterns of ZnT mRNAs in normal human tissues. cDNAs reverse transcribed from human normal tissue mRNAs (spinal cord, cerebral cortex, hippocampus, cerebellum, liver, kidney, lung, and pancreas) were analyzed by the TaqMan-based qPCR assay. Data are normalized to the amount of 18s ribosomal RNA (18s rRNA), and results are expressed as the fold increase compared with that in the spinal cord.

Fig. 2. ZnT protein levels in the spinal cords of ALS patients. The spinal cords of amyotrophic lateral sclerosis (ALS) patients and non-ALS controls were analyzed by western blotting using the indicated anti-ZnT antibodies. Data are normalized to the amount of GAPDH. Results are depicted as a dot plot (control, n = 14; ALS, n = 10). Asterisk represents a significant difference (Student's *t*-test, *$p < 0.05$ and **$p < 0.01$).

Fig. 3. Comparison of ZnT3 and 6 expression in spinal cords between ALS patients and controls. Coronal sections of the human spinal cord were subjected to DAB staining with anti-ZnT3 (A) and ZnT6 (B) antibodies (control, top; ALS, middle and bottom). Scale bars, 50 μm (20×, 61.5×). The arrowheads indicate neurons in the gray matter. The numbers of ZnT3 and ZnT6 immunopositive neurons were decreased in the spinal cords of the patients with ALS compared to those of controls, respectively (3A and B).

Fig. 4. Localization of ZnTs in the mouse spinal cord. Coronal sections of the murine spinal cord were subjected to immunofluorescence staining with anti-ZnT1, 3, 4, 5, 6, or 7 antibodies and with (A) anti-class III β-tubulin (Tuj-1), (B) anti-GFAP, (C) anti-CNPase, or (D) anti-ChAT antibodies. Scale bars, 200 μm (5×; left panels) and 50 μm (20×; right panels).

Fig. 5. ZnT3 and 6 protein levels in the spinal cords of *SOD1* (G93A) mutant Tg mice. The spinal cords of *SOD1* (G93A) mutant Tg mice (TG) and wild-type controls (WT) at the age of 10 weeks (before and after the onset of ALS symptoms), at the age of 14 weeks (after the onset of ALS symptoms, with degeneration of motor neurons), and at the endpoint (18–20 weeks) were analyzed by western blotting using anti-ZnT3 and anti-ZnT6 antibodies. Data are normalized to the amount of GAPDH (mean ± SEM; $n = 3$). Results are expressed as the fold increase compared with that in WT mice.