Oct3/4, Sox2, Klf4, and c-Myc, which are important for self-renewal of ESC, have been shown to reprogram both mouseand human somatic cells into ESC-like pluripotent cells, calledinduced pluripotent stem cells (iPSC) (Takahashi et al., 2007).

ESC and iPSC theoretically can differentiate into various cell-types of neural lineage, including dopaminergic neuron, neural crest, retina, cerebral cortex, and spinal motor neuron (Li et al., 2008). Using these differentiation methods ESC or iPSC derived neurons were applied to disease modeling (Li et al., 2008; Ebert et al., 2009; Lee et al., 2009).

Clinical Trials Using ESC-Derived Cells

The development of ESC and iPSC is attractive not only for disease mechanism research but also for cellular replacement therapy. Several clinical trials with ESC are ongoing (Table 25.1). In 2009, the Food and Drug Administration (FDA) gave the first approval for an ESC-based clinical trial, conducted by Geron Corporation. Geron planned to apply human ESC-derived oligodendrocyte progenitor cells (OPCs) to the treatment of spinal cord injury patients. In a rat model of spinal cord injury, transplantation of ESC-derived OPCs dramatically improved disability to almost a normal level (Sharp et al., 2010). The mechanism of the improvement was well assessed, demonstrating secreting nerve growth factor and remyelination. The safety of the process, especially in terms of tumorigenicity, was also evaluated. However, the FDA placed a hold on this trial because of cystic structure in animal model after transplantation. After additional safety-proving data, phase I clinical trials of ESC-derived OPCs were released in 2010 (Strauss, 2010).

Furthermore, Advanced Cell Technology, Inc. (ACT) received FDA clearance for a run of second clinical trials using ESC of age-related macular degeneration (AMD) in 2010. AMD is the most common form of macular degeneration and is intractable by existing medicine. ACT plans to transplant ESC-derived retinal pigment epithelial (RPE) cells to AMD patients and perform safety evaluation as a phase I clinical trial (Zhu et al., 2010). ACT is also approved for Stargardt's macular degeneration by transplanting ESC-derived RPE cells in 2010 November.

Regenerative Therapy for ALS

Cell replacement therapy promises to be powerful and attractive especially for CNS disorders, which are impossible to cure by conventional therapy. One of the most intractable CNS disorders is ALS.

ALS is a fatal neurodegenerative disease with late-middle age onset. ALS is clinically characterized by dysfunction of both upper and lower motor neurons, resulting in rapid progression of weakness and respiratory failure. Median survival of ALS patients without ventilators is only a few years. Numerous drugs have been attempted in clinical trials, but almost all failed to achieve even disease modification. The solely successful drug, riluzole, can only slow disease progression slightly.

The neuropathological hallmark of ALS is a massive loss of motor neurons in both the primary motor area and the anterior horn of the spinal cord. A recent study revealed that TAR DNA binding protein of 43 kDa (TDP-43) aggregates are observed not only in degenerative neurons but also in glial cells (Arai et al., 2006; Neumann et al., 2006). Unveiling the role of TDP-43 and relevant molecules would accelerate ALS research, but would still not be sufficient for a complete cure. Therefore, replacement therapy using stem cells is expected to be a potent candidate for modifying or recovering from the disease state. Here we review the recent progress and present future vision for cell replacement therapy for ALS, also emphasizing the hurdles to overcome before clinical trials can commence.

Table 25.1 Clinical trials using ESC in neurological disorders

Company	Disease	Cell type	Progress
Geron	Spinal cord injury	OPCs	Phase I 2010 October
ACT	Stargardt's macular degeneration	RPE	Phase I 2010 November
ACT	AMD	RPE	Phase I 2010 November
California stem cell	SMA type I	Motor neuron	Hold 2011 February

Transplantation Research for ALS

Before clinical trials can be initiated, basic research using animal models is necessary for evaluating their safety and efficacy. Almost all transplantation research was established using human superoxide dismutase 1 (SOD1), with G93A mutation, transgenic rats or mice. SOD1 is the most common causative gene of familial ALS, and SOD1 transgenic animals represent lower motor neuronal loss, mimicking symptoms of ALS patients (Gurney et al., 1994).

For cell resources, various kinds of stem cells are used for transplantation, e.g., rodent bone marrow cells, human mesenchymal stem cells (hMSC), neurotrophic-factor-secreting hMSC, human umbilical cord blood cells (hUCBC), neural progenitor cells (NPC), and ESC-derived glial precursors (Lepore et al., 2008; Thonhoff et al., 2009).

Transplantation routes also vary, e.g., direct injection into the spinal cord, intraperitoneal, intracerebroventricular, and intravenous injection. Direct injection into the spinal cord can localize transplanted cells and provide high survival efficacy. In contrast, intravenous injection can deliver transplanted cells widely, but it has lower efficacy of engraftment or can result in pulmonary embolism. Outcome results vary from research to research, and graft-modifying technique (e.g., molecular modification to secrete neurotrophic factors) can enhance the efficacy of engraftment (Thonhoff et al., 2009). To evaluate the optimal delivery efficacy and survival rate, Takahashi et al., (2010) compared each transplantation route (including lesion-direct, intrathecal and intravenous injection) with spinal cord injury mice model and luciferase imaging. They concluded that direct injection achieved the highest delivery efficacy and graft survival 6 weeks after injection.

Clinical Trials of Cell Transplantation for ALS

Recently, several clinical trials using somatic stem cell transplantation for ALS have been conducted. All of the published clinical trials were based on autologous MSC from bone marrow (Table 25.2). According to the published data, they successfully achieved a safety endpoint, but spinal cord swelling at the transplanted site is noted in some cases (Karussis et al., 2010). Therefore, the risk of tumorigenicity has not been excluded even in adult somatic stem cells. Emory University and Neural Stem Inc., received FDA approval in 2009, and they have already started an ALS phase I clinical trial by transplantation of fetal neural stem cells.

Hurdles in Transplantation Therapy

Basic research using animal models will help to shed light on problems needing to be overcome before clinical trials.

Ethical Issues

To obtain ESC culture, it is necessary to manipulate embryos for scientific use. However, among various moral and ethical issues involved, the catholic church identifies embryos at this stage as having the same rights as a developing human being.

Robust Supply

As described above (CLINICAL TRIALS), MCS are widely applied and are clinically easy to access from general hospitals. However, because they are not of neural lineage, their effectiveness as cell replacement is limited. Up-coming clinical trials with neural stem cells (NSC) of fetal spinal are expected to prove them as a suitable cell resource for neural replacement. However, the graft cell resource will depend on the fetal spinal cord, limiting the number of graft cells.

Somatic cells and MSC have a finite replicative lifespan, beyond which senescence will prevent division. In contrast, ESC or iPSC can proliferate indefinitely and make robust stable freeze stocks. Furthermore, transplant of ESC- or iPSC-derived NSC can provide both neuronal replacement and protective glial cells, modifying the ALS environment around remaining neurons.

Table 25.2 Clinical trials of cell transplantation in ALS

Country	Company/Center	Date	Cell source	Cell type	Route	No. enrolled	Trial	Results
Italy	Eastern Piedmont Univ.	(2010 publish)	Auto, BM	MSC	Upper Th	10	Phase I	Safe
Turkey	Akay Hospital	(2009 publish)	Auto, BM	MSC	C1-2	13?	Phase I	Safe, improved
Spain	Hospital Universitario Virgen de la Arrixaca	2007 Feb–2010 Feb	Auto, BM	MSC	Th5-6	11	Phase I	
Spain	Hospital Universitario Virgen de la Arrixaca	2010 Oct~	Auto, BM	MSC	Th5-6	63	Phase II	(Currently recruiting)
Spain	Autonomous University of Barcelona	(2010 publish)	Auto, nose/BM	OEC/MSC		20	N.A.	Safe, no effect
Israel	Hadassah Medical Organization	2010 Jan~	Auto, BM	MSC-NTF	muscle	12	Phase I	(Currently recruiting)
Israel	Hadassah Medical Organization	2010 Jan~	Auto, BM	MSC-NTF	CSF (lumbar puncture)	12	Phase II	(Currently recruiting)
U.S.A.	TCA Cellular Therapy	2010~	Auto, BM	MSC	CSF (lumbar puncture)	6	Phase I	(Currently recruiting)
U.S.A.	Emory University	2009~	Fetal Spinal Cord	NSC	Cervical/ Lumbar	12	Phase I	N.A.
U.S.A.	Unknown	in plannning	hESC	Glial cells (astro-cytes)	–	–	–	–

Safety

Self-renewal and plasticity features of ESC and iPSC are also characteristics of cancer cells. Sometimes graft stem cells can lose control of appropriate proliferation and develop tumor as an unacceptable side-effect. We can decrease tumorigenicity risk by (1) using well-maturated cells for transplantation or (2) characterizing and selecting ESC or iPSC that have low tumorigenicity. To achieve (1), we have to enhance the sophistication of the differentiation and purification techniques of target cells. The tumorigenic potential of ESC will be reduced after maturation. For investigating (2), Miura et al., (2009) clarified that ES and iPSC have different tendencies to form neural tumor or teratoma from clone to clone. To decrease tumorigenicity, a novel iPSC reprogramming technique, using L-Myc instead of c-Myc, was reported (Nakagawa et al., 2010). By mixing and balancing these evaluation techniques, we will be able to avoid or decrease tumorigenicity in the future.

Before transplantation, ESC and iPSC need to pass through many steps to reach an appropriate state for use. Throughout, we must prevent contamination risk by harmful components and meet the standard of "Good Manufacturing Practice". In detail, adequate screening of donor material for infectious diseases as well as possible genetic testing will be necessary. In addition, avoiding the use of nonhuman animal components (a potential source of unknown infection) will also be important.

Functional Efficacy

Even if appropriate numbers of graft-cells can survive, it is difficult to make a neural network with the remaining neurons. For example, of fetus mid-brain transplantation in PD, cell therapy could improve motor symptoms and decrease drug dosage. However, therapy cannot improve dyskinesia (inappropriate secretion of

neuronal transmitter) (Barker and Kuan, 2010). This phenomenon is explained by failure to make a synchronized network with the remaining neurons around engrafting sites (Carlsson et al., 2006) or by contamination of serotonergic neurons in graft (Barker and Kuan, 2010). In the case of ALS, engrafted cells have to expand their axons to muscle (target site), far away from the spinal cord. Several researchers successfully overcame this difficulty and observed that transplanted cells (human neural stem cells) innervated host animal muscle (Gao et al., 2005; Deshpande et al., 2006).

For choosing cell type for the optimal state of ALS transplantation therapy, we can mainly list neural precursors, motor neurons, astrocytes, oligodendrocytes, and microglial cells. Simply stated, ESC- or iPSC-derived motor neurons would be a most suitable candidate for treating motor neuron disease. However, when generating or purifying motor neurons from ESC or iPSC, it is difficult to maintain moderate differentiation efficiency. Neural precursors, including various subtypes of neurons, are expected to have the most powerful ability to regenerate or protect damaged tissue. On the other hand, neural precursors generally contain immature cells and can have high tumorigenicity. In contrast to the regenerative effect of neural transplantation, glial cell transplantation can exert a neuroprotective effect via secreting neurotrophic factors and improving inflammatory damage of ALS. Then, for maintaining an all-around sufficient efficacy and safety level, matured glial cells would also be favorable candidates for clinical trials. More preclinical research will be required to approach solutions to this problem (Papadeas and Maragakis, 2009).

We utilize animal models for the evaluation of transplantation efficacy, and rodent is usually employed as disease model and recipient. However, rodent is a distinctively different species from humans. As an example of a spinal cord injury model, mice recover their motor function only a few days after injury, without any treatment. Natural recovery (or compensation), commonly observed in rodent, is never seen in humans. This difference is ascribed to the difference in upper motor neural tract between rodent and humans. Then, we have to investigate other species that are closer to humans, such as canine ALS models (Awano et al., 2009).

Difficulty of Efficient Cell-Delivery into ALS Lesions

The pathological changes of PD patients are mostly localized in midbrain or basal ganglia (striatum), and it is easy to apply direct injection of stem cells. However, pathological changes, based on TDP-43 immunostaining, are widely observed in the whole CNS (Liscic et al., 2008). ALS is contemporarily recognized as a multisystem neurodegenerative disorder. To overcome difficulties regarding the "wide-spread lesions of ALS" and the blood-brain barrier, transplantation via cerebral ventricle might be a solution (Morita et al., 2008).

For Future Clinical Trials

From the series of long discussions between the FDA and Geron, we can understand that safety is considered to be critically important for successful clinical trials. The International Society for Stem Cell Research (ISSCR) recently issued guidelines regarding threshold safety and ethical criteria for clinical transplantation therapy (Hyun et al., 2008). The ISSCR guidelines deal not only with not just ESC research but also with other pluripotent stem cells, including iPSC. ISSCR guidelines also point out the need to assess the risks of tumorigenicity. Abiding by the guidelines, stem cell transplantation is expected to win approval smoothly and to maintain a constant level of quality.

Cell Resource

We have two pluripotent stem cells, ESC and iPSC, as graft resource. Today, a few clinical or preclinical trials are mostly based on ESC or ESC-derived precursor cells, but not on iPSC. iPSC have the ability of proliferation and differentiation like ESC, and they are considered to have a similar character to that of ESC. However, recent study revealed a difference between them at the level of genome methylation or gene expression (Bock et al., 2011). We can characterize and select iPSC clones that are epigenetically

identical to ESC. Then, the transplantation management of ESC is applicable to iPSC. Here we list the pros and cons of choosing ESC or iPSC in future clinical applications.

Generally, differentiation and transplantation research using ESC has a decade of history, and both usability and safety information have already accumulated to some extent. This information is a great advantage for transplantation therapy, which requires a very strict safety level (but not enough). In contrast, iPSC technology is in its nascent stages, but recent rapid advances in the field are expected to bridge the gap.

To prevent GVHD risk, recipients must continue immunosuppressant drugs after ESC transplantation. Moreover, GVHD reaction has been pointed out as raising focal inflammation at the transplant site and exacerbating degenerative progression (Kordower et al., 2008). Besides the GVHD harmful events, there is also an interesting discussion regarding GVHD possibly acting as a safety-lock against tumorigenicity, which is an intolerable side-effect. In other words, reactivated GVHD reaction, by discontinuing immunosuppressants, could eliminate "tumor" derived from ESC-graft. We need to evaluate carefully this two-sided character of GVHD mechanisms through animal model research.

In contrast, we can generate iPSC from adult somatic cells of a patient (transplantation donor) and return patient-derived iPSC-graft back to the same patient (transplantation recipient), without GHVD risk. In the future, if a quick, safe, and low-cost method for iPSC generation is developed, every patient will be able to receive his/her own iPSC-derived cells, as ultimately customized transplantation therapy. However, the reprogramming state of iPSC is known to differ from clone to clone (Miura et al., 2009). The selection of safe iPSC will be critical for safe transplantation. To overcome the described hurdles of both ESC- and-iPSC based research, HLA type characterized iPSC bank will enable us to minimize the risk for GVHD and lower the dosage of immunosuppressant drugs (Nakatsuji et al., 2008). Furthermore, by using iPSC bank, we can also circumvent the ethical issue of using ESC.

In conclusion, innovations in stem cell manipulation will accelerate transplantation therapy using stem cells. Basic research focusing on the safety of transplantation, in addition to therapeutic experiments, can lead to beneficial outcome in practical use.

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Clinical/Scientific Notes

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AN AUTOPSY CASE OF *SOD1*-RELATED ALS WITH TDP-43 POSITIVE INCLUSIONS

Approximately 20% of familial amyotrophic lateral sclerosis (FALS) cases are linked to mutations in the antioxidant enzyme, Cu/Zn superoxide dismutase (SOD1). Controversy exists as to whether *SOD1*-related FALS and sporadic ALS (SALS) share common mechanistic pathways, since very few *SOD1*-related FALS cases exhibit cytoplasmic inclusions composed of 43-kDa TAR DNA-binding protein (TDP-43), a pathologic hallmark of SALS and *SOD1*-unrelated FALS.[1,2,e1,e2] We describe prominent TDP-43 pathology in the spinal cord of a patient with *SOD1*-related ALS with *SOD1* I112T mutation, a relatively uncommon variant form leading to rapid disease progression.[3]

Case report. A 41-year-old man with no familial history of neurodegenerative disorder noted progressive weakness in his left leg. Four months after onset, a neurologic examination detected muscle weakness in the left lower extremity and fasciculation in all the extremities. Tendon reflexes were decreased and Babinski sign was negative bilaterally; EMG revealed a neurogenic pattern in the left extremities. Dysarthria and dysphagia were not evident. He was diagnosed with ALS. Within a year, he became bedridden and died at the age of 43 because of respiratory failure. Genomic DNA was extracted from the autopsied samples with informed consent from his family. DNA analysis revealed a missense mutation (I112T) in *SOD1* gene (figure 1A). Mutations were not found in other exons of *SOD1* gene or in the *TDP-43* gene. The prefixed brain weighed 1,400 g with no apparent abnormalities in external appearance. Microscopic examination revealed marked loss of motor neurons, with glial proliferation in the spinal cord and Lewy body–like hyaline inclusions found in the remaining motor neurons; Bunina bodies were not observed. Myelin pallor was evident in the corticospinal tract, the middle root zone of the posterior column, and the spinocerebellar tract (figure e-1 on the *Neurology*® Web site at www.neurology.org). Immunohistochemical analysis was performed with antibodies specific to anti-misfolded SOD1 (D3H5),[4,5] anti-SOD1 (SOD-100), anti-phosphorylated TDP-43

(pTDP-43), and anti-nonphosphorylated TDP-43 (TDP-43) (table e-1). Inclusions immunopositive for D3H5 or pTDP-43 were undetectable in the brain, except for the brainstem or spinal cord. D3H5 immunoreactivity was more intense and more diffusely distributed than that of SOD-100. Numerous D3H5-positive inclusions were observed in brainstem nuclei, including the hypoglossal nucleus and reticular formation of the medulla oblongata (figure 1, B and C). D3H5-positive inclusions were less frequently present in the anterior horn cells of the cervical spinal cord (figure 1D); however, they were not found in the lumbar spinal cord (figure 1E). In contrast, pathologic pTDP-43 immunoreactivity was not found in the brainstem (figure 1, F and G) or cervical spinal cord (figure 1H), whereas abundant pTDP-43-positive inclusions were present in the neuronal cytoplasm (figure 1I) or glial cells (figure 1, J and K) of the lumbar spinal cord. The topographic distributions of D3H5 and pTDP-43 immunoreactivity were apparently distinct, and double immunofluorescent staining revealed that D3H5 did not colocalize with pTDP-43 (figure 1, L–N) or TDP-43 (figure 1, O–Q).

D3H5-immunoreactive inclusions were not detectable in the lumbar anterior horn cells of a SALS case or 2 non-ALS disease control cases (figure e-2).

Discussion. The relative paucity of cytoplasmic TDP-43 inclusions in mutant *SOD1*-related FALS cases[2,e2] suggests that the 2 pathogenic pathways are unrelated. Nevertheless, the present ALS case with a mutation in *SOD1* exhibits typical TDP-43 pathology, including the presence of neuronal and glial cytoplasmic inclusions. Two previously reported *SOD1*-related FALS cases (C111Y and H48Q) have reported a few TDP-43 inclusions in the neuronal cytoplasm.[6,e3] In one case, TDP-43 colocalized with SOD1, suggesting that TDP-43 is sequestered into SOD1 aggregation[6]; while in the other study, the TDP-43 immunoreactivity was only present in a single neuron, with no reference to colocalization of TDP-43 with SOD1.[e3] The striking feature in our patient is that misfolded SOD1 and pTDP-43 aggregates were both present, though mutually exclusive.
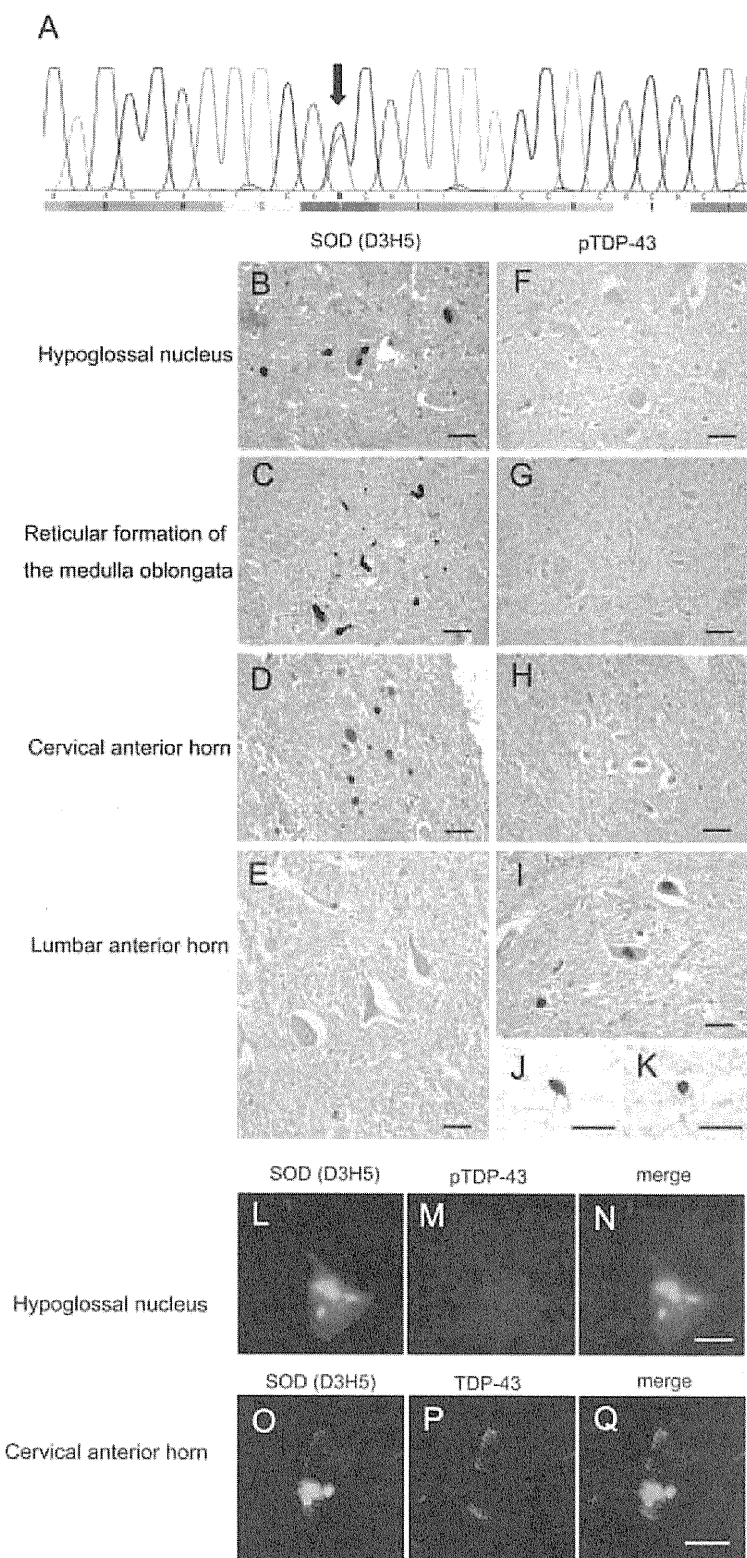
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Heterozygous point mutation (1,144 T>C) in exon 4, resulting in amino acid substitution of isoleucine to threonine at the 112th residue (A, arrow). Mutually exclusive patterns of D3H5 and pTDP-43 immunoreactivity in the brainstem and spinal cord areas indicated (B–K). Noncolocalization of D3H5 with pTDP-43 (L–N) or TDP-43 (O–Q) in the hypoglossal nucleus and cervical cord. Autofluorescence associated with lipofuscin granules (P, Q). Bars, 40 μm (B–H, L–Q) and 20 μm (I–K).

Given that misfolded SOD1 oxidized at C111 residue is found even in SALS,[7] it appears that pathogenesis of *SOD1*-related FALS and SALS might converge on a common pathway. Further genotype–phenotype correlation needs to be investigated to elucidate the linkage, if any, between the SOD1 and TDP-43 mechanistic pathways involved in ALS.

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PLoS one

Colocalization of 14-3-3 Proteins with SOD1 in Lewy Body-Like Hyaline Inclusions in Familial Amyotrophic Lateral Sclerosis Cases and the Animal Model

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Abstract

Background and Purpose: Cu/Zn superoxide dismutase (SOD1) is a major component of Lewy body-like hyaline inclusion (LBHI) found in the postmortem tissue of SOD1-linked familial amyotrophic lateral sclerosis (FALS) patients. In our recent studies, 14-3-3 proteins have been found in the ubiquitinated inclusions inside the anterior horn cells of spinal cords with sporadic amyotrophic lateral sclerosis (ALS). To further investigate the role of 14-3-3 proteins in ALS, we performed immunohistochemical analysis of 14-3-3 proteins and compared their distributions with those of SOD1 in FALS patients and SOD1-overexpressing mice.

Methods: We examined the postmortem brains and the spinal cords of three FALS cases (A4V SOD1 mutant). Transgenic mice expressing the G93A mutant human SOD1 (mutant SOD1-Tg mice), transgenic mice expressing the wild-type human SOD1 (wild-type SOD1-Tg mice), and non-Tg wild-type mice were also subjected to the immunohistochemical analysis.

Results: In all the FALS patients, LBHIs were observed in the cytoplasm of the anterior horn cells, and these inclusions were immunopositive intensely for pan 14-3-3, 14-3-3β, and 14-3-3γ. In the mutant SOD1-Tg mice, a high degree of immunoreactivity for misfolded SOD1 (C4F6) was observed in the cytoplasm, with an even greater degree of immunoreactivity present in the cytoplasmic aggregates of the anterior horn cells in the lumbar spinal cord. Furthermore, we have found increased 14-3-3β and 14-3-3γ immunoreactivities in the mutant SOD1-Tg mice. Double immunofluorescent staining showed that C4F6 and 14-3-3 proteins were partially co-localized in the spinal cord with FALS and the mutant SOD1-Tg mice. In comparison, the wild-type SOD1-Tg and non-Tg wild-type mice showed no or faint immunoreactivity for C4F6 and 14-3-3 proteins (pan 14-3-3, 14-3-3β, and 14-3-3γ) in any neuronal compartments.

Discussion: These results suggest that 14-3-3 proteins may be associated with the formation of SOD1-containing inclusions, in FALS patients and the mutant SOD1-Tg mice.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal, progressive neurodegenerative disease characterized by the degeneration of motor neurons in the motor cortex, brainstem and spinal cord. The vast majority of ALS patients are sporadic, and approximately 5–10% of ALS cases are familial ALS (FALS) [1]. Among the FALS patients, approximately 20% are linked to mutations in the antioxidant enzyme Cu/Zn superoxide dismutase (SOD1) [2]. Mutant SOD1 proteins aggregate and form Lewy body-like hyaline inclusions (LBHIs) in the anterior horn cells of the spinal cord [3].

Transgenic mice carrying several copies of human mutant SOD1 genes show ALS-like symptoms such as progressive motor disturbances and neurogenic amyotrophy, and develop a pathology resembling ALS [4]. In brief, these Tg mice demonstrate atrophy of the motor neuronal system, vacuolar degeneration of the motor neurons, and ubiquitinated neuronal hyaline inclusions which contain SOD1 in their cell bodies and swollen processes [5].

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SOD1 is a major constituent of LBHIs linked to FALS, and these LBHIs contain ubiquitin [6], phosphorylated neurofilaments [7], and a copper chaperone for superoxide dismutase [8].

The 14-3-3 proteins, a family of protein chaperones, are abundant in the brain, comprising approximately 1% of the total brain protein [9]. 14-3-3 proteins consist of seven different isoforms, named with Greek letters (β, ε, γ, η, θ, σ, and ζ). Each isoform forms homo- or hetero-dimers. 14-3-3 dimers can simultaneously bind two ligands, modulate different signaling molecules and participate in cell cycle control, cell adhesion, neuronal plasticity as well as various intracellular signal transduction pathways [10]. 14-3-3 proteins seem to control the subcellular localization of proteins and to function as adaptor molecules, stimulating protein-protein interactions. The regulation of this interaction usually involves the phosphorylation of the interacting proteins [11].

In our recent studies, several types of 14-3-3 proteins such as 14-3-3β, 14-3-3γ, 14-3-3ζ, 14-3-3θ, or 14-3-3ε have been found in the ubiquitinated inclusions of anterior horn cells from patients with sporadic ALS [12]. 14-3-3 mRNA was also demonstrated to be upregulated in the spinal cords with sporadic ALS [13].

However, the association of 14-3-3 proteins with FALS remains unknown. In this study, to investigate the role of 14-3-3 proteins and SOD1 in the pathogenesis of FALS, we performed immunohistochemical staining for 14-3-3 proteins and SOD1 in formalin-fixed, paraffin-embedded sections from patients with FALS. Transgenic mice which overexpress mutant human SOD1, transgenic mice which overexpress wild type human SOD1, and non-transgenic wild-type mice were also subjected to immunohistochemical analysis.

Methods

Ethics Statement

The protocols for genetic analysis and neuropathological procedures were approved by and performed under the guidelines of our institutional ethics committee. Informed consent was obtained from all individuals or their guardians before the analysis. The animal study was carried out in strict accordance with the guidelines for animal experimentation from the Animal Research Committee of our institution. The protocol was approved by the Animal Research Committee, Kyoto University (Permit Number: MedKyo10202).

Human FALS cases

We analyzed three cases of FALS (A4V SOD1 mutant). The clinicopathological backgrounds of these FALS cases have been previously reported [14]. These patients were members of the American "C" family. The three patients were males, and their ages at death were 39, 46 and 66 years. They were pathologically consistent with FALS with posterior column involvement [15].

Transgenic mice expressing G93A mutant human SOD1 and wild type human SOD1

We used transgenic mice expressing the G93A mutant human SOD1 gene (mutant SOD1-Tg mice) [B6SJL-TgN (SOD1-G93A) 1Gur] and wild-type human SOD1 gene (wild-type SOD1-Tg mice) [B6SJL-Tg (SOD1) 2Gur/J], which were originally obtained from the Jackson Laboratory [16]. The mutant SOD1-Tg mice develop signs of hind limb weakness at the age of 3 to 4 months. At the age of 5 to 6 months, they are not able to forage for food and water and then die. The wild-type SOD1-Tg mice show no motor symptoms [17]. We analyzed four-month-old mutant SOD1-Tg and wild-type SOD1-Tg mice (n = 4, each).

Human tissues

Human tissue blocks obtained from the different levels of spinal cords of FALS cases were embedded in paraffin. The blocks were sectioned with a microtome at 6 μm thickness for routine and immunohistochemical staining. Routine histological assessment was carried out with hematoxylin and eosin (H&E).

H&E-stained sections with LBHIs were photographed, decolorized with 70% ethanol and pretreated with 0.3% H_2O_2 in 0.1 mol/L phosphate-buffered saline (PBS) for 30 min at room temperature to inhibit any endogenous peroxidase activity. After washing with 0.1 mol/L PBS, these sections were blocked with 0.1 mol/L PBS plus 3% skim milk for 2 hours at room temperature. Then, the specimens were used for immunohistochemistry; this involved sequential incubation with primary antibody, appropriate biotinylated secondary antibody (Vector Laboratories, diluted 1:200), and avidin-biotin-peroxidase complex (ABC; Vector Laboratories, 1:200) in 0.1 mol/L PBS containing 0.3% Triton X-100 (PBST, pH 7.4). The sections were rinsed with PBST for 15 min between each step and finally visualized with 0.01% diaminobenzidinetetrahydrochloride and 0.005% H_2O_2 in 50 mmol/L Tris-HCl (pH 7.6).

Animal tissues

Mice were deeply anesthetized with sodium pentobarbital and were perfused transcardially with 0.01 mol/L PBS and then with a fixative containing 4% paraformaldehyde (PFA) and 0.2% picric acid in 0.1 mol/L phosphate buffer (PB, pH 7.4). Then, the brains and the spinal cords were removed. The tissues were post-fixed for 24 hours in 4% PFA and stored in 20% sucrose in 0.1 mol/L PB (pH 7.4). Serial lumbar spinal sections were cut into 20 μm thick sections on a cryostat, and immunohistochemical analysis was performed in the same way as human tissues described above.

Table 1. Primary antibodies.

Primary antibody				Company	Dilution
C4F6	mouse monoclonal			Reference [18]	1:1000
SOD1	goat polyclonal	C-17	SC-8637	Santa Cruz Biotechnology	1:1000
pan 14-3-3	mouse monoclonal	H-8	SC-1657	Santa Cruz Biotechnology	1:1000
14-3-3β	rabbit polyclonal	C-20	SC-628	Santa Cruz Biotechnology	1:2000
14-3-3γ	rabbit polyclonal	C-16	SC-731	Santa Cruz Biotechnology	1:2000
14-3-3ε	rabbit polyclonal	T-16	SC-1020	Santa Cruz Biotechnology	1:400
14-3-3η	goat polyclonal	E-12	SC-17287	Santa Cruz Biotechnology	1:400
14-3-3θ	rabbit polyclonal	C-17	SC-732	Santa Cruz Biotechnology	1:2000
14-3-3σ	goat polyclonal	C-18	SC-7683	Santa Cruz Biotechnology	1:400
14-3-3σ	goat polyclonal	N-14	SC-7681	Santa Cruz Biotechnology	1:400
14-3-3ζ	rabbit polyclonal	C-16	SC-1019	Santa Cruz Biotechnology	1:2000

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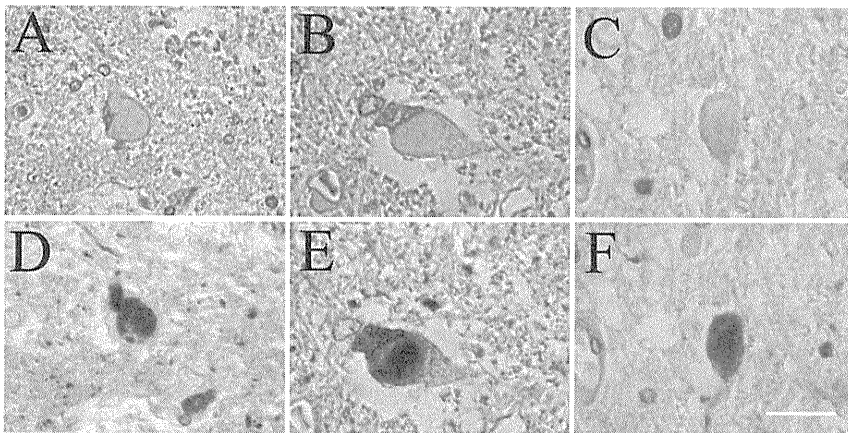
Figure 1. LBHIs immunopositive for 14-3-3 proteins in FALS patients. A, B, and C are the same sections as D, E, and F, respectively. The upper panels (A–C) are stained with H&E, and the lower panels (D–F) are immunostained with the anti-pan 14-3-3 antibody. LBHIs observed on H&E in the anterior horn cells are intensely immunopositive for pan 14-3-3. Bar indicates 100 μm in (A, D), and 50 μm in (B, C, E, F).
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Primary antibodies

As primary antibodies, we used anti-SOD1, anti-misfolded SOD1 (C4F6) [18], and anti- pan and isoform-specific 14-3-3 protein antibodies. The primary antibodies used were listed in Table 1.

Double labeling immunohistochemistry

To investigate the relationship between SOD1 and 14-3-3 proteins, spinal cord sections of the FALS cases were incubated with primary antibodies against SOD1 and pan 14-3-3, followed by incubation with FITC- or rhodamine-labeled appropriate secondary antibodies. For mouse tissues, anti-14-3-3 (β or γ) and C4F6-DyLight 488 antibodies were used. C4F6 was labeled with

DyLight Fluor 488 using a commercially available kit (DyLight Microscale Antibody Labeling Kits, Thermo Scientific).

Results

14-3-3 immunoreactivity in patients with FALS

In all the three FALS cases, LBHIs were observed inside the anterior horn cells (Figure 1A–C). All the LBHIs observed on H&E showed strong pan 14-3-3 immunoreactivity (Figure 1D–F). Using 14-3-3 isoform-specific antibodies, all the LBHIs detected by H&E (Figure 2A, B) were intensely immunopositive both for 14-3-3β (Figure 2C) and 14-3-3γ (Figure 2D).

Double immunofluorescent-stained sections showed that pan 14-3-3 was co-localized with SOD1 in the LBHI (Figure 3).
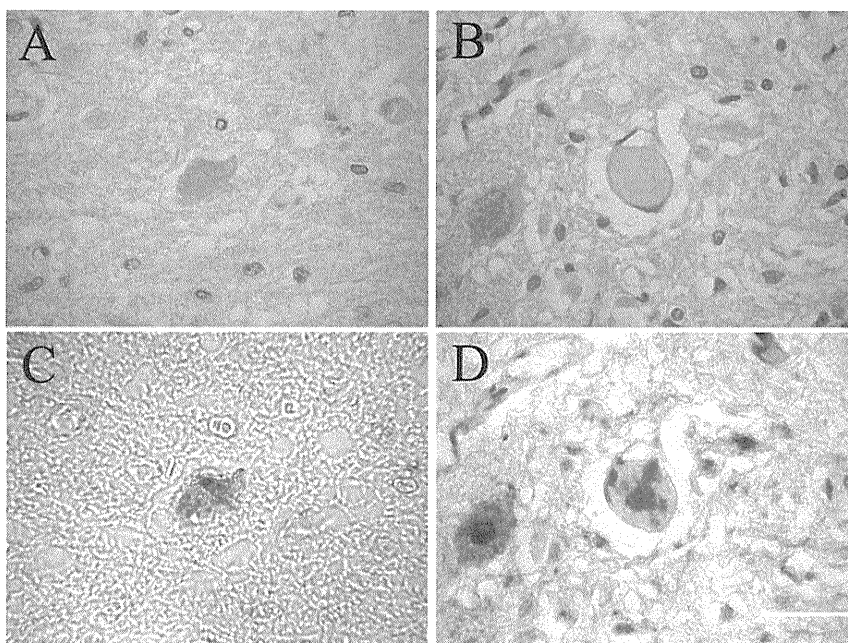
Figure 2. LBHIs immunopositive for 14-3-3β or 14-3-3γ in FALS patients. A and B are the same sections as C and D, respectively. The identical LBHIs observed on H&E (A, B) in the anterior horn cells are intensely immunostained with 14-3-3β (C) and 14-3-3γ (D). Bar indicates 50 μm.
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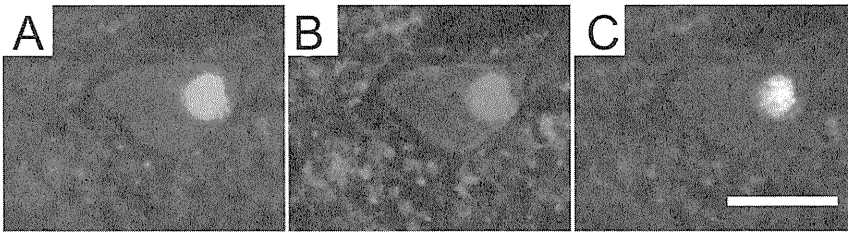
Figure 3. A LBHI double-positive for 14-3-3 and SOD1 in a FALS patient. A LBHI in an anterior horn cell is immunostained for pan 14-3-3 (A, green) and SOD1 (B, red), and the merged image is shown in C (yellow). Bar indicates 50 μm.
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Mutant SOD1 immunoreactivy in the mutant SOD1-Tg, the wild-type SOD1-Tg, and non-Tg wild-type mice

In mutant SOD1-Tg mice, C4F6 immunoreactivity was observed in the remaining anterior horn cells with cytoplasmic inclusions (Figure 4A). Immunoreactivity for C4F6 was restricted to the glial cells that were morphologically consistent with microglia in wild-type SOD1-Tg mice (Figure 4B) and absent in non-Tg wild-type mice (Figure 4C).

14-3-3 immunoreactivity in the mutant SOD1-Tg, the wild-type SOD1-Tg, and non-Tg wild-type mice

Pan 14-3-3, 14-3-3β and 14-3-3γ immunoreactivities were grossly different between the mutant SOD1-Tg and the wild-type SOD1-Tg or non-Tg wild-type mice (Figure 5).
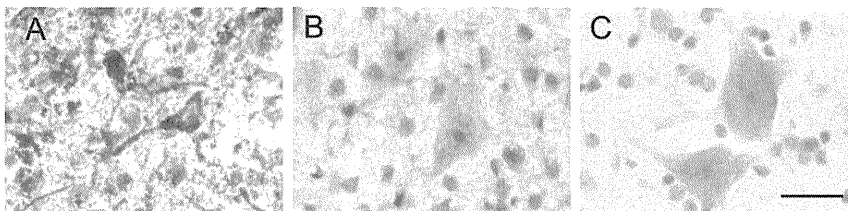
Such 14-3-3 immunoreactivities were strong in most of the remaining anterior horn cells of the mutant SOD1-Tg mice (Figure 5A, D, G), although they were not observed in the wild-type SOD1-Tg (Figure 5B, E, H) or non-Tg wild-type mice (Figure 5C, F, I).

Double immunofluorescent staining of C4F6 and 14-3-3β or 14-3-3γ in SOD1-Tg mice

As described above, the strong immunoreactivity of 14-3-3β and 14-3-3γ were observed in the mutant SOD1-Tg mice but not in the wild-type SOD1-Tg mice. Wherein, the distribution of immunoreactivity for C4F6, 14-3-3β and 14-3-3γ was analyzed.

All the three immunoreactivities were observed in the neuronal somata of the anterior horn cells. Furthermore, double immunofluorescent staining showed that both 14-3-3β and 14-3-3γ were partially co-localized with C4F6 in mutant SOD1-Tg mice (Figure 6).

In negative immunohistochemical controls, some sections were incubated with the primary antibody (0.2 μg/ml) preabsorbed with an excess amount of the antigenic peptides, pan 14-3-3, 14-3-3β and 14-3-3γ (10 μg/ml). No specific immunopositive staining was detected in these control sections.

Other 14-3-3 isoforms in the mutant SOD1-Tg mice and the wild-type SOD1-Tg mice

Immunoreactivitiy for 14-3-3ε, η, θ, σ, and ζ was observed in the neuronal somata and processes in the spinal cord. However, there were no remarkable differences in the distribution or intensity of the immunoreactivities between the mutant SOD1-Tg and wild-type SOD1-Tg mice.

Discussion

In this study, LBHIs in all FALS cases showed intense pan 14-3-3, 14-3-3β and 14-3-3γ immunoreactivities. Furthermore, the double immunofluorescent study showed 14-3-3 proteins were co-localized with SOD1 in LBHIs. Such distribution patterns were quite similar to those of the mutant SOD1-Tg mice. This is the first report that demonstrates a close relationship between 14-3-3 and SOD1 both in patients with FALS and mutant SOD1-Tg mice.

We have previously reported the localization of 14-3-3 proteins in the ubiquitinated intraneuronal inclusions in the anterior horn cells from patients with sporadic ALS [12]. We also already reported 14-3-3 immunoreactivity in the LBHIs in the anterior horn cells from a patient with FALS, with a two-base pair deletion in the SOD1 gene [19]; however, the co-localization of SOD1 and 14-3-3 was not assessed. Therefore the role of 14-3-3 proteins in LBHI formation with a SOD1 mutation has remained unclear. The co-localization of 14-3-3 and SOD1 in the LBHIs in both FALS patients and mutant SOD1-Tg mice suggested that 14-3-3 may play an important role in the formation of SOD1-containing LBHIs. The similar 14-3-3 positivity in the LBHI of sporadic ALS and FALS with SOD1 mutation further suggests that 14-3-3 is involved in the pathogenesis of ALS, irrespective of whether it is sporadic or familial.

Among the various isoforms of 14-3-3 protein, Kaneko and Hachiya proposed the possibility that a distinctive function of 14-3-3ζ might be as a sweeper for misfolded proteins, such as aggregates or inclusion bodies [20]. Santpere et al. suggested that
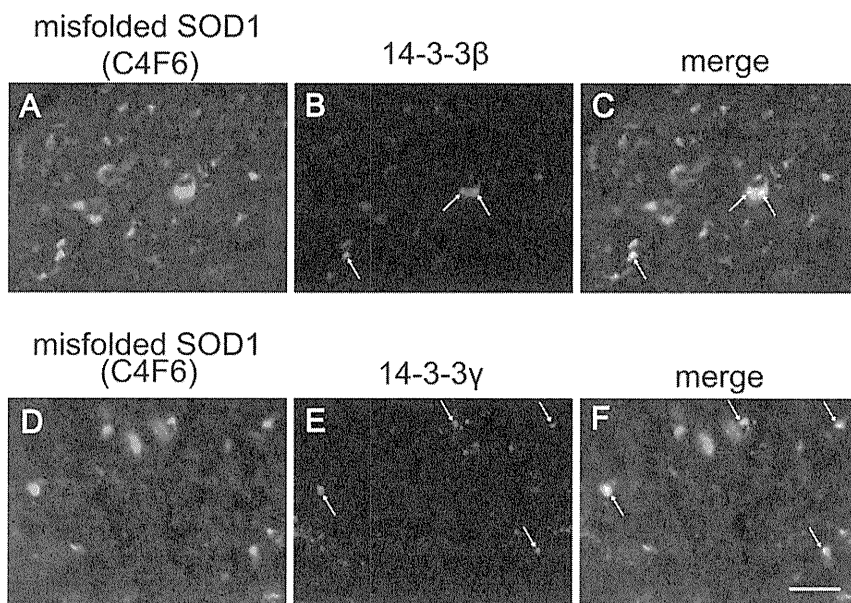

Figure 4. Neuronal inclusions immunopositive for C4F6 in mice. Strong immunoreactivity for C4F6 (A) was observed in the somatodendritic compartment with cytoplasmic inclusions in the mutant SOD1-Tg mice. Immunoreactivity for C4F6 was restricted to glial cells morphologically consistent with microglia in the wild-type SOD1-Tg mice (B) and absent in the non-Tg wild-type mice (C). Bar indicates 50 μm.
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Figure 5. Neuronal inclusions immunopositive for pan 14-3-3, 14-3-3β, and 14-3-3γ in the spinal cord of SOD1-Tg mice. In mutant SOD1-Tg mice, strong immunoreactivity for pan 14-3-3 (A), 14-3-3β (D), and 14-3-3γ (G) were observed in the neuronal cytoplasm or neuronal process of the lumbar anterior horn cells. Such immunoreactivities were not observed in the wild-type SOD1-Tg (B, E, H) or non-Tg wild-type mice (C, F, I). Bar indicates 50 μm.
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the 14-3-3γ and 14-3-3ζ isoforms may be the targets of oxidative damage in Alzheimer's disease [21], and some neurofibrillary tangles were reported to be immunolabeled with 14-3-3β and 14-3-3γ [22]. Similarly, 14-3-3 proteins have been co-localized in Lewy bodies [23] and in glial cytoplasmic inclusions from patients with multiple system atrophy [24]. In our recent study, 14-3-3β and 14-3-3γ were strongly expressed in the neuronal somata and processes of anterior horn cells in the spinal cord of mutant human α-synuclein (A53T)-Tg mice, an animal model of Parkinson's

disease (PD) [25]. Therefore, 14-3-3β and 14-3-3γ may be the key isoforms associated with the formation of α-synuclein- and SOD1-containing inclusions. This raises the possibility that there might be a common mechanism for inclusion formation at least between ALS and PD.

An insufficient function of the molecular chaperones may be directly involved in the loss of motor neurons in ALS [26,27]. Under non-pathological conditions, 14-3-3 proteins play important roles in signal transduction, apoptotic cell death and cell cycle

Figure 6. Neuronal inclusions double-positive for C4F6 and 14-3-3 in the spinal cord of mutant SOD1-Tg mice. Immunofluorescence for C4F6 (A, D, green), 14-3-3β (B, red), and 14-3-3γ (E, red), double immunofluorescence for C4F6 and 14-3-3β (C, merge), and double immunofluorescence for C4F6 and 14-3-3γ (F, merge) are shown in the anterior horn cells. Bar indicates 20 μm.
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control. 14-3-3 proteins inhibit apoptosis by binding to and inactivating pro-apoptotic proteins, including the mitochondrial Bcl-2 family member BAD, apoptosis signal-regulating kinase 1 (ASK1), and the Forkhead transcription factor FKHRL1 [10]. Therefore, the sequestration of 14-3-3 may cause neuronal dysfunction and thus contribute to cell death. Strong immunoreactivity for 14-3-3 in the LBHIs of FALS patients and in the mutant SOD1-Tg mice suggested that 14-3-3 proteins are trapped in the LBHIs, and this deficiency of the 14-3-3 proteins causes motor neuronal death in patient with FALS.

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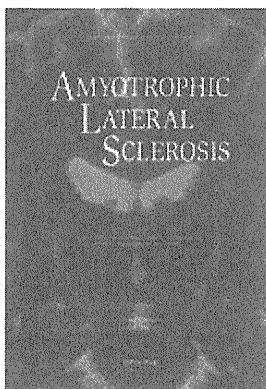
Acknowledgments

Author Contributions

Conceived and designed the experiments: MI HT HI SS RT. Performed the experiments: YO YS YK UM MO. Analyzed the data: YO MI. Contributed reagents/materials/analysis tools: SK HY AH. Wrote the paper: YO MI.

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Protein Disulfide Isomerase immunopositive Inclusions in patients with Amyotrophic lateral Sclerosis

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Protein Disulfide Isomerase immunopositive Inclusions in patients with Amyotrophic lateral Sclerosis

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Running title: PDI-immunopositive inclusions in patients with ALS

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Abstract

Objective: The major pathological hallmarks of amyotrophic lateral sclerosis (ALS) are neuronal cytoplasmic inclusions (NCIs) and swollen neurites. Superoxide dismutase (SOD) 1-immunopositive NCIs are observed in patients with familial ALS (FALS) and TAR DNA-binding protein 43kDa (TDP-43)-immunopositive NCIs are found in patients with sporadic ALS (SALS). Protein disulfide isomerase (PDI) is a member of the thioredoxin superfamily and is believed to accelerate the folding of disulfide-bonded proteins by catalyzing the disulfide interchange reaction, which is the rate-limiting step during protein folding in the luminal space of the endoplasmic reticulum. **Methods:** Post-mortem spinal cord specimens from five patients with SALS and one with FALS (I113T), and five normal controls were utilized in this immunohistochemical study. **Results:** We found PDI-immunopositive swollen neurites and NCIs in the patients with ALS. Furthermore, PDI was co-localized with TDP-43 and SOD1 in NCIs. The accumulation of misfolding proteins may disturb axon transport and make swollen neurites. As the motor neuron is the longest cell in the nervous system, the motor system may selectively be disturbed. **Conclusions:** We assume that PDI is S-nitrosylated in the affected neurons, which inhibits its enzymatic activity and thus allows protein misfolding to occur in ALS.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a major neurodegenerative disease but there is

currently no effective treatment available because the etiology or mechanism of ALS is

still unclear. In general, 90-95% patients with the disease are sporadic ALS (SALS) and

5-10% are familial ALS (FALS) patients (1). In a gene analysis study, approximately

20% of FALS patients had a mutation in Cu/Zn-superoxide dismutase (SOD) 1 (1). But

SOD1 deficiency does not make ALS, because SOD1-knockout mice do not reveal any

obvious phenotype (2). In contrast, transgenic mice expressing human SOD1 containing

a mutation develop a motor neuron disease similar to FALS (3). These results suggested

that the accumulation of SOD1 is toxic for neurons and is linked to the pathogenesis of

ALS. In fact, SOD1 transgenic mice are commonly used as a model for ALS.

The major pathological changes of ALS are neuronal cytoplasmic inclusions

(NCIs) and swollen axons, which are called spheroid. Lewy body-like hyaline

inclusions (LBHIs) in SALS and conglomerate inclusions in FALS are specific NCIs

in ALS and both are a major pathological hallmark of ALS. TAR DNA-binding protein

43kDa (TDP-43) is the major component of LBHIs and is seen in patients with ALS

(1). Recently, a human TDP-43 transgenic mouse has been reported (4). The transgenic

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mice, expressing high levels of human TDP-43, displayed severe neurological

alterations and died prematurely at 5 months of age. In addition, the mice developed

progressive accumulation of inclusions in neurons, but the inclusions were not

immunopositive for TDP-43, implying the involvement of other unknown factors than

TDP-43. TDP-43 is not only the protein to contributing to the pathology of SALS.

Protein disulfide isomerase (PDI) is a member of the thioredoxin superfamily

and is believed to accelerate the folding of disulfide-bonded proteins by catalyzing the

disulfide interchange reaction, which is the rate-limiting step during protein folding in

the luminal space of the endoplasmic reticulum (ER) (5,6). Such exchange reactions can

occur intramolecularly, leading to rearrangement of disulfide bonds in a single protein.

The second major function of PDI is as a chaperone to unfold cholera toxin (7).   This

is independent of its first function in disulfide exchange. NO-induced S-nitrosylation of

PDI inhibits its enzymatic activity, leading to the accumulation of polyubiquitinated

proteins in patients with SALS and ALS model mice (8). Furthermore, overexpression

of PDI decreased mutant SOD1 aggregation, inclusion formation, ER stress induction,

and toxicity, whereas small interfering RNA targeting PDI increased mutant SOD1

inclusion formation, indicating a protective role for PDI against SOD1 misfolding (8).

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