In this study, we identified anti-PDI-antibody immunopositive NCIs in the spinal cord of patients with ALS. Furthermore, we found PDI-immunopositive swollen neurites in the spinal cord of patients with ALS. In addition, TDP-43 and SOD1 were co-localized with PDI in NCIs of patients with ALS. The accumulation of misfolded proteins such as SOD1 and TDP-43 is toxic for motor neurons and PDI may be involved in NCIs.

Material and Methods

Tissue preparation

Postmortem spinal cord specimens from five patients with SALS and one with FALS (I113T), and five normal control were utilized in this study. All patients gave informed consent and this study was approved by the local ethics committee. The diagnosis of the patients was defined by pathological study. Specimens from the spinal cord and brainstem were obtained from the autopsied spinal cord of the normal control and ALS patients. All samples were fixed in 10% neutral formalin at room temperature. Several paraffin-embedded tissue blocks were prepared and cut into 7 μm-thick sections on a microtome. The paraffin-embedded sections were deparaffinized in xylene, followed by

rehydration in ethanol solutions of decreasing concentration as previously described (9,10).

Immunohistochemistry

Immunohistochemical staining was performed as previously described (9,10). Immunohistochemical staining for PDI was performed using polyclonal rabbit anti-PDI antibody that has been described before (11-13). Monoclonal mouse anti-TDP-43 antibody was purchased from Abnova (Taipei, Taiwan) and mouse anti-human SOD1 antibody was purchased from R&D systems, Inc. (Minneapolis, MN, USA). The sections were incubated in a microwave oven for a few minutes after which the sections were incubated in 0.3% hydrogen peroxide in 0.1 M PBS at room temperature for 30 min to block the endogenous peroxidase activity. After washing with PBS containing 0.3% Triton X100 (PBST) the sections were incubated overnight with the primary antibody diluted in PBST at room temperature. A dilution of 1:100 was used for the primary PDI antibody. After incubation with the primary antibody and washing, the sections were incubated with the secondary antibody (diluted 1:100). The sections were incubated with an avidin biotin complex, and were allowed to react with a solution containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.005% hydrogen

peroxide, and 0.6% nickel acetate in 0.05 M Tris/HCl buffer.

Double staining of PDI and human TDP-43 in tissue sections from SALS samples

To confirm the anatomical relationship between PDI and human TDP-43, we performed a double-staining study using mouse anti-TDP-43 antibody and rabbit anti-PDI antibody as described in (9). Briefly, sections of the spinal cords were incubated in medium containing anti-TDP-43 and anti-PDI antibodies (each diluted 1:100) in PBST overnight at room temperature. After washing, the sections were reacted with the secondary antibodies consisting of polyclonal goat anti-rabbit immunoglobulins/FITC (The Jackson Laboratory, Bar Harbor, ME, USA) and polyclonal swine anti-mouse immunoglobulins/TRITC (Cosmo Bio Science, San Diego, CA, USA) for 1 h at room temperature. After rinsing, the slides were mounted Vectashield (Vector Laboratories, Burlingame, CA, USA) and photographed using an Olympus Fr1000D: FV/IX81 (Olympus corporation, Tokyo, Japan) confocal laser scanning microscope. We selected 15 TDP-43-immunopositive NCIs in the immunostained sections of the spinal cord from each of three patients with SALS. We then counted the number of PDI-immunopositive NCIs in the selected TDP-43-immunopositive NCIs from each patient.

Double staining of PDI and SOD1 in FALS samples

Double staining using anti-PDI antibody and anti-SOD1 antibody was performed as described above. We selected 15 SOD1-immunopositive NCIs in the immunostained sections of the spinal cord from the patient with FALS. We then counted the number of PDI-immunopositive NCIs in the selected SOD1-immunopositive NCIs from the patient.

Results

PDI-immunopositive neuronal cells

In the control specimens, many neurons were immunopositive for the anti-PDI antibody. PDI immunoreactivity was typically observed in the neuronal bodies and dendrites, but nuclei were not stained (Figure 1, panels A,B). In addition, some of the oligodendrocytes were also immunopositive using anti-PDI antibody (Figure 1, panel B). Some of astrocytes were PDI-immunopositive, but they were stained weakly. In the tissue sections from patients with SALS and FALS, we detected numerous PDI-immunopositive NCIs (Figure 2, panels A,B). These PDI-immunopositive NCIs were observed in all patients with SALS and FALS. Furthermore, we found

anti-PDI-antibody-immunopositive swollen neurites in SALS and FALS samples (Figure 3, panels A,B). Other immunoreactivity of anti-PDI antibody was not markedly different between the normal and ALS patients.

Double staining of PDI and TDP-43 in SALS samples

Immunohistochemical double staining for PDI and TDP-43 showed that PDI and TDP-43 were co-localized in the NCIs of SALS samples (Figure 4). The number of NCIs labeled by antibodies to PDI was smaller than the number of TDP-43-immunopositive NCIs (Figure 4). A quantitative examination revealed that approximately 93% of the TDP-43-immunopositive NCIs were also PDI-immunoreactive. The proportion of PDI-immunopositive NCIs compared with TDP-43-immunopositive NCIs was not remarkably different among patients.

Double staining of PDI and SOD1 in FALS samples

Immunohistochemical double staining for PDI and SOD1 showed that PDI and SOD1 were co-localized in the NCIs of FALS samples (Figure 5). The number of NCIs labeled by antibodies to PDI was less than the number of SOD1-immunopositive NCIs. A quantitative examination revealed that approximately 73% of the

SOD1-immunopositive NCIs were also PDI-immunoreactive.

Discussion

The accumulation of misfolded, aggregated proteins and Ca^{2+} influx can cause ER stress in neurons (5). ER stress signaling, otherwise known as the unfolded protein response (UPR), is triggered by an increased load of misfolded proteins in the organelle. In SOD1 (L84V) transgenic mice, the aggregation of ER and numerous free ribosomes was observed associated with abnormal inclusion-like structures in spinal cord neurons at the presymptomatic stage (14). Furthermore, the induction of LBHIs *in vitro* by ER stress in neuroblastoma cells was revealed and the inclusions were closely similar to LBHIs in patients with SOD1-linked FALS (14). The accumulation of SOD1 can cause ER stress and that may cause apoptosis of neuronal cells in FALS.

Mutant SOD1 specifically interacted with Derlin-1, a component of ER-associated degradation (ERAD) machinery and triggered ER stress through dysfunction of ERAD (15). Mutant SOD1-induced ER stress activated apoptosis. Perturbation of binding between mutant SOD1 and Derlin-1 by Derlin-1-derived oligopeptide suppressed mutant SOD1-induced ER stress and motor neuron death (15). In addition, Derlin-1 overexpression reduced mutant SOD1-induced cell toxicity and

increased cell viability by suppressing the activation of the ER stress pathway factors (16). Interestingly, exogenous Derlin-1 resulted in a decrease in the amount of mutant SOD1, and a lesser decrease in that of wild-type SOD1 in transfected cells. In addition, reduced SOD1 protein expression was observed in the microsomal fraction of wild-type and mutant SOD1 cells (16). Furthermore, Chromogranins, components of neurosecretory vesicles, interact with mutant forms of SOD1 that are linked to ALS, but not with wild-type SOD1 (17). These results suggest that Derlin-1 and Chromogranins may act as chaperone-like proteins to promote the secretion of SOD1 mutants. In ALS, the mutant SOD1-binding protein could play an important role through the ER stress pathway. There are a lot of ER-related chaperone proteins in neurons. In these proteins, Derlin-1 and Chromogranin bind to mutant SOD1. These two proteins may be the link to co-localization of mutant SOD1 and PDI.

PDI is an ER-specific chaperone and is linked to the accumulation of misfolded proteins in many neurodegenerative diseases (5). In this study, we have shown the localization of PDI in neuronal cells. PDI prevents the neurotoxicity associated with ER stress and protein misfolding, but NO blocks the enzyme's protective effect through the S-nitrosylation of PDI. This inhibition of PDI leads to ER stress, which can induce apoptosis (5). Recently, S-nitrosylation of PDI in patients with ALS was reported (8).

The levels of S-nitrosylated PDI were increased in transgenic mutant SOD1 mouse and human SALS spinal cord tissues. Hence, despite upregulation, PDI is also functionally inactivated in ALS (8). NO-induced S-nitrosylation of PDI inhibits its enzymatic activity, leading to the accumulation of polyubiquitinated proteins in 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). Thus, PDI prevents the neurotoxicity associated with ER stress and misfolding in ALS. In addition, PDI was present in cerebrospinal fluid and was aggregated and widely distributed throughout the motor neurons of patients with SALS (18).

The accumulation of SOD1 is a link to the pathogenesis of FALS (3). Nevertheless, the accumulation of SOD1 is not observed in SALS. The mechanism of SALS may be different from FALS. TDP-43 is the major component of LBHIs and TDP-43 is seen in patients with SALS, but TDP-43 is not the only protein to contribute to the pathology of SALS. Further study is needed to elucidate the mechanism of progressive accumulation of TDP-43 in neurons.

Another pathological hallmark of ALS is swollen neurites. Axonal transport has two components: transport of vesicles and mitochondria by kinesin and related proteins (fast transport) and movement of the major structural components of the neuron, many enzymes, and other cytoplasmic proteins (slow transport). Slow transport can be divided into two components based on the rate of movement and containing the neurofilament proteins tubulin and actin, and containing tubulin, actin, and other cytoplasmic proteins (19). Retardation of slow axonal transport is a very early event in mice expressing the FALS-linked SOD1 (G37R) and SOD1 (G85R) mutations (19). In SOD1 (G85R) mutant mice, this is the earliest known abnormality, arising months before any pathological changes can be detected. Tubulin transport slows more dramatically at earlier stages, whereas the transport of neurofilaments and other cargo yet to be identified is affected at later time points, indicating a worsening of the defect, and presumably the underlying neuronal health and function, with time. This is consistent with the slow accumulation of damage over a long period, ultimately culminating in late onset of disease in both mice and humans. Further support for a disruption in slow axonal transport early in disease comes from the obvious proximal axon swellings in both SOD1 (G37R) and SOD1 (G85R) mice (19). The known neurofilament dependent slowing of axonal transport, combined with the accumulation

of neurofilaments in ALS, suggests that an important aspect of toxicity may arise from damage to transport (19).

In this study, we have revealed anti-PDI-antibody-immunopositive NCIs in the patients with SALS and FALS. Furthermore, PDI was co-localized with TDP-43 and SOD1 in NCIs. We assume that NO inhibited PDI and led to the accumulation of unfolded proteins in ALS. Abnormal TDP-43 and SOD1 or other proteins may be accumulated in NCIs and cause ER stress in ALS. In degenerated motor neurons, ER and other organelles are probably destroyed and injured. As PDI is working in neurons as a chaperone it may bind to TDP-43 or SOD1, and become included in NCIs. But the PDI in the NCIs may be a non-functional protein. We propose the mechanism of action of PDI recruitment to NCIs to be as follows. First, many unfolded proteins can accumulate in both cytosol and ER lumen of the motor neurons of patients with ALS, and these unfolded proteins can accumulate and make a mass of accumulated unfolded proteins. Second, this mass disrupts the organelle compartment, and as a result, many chaperone proteins including the ER-resident protein PDI, are involved in this mass. Since these accumulated proteins expose the hydrophobic surface, it is easy for PDI to interact with these unfolded proteins by hydrophobic interaction. The co-localization of PDI and TDP-43 or SOD1 in NCIs could be linked to the formation of these inclusions.

One of the great puzzles in the study of ALS is why the motor system, and particular subsets of motor neurons, is selectively targeted for toxicity. Because of the accumulation of misfolding proteins, axon transport may be disturbed and make swollen neurites. As the motor neuron is the longest cell in the human nervous system, the motor system may be selectively disordered by the accumulation of misfolding proteins. We observed PDI-immunopositive swollen neurites in the patients with ALS. PDI may leave the injured ER and become to be aggregated in swollen axons due to disturbance of axon transport. In the ALS model mouse, axonal swelling is one of the early events and PDI is accumulated in the swollen axons in human ALS samples. However, further study is needed to find out weather it is a primary event or a late event.

In summary, we found co-localized inclusions of PDI with mutant SOD1 and TDP-43 in patients with ALS. The ER-specific chaperone protein PDI may leave the ER and then accumulate with SOD1 or TDP-43 in the cytosol. Furthermore, we found PDI-immunopositive swollen neurites in patients with SALS and FALS. As neurites are parts of the motor neuron, they are also degenerated. PDI may also accumulate in the swollen neurites due to the disturbance of axon transport. But the function of PDI

may be lost. These results suggest that the increase in the PDI activity may be a promising therapeutic strategy in ALS.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure Legends

Fig. 1

(A) Neurons (arrows) in normal cervical spinal cord were immunopositive for PDI. (B) Oligodendrocytes (arrowhead) and neurites (arrows) in normal cervical spinal cord were also immunostained by the anti-PDI antibody. Scale bars: 20 μm.

Fig. 2

Anti-PDI-antibody-immunopositive NCI (arrow) from a patient with (A) SALS (cervical spinal cord) and (B) FALS (cervical spinal cord). Scale bars: 10 μm.

Fig. 3

(A) Anti-PDI-antibody-immunopositive swollen neurites (arrows) and degenerated neurons (arrowheads) from a patient with FALS (cervical spinal cord). (B) Anti-PDI-antibody-immunoreactive swollen neurites (arrow) from a patient with SALS (cervical spinal cord). Scale bars: 20 μm.

Fig. 4

Double immunostaining of NCIs from a patient with SALS (cervical spinal cord). TDP-43 and PDI are co-localized in NCIs (arrow). Green: anti-PDI antibody immunostaining (A). Red: anti-TDP-43 antibody immunostaining (B). Yellow: merged

immunostaining (C). *Differential interference contrast* (DIC): (D). Scale bar: 20 µm.

Fig. 5

Double immunostaining of NCI from a patient with FALS (cervical spinal cord). SOD1 and PDI are co-localized in NCI (arrow). Green: anti-PDI antibody immunostaining (A). Red: anti-SOD1 antibody immunostaining (B). Yellow: merged immunostaning (C). *Differential interference contrast* (DIC): (D). Scale bar: 20 µm.

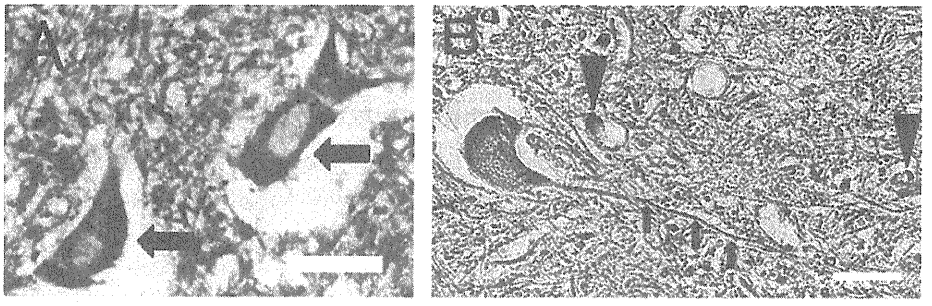

Figure 1
(A) Neurons (arrows) in normal cervical spinal cord were immunopositive for PDI. (B) Oligodendrocytes (arrowhead) and neurite (arrows) in normal cervical spinal cord were also immunostained by the anti-PDI antibody. Scale bars: 20 μm.

34x10mm (600 x 600 DPI)

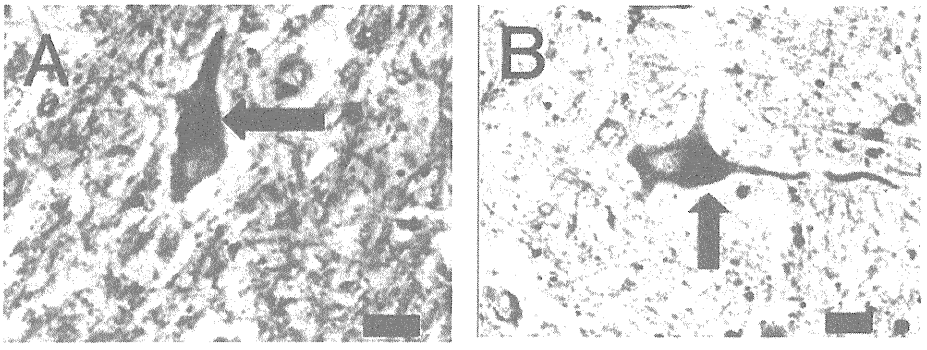

Figure2
Anti-PDI-antibody-immunopositive NCI (arrow) from a patient with (A) SALS (cervical spinal cord) and (B) Anti-PDI-antibody-immunoreactive NCI (arrow) from a patient with FALS (cervical spinal cord). Scale bars: 10 μm.
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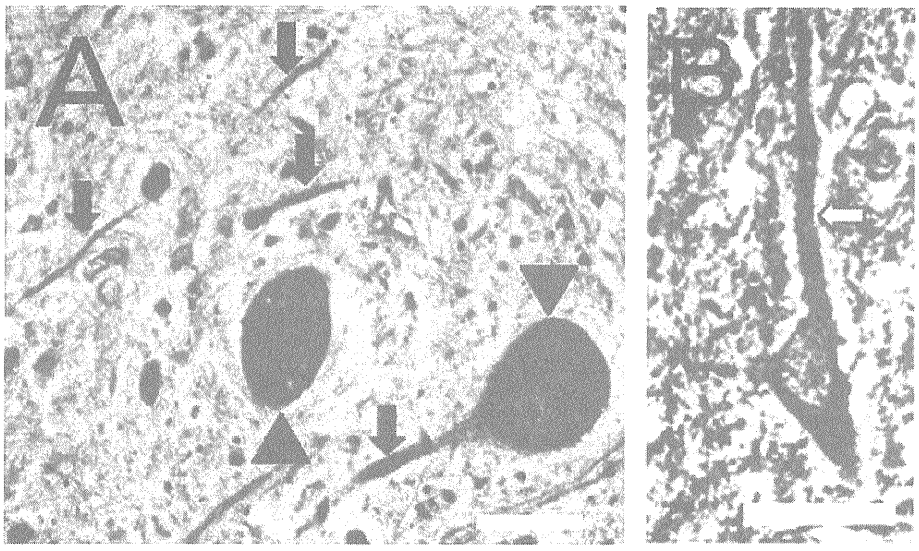

Figure 3
(A) Anti-PDI-antibody-immunopositive swollen neurites (arrows) and degenerated neurons (arrow heads) from a patient with FALS (cervical spinal cord). (B) Anti-PDI-antibody-immunoreactive swollen neurite (arrow) from a patient with SALS (cervical spinal cord). Scale bars: (A, B) 20 µm.

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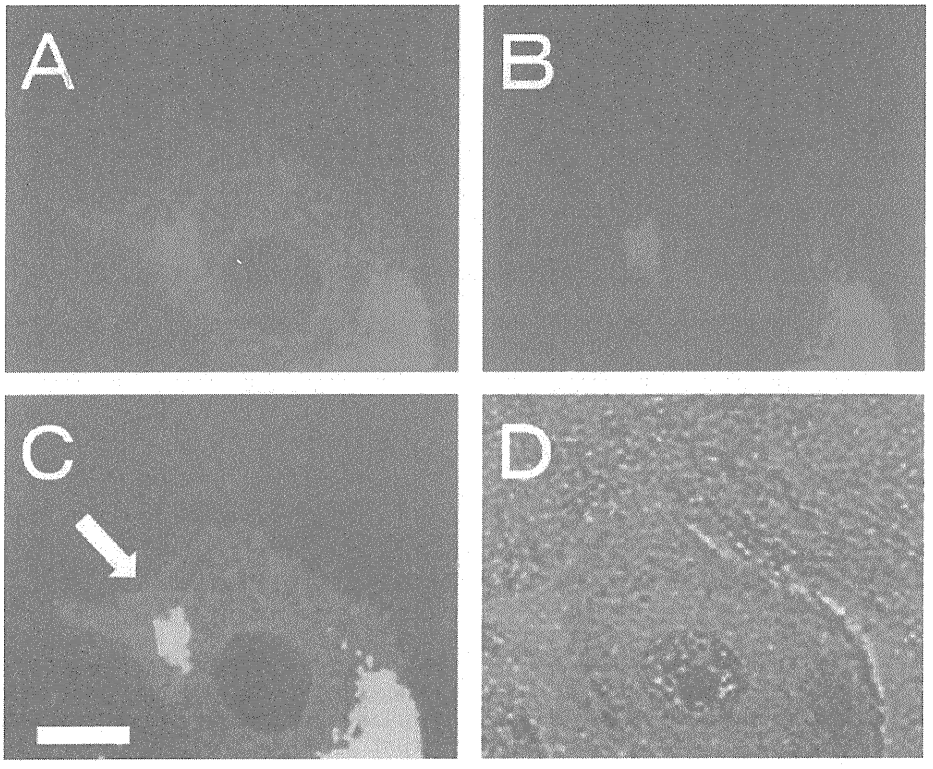

Figure 4
Double immunostaining of NCIs from a patient with SALS (cervical spinal cord). TDP-43 and PDI are co-localized in NCIs (arrow). Green: anti-PDI antibody immunostaining (A). Red: anti-TDP-43 antibody immunostaining (B). Yellow: merged immunostaining (C). Differential interference contrast (DIC): (D). Scale bar: 20 μm.
54x44mm (600 x 600 DPI)