The amount of Dorfin bound with VCP was saturated at even molar ratio *in vitro* (Fig. 3, *B* and *C*). Since VCP exists as a homohexamer (Fig. 3*D*), the *in vivo* observed size of ~600 kDa appears to be too small for the Dorfin-VCP complex if one VCP molecule binds to more than one Dorfin as shown in *in vitro* experiments. However, it is noteworthy that the size of molecules estimated by glycerol density gradient centrifugation analysis used in this study is not accurate and sufficient to discuss the molecular interaction of Dorfin and VCP in the cells. To date, various adaptor proteins, with which VCP forms multiprotein complexes, have been identified, such as Npl4, Ufd1 (18, 20), Ufd2 (34), Ufd3 (35), p47 (36), or SVIP (37). Although our *in vitro* study showed direct physical interaction between Dorfin and VCP, the environment with those adaptor proteins might reflect *in vivo* conditions. This also may explain the apparent discrepancy of the Dorfin-VCP binding fashions between *in vivo* and *in vitro* analyses.

Treatment with a proteasomal inhibitor causes the translocation of endogenous VCP and Dorfin to the aggresome in cultured cells (4, 15). Our results showed that these two proteins indeed colocalized perinuclearly in the aggresome following treatment with a proteasomal inhibitor (Fig. 4). Furthermore, we were able to demonstrate both Dorfin and VCP immunoreactivities in LB-like inclusions in ALS and LBs in PD (Fig. 5). In the majority of LBs, indistinguishable peripheral staining patterns were observed with both anti-Dorfin and anti-VCP antibodies. These results confirmed that both Dorfin and VCP are associated with the formation processes of aggresomes and inclusion bodies through physical interaction.

We showed here that co-expression of VCP^{K524A} resulted in a marked decrease of ubiquitylation activity of Dorfin compared with co-expression of VCP^{WT} or control. On the other hand, VCP^{K524A} failed to decrease autoubiquitylation activity of Parkin. VCP^{K524A} did not change the level of polyubiquitylated protein accumulation in the cell lysate in this study (Fig. 7). Knockdown experiments using the RNA interference technique showed accumulation of polyubiquitylated proteins (38). Combined with the observation that inhibition of VCP did not decrease the general accumulation of polyubiquitylated proteins, our results indicated that the E3 regulation function of VCP may be specific to certain E3 ubiquitin ligases such as Dorfin. VCP is an abundant protein that accounts for more than 1% of protein in the cell cytosol and is known to have various chaperone-like activities (39); therefore, it may function as a scaffold protein on the E3 activity of Dorfin. The localization of Dorfin and VCP in UBIs in various neurodegenerative disorders indicates the involvement of these proteins in the quality control system for abnormal proteins accumulated in the affected neurons in neurodegenerative disorders.

Since the unfolded protein response and ERAD are dynamic responses required for the coordinated disposal of misfolded proteins (40), the ERAD pathway can be critical for the etiology of neuronal cell death caused by various unfolded proteins. VCP is required for multiple aspects of the ERAD system by recognition of polyubiquitylated proteins and translocations to the 26 S proteasome for processive degradation through the VCP-Npl4-Ufd1 complex (18, 41). Our results suggest the involvement of Dorfin in the ERAD system, which is related to the pathogenesis of neurodegenerative disorders, such as PD or Alzheimer's disease. Further study including Dorfin knockout and/or knockdown models should examine the pathophysiology of Dorfin in association with the ERAD pathway or other cellular functions. Such studies should enhance our understanding of the pathogenetic role of Dorfin in neurodegenerative disorders.

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Journal of Neurochemistry, 2004, **89**, 64–72 doi:10.1046/j.1471-4159.2003.02289.x

Dorfin prevents cell death by reducing mitochondrial localizing mutant superoxide dismutase 1 in a neuronal cell model of familial amyotrophic lateral sclerosis

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Abstract

Dorfin is a RING-finger type ubiquitin ligase for mutant superoxide dismutase 1 (SOD1) that enhances its degradation. Mutant SOD1s cause familial amyotrophic lateral sclerosis (FALS) through the gain of unelucidated toxic properties. We previously showed that the accumulation of mutant SOD1 in the mitochondria triggered the release of cytochrome *c*, followed by the activation of the caspase cascade and induction of neuronal cell death. In the present study, therefore, we investigated whether Dorfin can modulate the level of mutant SOD1 in the mitochondria and subsequent caspase activation. We showed that Dorfin significantly reduced the amount of mutant SOD1 in the mitochondria, the release of cytochrome *c* and the activation of the following caspase cascade, thereby preventing eventual neuronal cell death in a neuronal cell model of FALS. These results suggest that reducing the accumulation of mutant SOD1 in the mitochondria may be a new therapeutic strategy for mutant SOD1-associated FALS, and that Dorfin may play a significant role in this.

Keywords: amyotrophic lateral sclerosis, Dorfin, mitochondria, neuronal cell death, superoxide dismutase 1, ubiquitin ligase.

J. Neurochem. (2004) **89**, 64–72.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease caused by selective death of motor neurons. Approximately 10% of ALS cases are familial (FALS). Missense mutations in the gene coding superoxide dismutase 1 (SOD1) are responsible for approximately 20% of FALS cases (Rosen *et al.* 1993; Hirano 1996) through the gain of unelucidated toxic properties (Yim *et al.* 1996).

Many reports have documented that the mitochondria are involved in the pathogenic process in mutant SOD1-associated FALS. Mitochondrial degeneration, including swelling, dilatation and vacuolization, is an early characteristic pathological feature of FALS and FALS transgenic (Tg) mice models with SOD1 mutations (Dal Canto and Gurney 1994; Wong *et al.* 1995; Hirano 1996; Kong and Xu 1998; Jaarsma *et al.* 2000; Higgins *et al.* 2003). Recently, it was demonstrated that SOD1, considered to be a cytosolic enzyme, exists in the mitochondria (Sturtz *et al.* 2001; Okado-Matsumoto and Fridovich 2001; Higgins *et al.* 2002), and that the mitochondrial vacuoles in mutant SOD1 Tg mice were lined with mutant SOD1 (Jaarsma *et al.* 2001; Higgins *et al.* 2003). Many studies have suggested that the programmed cell death (PCD) pathway contributes to motor neuron death in FALS (Durham *et al.* 1997; Martin 1999; Li *et al.* 2000; Pasinelli *et al.* 2000; Guégan *et al.* 2001; Kriz *et al.* 2002; Raoul *et al.* 2002; Zhu *et al.* 2002). Moreover, we previously reported that accumulation of mutant SOD1 in the mitochondria triggered the release of mitochondrial cytochrome *c*, which subsequently activated the caspase cascade and induced neuronal cell death (Takeuchi *et al.* 2002a). Taken together, these results suggest that the accumulation of mutant SOD1 in the mitochondria is critical in the pathogenesis of mutant SOD1-associated FALS.

Received September 23, 2003; revised manuscript received November 17, 2003; accepted November 24, 2003.

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Abbreviations used: ALS, amyotrophic lateral sclerosis; COX, cytochrome *c* oxidase; DMEM, Dulbecco's modified Eagle's medium; E3, ubiquitin ligase; EGFP, enhanced green fluorescent protein; FALS, familial amyotrophic lateral sclerosis; MTS, 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PCD, programmed cell death; PI, propidium iodide; SOD1, superoxide dismutase 1; Tg, transgenic.

Dorfin is the product of a gene that we cloned from the anterior horn tissue of the human spinal cord (Niwa *et al.* 2001); it contains a RING-finger/IBR motif (Niwa *et al.* 2001) at its N-terminus. It was reported that a distinct subclass of RING-finger/in-between RING-fingers (IBR) motif-containing proteins represents a new ubiquitin ligase (E3) family that interacts specifically with distinct ubiquitin-conjugating enzymes (Moynihan *et al.* 1999; Ardley *et al.* 2001). Dorfin is a juxtanuclearly located E3 that ubiquitylates various SOD1 mutants derived from patients with FALS, and enhances the degradation of mutant SOD1 (Niwa *et al.* 2002). Whether Dorfin can modulate the protein level of mutant SOD1 in the mitochondria, and the subsequent activation of the mitochondrial caspase cascade, is an important and interesting question.

Here we show that Dorfin significantly reduced the amount of mutant SOD1 in mitochondria, the release of cytochrome *c* from mitochondria into the cytosol and the subsequent activation of the caspase cascade, thereby preventing the eventual neuronal cell death in a neuronal cell model of FALS. These results suggest that reducing mutant SOD1 in the mitochondria may be a useful strategy for the treatment of mutant SOD1-associated FALS, and that Dorfin might play a significant role in this.

Materials and methods

Plasmids

Non-organelle-oriented plasmids expressing the enhanced green fluorescent protein (EGFP)-tagged human SOD1 (wild type, mutant G93A, and G85R) were described previously (Takeuchi *et al.* 2002a,b). These vectors express SOD1-EGFP fusion proteins ubiquitously in each organelle (Takeuchi *et al.* 2002a). They were designated Cyto-WT, Cyto-G93A and Cyto-G85R respectively. Mitochondria-oriented plasmids expressing EGFP-tagged human SOD1 (wil dtype, mutant G93A and G85R) with mitochondrial localizing signals were generated as described previously (Takeuchi *et al.* 2002a). These vectors express SOD1-EGFP fusion proteins mainly in the mitochondria (Takeuchi *et al.* 2002a). They were designated Mito-WT, Mito-G93A and Mito-G85R respectively. The plasmid pcDNA3.1/HisMax-Dorfin, which expresses Xpress-tagged Dorfin, was also described previously (Niwa *et al.* 2001). As a control, we used pCMV-β vector expressing LacZ (Clontech, Palo Alto, CA, USA). All constructs used here were confirmed by DNA sequence analysis.

Cell culture

Mouse neuroblastoma cell line Neuro2a cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen Corp.) as described previously (Takeuchi *et al.* 2002b). They were cultured on Laboratory-Tec II four-well chamber slides (Nalge Nunc International, Rochester, NY, USA) coated with poly-L-lysine (Sigma, St Louis, MO, USA). Transient expression of SOD1 plasmids (0.1 μg of DNA/well) and pcDNA3.1/His Max-Dorfin or pCMV-β (0.3 μg of DNA/well) in Neuro2a cells (2×10^4 cells/well) was accomplished with LipofectAMINE PLUS reagent (Invitrogen Corp.). After incubation for 3 h with transfection reagents, transfected cells were cultured in differentiation medium (DMEM supplemented with 1% fetal calf serum and 20 μM retinoic acid). To detect Xpress-Dorfin fusion protein, 0.5 μM proteasome inhibitor MG132 (Sigma) was added 16 h before collection, as described previously (Niwa *et al.* 2001).

Cell fractionation

At each time point (0, 24 and 48 h) after transfection, cells were collected and gently homogenized with a Dounce homogenizer in cold buffer [250 mM sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM EDTA and protease inhibitor cocktail (Complete Mini EDTA-free; Roche Diagnostics, Basel, Switzerland)]. Cell fractionation was performed as described previously (Takeuchi *et al.* 2002a). To verify the fractionation, each fraction was subjected to western blotting for cytochrome *c* oxidase (COX) as a mitochondrial marker using anti-COX subunit IV mouse monoclonal antibody (1 : 1000; Molecular Probes, Eugene, OR, USA), and β-actin as a cytosolic marker using anti-β-actin mouse monoclonal antibody (1 : 5000; Sigma).

Western blot analysis

The protein concentration was determined with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and western blotting was done as described previously (Takeuchi *et al.* 2002b). To evaluate the level of mitochondrially localized SOD1-EGFP fusion proteins, 20 μg protein from the mitochondrial fraction was loaded. For analyzing the release of cytochrome *c* from the mitochondria into the cytosol, 20 μg protein from the mitochondrial fraction or the cytosolic fraction was loaded.

To assess the levels of SOD1-EGFP fusion proteins, Xpress-Dorfin fusion proteins and the activation of caspase-9 and caspase-3, cells were collected at each time point (0, 24 and 48 h) after transfection, and lysed in TNES buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 0.1% sodium dodecyl sulfate and protease inhibitor cocktail) as described previously (Takeuchi *et al.* 2002a). For the analysis, 20 μg protein from the total lysate was loaded.

The primary antibodies used were as follows: anti-SOD1 rabbit polyclonal antibody (1 : 10 000; StressGen Biotechnologies, Victoria, BC, Canada), anti-Xpress mouse monoclonal antibody (1 : 5000; Invitrogen Corp.), anti-caspase-3 rabbit polyclonal antibody and anti-caspase-9 rabbit polyclonal antibody (1 : 1000; Cell Signaling, Beverly, MA, USA) and anti-cytochrome *c* mouse monoclonal antibody (1 : 1000; Pharmingen, San Diego, CA, USA). After overnight incubation with primary antibodies at 4°C, each blot was probed with horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG (1 : 5000; Amersham Biosciences, Piscataway, NJ, USA). Blots were then visualized with ECL Plus western blotting detection reagents (Amersham Biosciences). The signal intensity was quantified by densitometry using NIH Image 1.63 software.

Immunocytochemistry

At each time point (0, 24 and 48 h) after transfection, cells were fixed with 4% paraformaldehyde for 30 min on ice and then

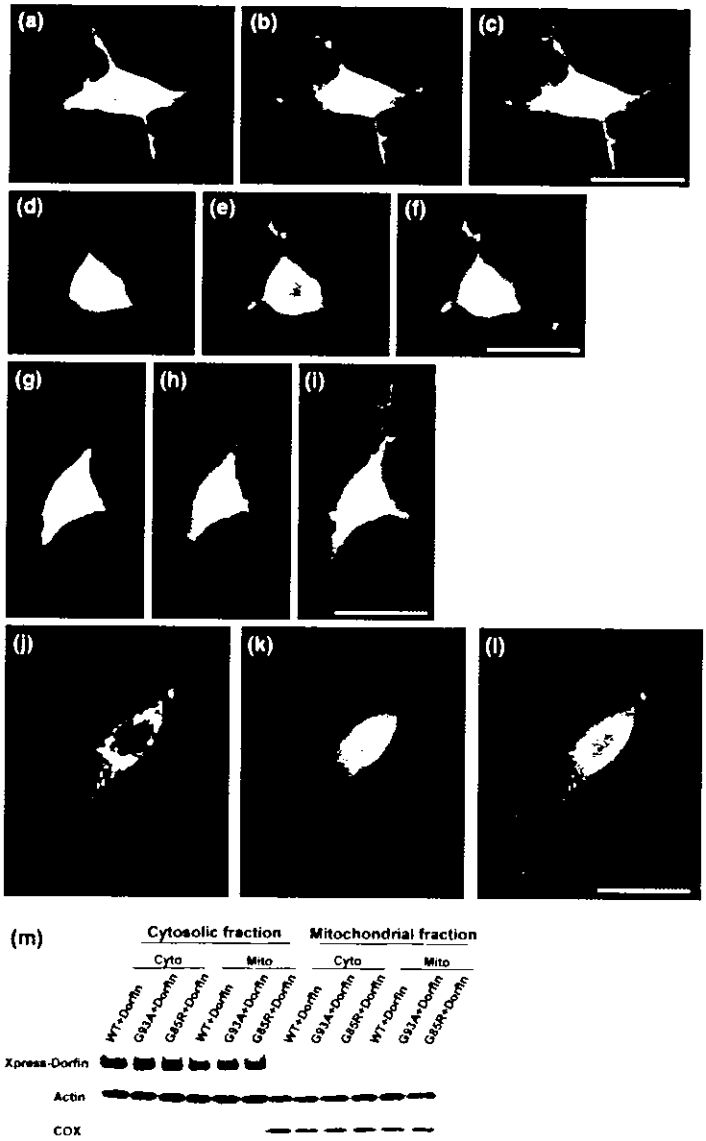

Fig. 1 Subcellular localization of SOD1-EGFP and Xpress-Dorfin in Neuro2a cells. (a–l) Confocal laser scanning microscopic images at 48 h after transfection. (m) Fractionation analysis of Xpress-Dorfin fusion protein. (a–c) Cyto-WT + Xpress-Dorfin, (d–f) Cyto-G93A + Xpress-Dorfin, (g–l) Cyto-G85R + Xpress-Dorfin; (j–l) Mito-G93A + Xpress-Dorfin. SOD1-EGFP fusion proteins (green; a, d and g) and Xpress-Dorfin fusion proteins (red; b, e and h) were observed ubiquitously in the cells with Cyto-SOD1 containing no organelle-oriented signals. SOD1-EGFP fusion proteins and Xpress-Dorfin fusion proteins were co-localized (yellow; c, f and i). In contrast, in the cells with Mito-SOD1, SOD1-EGFP fusion proteins were observed in the mitochondria (green; j) and Xpress-Dorfin fusion proteins (red; k) were observed mainly in the cytoplasm. They were not co-localized in the cells with Mito-SOD1 (l). Cells were counterstained with TO-PRO-3 (blue). Scale bars, 10 μm. Western blots also revealed that Xpress-Dorfin fusion proteins were absent in the mitochondrial fraction (m).

permeabilized with 0.05% Triton X-100 at room temperature for 10 min. They were stained with the anti-Xpress mouse monoclonal antibody (1 : 5000; Invitrogen Corp.) at 4°C overnight. They were subsequently stained with Alexa-568-conjugated secondary antibody (1 : 5000; Molecular Probes) at room temperature for 90 min. Then they were counterstained with 2 μg/mL TO-PRO-3 (Molecular Probes) at room temperature for 10 min, and mounted in Gelvatol. A confocal laser scanning microscope (MRC1024; Bio-Rad Laboratories) was used for the morphological analysis.

Quantitative assessment of mitochondrial impairment and cell death

To assess cell viability through mitochondrial impairment, we used the 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay with CellTiter 96 Aqueous one solution assay (Promega, Madison, WI, USA), as described previously (Takeuchi *et al.* 2002a). At each time point (0, 24 and 48 h) after transfection, MTS assays were carried out in six independent trials. Absorbance at 490 nm was measured in a multiple plate reader as described previously (Ishigaki *et al.* 2002).

Cell death was assessed by the dye exclusion method with propidium iodide (PI; Molecular Probes) as described previously (Takeuchi *et al.* 2002a). At each time point (0, 24 and 48 h) after transfection, cells were incubated with 2 μg/mL PI in DMEM for 15 min at room temperature and mounted in Gelvatol. More than 200 transfected cells in duplicate slides were assessed blindly in three independent trials under a conventional fluorescent microscope. The ratio of dead cells was calculated as a percentage of PI-positive cells among EGFP-positive cells.

Statistical analysis

All results were analyzed by two-way ANOVA with Tukey–Kramer post-hoc test, using Statview software version 5 (SAS Institute Inc., Cary, NC, USA).

Results

Dorfin reduces the levels of total, cytosolic and mitochondrial mutant SOD1

Confocal laser scanning microscopic images revealed that expression of both non-organelle-oriented Cyto-SOD1 plasmid and pcDNA3.1/HisMax-Dorfin was diffusely present in the cells. SOD1-EGFP fusion proteins were co-localized with Xpress-Dorfin fusion proteins (Figs 1a–i), consistent with our previous study (Niwa *et al.* 2002; Takeuchi *et al.* 2002a). In contrast, the expression of mitochondria-oriented Mito-SOD1 plasmid was observed in the mitochondria, as in our previous report (Takeuchi *et al.* 2002a), and was not co-localized with Xpress-Dorfin fusion proteins (Figs 1j–l). Western blots also revealed that Xpress-Dorfin fusion proteins were absent from the mitochondrial fraction (Fig. 1m). At 48 h after transfection, co-expression of Dorfin had reduced the total cell lysate level of SOD1-EGFP fusion proteins expressed by Cyto-G93A or Cyto-G85R by approximately 40%, whereas it did not affect those expressed by Cyto-WT (Fig. 2). In contrast, the amount of SOD1-EGFP fusion proteins expressed by Mito-SOD1 did not show any reduction even with co-expression of Dorfin (Fig. 2). In the cytosolic fraction, co-expression of Dorfin also reduced the level of SOD1-EGFP fusion proteins expressed by Cyto-G93A or Cyto-G85R by approximately 40%, whereas it did not affect those expressed by Cyto-WT (Fig. 3). As we described previously (Takeuchi *et al.* 2002a), cells with Mito-SOD1 showed very small amounts of SOD1-EGFP fusion proteins in the cytosolic fraction (Fig. 3). In the mitochondrial fraction, co-expression of Dorfin also reduced the level of SOD1-EGFP fusion proteins expressed by Cyto-G93A or Cyto-G85R by approximately 50%, whereas it did not affect those expressed by Cyto-WT (Fig. 4). This reduction in mitochondrial SOD1-EGFP was observed from 24 h after transfection, earlier than that of total or cytosolic SOD1-EGFP. In contrast, in the cells with Mito-SOD1, Dorfin did not reduce the amount of mitochondrial SOD1-EGFP fusion proteins (Fig. 4). The above results suggest that the mitochondrial accumulation of mutant SOD1 without organelle-oriented signals might be a result of mutant SOD1 in the cytosol, and we suggest that Dorfin, a cytosolic E3, reduced the accumulation of mutant SOD1 in the mitochondria by enhancing the degradation of mutant SOD1 in the cytosol, not in the mitochondria.

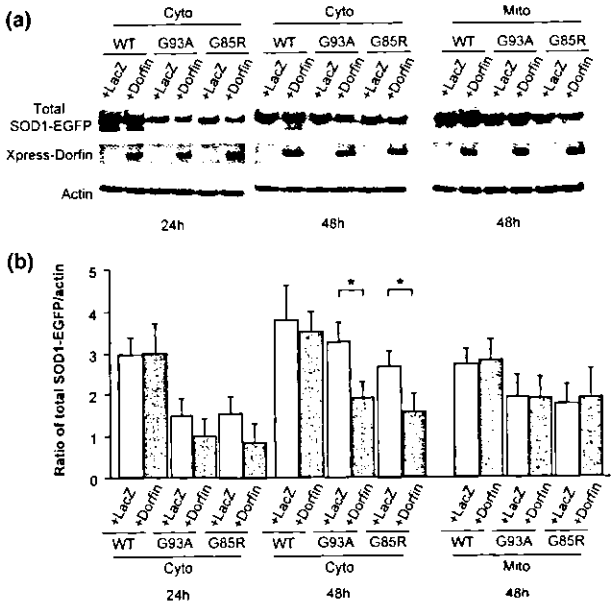

Fig. 2 Level of total SOD1-EGFP fusion protein. (a) Levels of total SOD1-EGFP fusion protein and Xpress-Dorfin fusion protein. (b) Densitometric analysis of total SOD1-EGFP fusion protein expressed as a ratio to actin. Dorfin significantly reduced the level of total SOD1-EGFP fusion protein expressed by Cyto-G93A or Cyto-G85R, whereas it did not reduce that expressed by Mito-SOD1. Values are mean ± SD ($n = 4$). *$p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

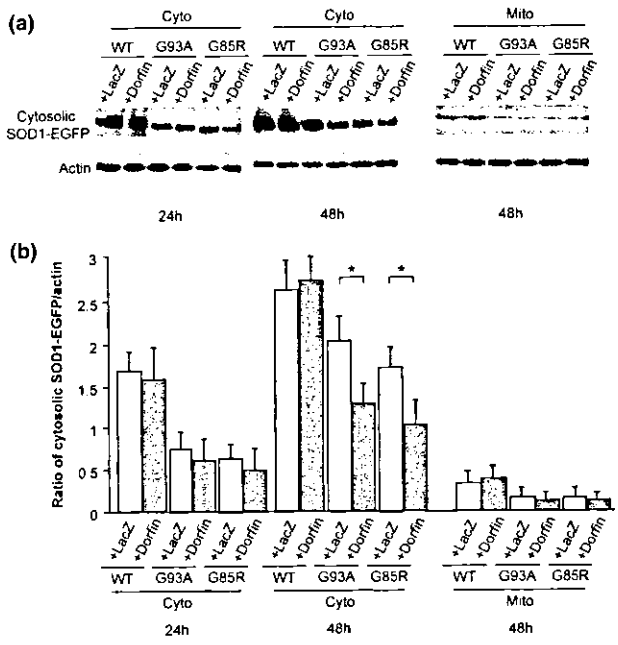

Fig. 3 Level of cytosolic SOD1-EGFP fusion protein. (a) Levels of cytosolic SOD1-EGFP fusion protein. (b) Densitometric analysis of cytosolic SOD1-EGFP fusion protein expressed as a ratio to actin. In the cytosolic fraction, Dorfin significantly reduced the levels of SOD1-EGFP fusion protein expressed by Cyto-G93A or Cyto-G85R. Mito-SOD1 showed very small amounts of SOD1-EGFP fusion proteins in the cytosolic fraction. Values are mean ± SD ($n = 4$). *$p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

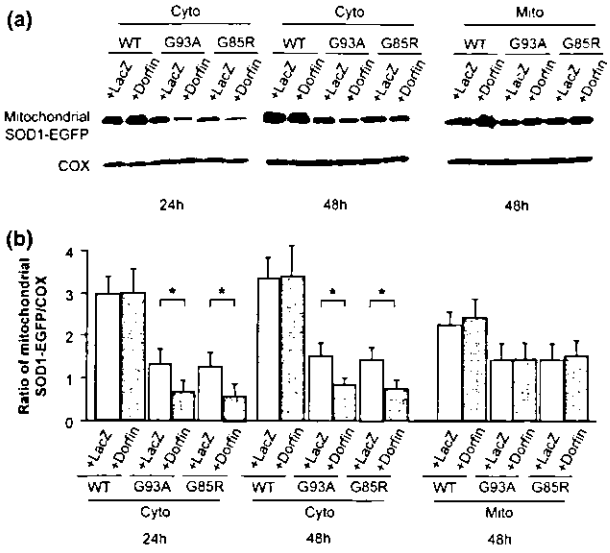

Fig. 4 Level of mitochondrial SOD1-EGFP fusion protein. (a) Levels of mitochondrial SOD1-EGFP fusion protein. (b) Densitometric analysis of mitochondrial SOD1-EGFP fusion protein expressed as a ratio to COX. In the mitochondrial fraction, Dorfin significantly reduced the level of SOD1-EGFP fusion protein expressed by Cyto-G93A or Cyto-G85R, whereas it did not reduce that expressed by Mito-SOD1. Values are mean ± SD ($n = 4$). $^*p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

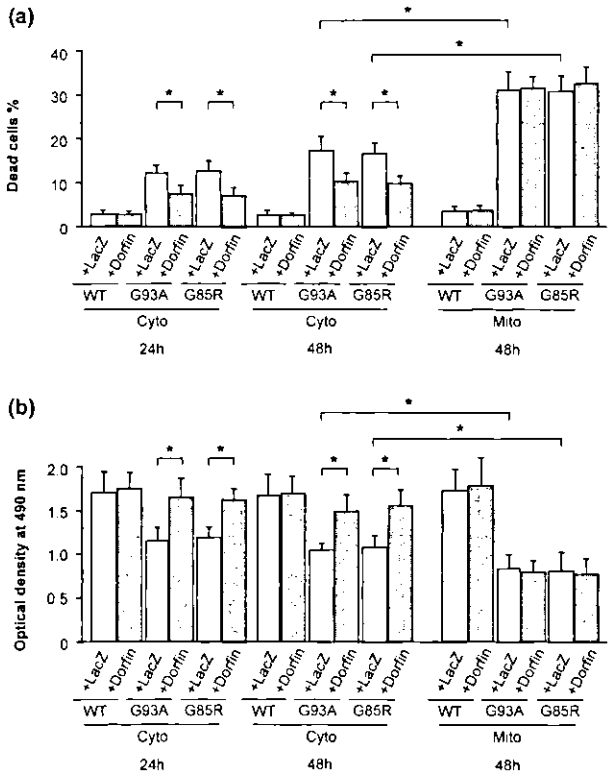

Fig. 5 (a) Frequency of dead cells and (b) mitochondrial impairment analyzed by MTS assay. The cells with Mito-G93A and Mito-G85R exhibited a significantly higher level of cell death and mitochondrial impairment than those with Cyto-G93A and Cyto-G85R. Dorfin significantly decreased cell death and mitochondrial impairment induced by Cyto-G93A and Cyto-G85R, whereas it did not affect those induced by Mito-SOD1. Values are mean ± SD ($n = 6$). $^*p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

Dorfin protects neuronal cells from mutant SOD1-mediated neurotoxicity by reducing mitochondrial mutant SOD1

As we demonstrated previously (Takeuchi *et al.* 2002a), the cells with Cyto-G93A and Cyto-G85R underwent cell death (Fig. 5a) and mitochondrial impairment (Fig. 5b), whereas those with Cyto-WT did not. The cells with Mito-G93A and Mito-G85R exhibited significantly more cell death and mitochondrial impairment than those with Cyto-G93A and Cyto-G85R, whereas those with Mito-WT did not (Fig. 5). Co-expression of Dorfin significantly ameliorated cell death and mitochondrial impairment induced by Cyto-G93A and Cyto-G85R (Fig. 5), as in our previous report (Niwa *et al.* 2002). In contrast, Dorfin did not affect cell death and mitochondrial impairment induced by Mito-SOD1 (Fig. 5), whose protein level Dorfin did not reduce. These findings suggest that Dorfin ameliorates mutant SOD1-mediated neurotoxicity by reducing the accumulation of mutant SOD1 in the mitochondria.

Dorfin reduces mitochondrial cytochrome *c* release and sequential activation of caspase-9 and caspase-3

We next assessed whether Dorfin reduced the mitochondrial death signal associated with the mutant SOD1-mediated cytotoxicity. Western blots revealed that Cyto-G93A and Cyto-G85R induced a gradual increase in the cytochrome *c* released from the mitochondria into the cytosol, whereas Cyto-WT did not (Fig. 6). The cells with Mito-G93A and Mito-G85R also exhibited a higher level of cytochrome *c* release than those with Cyto-G93A and Cyto-G85R, whereas those with Mito-WT did not (Fig. 6). Co-expression of Dorfin significantly reduced the release of cytochrome *c* from the mitochondria into the cytosol induced by Cyto-G93A and Cyto-G85R (Fig. 6). In the cells with Mito-G93A and Mito-G85R, however, Dorfin did not reduce the cytochrome *c* release from the mitochondria into the cytosol (Fig. 6).

Next, we examined whether Dorfin affected the downstream signal cascade of the activation of caspase-9 and caspase-3 following the release of mitochondrial cytochrome *c*. As we demonstrated previously (Takeuchi *et al.* 2002a), western blots revealed that Cyto-G93A and Cyto-G85R induced gradual activation of caspase-9 and caspase-3, whereas Cyto-WT did not (Figs 7 and 8). The cells with Mito-G93A and Mito-G85R exhibited a higher level of activation of caspase-9 and caspase-3 than those with Cyto-G93A and Cyto-G85R, whereas those with Mito-WT did not (Figs 7 and 8). Co-expression of Dorfin significantly reduced

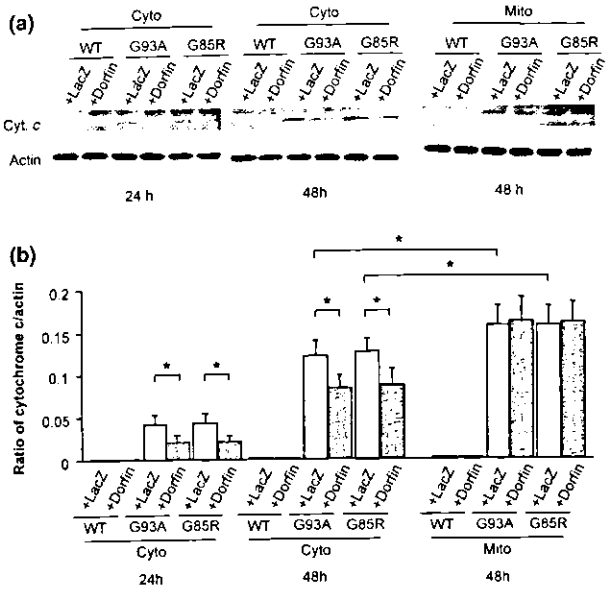

Fig. 6 Western blot analysis of cytochrome *c* release. (a) Time course of mitochondrial cytochrome *c* release into the cytosol. (b) Densitometric analysis of cytochrome *c* release expressed as a ratio to COX. The cells with Mito-G93A and Mito-G85R exhibited significantly more cytochrome *c* release than those with Cyto-G93A and Cyto-G85R. Dorfin significantly reduced the amount of mitochondrial cytochrome *c* released into the cytosol induced by Cyto-G93A and Cyto-G85R, whereas it did not affect that induced by Mito-SOD1. Values are mean ± SD ($n = 4$). $^*p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

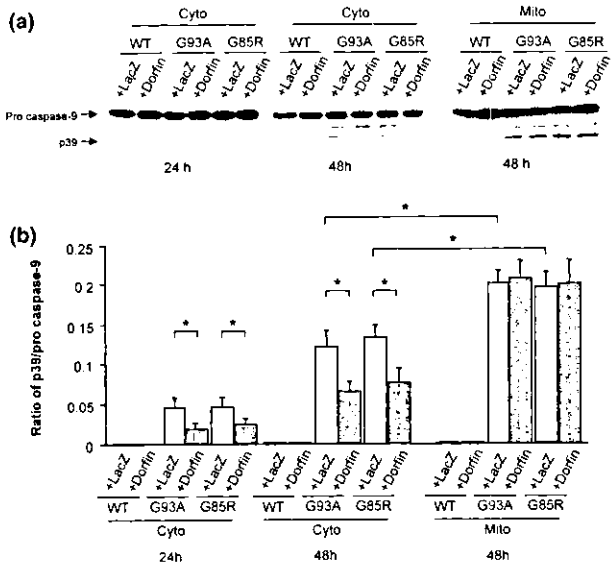

Fig. 7 Western blot analysis of caspase-9 activation. (a) Time course of the activation of caspase-9. (b) Densitometric analysis of caspase-9 activation. The cells with Mito-G93A and Mito-G85R exhibited significantly more activation of caspase-9 than those with Cyto-G93A and Cyto-G85R. Dorfin significantly reduced the activation of caspase-9 induced by Cyto-G93A and Cyto-G85R, whereas it did not reduce that induced by Mito-SOD1. Values are mean ± SD ($n = 4$). $^*p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

the activation of caspase-9 and caspase-3 induced by Cyto-G93A and Cyto-G85R (Figs 7 and 8). However, Dorfin did not reduce the activation of caspase-9 and caspase-3 induced by Mito-G93A and Mito-G85R (Figs 7 and 8), as it did not reduce the release of cytochrome *c* induced by Mito-G93A and Mito-G85R (Fig. 6). These findings combined with the aforementioned observations suggest that the reduction in the amount of mitochondrial mutant SOD1 due to Dorfin results in attenuated activation of the mitochondrial PCD pathway and prevents eventual cell death.

Discussion

In the present study, we first demonstrated that Dorfin, an E3 for mutant SOD1s, attenuated the activation of the mitochondrial PCD pathway and prevented eventual cell death in a neuronal cell model of FALS by reducing the amount of mutant SOD1 in the mitochondria. Dorfin reduced the levels of both cytosolic and mitochondrial mutant SOD1-EGFP fusion proteins that were expressed by Cyto-G93A and Cyto-G85R without organelle-oriented signals, whereas Dorfin did not affect the level of mutant SOD1-EGFP fusion protein that was expressed by Mito-G93A and Mito-G85R with mitochondrial localizing signals. The reduction in the level of

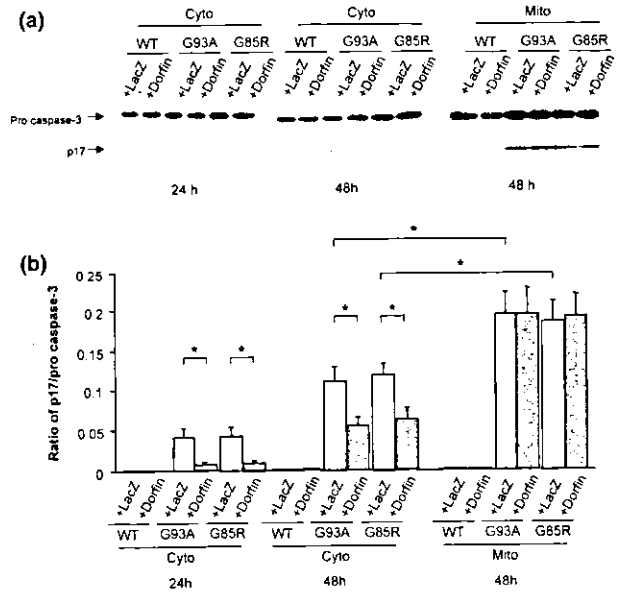

Fig. 8 Western blot analysis of caspase-3 activation. (a) Time course of activation of caspase-3. (b) Densitometric analysis of caspase-3 activation. The cells with Mito-G93A and Mito-G85R exhibited significantly more activation of caspase-3 than those with Cyto-G93A and Cyto-G85R. Dorfin significantly reduced the activation of caspase-3 induced by Cyto-G93A and Cyto-G85R, whereas it did not reduce that induced by Mito-SOD1. Values are mean ± SD ($n = 4$). $^*p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

mitochondrial SOD1-EGFP was observed earlier than that of total or cytosolic SOD1-EGFP. Moreover, Dorfin was present in the cytosol, not in the mitochondria. These findings indicated that the mitochondrial mutant SOD1 without organelle-oriented signals (Cyto-G93A and Cyto-G85R) might be translocated from the cytosol, and we suggest that Dorfin reduces the mitochondrial accumulation of mutant SOD1 by enhancing the degradation of mutant SOD1 in the cytosol through the ubiquitin–proteasomal pathway, thereby reducing the uptake of mutant SOD1 into the mitochondria.

Many reports have documented mitochondrial involvement in ALS and FALS. Mitochondrial degeneration with vacuolization or membrane disintegration in motor neurons is one of the earliest pathological findings in FALS Tg mice (Dal Canto and Gurney 1994; Wong *et al.* 1995; Hirano 1996; Kong and Xu 1998; Jaarsma *et al.* 2000; Higgins *et al.* 2003). Moreover, mitochondrial dysfunction such as altered calcium homeostasis (Carrì *et al.* 1997; Menzies *et al.* 2002b), decreased respiratory chain complex activity (Mattiazzi *et al.* 2002; Menzies *et al.* 2002a), alteration of mitochondria-related gene expression (Yoshihara *et al.* 2002) and an increase in reactive oxygen species (Beretta *et al.* 2003) have been reported in *in vitro* and *in vivo* models of FALS. Several studies have documented that SOD1, which has been considered a cytosolic enzyme, also exists in the mitochondrial intermembrane space (Okado-Matsumoto and Fridovich 2001; Sturtz *et al.* 2001; Higgins *et al.* 2002) and that the mitochondrial vacuoles are lined with mutant SOD1 in a FALS Tg mice model (Jaarsma *et al.* 2001; Higgins *et al.* 2003). Although the mitochondria-oriented vector we used here is designed to localize proteins to the mitochondrial matrix, we predict that SOD1-EGFP also exists in the mitochondrial intermembrane space through the process of its uptake into the mitochondrial matrix in our model, although were not able to confirm this. Recent studies also revealed that SOD1 in the mitochondria originates from the uptake of SOD1 in the cytosol (Sturtz *et al.* 2001; Okado-Matsumoto and Fridovich 2002; Field *et al.* 2003). At least our result provided enough evidence that Dorfin interacts with mutant SOD1 in the cytosol, not in the mitochondria. Thus we suggest that Dorfin indirectly reduces the mitochondrial accumulation of mutant SOD1 by reducing the uptake of mutant SOD1 into the mitochondria.

Previous studies demonstrated that the mitochondrial PCD pathway, cytochrome *c* release and subsequent caspase activation, might contribute to the motor neuron cell death in FALS (Durham *et al.* 1997; Martin 1999; Li *et al.* 2000; Pasinelli *et al.* 2000; Guégan *et al.* 2001; Kriz *et al.* 2002; Zhu *et al.* 2002). Thus, inhibiting the activation of the mitochondrial PCD pathway is potentially useful in the treatment of FALS. Methods for this include inhibition of cytochrome *c* release by minocycline (Zhu *et al.* 2002; Kriz *et al.* 2002), co-expression of bcl-2 (Lee *et al.* 2001) or X-chromosome-linked inhibitor of apoptosis protein (Ishigaki *et al.* 2002), and treatment with a broad caspase inhibitor zVAD-fmk (Pasinelli *et al.* 2000; Takeuchi *et al.* 2002a) or a caspase-9 specific inhibitor zLEHD-fmk (Takeuchi *et al.* 2002a). In this study, we demonstrated that Dorfin reduces the amount of mitochondrial mutant SOD1, attenuates the activation of the mitochondrial PCD pathway and prevents eventual neuronal cell death. It is therefore possible that reducing the amount of mutant SOD1 in the mitochondria may be adopted as a new therapeutic strategy for mutant SOD1-associated FALS.

Recent studies have suggested that some E3s, including Dorfin, act in a quality-control system to degrade cytosolic or transmembranous unfolded abnormal proteins (Moynihan *et al.* 1999; Fang *et al.* 2001; Meacham *et al.* 2001; Murata *et al.* 2001; Yoshida *et al.* 2002). The mitochondria also have a quality-control system that depends on mitochondria-specific molecular chaperones and ATPases associated with diverse cellular activities (AAA) proteases such as chaperonin 60 (Gottesman *et al.* 1997), mitochondrial heat-shock protein 70 (Savel'ev *et al.* 1998), and homologs of Lon, Yme1p, ClpP and ClpX (Wang *et al.* 1993; Suzuki *et al.* 1997; Langer 2000; Shah *et al.* 2000; Kang *et al.* 2002; Röttgers *et al.* 2003). A recent study documented that the accumulation of unfolded abnormal proteins in the mitochondria itself up-regulated the nuclear gene expression encoding mitochondrial-specific molecular chaperones (Zhao *et al.* 2002). Even though the mitochondria are able to dispose of abnormal proteins, they appear to have limited capacity to do this. They also seem to release death signals when abnormal proteins overflow their disposing capacity. Combination therapy such as Dorfin and mitochondria-specific molecular chaperones or AAA proteases thus seems more effective. Further investigations are needed to develop this therapeutic avenue.

There remains the problem of how the mutant SOD1 induces the mitochondrial PCD pathway. One of our previous studies revealed that bcl-2 family pro-apoptotic proteins, such as Bax, Bak, Bid, Bad and Bim, and other mitochondrial death signals such as apoptosis-inducing factor (AIF) and second mitochondria-derived activator of caspase (Smac) were not involved in the neuronal cell death in our model (Takeuchi *et al.* 2002a). Other studies have reported that translocation of Bax and cleavage of Bid were associated with neuronal cell death in the FALS Tg mouse model (Guégan *et al.* 2001; 2002), but there is a possibility that the surrounding environment of motor neurons such as astrocytes, microglia or dying neurons might have been affected in these models. Moreover, we have indicated that a non-apoptotic form of PCD might contribute to neuronal cell death through the mitochondrial PCD pathway in our model (Takeuchi *et al.* 2002a). Another report also mentioned that a non-apoptotic type of PCD acting through the mitochondrial PCD pathway might underlie mutant SOD1-related neurotoxicity (Guégan and Przedborski 2003). Further *in vivo*

investigations are needed to shed light on the mechanism of mutant SOD1-mediated neuronal cell death.

In this study we demonstrated that Dorfin, an E3 for mutant SOD1s, significantly reduced the level of mutant SOD1 in the mitochondria, attenuated the subsequent activation of the mitochondrial PCD pathway and prevented eventual neuronal cell death in a neuronal cell model of FALS. Reducing the accumulation of mutant SOD1 in the mitochondria may have an important place in the therapeutic strategy for mutant SOD1-associated FALS, and Dorfin may play a key role in this.

Acknowledgements

We are grateful to Dr Keiji Tanaka (Department of Molecular Oncology, The Tokyo Metropolitan Institute of Medical Science) for his helpful comments. This work was supported by grants from the Ministry of Health, Labor and Welfare of Japan, and a Center of Excellence grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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筋萎縮性側索硬化症

神経栄養因子HGFの髄腔内投与による筋萎縮性側索硬化症治療法の開発

青木正志*　永井真貴子*
石垣あや*　糸山泰人**

要　旨

筋萎縮性側索硬化症（ALS）に対する治療法の開発のために，我々はトランスジェニックラットによるALSモデルの開発に成功した．このラットは従来のマウスに比較して約20倍の大きさを持ち，脊髄や脊髄腔に対するアプローチが容易である．肝細胞増殖因子（HGF）はその強力な運動ニューロン栄養作用が注目されているが，本稿ではこのALSラットに対するHGFの脊髄腔内への持続投与による治療法の開発を紹介する．

はじめに

筋萎縮性側索硬化症（ALS）は，上位および下位運動ニューロンを選択的かつ系統的に障害し，呼吸筋を含む全身の筋萎縮を来す進行性疾患である．加えて現在までに有効な治療薬や治療法がほとんどないため，ALSは神経疾患の中で最も過酷な疾患とされ，早期に病因の解明と有効な治療法の確立が求められている．遺伝学的解析法の進歩により，1993年に家族性ALSにおいてその一部の原因遺伝子がCu/Znスーパーオキシドジスムターゼ（Cu/Zn SOD）であることが明らかになり[1-3]，さらにはこのCu/Zn SOD遺伝子の突然変異をマウスに導入することにより，ヒトALSの病態を非常によく再現することに成功した[4-7]．ALSに対する治療法の開発には，① 臨床型および病理像を忠実に再現した動物モデルの作製とその病態機序の解明，および ② その動物モデルに対する治療法の開発の2つのステップが重要である．我々は動物モデルにおける脊髄や脊髄腔に対する治療的なアプローチを可能とするために，世界に先駆けて変異Cu/Zn SOD導入トランスジェニックラットによるALSモデルの作製に成功した．さらには，このラットによるALSモデルを用いて，神経栄養因子の脊髄腔内への持続投与による新しい治療法の開発を行っているので紹介したい．

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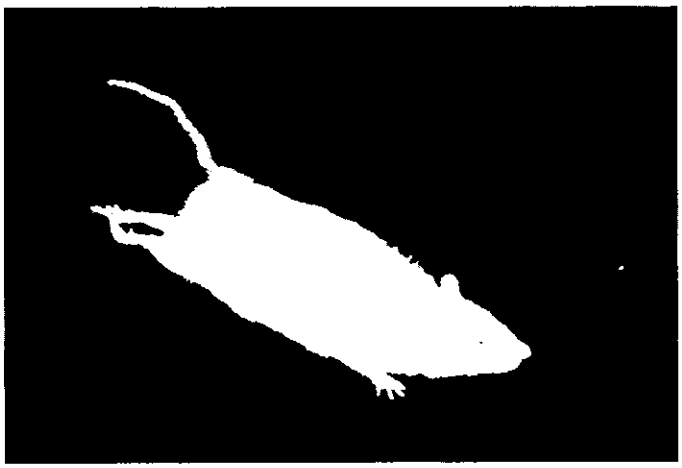
キーワード：遺伝子変異，肝細胞増殖因子，筋萎縮性側索硬化症，トランスジェニックラット，神経栄養因子

図1　両後肢に痙性麻痺の出現が認められたトランスジェニックラット



尾のトーヌスも亢進している.

トランスジェニックラットによる新しい筋萎縮性側索硬化症（ALS）モデル

Cu/Zn SOD 遺伝子変異による家族性 ALS の発症メカニズムはまだ十分には解明されていないが，変異による SOD 活性の低下が直接の原因ではなく，変異 Cu/Zn SOD が新たに獲得した“gain of toxic function”によるものと考えられている[8]．その最大の根拠は，変異 Cu/Zn SOD を導入したトランスジェニックマウスが，ヒト ALS に極めてよく似た表現型と病理所見を示す一方で[4~7]，Cu/Zn SOD 遺伝子をノックアウトしても ALS 症状は示さないことである[9]．これまでに数種類のヒト変異 Cu/Zn SOD 遺伝子を導入したマウスが報告され[4~7]，その一部は米国 Jackson Laboratory により世界中に供給されている．しかしながらマウスによる動物モデルは，特に病態の主座である脊髄の解析には，その個体の大きさによる研究上のさまざまな制約があった．さらには，脊髄の運動ニューロンに対して効率良くしかも副作用を回避できる薬物の投与ルートとして髄腔内投与が注目されており，実際に米国では ALS 患者への持続注入ポンプを用いた神経栄養因子の髄腔内投与が試みられている．日本でも岡山大学神経内科において IGF-I の髄腔内投与による臨床試験が進行中である[10]．そこで東北大学の永井真貴子（現 米国コロンビア大学へ留学中）らは，動物モデルにおける脊髄や脊髄腔に対する治療的なアプローチを可能にするために，世界に先駆けて変異 Cu/Zn SOD 導入トランスジェニックラットによる ALS モデルの開発を行った（図1）[7,11]．

東北大学では，ヒト家族性 ALS 家系において非常に緩徐な臨床経過が報告されている H46R 変異[1,12] および，経過が一般的でトランスジェニックマウスが世界的に供給されている G93A 変異[9] を持つ Cu/Zn SOD 遺伝子をそれぞれ SD ラット受精卵にマイクロインジェクションすることにより，トランスジェニックラットの作製を行った[7,11]．H46R 変異および G93A 変異を持つトランスジェニックラットともに，導入された変異ヒト Cu/Zn SOD タンパク質が多く発現した系統（H46R-4 および G93A-39）において，運動ニューロン病の症状の発現が認められてい

表1　トランスジェニックラットにおける発症と経過（発症から死亡まで）の関係

	発症（日）	経過（日）
H46R-4	144.7±6.4	24.2±2.9
G93A-39	118.6±14.1	8.3±0.7

る（表1）．病理所見では，脊髄前角の運動ニューロンに選択的な変性・消失が見られ，ヒト ALS 患者に特徴的に認められる Lewy 小体様封入体に類似した封入体が認められている[13]．発症は，2つの変異を導入したトランスジェニックラットとも後肢の筋力低下で始まり，対麻痺，四肢麻痺へと進行し死に至った（図1）．H46R-4 は 144.7 日で発症し，24.2 日の経過で死亡した．G93A-39 は 118.6 日で発症し，8.3 日の経過で死亡した．変異 Cu/Zn SOD タンパク質の発現量は G93A-39 のほうが H46R-4 に比較して少ないにもかかわらず，G93A-39 はより早期に発症し，かつ非常に急速な進行を示している（表1）．このことは，各点突然変異によるヒト ALS の罹病期間の違いもよく再現している[7][11]．

今回作製されたトランスジェニックラットでは，従来のマウスに比較して約 20 倍の大きさを持つために，脳脊髄液（髄液）の採取および解析ならびに薬剤や遺伝子治療用のベクターの髄腔内投与が極めて容易である．また電気生理的に運動単位推定（motor unit number estimate：MUNE）も施行可能であり，治療法の評価に使用できる．将来的な遺伝子治療や外来の神経幹細胞の髄腔内投与や脊髄への直接移植による cell replacement therapy[14][15] も含めた新しい治療法開発のために，非常に有用なモデルとなることが期待される．

肝細胞増殖因子（HGF）を用いた ALS の治療

1．新しい運動神経栄養因子としての HGF

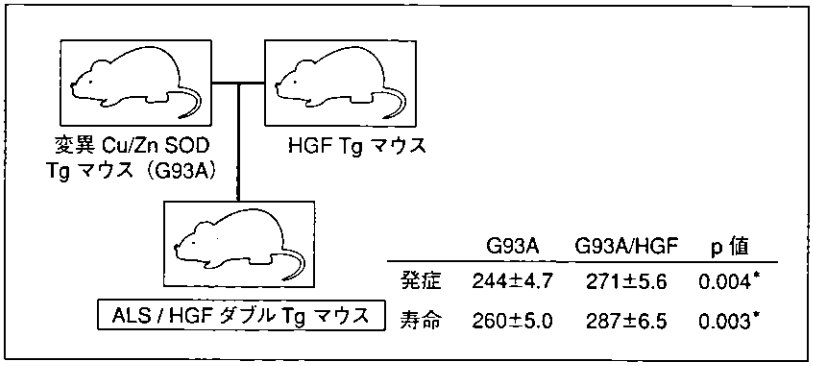
肝細胞増殖因子（HGF）は，我が国の中村敏一らによってクローニングされた新しい増殖因子である．HGF は4つのクリングル構造を持つ α 鎖とセリンプロテアーゼ様構造を持つ β 鎖からなるヘテロ2量体で，1本鎖の不活性型 pro HGF として産生され，その後プロセシングを受けて2本鎖活性型 HGF となる．活性型 HGF はチロシンキナーゼ受容体（c-Met）に結合することにより細胞内シグナル伝達を行う[16][17]．HGF は当初，培養肝細胞の増殖活性を指標に同定されたが，その後の研究の結果，HGF は肝細胞に限らず種々の細胞に対して増殖促進活性を示すのみならず，分化，遊走，形態形成誘導，抗アポトーシス，血管新生などの多様な生物活性を示すことが明らかになった．実際に HGF は閉塞性動脈硬化症，劇症肝炎，虚血性心疾患への臨床応用が進んでおり，大阪大学医学部では閉塞性動脈硬化症の患者への遺伝子治療が HGF 遺伝子プラスミド投与の形で開始され，その効果が確認されている．

HGF は海馬，大脳皮質，運動，感覚，小脳顆粒細胞などの神経細胞に対しても新しい神経栄養因子として作用することが明らかになったが，中でも HGF の培養運動ニューロンに対する神経生存促進活性は非常に強力である．その活性は既知の運動神経栄養因子の中でも強力とされ，ALS に対する臨床試験が行われたグリア細胞由来神経栄養因子（GDNF）や脳由来神経栄養因子（BDNF）に全くひけをとらないとされる[16][17]．

2．HGF 遺伝子導入による ALS トランスジェニックマウスモデルの寿命の延長

そこで，大阪大学分子組織再生分野の船越

図2 神経特異的HGF発現トランスジェニック（Tg）マウスおよびG93A変異導入トランスジェニックマウス（ALSマウス）の交配によるHGF/ALSダブルトランスジェニックマウスの作製



	G93A	G93A/HGF	p 値
発症	244±4.7	271±5.6	0.004*
寿命	260±5.0	287±6.5	0.003*

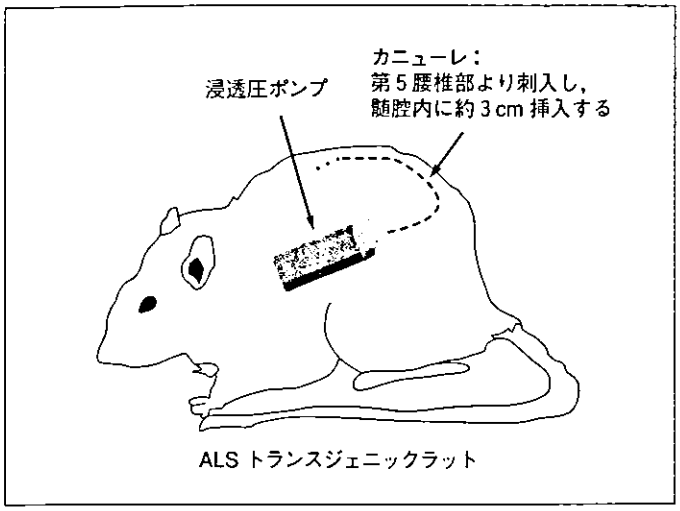
HGF/ALSダブルトランスジェニックマウスにおいては，ALSマウスに比較して有意に発症および寿命の延長が認められている．* t-testによる有意差検定
略語：巻末の「今月の略語」参照

洋らは，ニューロン特異的エノラーゼプロモーターを用いてHGF遺伝子を神経特異的に発現するトランスジェニックマウスを作製し，ALSトランスジェニックマウスとのダブルトランスジェニックマウスを作製した．すなわちALSトランスジェニックマウスの運動ニューロンにHGFを長期間にわたって発現させることにより，HGFのALSトランスジェニックマウスに対する効果を確認した（図2左）[16,18]．その結果，HGFを発現させることにより，後肢反射テストおよびフットプリントテストにおけるALSトランスジェニックマウスの運動機能が大幅に改善した．さらにはHGF/ALSダブルトランスジェニックマウスにおいては，ALSトランスジェニックマウスと比較して麻痺の発症時期と寿命が約1ヵ月延長した（図2右）．この効果はヒトALS患者に換算すると約6年の寿命延長効果に相当する．すなわちHGFは，その投与法を確立させればALSに対する有効な治療となることが証明された．船越らは，HGFのALS病態に対する効果は，脊髄運動ニューロンに対して直接作用してカスパーゼ1の誘導を抑制する神経栄養作用のみならず，反応性アストロサイトのグルタミン酸トランスポーター（EAAT2）の発現低下[19]の阻止を介する間接作用も想定している[16,18]．

髄腔内持続投与による新しい治療法の開発

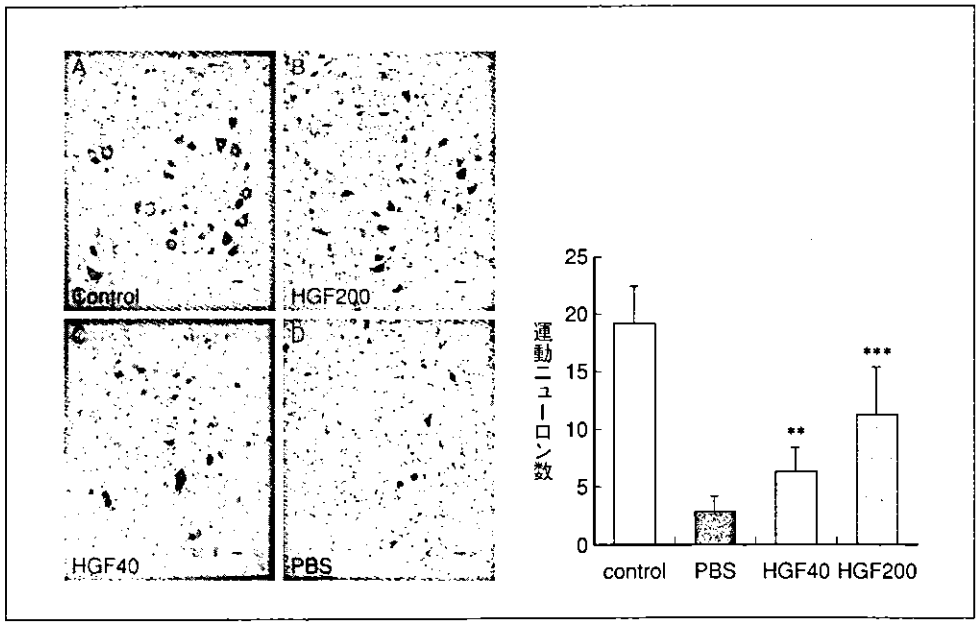
前述のように，ALSの病態の主座である脊髄の運動ニューロンに対して効率良く，しかも全身に対する副作用を回避できる薬物の投与ルートとして，髄腔内投与が注目されている．トランスジェニックラットによるALSモデルはこのルートによる薬剤供給が可能である（図3）．これまでに東北大学では，G93Aトランスジェニックラットに対して浸透圧ポンプ（Alzet Model 2004）を用いて髄腔内に遺伝子組み換え型ヒトHGFタンパク質の持続投与を行った．vehicle（PBS）および40μg，200μg（総量）の3群のトランスジェニックラットにそれぞれHGFを発症前の100日齢から1ヵ月間にわたり投与し，灌流固定後パラフィン包埋切片を作成してNissl染色標本で腰髄1切片当たりの運動ニューロン数を定量した．その結果，HGF投与群においてはvehicle投与群に比較して

図3　トランスジェニックラットに対する皮下への浸透圧ポンプの植え込みの模式図（東北大学神経内科 石垣あや作図）



吸入麻酔下に第3腰椎背側より椎弓小切除を行い，同部位よりカテーテルを挿入，ポンプは皮下に留置して術創を閉鎖している．

図4　ALS トランスジェニックラットに対する HGF 髄腔内継続投与の効果



vehicle を投与した G93A トランスジェニックラット（PBS）では，同胞の非トランスジェニックラット（control）に比べて運動ニューロン脱落が明らかである．これに対し HGF を投与した G93A トランスジェニックラットでは，用量依存性に運動ニューロン脱落の抑制効果が認められた．
HGF40：40μg 投与群，HGF200：200μg 投与群，$^{**}p<0.01$，$^{***}p<0.001$ 対 PBS 投与群
略語：巻末の「今月の略語」参照

有意に腰髄運動ニューロン数が保たれていることが明らかとなり，このことは HGF 投与量に依存的であった（図4）．さらには，高用量（200μg）HGF 投与群では寿命の延長も確認されている．今後は HGF の投与開始時期および至適用量の設定，安全性の確認などがクリアーされれば，この遺伝子組み換え型 HGF タンパク質投与はすぐにでも臨床への応用が可能であり，新しい ALS の治療法として期待される．他の多くの神経栄養因子とは異なり，日本で発見された HGF はその特許も国内にあり，開発を国内で進めることができ，日本発で世界に発信する治療法の確立が可能である．

おわりに

これまでに ALS に対しては，さまざまな神経栄養因子が治療薬の候補として臨床試験が行われたが，いずれも失敗に終わっている．この結果の解釈は慎重であるべきで，果たして十分量の薬剤が運動ニューロンへ到達しているかどうかを検証する必要がある．また今後の「神経再生医療」の展開には，神経再生に必要な場を作り出すための神経栄養因子の効率的な利用が必須であると思われる[14]．おそらく将来的には，運動ニューロン特異的なウイルスベクターによる遺伝子導入が最も効率的に神経栄養因子の供給法となることが想定されるが，私たちはこれらの方法を含め，脊髄の運動ニューロンに対する薬剤の効率的な供給法を，トランスジェニックラットによる ALS モデルを通じて検討していきたいと考えている．

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Intrathecal Administration of Hepatocyte Growth Factor in ALS Transgenic Rats

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