Mitochondrial Localization of Mutant Superoxide Dismutase 1 Triggers Caspase-dependent Cell Death in a Cellular Model of Familial Amyotrophic Lateral Sclerosis*

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The mutations in superoxide dismutase 1 (SOD1) cause ~20% of familial amyotrophic lateral sclerosis cases. A toxic gain of function has been considered to be the cause of the disease, but its molecular mechanism remains uncertain. To determine whether the subcellular localization of mutant SOD1 is crucial to mutant SOD1-mediated cell death, we produced neuronal cell models with accumulation of SOD1 in each subcellular fraction/organelle, such as the cytosol, nucleus, endoplasmic reticulum, and mitochondria. We showed that the localization of mutant SOD1 in the mitochondria triggered the release of mitochondrial cytochrome c followed by the activation of caspase cascade and induced neuronal cell death without cytoplasmic mutant SOD1 aggregate formation. Nuclear and endoplasmic reticulum localization of mutant SOD1 did not induce cell death. These results suggest that the localization of mutant SOD1 in the mitochondria is critical in the pathogenesis of mutant SOD1-associated familial amyotrophic lateral sclerosis.

Amyotrophic lateral sclerosis (ALS)1 is a paralytic and lethal disease caused by selective death of motor neurons. Approximately 10% of ALS cases occur in familial (FALS) form. The mutations in superoxide dismutase 1 (SOD1) cause ~20% of FALS cases (1, 2), and there is overwhelming evidence of a toxic gain of function as the cause of the disease (3, 4). However, it remains unclear how mutant SOD1 causes the cell death of motor neurons.

Mitochondrial degeneration such as swelling, dilatation, and vacuolation and cytoplasmic aggregate formation containing mutant SOD1 are characteristic pathological features of FALS cases and FALS transgenic mice models with SOD1 mutations (2, 4–9). The major question is where and how mutant SOD1 exerts its toxic function in motor neuron degeneration. In polyglutamine diseases, nuclear localization of mutant proteins with expanded polyglutamine tract is considered an essential process in causing neuronal cell death (10–14). Endoplasmic reticulum (ER) stress by unfolded protein accumulation in the ER is considered to cause familial Alzheimer’s disease (15, 16) or atubosomal recessive juvenile Parkinsonism (17). These reports suggest that the subcellular localization of mutant or modified protein is crucial to neuronal cell degeneration. In mutant SOD1-associated FALS, many reports have documented that the mitochondria is involved in the pathogenic process (18–26). Moreover, it has been demonstrated that SOD1, considered a cytosolic enzyme, exists in the mitochondria (27–29), suggesting that the mitochondria is the important site in pathogenesis of FALS. On the other hand, it remains controversial as to whether cytoplasmic mutant SOD1 aggregates are toxic (30) or not (31–33). Previous studies have demonstrated that the inhibition of cytoplasmic aggregate formation by heat shock proteins assure cell survival at an early stage but is unable to prevent eventual cell death at the late stage in the in vitro models of FALS (34, 35). Thus, it is important to determine in which organelle of the neuron mutant SOD1 triggers neuronal cell death. To examine this issue, we have produced neuronal cell models with the obligatory accumulation of SOD1 in the subcellular fraction/organelle of the cytosol, nucleus, ER, and mitochondria. In the present study, we provided unequivocal evidence that localization of mutant SOD1 in the mitochondria is the primary cause of mutant SOD1-mediated neuronal cell death, triggering the release of mitochondrial cytochrome c followed by the activation of caspase cascade. Furthermore, we demonstrated that cytoplasmic mutant SOD1 aggregate formation is independent of mutant SOD1-mediated neuronal cell death.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The non-organelle-oriented vectors expressing the fusion proteins of human SOD1 (wild type, mutant G93A, and G85R) and enhanced green fluorescent protein (EGFP) were generated with pEGFP-N1 vector (Clontech) as described previously (35). These vectors were designated Cyto-wtSOD1, Cyto-mSOD1G93A, and Cyto-mSOD1G85R, respectively. After these constructs were digested with Xhol and NotI, EGFP-tagged human SOD1 (wild type, mutant G93A, and G85R) was subcloned into the Xhol/NotI site of pShooter vectors (pCMV/myc/nuc, pCMV/myc/ER, and pCMV/myc/mito; Invitrogen). The nucleus-oriented vectors with nuclear localizing signals were designated Nuc-wtSOD1, Nuc-mSOD1G93A, and Nuc-mSOD1G85R, respectively. The ER-oriented vectors with ER retention signals were designated ER-wtSOD1, ER-mSOD1G93A, and ER-mSOD1G85R, respectively. The mitochondria-oriented vectors with mitochondrial localizing signals were designated Mito-wtSOD1, Mito-mSOD1G93A, and Mito-mSOD1G85R, respectively. As controls, we used LacZ subcloned into pEGFP-N1 vector or EGFP-tagged LacZ subcloned into pShooter vector. All of the constructs used here were confirmed by DNA sequence analysis.
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Cell Culture—Mouse neuroblastoma cell line Neuro2a cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) as described previously (35). They were cultured in Lab-Tec II 4-well chamber slides (Nalge Nunc International) coated with rat tail collagen (Roche Diagnostics). Transient expression of each vector (0.4 μg of DNA/well) in Neuro2a cells (2 × 10⁵ cells/well) was accomplished with LipofectAMINE Plus reagent (Invitrogen). After a 3-h incubation with transfection reagents, the transfected cells were cultured in differentiation medium (Dulbecco’s modified Eagle’s medium supplemented with 1% fetal calf serum and 20 μM retinoic acid). For treatment with the broad caspase inhibitor (zVAD-fmk; Promega) and the caspase-9-specific inhibitor (zLEHD-fmk; Calbiochem), either 20 μM zVAD-fmk or 20 μM zLEHD-fmk was added at this time.

Cell Fractionation—At each time point (12, 24, and 48 h) after transfection, the 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 MgCl₂, 2 mM EDTA, and protease inhibitor mixture (Complete Mini EDTA-free; Roche Diagnostics)). The homogenates were centrifuged (600 g, 10 min), and the pellets were designated as the nuclear fractions. The supernatants were centrifuged (10,000 g, 10 min), and the resulting pellets were designated as the mitochondrial fractions. The supernatants were centrifuged (100,000 g, 60 min), and the resulting supernatants were designated as the microsomal fractions. Each pellet was resuspended in TNES buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 0.1% SDS, and protease inhibitor mixture (Complete Mini EDTA-free; Roche Diagnostics)). The insoluble debris was briefly pelleted. To verify the fractionation, each fraction was subjected to Western blotting for Sp1 as a nuclear marker using anti-Sp1 rabbit polyclonal antibody (1:1,000, Santa Cruz), cytochrome c oxidase (COX) as a mitochondrial marker using a anti-COX subunit IV mouse monoclonal antibody (1:1,000, Molecular Probes), GRP78 as a microsomal marker using anti-GRP78 goat polyclonal antibody (1:1,000; Santa Cruz), and β-actin as a cytosolic marker using anti-β-actin mouse monoclonal antibody (1:5,000; Sigma).

Western Blot Analysis—The protein concentration was determined with a DC protein assay kit (Bio-Rad), and Western blotting was processed as described previously (35). To verify the subcellular localization of SOD1-EGFP fusion proteins, 20 μg of protein from each fraction was loaded. For analyzing the release of cytochrome c, apoptosis-inducing factor (AIF), and second mitochondria-derived activator of caspase (Smac) from the mitochondria into the cytosol and for analyzing the translocation of Bax, Bak, Bim, and Bid from the cytosol into the mitochondria, 20 μg of protein from the mitochondrial fraction or the cytosolic fraction was loaded, and the cells incubated with 10 μM staurosporin for 24 h served as a positive control.

To assess the protein levels of SOD1-EGFP fusion proteins and the activation of caspase-9 and -3, cells were collected at each time point (12, 24, and 48 h) after transfection and lysed in TNES buffer. Insoluble debris was pelleted, and 20 μg of protein was loaded.

The primary antibodies used here were as follows: anti-SOD1 rabbit polyclonal antibody (1:10,000; StressGen Biotechnologies), anti-caspase-3 rabbit polyclonal antibody, anti-caspase-9 rabbit polyclonal antibody (1:1,000; Cell Signaling), anti-cytochrome c mouse monoclonal antibody (1:1,000; BD PharMingen), anti-AIF rabbit polyclonal antibody, anti-Smac goat polyclonal antibody (1:500; Santa Cruz Biotechnology), anti-Bax rabbit polyclonal antibody, anti-Bak rabbit polyclonal antibody, anti-Bid rabbit polyclonal antibody, and anti-Endothelial nitric oxide synthase polyclonal antibody (1:200; Santa Cruz Biotechnology). After overnight incubation with primary antibodies at 4 °C, each blot was probed with horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG (1:5,000; Amersham Biosciences) or anti-goat IgG (1:5,000; Santa Cruz Biotechnology). Then they were
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visualized with ECL Plus Western blotting detection reagents (Amersham Biosciences). The signal intensity was quantified by densitometry using NIH Image 1.59 software.

**Quantitative Assessment of Cytoplasmic Aggregates, Mitochondrial Impairment, and Cell Death**—At each time point (12, 24, and 48 h) after transfection, the cells were fixed with 4% paraformaldehyde for 15 min on ice and then permeabilized with 0.05% Triton X-100 at room temperature for 10 min. Next, they were counterstained with 2 μg/ml propidium iodide (PI; Molecular Probes) at room temperature for 10 min and mounted in Gelvatol. A laser confocal scan microscope (MRC1024, Bio-Rad) was used for the morphological analysis, quantitative assessment of aggregates and mitochondrial membrane potentials, and terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) assay.

For quantitative assessment of aggregates, more than 200 transfected cells in duplicate slides were assessed blindly in three independent trials. The ratio of aggregate-positive cells was calculated as a percentage of such cells among EGFP-positive cells as described previously (25, 35).

For assessment of mitochondrial membrane potential, we used 5,5′,6,6′-tetraethylbenzimidazolocarbocyanine iodide (JC-1; Molecular Probes) according to the manufacturer’s protocol at each time point (12, 24, and 48 h) after transfection. More than 200 transfected cells in duplicate slides were assessed blindly in three independent trials.

For TUNEL assay, we used the *in situ* cell death detection kit, TMR red (Roche Diagnostics). At each time point (12, 24, and 48 h) after transfection, the TUNEL assay was carried out according to the manufacturer’s protocol. As a positive control, we used the EGFP-LacZ transfected cells that were incubated with 10 μM staurosporin for 24 h. More than 200 transfected cells in duplicate slides were assessed blindly in three independent trials.

Cell death was assessed by the dye exclusion method with PI as described previously (25, 35). At each time point (12, 24, and 48 h) after transfection, the cells were incubated with 2 μg/ml PI in Dulbecco’s modified Eagle’s medium for 15 min at 37 °C 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.

For assessment of 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). At each time point (12, 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 and mounted in Gelvatol.

**Statistical Analysis**—All of the results were analyzed by two-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc test using Statview software version 5 (SAS Institute Inc.).

**RESULTS**

**Mito-mSOD1 Induces Significant Cell Death with Lack of Aggregate Formation**—Laser confocal microscopic images and Western blots demonstrated that the subcellular localization of SOD1-EGFP fusion proteins expressed by Cyto-SOD1 was comparably ubiquitous among the subcellular fractions (Figs. 1, A–C, and 2B). In contrast, each transient expression of organelle-oriented SOD1 (Nuc-, ER-, and Mito-SOD1) was mainly observed in each organelle (Figs. 1, D–L, and 2B). The protein level of SOD1-EGFP fusion proteins was much higher than endogenous SOD1 (Fig. 2A). The protein level of the mutant SOD1-EGFP was consistently less than that of wild type SOD1-EGFP (Fig. 2A). As reported previously (32, 35, 36), SOD1 immunoreactive, ladder-like, slowly migrating masses speculated as SOD1-EGFP oligomers were observed through the gels (Fig. 2A, asterisk), but no constant relation was presented between wtSOD1 and mSOD1 of all vectors. These ladder-like masses were most clearly observed in the lanes of ER-SOD1s, especially ER-wtSOD1 (Fig. 2A).

As we previously demonstrated (35), the cells with Cyto-mSOD1 developed cell death and cytoplasmic aggregates of SOD1-EGFP fusion proteins, whereas those with Cyto-wtSOD1 did not (Figs. 1, A–C, and 3). The cells with Mito-mSOD1 exhibited a more significant level of cell death and mitochondrial impairment than those with Cyto-mSOD1 but did not develop cytoplasmic aggregates (Figs. 1, K and L, and 3). The cells with Nuc-wt and mSOD1, ER-wt and mSOD1, or Mito-wtSOD1 did not develop cytoplasmic aggregates or show cell death (Figs. 1, D–J, and 3). Expression of either the empty vector or the control vector alone did not induce cytoplasmic aggregates and cell death (data not shown). These findings suggest that the localization of mSOD1 in the mitochondria plays a critical role in mSOD1-mediated cytotoxicity, whereas cytoplasmic aggregate formation or oligomeric formation does not. Furthermore, Cyto-mSOD1-induced cell death, which was less significant than Mito-mSOD1-induced cell death, could be mediated by mitochondrial localization of mSOD1 because a moderate amount of mSOD1 was present in the mitochondria.

**Mito-mSOD1 Induces Mitochondrial Cytochrome c Release Followed by Sequential Activation of Caspase-9 and -3**—We assessed which mitochondrial death signal was involved in mSOD1-mediated cytotoxicity. Western blots revealed that cytochrome c release from the mitochondria into the cytosol occurred in the cells with Mito-mSOD1 (Fig. 4A). The cells with Cyto-mSOD1 also elicited less cytochrome c release (Fig. 4A), whereas it did not occur in the cells with Nuc-mSOD1 or ER-mSOD1 (data not shown). The time course of densitometric analysis revealed that the cytochrome c release in the cells
with Mito-mSOD1 increased gradually and was significantly stronger than those with Cyto-mSOD1 (Fig. 4B). Because AIF and Smac are also known as the signal proteins released from the mitochondria into the cytosol that promote apoptosis (37, 38), we examined the release of these mitochondrial proteins into the cytosol in this model. Western blots were not able to detect the release of AIF and Smac in the cells with either Cyto-mSOD1 or Mito-mSOD1 (Fig. 4C). Thus, AIF and Smac did not seem to be involved in the neuronal cell death in our model.

Then we examined the downstream signal cascade of the activation of caspase-9 and -3 following the mitochondrial cytochrome c release. Western blots demonstrated that the caspase-9 and -3 were activated in the cells with Cyto-mSOD1 and those with Mito-mSOD1, whereas they were not activated in the cells with Cyto-wtSOD1 and those with Mito-wtSOD1 (Fig. 5A, lanes 1–18). The time course of densitometric analysis revealed that caspase-9 and -3 were activated gradually and sequentially, and both activations were significantly stronger in the cells with Mito-mSOD1 than those with Cyto-mSOD1 (Fig. 5B and C).

Bcl-2 Family Pro-apoptotic Proteins Are Not Involved in Cyto-mSOD1 and Mito-mSOD1-induced Cell Death—Bcl-2 family pro-apoptotic proteins such as Bax, Bak, Bid, Bad, and Bim were considered to be translocated from the cytosol to the mitochondria during apoptosis and to promote the mitochondrial cytochrome c release to the cytosol (37–39). Thus, we examined whether Bax, Bak, Bid, Bad, and Bim were involved in the cytochrome c release in this model. Western blots, however, did not show the translocation of these proteins to the mitochondria (Fig. 5D). We also assessed the alteration of mitochondrial membrane potential with JC-1 but were unable to detect a significant difference in the ratio of JC-1 monomer and J aggregates between the cells with wtSOD1 and those with mSOD1 of any vectors (data not shown). These data suggested that mitochondrial localization of mutant SOD1 elicited cytochrome c release from the mitochondria to the cytosol followed by caspase activation without involvement of Bcl-2 family pro-apoptotic protein translocation and mitochondrial membrane potential alterations.

Caspase-9-specific Inhibitor and Broad Caspase Inhibitor Prevented Mitochondrial-localized Mutant SOD1-mediated Cell Death—To determine whether the mitochondrial-dependent caspase cascade activation plays a role in the mSOD1-mediated cell death, we examined the effect of treatments with the broad caspase inhibitor (zVAD-fmk) and the caspase-9-specific inhibitor (zLEHD-fmk). Western blots indicated that treatment with 20 \( \mu \)M zVAD-fmk or 20 \( \mu \)M zLEHD-fmk completely blocked the activation of caspase-9 and -3 (Fig. 5A, lanes 19–30) and diminished mSOD1-mediated cell death and mitochondrial impairment (Fig. 6) in the cells with Mito-mSOD1 as well as Cyto-mSOD1. No significant difference was observed in the inhibitory effect of zVAD-fmk and zLEHD-fmk (Fig. 6). These findings suggest that the pathway from release of cytochrome c to activation of caspase-9 and caspase-3 is the main process of mSOD1-mediated neuronal cell death.

Despite the activation of caspase-3, the cells with Cyto-mSOD1 or Mito-mSOD1 showed TUNEL-negative staining as
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Fig. 5. Western blot analysis of caspase-9 and -3 and Bcl-2 family pro-apoptotic proteins. A, time course of the activation of caspase-9 and -3. B, densitometric analysis of caspase-9 activation. C, densitometric analysis of caspase-3 activation. D, subcellular localization of Bcl-2 family pro-apoptotic proteins 48 h after transfection. COX and β-actin were used as markers of the mitochondrial and cytosolic fraction, respectively. WT, wtSOD1; G93A, mSOD1G93A; G85R, mSOD1G85R; Cyto, Cyto-SOD1; Mito, Mito-SOD1. ○, Cyto-wtSOD1; △, Cyto-mSOD1G93A; □, Cyto-mSOD1G85R; ●, Mito-wtSOD1; ▲, Mito-mSOD1G93A; ■, Mito-mSOD1G85R. *, p < 0.001 versus wt-SOD1 of each vector; **, p < 0.05; versus Cyto-mSOD1 (by two-way ANOVA with Tukey-Kramer post-hoc test). The values are the means ± S.D. (n = 3).

well as those with Cyto-wtSOD1 or Mito-wtSOD1 (Fig. 7, A–F), whereas cells treated with staurosporin exhibited obvious TUNEL-positive staining and nuclear pycnosis (Fig. 7, G–I).

DISCUSSION

Here we provided unequivocal evidence that mitochondrial localization of mutant SOD1 is the essential part of mutant SOD1-mediated neurotoxicity in a cellular model of FALS. First, neuronal cell death was elicited when mutant SOD1 was localized in the mitochondria, not in the nucleus nor ER, and was not associated with cytoplasmic aggregate formation. Second, the extent of cytochrome c release and following caspase-9 and -3 activation were markedly enhanced by accumulation of mutant SOD1 in the mitochondria. Third, Bcl-2 family pro-apoptotic proteins such as Bax, Bak, Bid, Bad, and Bim and the mitochondrial membrane potential alterations were not involved in the cytochrome c release from the mitochondria into the cytosol in our model. Fourth, a caspase-9-specific inhibitor zLEHD-fmk as well as a broad caspase inhibitor zVAD-fmk diminished mutant SOD1-mediated neuronal cell death. Thus, the localization of mutant SOD1 in the mitochondria triggers the cytochrome c release followed by caspase-dependent neuronal cell death independent of Bcl-2 family pro-apoptotic proteins and alteration of mitochondrial membrane potentials. Furthermore, mutant SOD1-mediated cell death was independent of cytoplasmic aggregate formation. A previous study reported that Bax translocation from the cytosol to the mitochondria was associated with cytochrome c release from the mitochondria into the cytosol in the FALS transgenic mice model (20), but there is a possibility that the surroundings of motor neurons such as astrocytes or dying neurons might affect Bax translocation in the model used.

Mitochondrial involvement in ALS and FALS has been documented (18–26). Mitochondrial degeneration of vacuolation or membrane disintegration in motor neurons is one of the earliest pathological findings in FALS transgenic mice (2, 5, 8, 9). Moreover, mitochondrial dysfunctions such as altered calcium homeostasis (18), decrease in respiratory chain complex activity (22, 23), alteration of the mitochondria-related gene expression (24), and increase in reactive oxygen species (39) have been reported in in vitro and in vivo models. Recent studies revealed that SOD1, which has been considered to be a cytosolic enzyme, also exists in the mitochondria (25–27) and that
mutant SOD1 was present in the vacuolated mitochondria of FALS transgenic mice model (27). Previous studies also revealed that cytochrome c release and subsequent caspase activation occurred (20, 21, 25, 40–43) and that inhibition of cytochrome c release by minocycline (21), coexpression of X chromosome-linked inhibitor of apoptosis protein (25), and treatment with a broad caspase inhibitor zVAD-fmk (42) inhibited cell death in the in vitro and in vivo models of FALS. In this study, we unequivocally demonstrated that mitochondrial localization of mutant SOD1 itself is primary and crucial to elicit the following mitochondrial death signals for mutant SOD1-mediated neuronal cell death. The cells with Cyto-mSOD1 showed a lesser extent of cell death than those with Mito-SOD1, probably because mutant SOD1 by Cyto-mSOD1 accumulated less in the mitochondria than that by Mito-SOD1.

In the present study, similar to previous reports (32, 35, 36), SOD1 immunoreactive, ladder-like, slowly migrating masses speculated to be SOD1-EGFP oligomers were observed on Western blots (Fig. 2A, asterisk), but no significant difference was detected between wtSOD1 and mSOD1 of all vectors. These ladder-like masses were most clearly observed in the ER-SOD1s, probably because SOD1-EGFP fusion proteins with ER retention signals may avoid proteasome-mediated degradation and tend to accumulate in the ER as an unfolded form. The level of these ladder-like, slowly migrating masses was not associated with neuronal cell death, but there remains the possibility that localization of mutant SOD1 oligomers in the mitochondria may cause neurotoxicity. The mitochondrial quality control system depends on ATP-dependent protease complexes such as homologues of Lon and Hsp70 (44–46). However, the capacity of mitochondria to deal with abnormal proteins might be rather limited, and the mitochondria seem to release death signals when abnormal proteins overflowed. Further investigations are needed to give an answer to this issue.

Cytoplasmic aggregate formation containing mutant SOD1 is a hallmark of mutant SOD1-associated FALS, and it has been demonstrated in the in vitro and in vivo FALS models (2, 4–9). These aggregates have been considered to participate in

**Fig. 6.** Frequency of dead cells and MTS assay after treatment with caspase inhibitors. A, frequency of dead cells. B, MTS assay. White columns, wtSOD1; gray columns, mSOD1G93A; black columns, mSOD1G85R; Cyto, Cyto-SOD1; Mito, Mito-SOD1. *, p < 0.001 versus untreated mSOD1 of each vector (by two-way ANOVA with Tukey-Kramer post-hoc test). The values are the means ± S.D. (n = 6).

**Fig. 7.** TUNEL assay. Overlays of two images were taken by laser confocal microscopy 48 h after transfection. A, Cyto-wtSOD1; B, Cyto-mSOD1G93A; C, Cyto-mSOD1G85R; D, Mito-wtSOD1; E, Mito-mSOD1G93A; F, Mito-mSOD1G85R. G–I, EGFP-LacZ transfected cells incubated with 0.01 μM staurosporin for 24 h as positive controls (red nuclear staining in pyknotic cells). The cells with Cyto-mSOD1 (B and C) or Mito-SOD1 (E and F) showed negative staining as well as those with wtSOD1 (A and D). Scale bar, 30 μm.
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a pathogenic process (30), although this has been disputed (31–33, 35). In this study, we conclusively demonstrated that
cytoplasmic aggregates are not directly associated with cell
death, similar to the in vitro models of polyglutamine diseases,
suggesting that the subcellular localization of mutant protein
itself, rather than aggregate formation, exerts toxicity (14, 30).
Controversy surrounds the issue of whether the motor neu-
ronal cell death in mutant SOD1-associated ALS is apoptosis
(47, 48) or not (49). A previous study on an in vitro model
reported that activated caspase-3-positive motor neurons were
observed, but TUNEL-positive motor neurons were not (43).
Similarly, we were unable to find TUNEL-positive cells despite
obvious activation of caspase-3. Recently, at least three types of
programmed cell death (PCD) have been proposed: apoptosis,
apoptosis-like PCD, and necrosis-like PCD (50). Apoptosis-like
PCD is cell death with less chromatin condensation than in
apoptosis or any degree and combination of apoptotic features
such as caspase activation, cytoplasmic shrinkage, and plasma
membrane blebbing (50). Previous reports suggested that
apoptosis-like PCD may be a type of neuronal cell death in neuro-
degenerative diseases including ALS (51, 52). The present
study also indicated the possibility that this apoptosis-like
PCD is a type of neuronal cell death through the mitochondrial
signal pathway in motor neurons with mutant SOD1. However,
further investigations are needed to shed light on the mecha-
nism of mutant SOD1-mediated neuronal cell death.

In conclusion, in this study we demonstrated that mitochon-
drial localization of mutant SOD1 itself triggers cytochrome
release from the mitochondria into the cytosol that initiates
the caspase-dependent cell death cascade in a FALS model. Inhi-
bition of mitochondrial localization of mutant SOD1 may well
be a candidate for a therapeutic approach to FALS.

Acknowledgments—We are grateful to Dr. Keiji Tanaka (Department of Molecular Oncology, The Tokyo Metropolitan Institute of Medical Science) and Dr. Jun-ichi Niwa (Department of Neurology, Nagoya University Graduate School of Medicine) for helpful discussion. We also thank Dr. Kumi Kawai (Department of Pathology, Nagoya University Graduate School of Medicine) for technical assistance.

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J. Biol. Chem. 2002, 277:50966-50972.
doi: 10.1074/jbc.M209356200 originally published online October 21, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209356200

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Mitochondrial localization of mutant superoxide dismutase 1 triggers caspase-dependent cell death in a cellular model of familial amyotrophic lateral sclerosis.

Incorrect immunoblots were used to assemble Figs. 2B, 4, A–C, and 5, A and D. As the original data were no longer available, these experiments were repeated, and the data from the replicated experiments are shown. These corrections do not affect the results or the conclusions of this work.
Additions and Corrections

A

|        | Cyto | Mito |        | Cyto | Mito |        | Cyto | Mito |
|--------|------|------|--------|------|------|--------|------|------|
| WT     | G83A | G85R | WT     | G83A | G85R | WT     | G83A | G85R |
| Cyto   |      |      | Mito   |      |      | 12 h   |      |      |
|       |      |      |        |      |      |       |      |      |
|        |      |      |        |      |      | 24 h   |      |      |
|       |      |      |        |      |      |       |      |      |
|        |      |      |        |      |      | 48 h   |      |      |
|       |      |      |        |      |      |       |      |      |

Cytochrome c →

Actin →

12 h

24 h

48 h

B

Ratio of cytochrome c/actin

0

0.05

0.1

0.15

0.2

0.25

12 h

24 h

48 h

C

|        | Cytosol | Mitochondria |
|--------|---------|--------------|
|        | Cyto    | Mito         | Cyto    | Mito   |
| WT     | G83A    | G85R         | WT      | G83A   |
| ALF    |          |              | Smac    |        |
| Actin  |          |              | COX     |        |

Fig. 4
Additions and Corrections

A

Pro caspase-9

p39

Pro caspase-3

p17

Cyto  Mitochondria

12 h  24 h  48 h  48 h  48 h

WT  G38A  G38A  WT  G38A  G38A  WT  G38A  G38A

B

Ratio of p39/pro caspase-9

0 0.05 0.1 0.15

12 h  24 h  48 h

C

Ratio of p17/pro caspase-3

0 0.02 0.04 0.06 0.1

12 h  24 h  48 h

D

Cytosol  Mitochondria

WT  G38A  G38A  WT  G38A  G38A  WT  G38A  G38A

Bax  Bak  Bid  Bad  Bim  Actin  COX

Fig. 5