A cysteine residue affects the conformational state and neuronal toxicity of mutant SOD1 in mice: relevance to the pathogenesis of ALS

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Abstract

We previously showed by in vitro experiments that the cysteine residue (Cys111) near the dimer interface is critical for monomerization and resultant aggregate formation of mutant Cu, Zn-superoxide dismutase (SOD1) protein, which is toxic to motor neurons in familial amyotrophic lateral sclerosis (ALS). To verify the importance of Cys111 in the mutant SOD1-associated ALS pathogenesis in vivo, we analyzed the disease phenotype of SOD1 transgenic mice harboring H46R mutation alone (H46R mice) or H46R/C111S double mutations (H46R/C111S mice). Behavioral, histological and biochemical analyses of the spinal cord showed that the onset and progression of the disease phenotype were delayed in H46R/C111S mice compared with H46R mice. We found that peroxidized Cys111 of H46R SOD1 plays a role in promoting formation of high molecular weight insoluble SOD1 species that is correlated with the progression of the motor neuron disease phenotype. These results support that Cys111 is a critical residue for the neuronal toxicity of mutant SOD1 in vivo, and the blockage of peroxidation of this residue in mutant SOD1 may constitute a future target for developing ALS treatment.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal disease that causes the degeneration of both upper and lower motor neurons. Approximately 10% of ALS occurs in a familial trait, 20–25% of which is caused by mutation of the Cu, Zn-superoxide dismutase (SOD1) gene (1). So far, >150 different pathogenic mutations of the SOD1 gene have been identified in familial ALS patients, which are scattered throughout the entire sequence of the gene regardless of particular functional domains. Transgenic mice overexpressing human SOD1 bearing a disease-causing mutation have been shown to develop spinal motor neuron degeneration with varying severity according to the expression level of mutant SOD1, whereas wild-type SOD1 transgenic or SOD1-deficient mice have no disease phenotype (2,3). These results indicate that the neuronal toxicity of mutant SOD1 is derived from an aberrantly acquired toxic function, rather than the loss of function of the protein. So far, the nature of the mutant SOD1 toxicity has not been fully uncovered. Previous reports have shown that SOD1-positive inclusion bodies are observed in degenerating motor neurons in spinal cords of familial ALS patients bearing an SOD1 mutation, as well as mutant SOD1 transgenic mice.
versely, replacement of cysteine residues, especially of Cys6 and Cys111, on thiol-mediated aggregate formation and oxidized/monomerized SOD1 species may cause cellular toxicity (9). On the other hand, the sulfhydryl moiety of Cys111 in human wild-type SOD1 is oxidized by redox substrates, such as glutathione or peroxides to form S-S or S-O covalent modifications, respectively (12–16). These oxidative modifications of Cys111, which are located on the edge of the dimer interface, can also change the configuration of the SOD1 dimer to cause monomerization.

Several reports have shown that the aberrant redox status of cysteine residues in familial ALS-associated mutant SOD1 contributes to the pathogenesis of the ALS models in vitro and in vivo. Mutant SOD1 exhibits abnormal vulnerability to reducing reagents, which weaken the intra-subunit disulfide bond between Cys57 and Cys146 to destabilize the SOD1 dimer (17). The dimer dissociation results in the exposure of the hydrophobic region of the SOD1 subunit, which consequently promotes aggregation of the protein (18,19). Furthermore, it has been shown that insoluble SOD1 oligomers crosslinked via intermolecular disulfide bonds at Cys57 and Cys146 were detected in spinal cords at the symptomatic stage of mutant SOD1 transgenic mice (18,19). Insoluble mutant SOD1 oligomers can also be formed in vitro by disulfide scrambling of all four cysteine residues (22,23). Conversely, replacement of cysteine residues, especially of Cys6 and Cys111, decreased disulfide-crosslinked mutant SOD1 oligomers and aggregate formation in cultured cells (24,25). Alternatively, the peroxidized forms (−SO₂H or −SO₃H) of Cys111 sulfhydryl moieties are detected in aggregates of mutant SOD1 in spinal cords of G93A SOD1 transgenic mice, suggesting Cys111 as a primary target of oxidative modification (12).

We have previously analyzed biochemical characteristics of mutant SOD1 using immobilized copper affinity chromatography and found that a fraction of mutant SOD1 is oxidatively modified at Cys111 and monomerized (14,26). We hypothesized that these oxidized/monomerized SOD1 species may cause cellular toxicity to mutant SOD1-expressing neurons by generating oxidative stress and disturbing the degradation of the protein. To establish the role of Cys111 on thiol-mediated aggregate formation and neuronal toxicity in familial ALS-causing mutant SOD1, we generated transgenic mice expressing human SOD1 bearing H46R mutation, as well as mice expressing H46R/C111S double mutant SOD1. We found that the disease phenotype of motor paralysis was retarded by Cys111 replacement in H46R SOD1-expressing mice, suggesting that Cys111 plays a significant role in the pathogenesis of mutant SOD1-related familial ALS.

### Results

#### Generation of H46R and H46R/C111S mutant SOD1 transgenic mice

To analyze the mutant SOD1-associated ALS phenotype in model mice, we created transgenic mice overexpressing human SOD1 bearing H46R mutation (H46R mice) that are known to develop degeneration of motor neurons (27). To investigate the role of Cys111 on the neuronal toxicity of mutant SOD1 in ALS, we also generated SOD1 transgenic mice with H46R together with C111S mutation (H46R/C111S mice), and aimed to compare their phenotype with that of H46R mice. We obtained 13 and 5 mice as founders, from which 9 and 5 lines were established for H46R and H46R/C111S mice, respectively.

Among them, 3 lines for each mutant strand, with varying copies of transgene (Table 1), developed motor paralysis starting from the hind limbs, spreading to the rest of the body, and ultimately resulting in shorter lifespan than that of wild-type mice (−800 days on average for BDF1 background (28)). Survival time of the affected mice was shortened according to the transgene dosage (Table 1 and Fig. 1A), suggesting that the disease phenotype is defined by the mutant SOD1 expression level, as described previously (8). H46R/C111S mice also developed the motor neuron disease phenotype, which was enhanced by increased gene copy number. However, their disease onset was later and the disease progression was slower compared with H46R mice expressing comparable or less transgene copy number (e.g. see H- and Q-lines). These results suggest that the existence of Cys111 is important to mediate the neuronal toxicity of H46R mutant SOD1 in vivo. The H-line, in which the transgene was transmitted to all the males, but no females, and was supposed to be incorporated into the Y chromosome, was excluded from subsequent analyses to avoid inconsistency of the hereditary manner.

<table>
<thead>
<tr>
<th>Line</th>
<th>Copy no.</th>
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<th>Copy no.</th>
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<tr>
<td>H</td>
<td>29–42</td>
<td>N</td>
<td>25–38</td>
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<tr>
<td>I</td>
<td>51–67</td>
<td>P</td>
<td>24–36</td>
</tr>
<tr>
<td>L</td>
<td>39–49</td>
<td>Q</td>
<td>39–48</td>
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The indicated copy numbers represent the ratio of the level of human SOD1 gene to that of internal mouse control gene in the genomic DNA of each mouse determined by semi-quantitative PCR.

| H46R/C111S SOD1 showed decreased toxicity in vivo compared with H46R SOD1 |

To examine the different toxic traits of H46R SOD1 and H46R/C111S SOD1 in motor neurons in detail, we chose to compare the disease phenotype of the following: offspring of L-line with those from Q-line and offspring of I-line with homozygous progeny of P-line, since the transgene copy numbers are comparable in each combination. Expression levels of transgene-derived proteins in Q- and homozygous P-lines were confirmed to be comparable with that in I-line and higher than that in L-line by immunoblot analysis at the pre-symptomatic stage (12 weeks of age) (Fig. 1B and C), which warranted the comparison of these sets of mouse lines. SOD1 enzymatic activity was also determined by semi-quantitative PCR.
measured in livers and spinal cords of H46R (L-line) and H46R/C111S (Q-line) mice (Supplementary Material, Fig. S1). As indicated previously (29), H46R SOD1 showed minimal activity due to the impairment of copper binding at the active site. There were no apparent differences of the activity between the liver and the spinal cord, or H46R and H46R/C111S mice, meaning that the SOD1 activity does not correlate with the disease phenotype.

Figure 1. H46R/C111S mutant SOD1 was less toxic than H46R SOD1 in transgenic mice. (A) Cumulative probabilities of lifespan in H46R and H46R/C111S mice. The numbers in parentheses indicate the analyzed number of mice in each line. Each line of H46R mice had a significantly shorter lifespan than any line of H46R/C111S mice (P < 1 × 10⁻¹⁰ by log-rank test). (B) Expression of human SOD1, mouse GAPDH and mouse SOD1 in spinal cords of each mouse line detected by immunoblot analysis. (C) The expression level of human SOD1 normalized by GAPDH and mouse SOD1 expression level (mean ± SD; n = 3). Mice from the I-line expressed the similar level of SOD1 to Q- and homozygous P-lines. L line showed less expression than Q- and homozygous P-lines, and the human SOD1/mouse SOD1 ratio between L- and Q-lines was significantly different (P < 0.05 by two-tailed t-test). (D and E) Cumulative probabilities of the disease onset (D) and lifespan (E) in H46R (I and L) and H46R/C111S (Q and homozygous P) mice. The numbers in parentheses indicate the analyzed number of mice in each line. Each line of H46R mice had significantly earlier onsets and shorter lifespans than either line of H46R/C111S mice (P < 1 × 10⁻¹⁰ by log-rank test). (F and G) Time courses of rotarod scores (F) and body weights (G) in non-transgenic, H46R and H46R/C111S mice (mean ± SD). The numbers in parentheses indicate the analyzed number of mice in each line. Rotarod scores of mice in I- and L-lines started to decrease at 18 and 30 weeks of age, respectively, while the scores were preserved in H46R/C111S mice (P < 0.01 for I- and L-lines at 21 and 33 weeks of age and older, respectively, compared with each line of H46R/C111S mice by two-tailed t-test). Mice of I- and L-lines also lost weights from 16 and 29 weeks of age, respectively.
C111S mice showed significantly delayed onset and extended duration compared with H46R mice. As a result, the H46R/C111S mice showed extended survival compared with H46R mice.

Table 2. Onset, lifespan and disease duration in each line of H46R and H46R/C111S mice

<table>
<thead>
<tr>
<th>Line</th>
<th>Onset time (days)</th>
<th>Lifespan (days)</th>
<th>Duration (days)</th>
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<tr>
<td>H46R</td>
<td></td>
<td></td>
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<tr>
<td>L</td>
<td>d(22)</td>
<td>145.9 ± 7.2</td>
<td>177.4 ± 9.7</td>
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<td></td>
<td>q(22)</td>
<td>157.3 ± 8.8</td>
<td>181.5 ± 7.1</td>
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<tr>
<td>L</td>
<td>d(23)</td>
<td>239.1 ± 16.7</td>
<td>273.9 ± 20.8</td>
</tr>
<tr>
<td></td>
<td>q(22)</td>
<td>238.9 ± 13.8</td>
<td>272.0 ± 10.8</td>
</tr>
<tr>
<td>H46R/C111S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>q(6)</td>
<td>705.0 ± 3.0</td>
<td>N.A.</td>
</tr>
<tr>
<td>P homozygote</td>
<td>d(4)</td>
<td>367.3 ± 54.1</td>
<td>405.0 ± 39.1**</td>
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<tr>
<td></td>
<td>q(6)</td>
<td>350.3 ± 41.9**</td>
<td>402.7 ± 41.0**</td>
</tr>
<tr>
<td>Q</td>
<td>d(8)</td>
<td>406.8 ± 60.3**</td>
<td>451.1 ± 61.7**</td>
</tr>
<tr>
<td></td>
<td>q(7)</td>
<td>363.9 ± 73.1**</td>
<td>426.0 ± 97.8**</td>
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The onset of the disease phenotype was determined as described in the ‘Materials and methods’. The numbers in parentheses indicate the analyzed number of mice in each line.

We also found that TA muscle fibers showed typical neuropathic changes (atrophy and infiltrated by inflammatory cells) in H46R mice, while muscle tissues from H46R/C111S mice appeared comparable to those of non-transgenic mice (Fig. 2D). Together, these results indicate that H46R/C111S mice are protected from the neurodegenerative changes in the spinal cord observed in H46R mice.

Previous studies have shown that the activation of microglia and astrocytes in spinal cords precedes the degeneration of motor neurons and modifies the progression of the disease in other mutant SOD1 mouse strains (30,31). To examine whether H46R/C111S mice show such a pre-symptomatic disease phenotype, we analyzed whether these glial cells were activated in spinal cords of H46R and H46R/C111S mice at the disease end point of H46R mice. We found that immunoreactivity for Iba1 and GFAP was significantly increased in H46R mice, suggesting the activation of microglia and astrocytes in these mice. In contrast, immunoreactivity for Iba1 and GFAP was only slightly increased in spinal cords from H46R/C111S mice (Fig. 2F). These results suggest that only the pre-symptomatic activation of these cells is initiated in H46R/C111S mice at 40 weeks of age.

To analyze qualitative modification of degenerative changes in H46R mice by C111S expression, we also analyzed lumbar spinal cords of H46R mice at 40 weeks of age. We also found that TA muscle fibers showed typical neuropathic changes (atrophy and infiltrated by inflammatory cells) in H46R mice, while muscle tissues from H46R/C111S mice appeared comparable to those of non-transgenic mice (Fig. 2D). Together, these results indicate that H46R/C111S mice are protected from the neurodegenerative changes in the spinal cord observed in H46R mice.

Cys111-oxidation and misfolding/aggregation of SOD1 in spinal cords of H46R and H46R/C111S mice

We previously showed that a fraction of mutant SOD1, but not wild-type SOD1, is oxidized at Cys111 in cultured neuronal cells (14). The oxidation of Cys111 affects the conformation of SOD1 and/or its dimer structure, making it unstable and likely to monomerize (13–16). To examine Cys111 oxidation status of the mutant SOD1 directly in H46R mouse tissues and correlate the expression with the disease progression, immunoblot analysis...
with a specific antibody to Cys111-sulfonylated (−SO₃H) SOD1 (12), an end product of peroxidized SOD1, was performed in immunoprecipitates with anti-pan SOD1 antibody. We were able to detect the Cys111-peroxidized SOD1 in H46R mice at all time points examined, while it was only minimally visible in wild-type SOD1 transgenic mice (Fig. 3A–C). Cys111-peroxidized SOD1 was detected not only in spinal cords but also in livers of H46R mice; and it was present considerably at the pre-symptomatic stage, suggesting that the peroxidation of Cys111 takes place systemically from the early stage of the disease in H46R mice.

Peroxidized-Cys111 SOD1 was not seen in H46R/C111S mice at any stage, whereas the same experimental procedure detected peroxidized-Cys111 in recombinant human wild-type SOD1 with a dose-dependent manner of hydrogen peroxide (Fig. 3A–C and E), confirming the specificity of the antibody used in this study. These results indicate that Cys111 of H46R SOD1 is prone to oxidization in vivo. The oxidation of other thiol substrates, such as glutathione, was not altered in the spinal cords of H46R or H46R/C111S mice (Supplementary Material, Fig. S3A), suggesting that general oxidative stress against thiols is not the cause of Cys111 oxidation in H46R SOD1.

To examine whether the Cys111 oxidation of H46R SOD1 is correlated with its misfolding status, we examined levels of misfolded SOD1 by immunoblot and immunohistochemistry using antibodies specific to misfolded SOD1. We performed immunoprecipitation from liver and spinal cord homogenates using C4F6 antibody (32), followed by immunoblot analysis with anti-pan SOD1 antibody. In the spinal cords of L-line of H46R mice,
misfolded SOD1 was detected at the symptomatic age (9 months of age), though it was not detectable prior to development of the disease (Fig. 3A). In H46R/C111S mouse spinal cords, on the other hand, misfolded SOD1 was only marginally detected at 9 months of age, while it became more intense at the symptomatic stage (15 months of age). We did not detect misfolded SOD1 in wild-type SOD1 transgenic mice (Figs. 3A, B and D). We also performed immunohistochemistry using D3H5 antibody (33) to detect misfolded SOD1 in the lumbar spinal cord of each strain (Fig. 3F). We found immunoreactivity in the remaining motor neurons of the ventral horn in H46R L-line mice at 9 months of age, but no signal with this antibody was observed in age-matched H46R/C111S Q-line mice or non-transgenic mice. Misfolded SOD1 in H46R mice was located on and in proximity of SOD1-positive inclusions stained with anti-pan SOD1 antibody in motor neurons (Fig. 3F, arrowheads).
reflecting the biochemical detection of misfolded SOD1 at that time point (Supplementary Material, Fig. S3B). Together, these results suggest that mutant SOD1 misfolding in vivo is observed only in tissues affected by the disease and is promoted by oxidation at Cys111.

Insoluble SOD1 formation is correlated with ALS phenotype and is formed independently of intermolecular disulfide bonds of SOD1

High molecular weight, insoluble SOD1 aggregates are known to increase in spinal cords with the progression of motor symptoms in mutant SOD1-overexpressing mice (34). It is reported that in spinal cords of the mutant SOD1-transgenic mice, mutant SOD1 forms intermolecular disulfide-crosslinked oligomers with two or more cysteine residues (20,21,35). To analyze the role of Cys111 in the formation of high molecular weight SOD1 aggregates, we examined SOD1 aggregate formation in each transgenic strain. By using immunoblot analysis, we found reducing reagent (DTT)-resistant high molecular weight SOD1 species in the insoluble fraction of spinal cord lysates from symptomatic H46R mice (both I- and L-lines), but not from age-matched wild-type SOD1 transgenic mice or asymptomatic H46R/C111S Q-line mice (Fig. 4B). High molecular weight insoluble SOD1 species became detectable in Q- or homozygous P-line in older ages after the appearance of motor symptoms. Although a single band was detected around the molecular weight of SOD1 dimer in livers and spinal cords of Q-line (Fig. 4A and B, arrowheads), we considered it to be non-specific since it was distributed mostly in the soluble fraction and equally between asymptomatic and symptomatic mice. We also found high molecular weight SOD1 immunoreactivity upon immunoblot analysis of spinal cord lysates from both H46R mice and H46R/C111S mice at all the examined time points under DTT (−) condition. This suggests that the formation of reducing reagent-sensitive intermolecular disulfide bonds in these mice is not mediated by Cys111, and that it is unrelated to the toxic property of mutant SOD1 on motor neurons. Collectively, these results suggest that Cys111 residue plays a role in promoting formation of high molecular weight insoluble SOD1 species that is correlated with the progression of the motor neuron disease phenotype, independently of intermolecular disulfide crosslinking.

No improvement of the motor phenotype of H46R mice was achieved by thioredoxin expression

Our current results demonstrate that the oxidative modification of Cys111 promotes the motor neuron pathology in H46R mice. Therefore, inhibiting the oxidative modification either for intermolecular disulfide crosslinking or for peroxidation of Cys111 may help delay the onset and/or progression of the disease phenotype in H46R mice. Thioredoxin (TRX) is a protein with two redox-active cysteine residues and catalyzes the reduction of cysteine disulfides in proteins through thiol-disulfide exchange reactions to protect them from oxidative stress (36). In addition, while sulfonylation (−SO3H) is an irreversible peroxidative modification that is difficult to reduce physiologically, sulfinylation (−SOH), the first step to oxidize cysteine residues to sulfonic (−SO2H) and sulfonic forms, is potentially reduced by the TRX redox system (8). Indeed, previous reports have shown that transgenic mice overexpressing TRX1, the cytosolic form of TRX, express increased TRX1 protein in neurons and thereby show tolerance to pathological states of neurons caused by oxidative stress such as ischaemia, indicating that the redox

![Figure 4. Reducing agent-resistant insoluble SOD1 complexes are associated with the disease progression of mutant SOD1 mice.](image-url)
environments in mouse neurons can be regulated (37). To gain insights as to whether TRX can prevent the progression of pathology in H46R mice, we crossed H46R I-line mice with the TRX1 transgenic mice (TRX1 mice) and examined whether the motor neuron disease phenotype is modified by TRX1 expression in H46R SOD1 and TRX1 double transgenic mice (H46R/TRX1 mice). Immunohistochemical analysis revealed that, in the ventral horn of the TRX1 mouse lumbar spinal cord, human TRX1 was expressed mainly in motor neurons and minimally in astrocytes, which is consistent with the previous report (Fig. 5A) (37). Human TRX1 was also detected in the spinal cords of H46R/TRX1 mice. The expression of endogenous mouse TRX1 was not altered in these mice (Fig. 5B). As a result, TRX activity was significantly increased in the spinal cords of H46R/TRX1 mice compared with those in H46R mice, indicating that the expressed human TRX1 functions in the tissue (Fig. 5C). The age of the disease onset and lifespan, monitored as mentioned above, were not statistically different between H46R mice and H46R/TRX1 mice (Table 3 and Fig. 5D and E). By performing rotarod tests, we also found that motor dysfunction was similarly observed in both H46R mice and H46R/TRX1 mice (Fig. 5F). TRX1 mice did not show any detectable changes in rotarod testing. We also monitored body weights of the mice as a way to detect the onset of the disease phenotype and found that both H46R mice and H46R/TRX1 mice showed similar profiles of body weight decrease during the observed time period (Fig. 5G). These results suggest that TRX1 expression does not change overall disease progression of H46R mice.

To examine whether the TRX1 protein overexpression affected mutant SOD1 misfolding, we performed protein-level analysis by immunoblot. We examined misfolded SOD1 expression in spinal cords of H46R mice and H46R/TRX1 mice at the late-symptomatic stage (24 weeks of age) by immunoprecipitation with CAF6 antibody followed by immunoblot analysis to detect misfolded SOD1 (Fig. 5H and I). We found no differences in the amount of misfolded SOD1 levels between mouse lines. The amount of Cys111-sulfonylated SOD1 was also analyzed in spinal cords at the same age (Fig. 5H and I). Again we found no differences in the amount of Cys111-peroxided SOD1. These results suggest that TRX1 cannot inhibit the peroxidization of Cys111 in mutant SOD1 that may be correlated with the formation of misfolded SOD1.

**Discussion**

We have shown here that the substitution of Cys111 in mutant SOD1 is sufficient to cause delay in the onset of the ALS phenotype and extend the survival of H46R mice. While a number of previous works have demonstrated pathological significance of different residues of SOD1 upon mutation in FALS, a mechanistic link between the mutation and pathogenesis has remained unclear. Our study here shows that Cys111 drives the pathogenicity of mutant SOD1 by demonstrating for the first time that substitution of a single residue in mutant SOD1 significantly reduces the disease phenotype of a FALS mouse model. Cys111 of H46R SOD1 is peroxided and plays a role in promoting misfolding of mutant SOD1 molecules to generate reducing reagent-resistant, high molecular weight, insoluble SOD1 species. These in vivo results in the present study are consistent with our previous findings in vitro that a fraction of mutant SOD1 is oxidatively modified at Cys111, and support our hypothesis that the modification of Cys111 plays a key role in the conformational changes of mutant SOD1 to misfold, monomerize and aggregate through the destabilization of the dimer structure and become toxic to motor neurons (14,26).

The peroxidative modification of SOD1 at Cys111 appeared considerably prior to the onset of the disease in spinal cords, as well as in livers of H46R mice. This may suggest that mutant SOD1 is more prone to be attacked by oxidants such as hydrogen peroxide or hydroxyl radicals than wild-type SOD1 due to a slight structural difference. In fact, the altered reactivity of Cys111 thiol has recently been documented in recombinant mutant SOD1 (38). On the other hand, misfolding of H46R SOD1 seemed to be promoted by Cys111 oxidation, but it was observed only in affected tissues (spinal cords), as reported previously (39), and only after the onset of motor neuron pathology. These results may suggest the contribution of other factors to help maintain the structure of mutant SOD1 to prevent misfolding even when Cys111 is peroxided. Indeed, previous reports suggested that the expression of such factors, e.g. heat shock factor-1, a transcriptional regulator of heat shock proteins, is reported to be relatively low in motor neurons (40). This restricted misfolding of mutant SOD1 may explain the region-specific deposition of protein aggregates and degenerative pathology of ALS.

We found that misfolding of mutant SOD1 eventually occurred in H46R/C111S mice, although its appearance was delayed compared with H46R mice. This means that, while Cys111 plays a role in promoting misfolding, it is not the only residue to mediate misfolding/aggregation of mutant SOD1. Given that the reducing reagent-resistant insoluble SOD1 smears/bands were formed in H46R/C111S mice in a pattern similar to that observed in H46R mice, Cys111 itself may not be involved in crosslinking for aggregates as several reports have suggested (41–43). H46R mutation disrupts metal binding capacity at the active site in SOD1 (29), and metal loss causes oligomerization of SOD1 (11). Considering that H46R/C111S SOD1 had minimal SOD1 activity as well as H46R SOD1, impairment of metal binding at the active site may be related to misfolding/aggregation of H46R/C111S SOD1.

It is reported that mutant SOD1 is liable to be reduced at the intra-subunit disulfide bond between Cys57 and Cys146 and easily dissociated (17,44). Moreover, two or more cysteine residues including Cys57 and Cys146 are known to be involved in crosslinking with intermolecular disulfide bonds to form an abnormal complex of reducing reagent-sensitive, insoluble SOD1 oligomers in spinal cords of mutant SOD1 mice (20,21,35). In our current analysis, we did not see any difference in the amount of intrasubunit disulfide-reduced or intermolecular disulfide-crosslinked mutant SOD1 between H46R and H46R/C111S mice. This implies that these mutant SOD1 species are formed independently of Cys111, despite previous reports hinting to the involvement of this residue (22–25). Considering that the ALS phenotype is mitigated by Cys111 replacement in H46R mice, the contribution of the disulfide crosslinking to ALS pathology might be limited (35,42). However, we cannot exclude the possibility that other cysteine residues (Cys6, 57 and 146) cause toxicity to motor neurons in the disulfide crosslinking-dependent manner. Further studies will be needed to explore this possibility.

The phenotype of motor neuron disease in H46R mice was not ameliorated by overexpression of TRX1, a key regulator of sulfhydryl/disulfide redox status of proteins (36). It is likely that TRX1 cannot act on reducing peroxided Cys111 of mutant SOD1, since no change of Cys111-sulfonylated SOD1 level was observed in the presence of TRX1. Considering this finding, other approaches to specifically target the peroxided cysteines, e.g. using chemicals coupled with dimerone, a trapping reagent of sulfenylated cysteines (8), may be necessary to protect motor neurons against degeneration in mice.
It has been previously shown that ALS-associated mutant SOD1 simultaneously harboring mutations in all metal-binding residues and free cysteines (Cys6 and 111) showed no toxicity in vivo by using a transgenic mouse model (45). However, it is difficult to confirm the contribution of Cys111 in this work, because the molecular circumstances surrounding SOD1 tend to be...
complex (metal loss etc.) and the expression level of the protein in these mice was relatively low compared with that in other transgenic mouse strains. Here we showed unequivocal evidence with Cys111 substitution in vivo that indicated the critical role of Cys111 in disease progression. Moreover, considering that C111S substitution also protects wild-type SOD1 against destabilization (46), and Cys111-oxidized and misfolded wild-type SOD1 has been detected in spinal cords of sporadic ALS patients (47), the pathogenic mechanism we have proved for mutant SOD1 might be shared by wild-type SOD1 in sporadic ALS. The removal or inhibition of Cys111 oxidative modification could be a promising treatment strategy for SOD1-related ALS patients.

Materials and Methods

Animal experiments

All animals were maintained in accordance with the guidelines of the National Center of Neurology and Psychiatry. The technical protocols for animal experiments in this study were approved by the Small Animal Welfare Committee in the National Center of Neurology and Psychiatry.

Generation and genotyping of transgenic mice

The human wild-type SOD1 genomic DNA construct containing its own promoter was a generous gift from Dr C. J. Epstein (University of California, San Francisco, USA) (48). H46R and H46R/C111S mutations were introduced by a PCR-based site-directed mutagenesis with Pfu-X DNA polymerase (Greiner bio-one). After confirming the sequences of all five exons, the whole mutated SOD1 gene (12 kb) was excised out with restriction enzymes and injected into BDF1 mouse embryos using a standard procedure. Mice were genotyped by PCR of genomic DNA extracted from their tails using primer sets according to the protocol established by the Jackson Laboratory (http://jaxmice.jax.org/literature/factsheet/working_with_ALS_mice.pdf). The end point of the disease was defined as the inability to stand up within 20 s after being placed on its side in the cage.

Behavioral tests

The motor function of mice was monitored once a week by observing the spread of hind limbs when lifting them by their tails beginning at 16 weeks of age for the comparison of H46R and H46R/C111S mice, and at 12 weeks of age for the comparison of H46R and H46R/TRX1 littermates. The onset was determined by observation of partial collapse of leg extension towards lateral midline or trembling of hind limbs (Score of 1 by ALS Therapy Development Institute) (http://jaxmice.jax.org/literature/factsheet/working_with_ALS_mice.pdf). The end point of the disease was defined as the inability to stand within 20 s after being placed on its side in the cage.

The copy number of mutant SOD1 transgene was determined by real-time PCR system (Applied Biosystems 7300) using FastStart Universal SYBR Green Master (Roche Diagnostics) with primer sets described above, and the ratio was calculated from the Ct values with each primer set. The copy number of the founder mouse harboring the least transgene was regarded as 1.

TRX1 transgenic mice were generated in C57BL/6 background with the transgene of human TRX1 cDNA controlled by the j-actin promoter (37). To obtain H46R SOD1/TRX1 double transgenic mice, H46R SOD1 I-line male mice were crossed with hemizygous TRX1 female mice, and the littermates were used for analysis. Genotyping of TRX1 progeny was performed by PCR with genomic DNA from tails using a primer set as follows: human TRX1 forward: AAGCA GATCGAGAGCAAGACT, reverse: TCCCTTATGGCTCCAGAAAT.

Immunoblotting and SOD1 activity assay

Tissue samples were lysed in a buffer containing 50 mM HEPES (pH 7.2), 0.1 mM EDTA and protease inhibitor cocktail (Nakarai Tesque). After centrifugation for 5 min at 16 000 g, the supernatant (0.5 and 20 µg of protein for SOD1 and TRX1, respectively) was subjected to immunoblot. For the immunoblot, the following primary antibodies were used: sheep anti-SOD1 (574 597, 1:4000 dilution, EMD Millipore); mouse anti-human TRX1 (1:1000 dilution) (37); and rabbit anti-mouse TRX1 (1:1000 dilution) (37). The immunoblot of Cys111-peroxidized SOD1 was done with rabbit anti-sulfonlated-Cys111 SOD1 antibody at 1:1000 dilution (12). Immunoreactivity was visualized by using HRP-conjugated secondary antibodies and chemiluminescent substrate (Wako). The chemiluminescence was captured by LAS-4000 mini (GE Healthcare), and the intensity was quantified using ImageJ (NIH). Soluble and insoluble fractions of homogenates were separated according to the previously described method (21). SOD1 enzymatic activity was assayed with a native-PAGE gel according to the method reported previously (49). One hundred micromgrams of the supernatant protein prepared above were used for the assay.

Table 3. Onset, lifespan and disease duration in each line of H46R and H46R/TRX1 mice

<table>
<thead>
<tr>
<th>Line</th>
<th>Onset time (days)</th>
<th>Lifespan (days)</th>
<th>Duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H46R (♂5, ♀6)</td>
<td>157.7 ± 7.2</td>
<td>188.2 ± 5.0</td>
<td>30.5 ± 6.8</td>
</tr>
<tr>
<td>H46R/TRX1 (♂4, ♀10)</td>
<td>156.6 ± 4.1</td>
<td>181.0 ± 9.8</td>
<td>24.4 ± 7.0</td>
</tr>
</tbody>
</table>

The the onset of the disease phenotype was determined as described in the ‘Materials and methods’. The numbers in parentheses indicate the analyzed number of mice in each line. No statistically significant difference was observed between H46R and H46R/TRX1 mice by two-tailed t-test.
Immunoprecipitation

Immunoprecipitation of misfolded SOD1 was performed with the established antibody according to the protocol (33) with minor modifications. Briefly, spinal cords were homogenized in TNG-T lysis buffer [50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 10% glycerol, 1% Triton-X] containing protease inhibitor cocktail and centrifuged for 10 min at 9000 g, 4°C. Afterwards, 300 µg of supernatant was incubated with 1 µl of anti-misfolded SOD1 antibody (C4F6) overnight at 4°C; the protein was captured by adding 20 µl of Dynabeads Protein G (Life Technologies) and incubated for 2 h at 4°C. The beads were washed three times with 50 mM Tris–HCl (pH 7.4) and 100 mM NaCl, and the protein was extracted by boiling for 10 min with 60 µl of SDS loading buffer. Fifteen microliters of the precipitated samples were subjected to immunoblot of anti-SOD1 antibody (SOD-100, Enzo Life Sciences) in the same procedure as above, except that 100 µg of supernatant was used.

Immunoprecipitation of whole SOD1 was done with rabbit anti-SOD1 antibody (SOD-100, Enzo Life Sciences) in the same procedure as above, except that 100 µg of supernatant was used.

Histological examinations

TA muscles were dissected from mice and rapidly frozen in 2-methylbutane iced with liquid nitrogen. Mice were then fixed by transcardiac perfusion of 4% paraformaldehyde/PBS. Lumbar spinal cords were removed, post-fixed for 72 h in 4% paraformaldehyde and cryoprotected in 30% sucrose at 4°C. Twenty-micrometer sections were cut on a cryostat (HM 550, Carl Zeiss Microscopy). Sections of TA muscles were stained with hematoxylin and eosin (H & E). For motor neuron counts, every fifth lumbar spinal cord cross-section was stained with 1% cresyl violet. The number of neurons having >200 µm² cell bodies with a nucleus and nucleolus was counted.

Immunohistochemistry, the following primary antibodies were used: mouse SMI-32 (SMI-32P, 1:1000 dilution, Covance); rabbit anti-Iba1 (019–19741, 1:1000 dilution, Wako); mouse anti-GFAP (MAB3402, 1:1000 dilution, EMD Millipore); rabbit anti-SOD1 (SOD-100, 1:1000 dilution, Enzo Life Sciences); mouse anti-misfolded SOD1 (D3H5, 1:500 dilution, EMD Millipore); rabbit anti-SOD1 (SOD-19741, 1:1000 dilution, Wako); mouse anti-GFAP (MAB3402, 1:1000 dilution, EMD Millipore); rabbit anti-SOD1 (SOD-100, 1:1000 dilution, Enzo Life Sciences); mouse anti-misfolded SOD1 (D3H5, 1:500 dilution); mouse anti-human TRX1 (37); rabbit anti-class III β-tubulin (PRB435-P, 1:2000 dilution, BioLegend); and rabbit anti-glutamine synthetase (G2781, 1:1000 dilution, Enzo Life Sciences). The number of neurons with >200 µm² cell bodies with a nucleus and nucleolus was counted.

Statistical analyses

Results are shown as mean ± SD. Differences between groups were examined for statistical significance using two-tailed t-test except that the differences of the onset and survival probabilities were determined by log-rank test using Statcel3 (OMS Ltd.).

Supplementary Material

Supplementary material is available at HMG online.

Conflict of Interest statement. None declared.

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References


or its derived Fab fragment against misfolded forms of SOD1 mutant delays mortality in a mouse model of ALS. 


