Chapter 5

Role of Cysteine Residue of Mutant Cu, Zn-Superoxide Dismutase (SOD1) in the Pathogenesis of Amyotrophic Lateral Sclerosis (ALS)

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Abstract

Mutations of *Cu, Zn-superoxide dismutase (SOD1)* gene have been identified in a subset of familial amyotrophic lateral sclerosis (ALS). Conformational change, that is, misfolding, of mutant SOD1 underlies its toxic gain of function for motor neuronal degeneration. Mutant SOD1 is prone to cause oxidative stress through the copper exposed on the protein by misfolding. The protein structure of SOD1 is critically affected by the redox state of cysteine residues, especially of Cys111. Oxidative modification of Cys111, which is enhanced in mutant SOD1, causes destabilization of the dimer interface to promote misfolding and aggregation of the protein. Substitution of Cys111 to serine alleviated the degeneration of motor neurons as well as the misfolding and aggregate formation of mutant SOD1 in the spinal cord of transgenic mice. It indicates that Cys111 is a crucial residue for the pathogenesis of ALS by mutant SOD1.

Keywords: amyotrophic lateral sclerosis, Cu, Zn-superoxide dismutase, cysteine residues, oxidative stress, monomerized SOD1

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal degenerative disease of motor neurons, which affects skeletal muscle strength of the whole body. About 10% of ALS cases are affected in a familial trait, 25–30% of which are caused by mutations of *Cu, Zn-superoxide dismutase (SOD1)* gene [1]. Although many causative genes of ALS have been identified so far, *SOD1* is still the second frequent responsible gene for ALS next to *C9orf72* [2]. Since the identification of the gene in 1993, research emphasis for ALS has been placed on uncovering the pathogenic

mechanism of motor neuronal death by the disease-causing mutant SOD1. We review the recent concept of neuronal toxicity by mutant SOD1 in relation to the posttranslational modification of SOD1 at cysteine residues, especially at Cys111, which is closely related to its conformational change, in ALS pathogenesis.

2. Conformational change and copper-mediated oxidative toxicity of mutant SOD1

SOD1 is a metal-binding antioxidant enzyme expressed ubiquitously in the body, and it functions to convert prooxidant superoxide anions to hydrogen peroxide and oxygen to remove the oxidative stress [3]. SOD1 forms a homodimer to accomplish its full enzymatic activity. Each subunit binds one atom each of copper and zinc. Copper works for the enzymatic activity, whereas zinc has an important role in maintaining the stable structure of SOD1 protein.

More than 150 different mutations of *SOD1* gene have been found in familial ALS patients so far, and they are scattered throughout the entire coding sequence of the gene regardless of specific functional domains. The enzymatic activity of mutant SOD1 is not necessarily reduced compared to that of wild-type SOD1 [4]. Moreover, mice deficient of *Sod1* gene do not cause symptoms of motor neuronal dysfunction, whereas transgenic mice that express mutant SOD1, but not wild-type SOD1, develop progressive motor paralysis and degeneration of motor neurons depending on the expression level of mutant SOD1 in the spinal cord [5, 6]. It means that the pathogenic mechanism of mutant SOD1 for the degeneration of motor neurons is derived from the gain of a specific aberrant function rather than a decrease in the enzyme activity of the protein.

What is the determinant of motor neuronal toxicity by mutant SOD1? Although the nature of mutant SOD1 toxicity has not been fully elucidated, conformational abnormality, that is, misfolding, of mutant SOD1 protein is deeply involved in the pathogenesis of familial ALS [7]. Misfolded SOD1 is subject to dissociate into monomers, which binds together abnormally to form oligomers or further high molecular weight aggregates in cells. Abnormal protein accumulation in neurons can impair their important cellular functions such as axonal transport [8] and degradation machinery of proteins [9]. SOD1-positive inclusion bodies have been actually detected in degenerating motor neurons in the spinal cords of familial ALS patients and mutant SOD1 transgenic mice [10, 11]. Therefore, it is important to understand the common mode of conformational change in mutant SOD1, which will lead to suppress the onset of ALS derived from SOD1 mutations.

Once SOD1 is misfolded, copper or zinc bound to the subunit of SOD1 is also prone to be exposed from the compact dimer structure. Because copper is catalytically redox-active, abnormal chemical reactions can occur to generate reactive oxygen (ROS) or nitrogen (RNS) species apart from the original SOD1 activity. Mutant SOD1, unlike wild-type SOD1, has a potential to generate ROS and RNS such as hydroxyl radicals [12, 13] and peroxynitrite [14] in

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a copper-dependent manner *in vitro*, which can be inhibited by copper chelators in cultured cells [15].

We first thought that mutant SOD1 is involved in the degeneration of motor neurons by causing oxidative stress through an adverse enzymatic reaction with copper on the protein, and we examined therapeutic effects of G93A mutant SOD1 transgenic mice by removal of the oxidative stress. As a result, a copper-chelating agent trientine and an antioxidant ascorbic acid showed a protective effect either alone, yet a higher beneficial effect was achieved by the combined use of these reagents [16, 17]. Furthermore, to confirm the validity of this hypothesis, the mutant SOD1 mice were bred with metallothionein I/II-deficient mice and the impact of the gene on motor paralysis was analyzed. Metallothionein is an endogenous protein that binds copper to prevent it from being prooxidant in cells. The decrease or halt of metallothionein I/II expression exacerbated the ALS symptoms in a gene dosage-dependent manner [18]. Copper-mediated toxicity in mutant SOD1 was also reinforced with other reports that decreasing intracellular copper, by treatment with copper chelators or by genetic reduction of copper uptake, alleviated ALS phenotype in mutant SOD1 transgenic mice [19–21]. Moreover, the upregulation of metallothionein expression has been shown to attenuate the disease course in mice [22, 23]. These data suggest that copper-mediated oxidative chemistry underlies the pathogenesis of familial ALS linked to mutations of *SOD1* gene, possibly triggered by misfolding of the mutant protein.

3. Conformational change and oxidation of cysteine residues in mutant SOD1

The subunit structure and dimer formation of SOD1 is critically affected by the binding state of copper and zinc, as well as the redox state of cysteine residues in the protein [24]. Human SOD1 has four cysteine residues (Cys6, Cys57, Cys111, and Cys146) in a subunit. Cys57 and Cys146 form an intra-subunit disulfide bond that maintains the rigid structure and enzymatic activity of SOD1 protein, whereas Cys6 and Cys111 are present in a reduced state having free sulfhydryl groups. Cys6 is deeply buried in the core of the subunit and less accessible by other molecules, while Cys111 is located on the protein surface. The intra-subunit Cys57-Cys146 disulfide bond of SOD1 is physiologically formed by copper chaperone for SOD1 (CCS) coupled with copper incorporation into the enzymatic active site, meaning that the metal coordination and disulfide formation are mechanistically related to each other for the conformation of SOD1 protein [25]. Reduction of the Cys57-Cys146 disulfide bond and/or deprivation of metals make human wild-type SOD1 liable to misfold, resulting in monomerization [26].

Modification of amino acid residues, especially by oxidative stress, can be a critical factor to enhance the misfolding of proteins [27]. Cysteine is in particular susceptible to oxidative modification, since its sulfhydryl moiety is readily attacked by redox-active substrates such as glutathione or peroxides to form S-S or S-O covalent modification. Sulfhydryl groups also crosslink each other to form intra- or intermolecular disulfide bond, which have important roles to maintain or disrupt physiological conformation of proteins.

Oxidative reactivity and modification of Cys111, such as glutathionylation [28, 29] and peroxidation [30], is documented with human or chick wild-type SOD1. Because Cys111 is located on the edge of the dimer interface of each subunit, the modification of Cys111 can interrupt the dimer contact at the interface stereochemically and cause the dissociation of SOD1. Molecular dynamic simulations of SOD1 imply that the region including Cys111 is important for the residue interaction network in the protein and is likely to affect the dimer interface through the network and may disrupt their coupled motions [31]. Indeed, it was noted *in vitro* that the Cys111 modification caused wild-type SOD1 liable to monomerize and decrease its enzymatic activity [32]. On the other hand, substitution of Cys111 to serine (C111S) is known to increase the structural stability and resistance to heat inactivation of wild-type SOD1 [33], also implying that the mode of Cys111 may regulate the conformational state of SOD1.

Changes in the redox state of cysteine residues have been reported in ALS-linked mutant SOD1. Mutant SOD1 exhibits aberrant vulnerability to mild reducing conditions, which cleave the intra-subunit Cys57-Cys146 disulfide bond to destabilize the SOD1 dimer [34]. The dimer dissociation results in the exposure of the hydrophobic region of the SOD1 subunit and promotes aggregation of the protein [35]. Alternatively, insoluble mutant SOD1 oligomers can be formed by crosslinking via inter-subunit disulfide bonds at Cys57 and Cys146 [26] or by disulfide scrambling of all four cysteine residues [36]. Such insoluble SOD1 oligomers were also detected in the spinal cord of mutant SOD1 transgenic mice in parallel to the disease onset [37]. These oligomers were mostly reversed by a reducing reagent, supposing that disulfide-mediated crosslinking at cysteine residues is a major factor for mutant SOD1 to form aggregates and ALS phenotype. Conversely, replacement of cysteine residues, especially of Cys6 and Cys111, decreased disulfide-crosslinked mutant SOD1 oligomers and aggregate formation, and improved cell viability in cultured cells [38, 39]. Glutaredoxins, which specifically catalyze the reduction of protein-SSG-mixed disulfides, significantly increased the solubility of mutant SOD1 and protected neuronal cells [39, 40]. On the other hand, the intermolecular disulfide binding at cysteines is shown to have a limited effect on the aggregation of mutant SOD1 [41].

With regard to Cys111, posttranslational modifications of Cys111 per se are also known in mutant SOD1. The change of the protein structure, which would affect the hindrance of Cys111 near the dimer interface, can enhance oxidative modification of Cys111 at the sulfhydryl moiety by substrates in mutant SOD1. Mutant SOD1 is commonly glutathionylated at Cys111 [42], and Cys111-peroxidized SOD1 is detected in the inclusion bodies of spinal motor neurons in G93A mutant SOD1 transgenic mice [30]. Those indicate the pathogenic significance of Cys111-oxidized SOD1 for misfolding and aggregation to acquire neuronal toxicity. Moreover, even in the spinal cord of sporadic ALS patients without SOD1 mutation, misfolded SOD1 deposits have been detected and the SOD1 species are peroxidized at Cys111, indicating that misfolding and aggregation of wild-type SOD1 may also be a factor in the pathogenesis of sporadic ALS [43]. However, in the vast majority of sporadic ALS, an RNA-binding protein TDP-43 is well known to mislocalize from the nucleus and deposit in the cytoplasm [44, 45]. SOD1 does not interact or co-localize with TDP-43 in general in such cases [46], which is

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inconsistent with the hypothesis mentioned above. Although the involvement of Cys111-mediated misfolded SOD1 may be limited in sporadic ALS, the theory is attractive and further investigation will be needed.

4. Molecular link between copper-mediated chemistry and cysteine oxidation in mutant SOD1

Then, what is the molecular mechanism by which mutant SOD1 causes the copper-mediated oxidative stress in relation to misfolding of the protein? In case that mutant SOD1 enhances an aberrant side reaction through the discoordinated copper due to the abnormality of the protein structure, we can develop treatment strategies by identifying the responsible site and its conformational state in the protein. To clarify a possible aberrant interaction of mutant SOD1 with copper, we fractionated cell lysates from the spinal cord of SOD1 transgenic mice and SOD1 expressing cultured cells by immobilized metal affinity chromatography (IMAC), a method that separates proteins based on their affinities with an immobilized metal such as copper [47]. Mutant SOD1 was eluted commonly in an aberrant fraction with high affinity for copper, in addition to that with low affinity for copper seen in wild-type SOD1 as well. Considering that mutant SOD1 is separated into two distinct fractions and the interaction of proteins on IMAC is determined by topology of metal-coordinating residues on solvent-facing surfaces [48], conformational transition from the native to non-native state is implicated in the high-affinity fraction for the copper of mutant SOD1.

Therefore, we further analyzed mutant SOD1 in the high-affinity fraction for copper to know its biochemical characteristics compared to that in the low-affinity fraction. Existence of Cys111 was critical to the appearance of the high-affinity fraction species, and mutant SOD1 was in a monomer state and oxidatively modified at Cys111 in this fraction [49]. Peroxidation of wild-type SOD1 forced by oxidants such as hydrogen peroxide made it to monomerize and generate the high-affinity fraction species by copper IMAC. Furthermore, these mutant SOD1 and Cys111-peroxidized wild-type SOD1 showed higher thiol oxidase activity, an adverse side activity reported in SOD1 [50], than untreated wild-type SOD1. These results indicate that mutant SOD1 is labile to be monomerized by peroxidaton of Cys111 near the dimer interface, which will expose the copper of the protein and raise its reactivity to cause oxidative stress, and eventually forms intracellular aggregates or inclusion bodies to cause neurodegeneration (**Figure 1**).

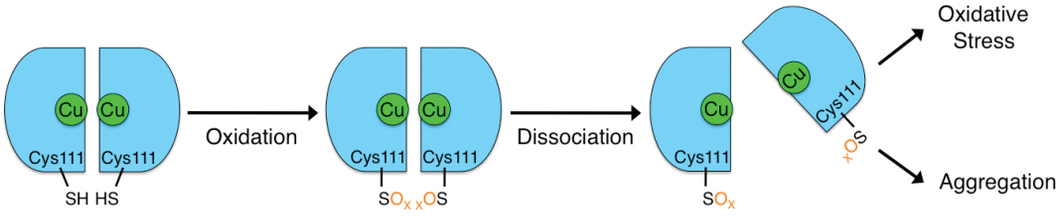

Figure 1. Proposed model of mutant SOD1 toxicity.

Oxidation of Cys111 leads mutant SOD1 dimers to dissociate into monomers, which causes oxidative stress through the copper and aggregate formation of the monomers.

5. Role of cysteine-mediated conformational change of mutant SOD1 in ALS pathogenesis

Although we determined the importance of oxidative modification of Cys111 for conformational change and copper-mediated aberrant chemistry of mutant SOD1, it has not been proved whether the modification of Cys111 is actually relevant to the pathogenesis of ALS by mutant SOD1 *in vivo*. Transgenic mice of mutant SOD1 that has simultaneous substitutions of all metal-coordinating residues and free cysteines (Cys6 and Cys111) were generated and had no pathology or motor symptoms [51]. However, it is difficult to confirm the direct contribution of Cys111 in these mice. To verify the importance of Cys111 solely, we conducted the study of transgenic mice expressing ALS-linked mutant SOD1 (H46R) with or without substitution of Cys111 to serine [52]. Both lines of transgenic mice (H46R and H46R/C111S SOD1 mice) were created, respectively, and were observed whether to obtain motor paralysis and the degeneration of motor neurons.

As a result, H46R SOD1 mice developed motor paralysis most quickly at 5 months of age, and reached the lifetime at 6 months of age. On the other hand, the onset of motor paralysis in H46R/C111S SOD1 mice was late at about 12 months of age, and their lifetime was extended to about 14 months after birth (**Figure 2**). Disease duration from the onset to lifetime was also significantly prolonged in H46R/C111S SOD1 mice than that in H46R SOD1 mice. The number of spinal motor neurons was decreased and the tibialis anterior muscle was atrophic at the time of endpoint in H46R SOD1 mice, while these indexes were preserved at the same age of H46R/C111S SOD1 mice. Activation of astrocytes and microglia, the phenomenon seen in the spinal cord of other mutant SOD1 transgenic mice as a modifying factor of neurodegeneration [53, 54], was also observed at endpoint in the spinal cord of H46R SOD1 mice, whereas it was not apparent in H46R/C111S SOD1 mice at the same time point.

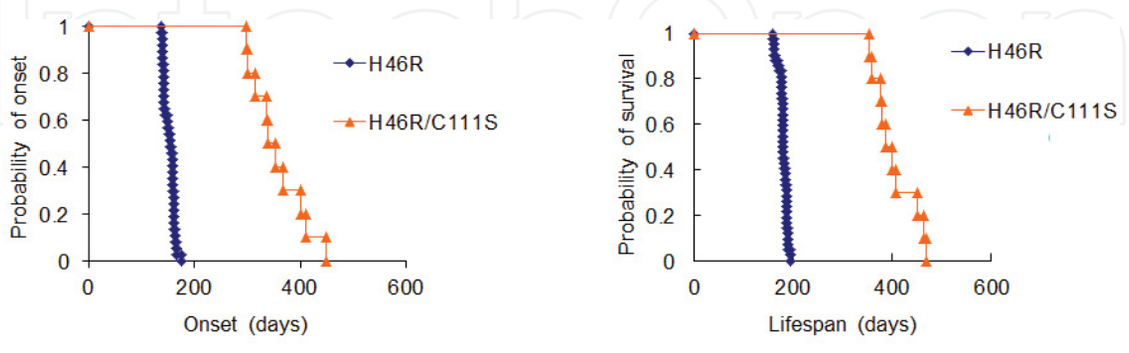

Figure 2. Kaplan-Meier curves of the onset and lifespan of H46R and H46R/C111S SOD1 mice.

Both the time at onset and lifespan were significantly extended in H46R/C111S SOD1 mice compared to those in H46R SOD1 mice.

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Next, we examined the redox state of Cys111 in SOD1 and the presence of misfolded/insoluble SOD1 in the liver and spinal cord of these mice as well as of wild-type SOD1 transgenic mice. Cys111-peroxidized SOD1 was detected in H46R SOD1 mice from the early presymptomatic age regardless of organs, although it was trivial in wild-type SOD1 transgenic mice. On the other hand, misfolded and insolubly aggregated SOD1 was found only in the spinal cord in parallel to the disease onset of the mice. The SOD1 species was not seen at the same age of H46R/C111S SOD1 mouse; however, it was observed in the same way as H46R SOD1 mice at endpoint. These results indicate that mutant SOD1 is more prone to be attacked at Cys111 by oxidants than wild-type SOD1 due to a slight structural difference, and peroxidation of Cys111 is important to push the mutant SOD1 into misfolding at the early phase of the disease. However, considering that misfolding and aggregation of Cys111-peroxidized mutant SOD1 are defined to the spinal cord, other factors may exist to enhance or suppress the misfolding of the SOD1 in the spinal cord or liver, respectively. In fact, the expression of an important protective factor, for example, heat shock factor-1, is reported to be relatively low in motor neurons [55]. The difference in clearance efficiency of the SOD1 protein in each organ or cell type may also explain the specificity of mutant SOD1 misfolding and aggregation.

As mentioned before, insoluble high molecular weight species of mutant SOD1 is likely to consist of aggregates crosslinked with inter-subunit disulfide bonds of cysteine residues including Cys111. To verify the significance of this phenomenon in our ALS model, we analyzed the reactivity of the insoluble aggregates to a reducing reagent in the spinal cord of H46R SOD1 mice. The majority of the insoluble aggregates were maintained even in the presence of the reagent. We further examined H46R SOD1 mice by mating with thioredoxin 1 transgenic mice, to see whether the motor symptoms could be alleviated according to inhibition of the disulfide bond-mediated SOD1 crosslinking. Thioredoxin 1 is an antioxidative protein present in the cytoplasm as well as SOD1, and has an effect to revert oxidatively formed protein disulfide bonds to sulfhydryls by reducing reaction. We did not see any change in the course of motor paralysis, decrease of motor neurons, glial activation, or deposition of SOD1 aggregates in H46R SOD1 mice with hemizygous thioredoxin 1 transgenic background. No suppressive effect of the disease was observed even in the SOD1 mice with homozygous thioredoxin 1 transgenic background, excluding the possibility that the expression level of thioredoxin 1 was insufficient to have the effect (Nagano S, unpublished data). It indicates that the involvement of inter-subunit disulfide bonds of cysteine residues may be limited in our H46R mutant SOD1 disease model. More intense study will be needed in other mutant SOD1 mouse models to know the role of inter-subunit disulfide crosslinking in the mutant SOD1 neurotoxicity.

6. Conclusions

We have shown that Cys111 drives the pathogenicity of mutant SOD1 by demonstrating that the substitution of a single residue in mutant SOD1 significantly reduces the disease phenotype of ALS model mice. Cys111 of mutant SOD1 is peroxidized and promotes misfolding of the protein to generate reducing reagent-resistant, high molecular weight insoluble SOD1 species.

It is promising to create a new therapeutic strategy for mutant SOD1-related ALS by developing reagents that inhibit the modification of Cys111 or subsequent monomerization of the mutant SOD1. Dimedone, a trapping reagent of sulfenylated (-SOH) cysteines to block further peroxidation [56], or bis-maleimidoethane, a crosslinker that is shown to crosslink between Cys111 of each SOD1 subunit to inhibit monomerization [49], may be candidates, but the problem is that these reagents have no specificity for SOD1. The reagent that binds specifically to a pocket of SOD1 dimer interface was developed by in silico drug screening approach and had a suppressive effect for monomerization of mutant SOD1 *in vitro* [57]. Clinical application will be achieved by developing a drug having higher effect and permeability into the central nervous system using the reagent as a lead compound. Alternatively, considering that misfolded wild-type or mutant SOD1 is likely to be propagated to the neighboring neurons [58] to cause further misfolding of SOD1 and the spread of the disease, antibody therapy targeting Cys111 or dimer interface of SOD1 may also be effective to inhibit the progress of ALS.

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