This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2003)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2003 Term

Instructors:
GARRY R. BUETTNER, Ph.D.
LARRY W. OBERLEY, Ph.D.

with guest lectures from:
Drs. Freya Q . Schafer, Douglas R. Spitz, and Frederick E. Domann

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ALS

by

Sharon Hu

B-180 Medical Laboratories
Free Radical and Radiation Biology Program
The University of Iowa
Iowa City, IA 52242-1181

For 77: 222, Spring 2003

May 8, 2003
Paper V

Abbreviations

AGE: advanced glycation end products
ALE: advanced lipoxidation end products
ALS: amyotrophic lateral sclerosis
CML: carboxymethyl-lysine
EAAT: excitatory amino acid transporter
FALS: familial amyotrophic lateral sclerosis
HNE: 4-hydroxy-2-nonenal
IF: intermediate filaments
ICE: Caspase-1
MDA: malondialdehyde
NFH: heavy neurofilaments
NFL: light neurofilaments
NFM: middle neurofilaments
SALS: sporadic amyotrophic lateral sclerosis
SOD1: Cu,Zn superoxide dismutase

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Abstract

Amyotrophic lateral sclerosis (ALS), is also called Lou Gehrig's disease or Charcot's disease. In ALS, there is degeneration of the motor neurons of the spinal cord, brain stem, and cerebral cortex. ALS has a prevalence of 2 ~3 per 100,000 people, and is generally fatal within 1 ~5 years of onset. Cu, ZnSOD (SOD1) is an antioxidant enzyme that primarily catalyzes the dismutation of superoxide anions ($O_2^{\bullet-}$) to oxygen (O_2) and hydrogen peroxide (H_2O_2). Mutations at various sites in SOD1 may open the loops that expose the active site to reaction with other oxidants, to generate higher oxidants, or to react with peroxynitrite to form nitrated tyrosines. More than 90 mutations are known. In this report, I review the current hypotheses on mechanisms of ALS disease with emphasis on toxicity of SOD1 mutants.

Introduction

"Amyotrophic" refers to the muscle atrophy, weakness, and fasciculation that signify disease of the lower motor neurons. "Lateral sclerosis" refers to the hardness to palpation of the lateral columns of the spinal cord in autopsy specimens [1]. The pathological hallmarks of amyotrophic lateral sclerosis (ALS) are degeneration and loss of motor neurons with astrocytic gliosis. Mitochondrial abnormalities and fragmentation of the Golgi apparatus were found in patients with ALS. Also, intraneuronal inclusions are seen in degenerating neurons and glia [1]. Table 1 shows possible therapies for ALS. Riluzole prolonged survival by three to six months in two therapeutic trials [1]. The efficacy of riluzole supported the excitotoxic-glutamate theory of the pathogenesis of ALS [1]. Gabapentin, like riluzole, extended survival but did not significantly affect the onset of clinical disease [1]. In contrast, vitamin E delayed the onset and the progression of the disease but failed to extend survival [1].

Class	Drug or Preparation
Glutamate antagonists	Riluzole*
	Lamotrigine†
	Dextromethorphan†
	Gabapentin†
	Branched-chain amino acids†
Antioxidants	Vitamin E†
	Acetylcysteine†
	Selegiline†
	Creatine‡
	Selenium
	Coenzyme Q10‡
Neurotrophic factors	Brain-derived neurotrophic factor†
	Insulin-like growth factor 1†
	Glial-derived neurotrophic factor†
	Xaliproden‡
	Thyrotropin-releasing hormone†
Immunomodulatory agents or approaches	Gangliosides
	Interferon
	Cyclophosphamide†
	Plasmapheresis
	Intravenous immune globulin
	Levamisole†
	Transfer factor†
Antiviral agents	Amantadine†
	Tiloronе†
Other agents	Snake venom

Table 1. The therapy for ALS [1].

ALS is subclassified into sporadic ALS (SALS) (90~95%), familial ALS (FALS) (5~10%) and western Pacific (the rest) forms. FALS includes some variants linked to chromosomes 21q22.2-22.2 (autosomal dominant), 2q33-35 (autosomal recessive), 9q34 (autosomal dominant) and 15q12-21 (autosomal recessive), and sex chromosome (X-linked dominant). Of these variants, the chromosome 21-linked variant has been investigated most intensively, because this variant is associated with mutations in the Cu,Zn superoxide dismutase (SOD1) gene [2].

SOD1 mutants

Table 2 shows the antioxidant and prooxidant reactions of wtSOD1. wtSOD1 is an antioxidant enzyme that primary catalyzes the dismutation of superoxide anions ($O_2^{\bullet -}$) to oxygen (O_2) and hydrogen peroxide (H_2O_2). In addition to this dismutase activity, wtSOD1 also exhibits prooxidant activities, which include the peroxidase activity, hydroxyl radical generating activity and nitration of tyrosine. wtSOD1 increases oxidative injury by mechanisms involving the increased generation of hydroxyl radical, increased peroxidase activity, or increased nitration of tyrosine by peroxynitrite [3].

Table 2. Free radical reactions of wtSOD1 [3].

Dismutase activity

1 $SOD-Cu^{2+} + O^{\cdot}_2 \rightarrow SOD-Cu^{+} + O_2$

2 $SOD-Cu^{+} + O^{\cdot}_2 \rightarrow SOD-Cu^{2+} + H_2O_2$

3 $O^{\cdot}_2 + O^{\cdot}_2 + 2H^{+} \xrightarrow{SOD} H_2O_2 + O_2$

Peroxidase activity

4 $SOD-Cu^{2+} + H_2O_2 \rightleftharpoons SOD-Cu^{+} + O^{\cdot}_2 + 2H^{+}$

5 $SOD-Cu^{+} + H_2O_2 \rightarrow SOD-Cu^{2+}-{}^{\cdot}OH + OH^{-}$

6 $SOD-Cu^{2+}-{}^{\cdot}OH + His\text{-}61 \rightarrow His^{\cdot} + SOD-Cu^{2+} + H_2O$

7 $SOD-Cu^{2+}-{}^{\cdot}OH + HCO_2^{-} \rightarrow SOD-Cu^{2+} + CO^{\cdot}_2 + H_2O$

8 $SOD-Cu^{2+}-{}^{\cdot}OH \rightarrow$ inactive SOD ($Enz-Cu^{2+}$ + 2-oxohistidine)

Hydroxyl radical generating activity

9 $Enz-Cu^{2+} + H_2O_2 \rightarrow Enz-Cu^{+} + O^{\cdot}_2 + 2H^{+}$

10 $Enz-Cu^{+} + H_2O_2 \rightarrow Enz-Cu^{2+} + {}^{\cdot}OH + OH^{-}$

Nitration of tyrosine

11 $SOD-Cu^{2+} + ONOO^{-} \rightarrow SOD-CuO...NO_2^{+}$

12 $SOD-CuO...NO_2^{+} + Tyr \rightarrow SOD-Cu^{2+} + OH^{-} + NO_2\text{-}Tyr$

The SOD1 polypeptide contains 153 amino acids. X-ray crystallographic studies revealed that the active channel of SOD1 is positively charged with amino acid residues, Arg-141 inside and Lys-120 and Lys-134 at the top of the channel. The positively charged channel is responsible for favorable electrostatic guidance to the active site for the anionic substrate $O_2^{\bullet-}$ to yield the usually rapid catalytic rate of superoxide dismutation [4].

Figure 1 shows the mutations in the SOD1 polypeptide known to cause ALS. The positions of these mutations are scattered throughout the protein - at turns of the β-barrel, some are within β-sheets of the β-barrel, within the active site channel, at the dimer interface and at copper-coordinating residues in the active sites. These mutants have different SOD1 activity levels. For example, G93A has elevated SOD1 activity, G37R retain full specific activity, and G85R has low SOD1 activity [5].

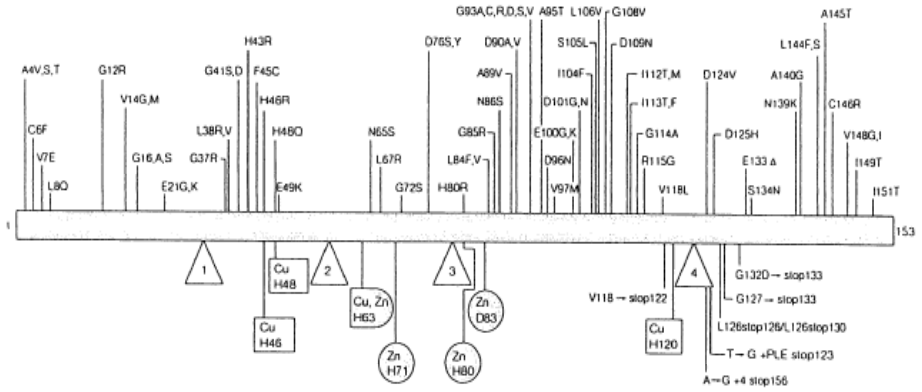

Figure 1. ALS-causing mutations lie throughout the SOD1 polypeptide [5].

Mechanism of toxicity

Proposed mechanisms of motor neuron degeneration such as oxidative injury, peroxynitrite toxicity, cytoskeletal disorganization, glutamate excitotoxicity, disrupted calcium homeostasis, SOD1 aggregation, carbonyl stress, as shown in figure 2 [2].

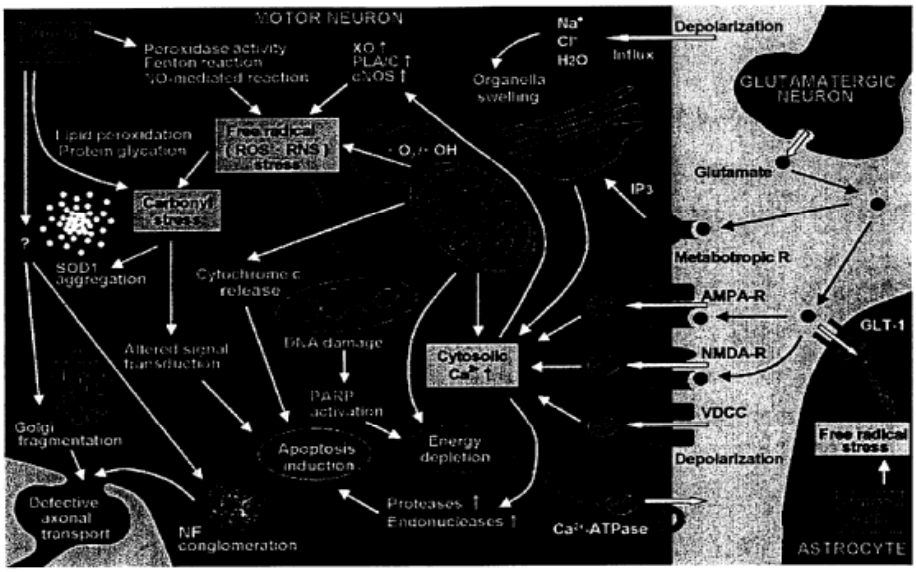

Figure 2. Proposed mechanisms of motor neuron degeneration with SOD1 mutation [2].

Oxidative injury and peroxynitrite toxicity

Increased hydroxyl radical $HO^{\bullet}$ generation may occur as a consequence of enhanced peroxidase activity or decreased Cu-binding affinity of mutant SOD1 [2]. Normally, SOD1 catalyzes the conversion of toxic $O_2^{\bullet-}$ to H_2O_2. Mutations in SOD1 gene may reverse this reaction, leading to the production of toxic $HO^{\bullet}$, or promote the use of other abnormal substrates such as peroxynitrite ($ONOO^-$), ultimately leading to the aberrant nitration of tyrosine residues (Tyr) in proteins, as shown in Figure 3 [1].

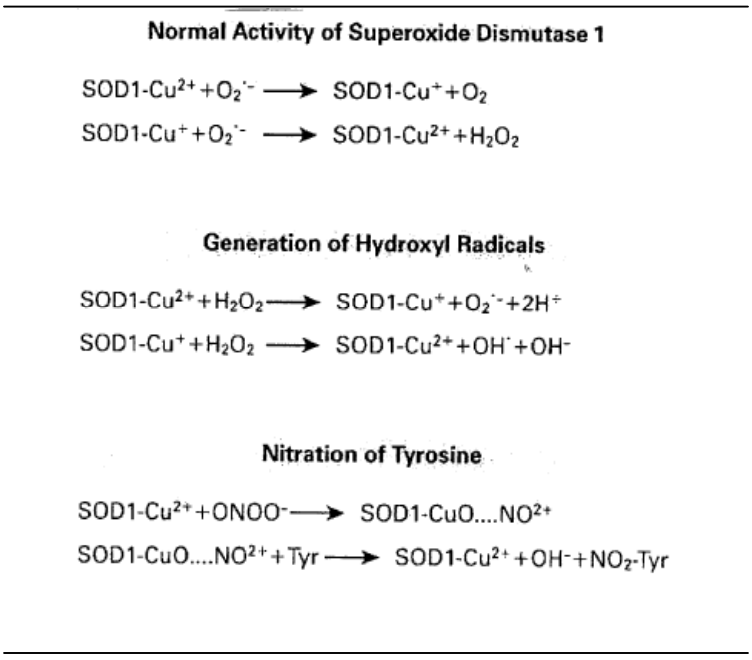

Figure 3. Copper-mediated oxidative reactions catalyzed by SOD1 [1].

Some investigators disagree with a potential implication for oxidative damage to the motor neurons in SOD1 mutant subjects, because there is no increased $HO^{\bullet}$ generation in G37R mice, and no efficacy of anti-oxidant N-acetylcysteine in G93A mice. Also, there is no accumulation of 8-hydroxy-2′-deoxyguanosine as one of the oxidative modification products of DNA in the nucleus of the motor neurons in ALS patients. Some studies on mutant SOD1 mice showed increased tyrosine levels in the cytoplasm of the motor neurons. The nitrotyrosine accumulates in these mice is a free form, not a protein bound form, so the target protein of the tyrosine nitration needs to be identified [2].

Cytoskeletal disorganization

Neurofilaments are intermediate cytoskeletal fibrils composed of heavy (NFH, 115kDa), middle (NFM, 90 kDa) and light (NFL, 61 kDa) subunits. Phosphorylated neurofilaments often accumulate in the chromatolytic neurons and swollen axons. There may be certain interactions of mutant SOD1 with NFL subunit, because knockout of the endogenous wt NFL subunit in G85R mice delays the onset of motor neuron disease [2]. In addition to mutant mutant SOD1, identified deletions or insertions of NFH also found in some ALS cases, as shown in Figure 4. The known neurofilament sequence variants are not capable of provoking disease. Thus, the variants in neurofilaments are risk factors for sporadic disease [6].

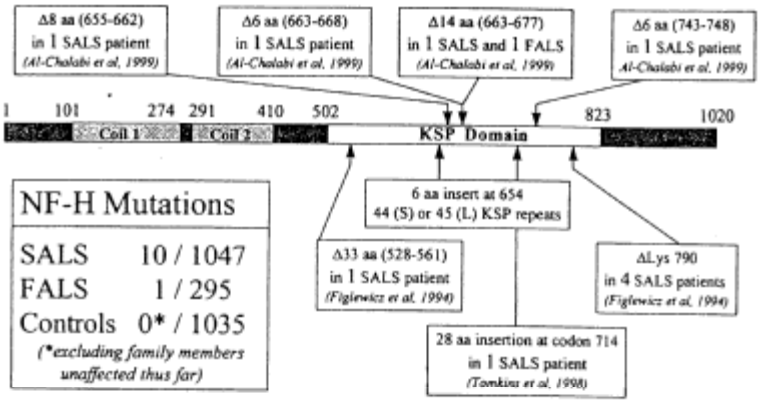

Figure 4. Mutations in SALS and FALS in the gene encoding the large neurofilament subunit NFH [6].

In both SALS and FALS, abnormal intermediate filaments (IF) accumulations are also found in the perikaryon and axon of motor neurons. Figure 5 shows different types of IF inclusions

with disparate properties. The overexpression of wt NFH proteins in mice induces the formation of large neurofilament accumulations in the motor neuron, but such perikaryal swellings don't cause cell death. The overexpression of peripherin transgenes caused motor neuron death in a contex of NFL deficiency. Thus, the presence of abundant IF inclusions in motor axons could impede axonal transport [7].

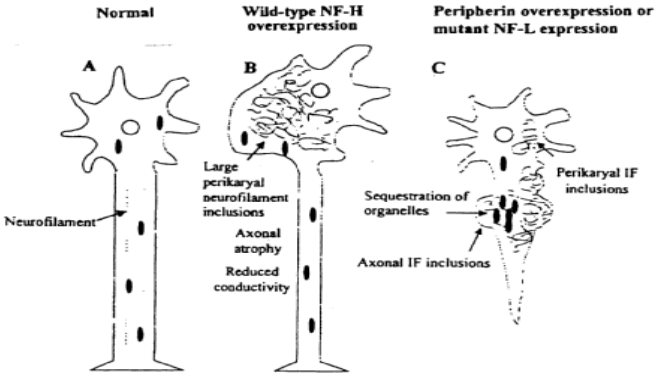

Figure 5. Different types of IF inclusions with disparate properties [7].

Glutamate excitotoxicity and disrupted calcium homeostasis

Glutamate-induced excitoxicity is another potential contributor to ALS. Excess activation of neuronal glutamate receptors can cause cell death via alternations in cytosolic free Ca^{2+} homeostasis. After activation of neuronal glutamate receptors, depolarization of the neuronal membrane activates voltage-dependent Ca^{2+} channels, allowing Ca^{2+} entry into the cell. Loss of glutamate transporter EAAT2 could lead to increased extracellular concentrations of glutamate and excitotoxic degeneration of motor neurons. The majority of SALS (~ 65%) have a reduction

in the astroglial EAAT2 in motor cortex and spinal cord. The glutamate-induced excitotoxicity resulting from the loss of astroglial EAAT2 may be a secondary effect, because germline mutations in the EAAT2 gene are very rare and they do not explain the existence of variant EAAT2 mRNAs in ALS [7].

Increased cytosolic Ca^{2+} levels activate autotoxic enzymes such as xanthine oxidase, phospholipase A_2/C, constitutive Ca^{2+} / calmodulin-dependent NO synthase, proteases, and endonucleases. Xanthine oxidase and phospholipase produce $O_2^{\bullet-}$, which forms $HO^{\bullet}$ via Fenton reactions and react with $NO^{\bullet}$, then producies $ONOO^-$. An excess of $ONOO^-$ may overproduce NO_2 and $HO^{\bullet}$ and promote tyrosine nitration as mentioned before. The highly reactive $HO^{\bullet}$ attacks nucleic acid, protein and lipid. Activated endonucleases also cause DNA fragmentation. Thus, increased cytosolic Ca^{2+} concentration mediates cell death [2].

SOD1 aggregation

Mutant SOD1 toxicity may result from aberrant oxidative chemistry (Figure 6a), as I mentioned above. Another mechanism for mutant SOD toxicity is that mutant proteins misfold and form intracellular aggregates. The aggregates are intensely immunoreactive with antibodies against SOD1. There are three related hypothesis. First, diffuse mutants SOD1 aggregates may sequester other protein components required for neuronal function (Figure 6b). Second, aggregates may reduce the availability of protein-folding chaperons to catalyze folding of other proteins (Figure 6c). Third, aggregates may reduce proteosome activity needed for normal protein turnover (Figure 6d) [8]. On the other hand, aggregates may protect the cell from the toxicity of the mutant protein, because the formation of visible SOD1 mutant G85R aggregates is

not required as an initial trigger of motor neuron death. Ongoing investigations could continue to determine why the mutant proteins misfold and whether the resulting aggregates are neurotoxic [9].

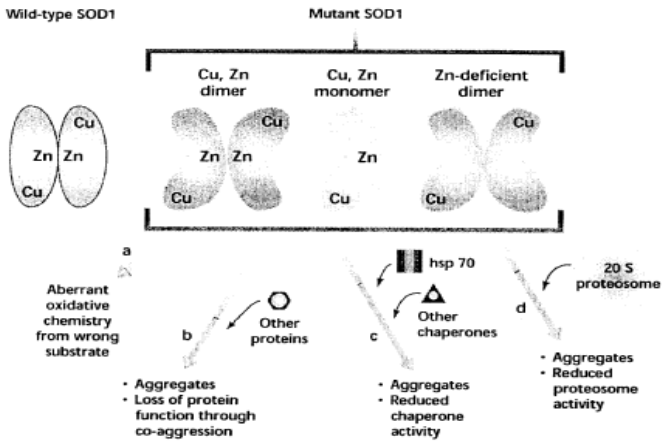

Figure 6. Proposed models for SOD1- mediated toxicity linked to altered conformation and aggregation of mutant SOD1 [8].

Carbonyl stress

Increased carbonyl content was only found in SALS. SALS patients showed increased levels of pentosidine, N-carboxymethyl-lysine (CML), malondialdehyde (MDA)-lysine and 4-hydroxy-2-nonenal (HNE)-histidine in the cytoplasm of the lower motor neurons. These observations may indicate that increased oxidative damage is closely related to SALS but not to FALS. As shown in Figure 7, carbonyls are subclassified into free form and protein-bound form. Free carbonyls are aldehyde intermediates of protein glycation or lipid peroxidation, and are highly reactive with amino groups of protein amino acid residues to form protein-bound carbonyls as post-

translational protein modification. Thus, they may form protein aggregation and alter signal transduction and induction of apoptosis. Protein-bound carbonyls derived from protein glycation are called advanced glycation end products (AGE), and those from lipid peroxidation are called advanced lipoxidation end products (ALE). Both AGE and ALE are deposited in tissue readily because of their protein-cross-linking property, decreased solubility or decreased resolvability [2].

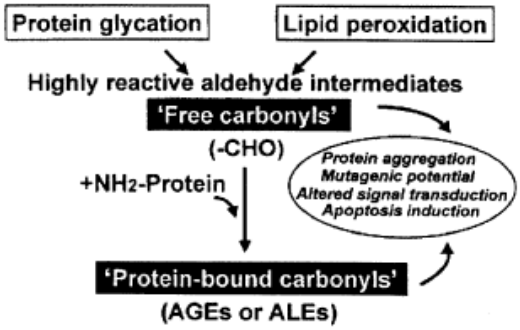

Figure 7. Formation processes and toxic effects of carbonyls [2].

Apoptotic cell death

Alternations in expression of a number of Bcl-2 family proteins are seen in mutant SOD1 transgenic mice spinal cord. In a recent, recombinant adeno-associated virus was employed to deliver Bcl-2 to spinal cord motor neurons of FALS transgenic mice, resulting in protection from cell degeneration and delayed motor deficits. Also, mutant SOD1 induced apoptosis is achieved through the activation of Caspase-1 (ICE). However, although mutant SOD1 is required for ICE cleavage, it is not sufficient to activate the cell death process. Thus, additional stimulus such as oxidative stress is necessary. Other proteins such as Bax also have been proposed as potential

targets for therapy. Bax upregulation may exert its effects through alternation of mitochondrial function [10].

Hypothesis and experiments

There are some puzzling results, where mutant SOD1-transgenic mice were crossed with mice lacking the neurofilament light subunit (NF-L), overexpressing NF-L or overexpressing the neurofilament heavy subunit (NF-H), an extension of life span was observed. I don't think these transgenic mice display compromised cellular functions of motor neurons. These compound mice are charactized by abundant abnormal perinuclear inclusions of neurofilaments. I don't think these inclusions may protect motor neurons by serving as a phosphorylation sink. I think we should consider h/m SOD1 ratio in every SOD1 transgenic models before crossing. We should consider the h/m SOD1 ratio and onset of disease, than find the proper ratio that reflects the onset of disease in ALS patients for each mutant case. Maybe one should prevent using m-mutants alone. I think we should use both h-mutant and m-mutant, and make sure the onset of disease has the similarity before crossing. The transgenic animals should be examined on a weekly basis for general health such as grooming and general activity. Onset of disease could be measured by motor function. Motor function or motor capacity can be assessed by measuring the grip strength of each animal.

Exp1. Construction of transgenic mice expressing wild-type and mutant SOD1.

One should find the proper promoter region that can drive high levels as motor neuron or astrocyte-specific expression. In addition, one may construct transgenic mice expressing wild-type and human or mice mutant SOD1.

Exp2. Limb grip strength measurement and four stages of muscle strength change in mice.

Mice are allowed to grab onto a vertical wire (2mm in diameter) with a small loop at the lower end. A vertical wire allows mice to use both fore- and hindlimbs to grab onto the wire. Both the fore- and hindlimbs contribute to the measured muscle strength. Mice could be tested once a week starting when they are 90d old. One can construct a figure that contain the weeks and hanging tine (sec). Thus, one can find the pre-muscle weakness stage (PMW), a rapid declining stage (RD), a slow declining stage (SD), and a paralysis stage (Para).

Exp3. SOD1 transgenic models

Transgenic line	h/m SOD1 ration	Onset of disease
eg. h-G37R-001	maybe from 0 to 15	maybe no disease or up to 1 year
h-37R-002		
h-37R-003		
m-G37R-001		
m-G37R-002		
m-G37R-003		
h-85R-(001~003)	maybe restricted to astrocytes	maybe no disease
m-G85R-(001~003)		

Exp4. Measure the size of motor neurons in L4 and L5 ventral roots.

Both L4 and L5 ventral roots can be examined because axonal degeneration is often an early sign of neuronal deneration. Once one find the onset of disease, one can also measured the size and divided into two groups (0~5 μm and >5 μm) of motor neurons in L4 and L5 ventral roots. Thus, one can find the relationship of the onset of disease and the degree of motor neuron loss.

The presence of the mutant protein in motor neurons might not be sufficient to lead to the development of a neurodegenerative disease. For example, G85R mouse model showed a 50 % decrease in the astrocyte-specific glutamate transporter GLT-1, which is the major glutamate transporter in the spinal cord. Thus, loss of astrocytic function or loss of glutamate uptake may be important in the ALS disease development. Astrocytes may be involved in the motor neuron cell death. Also, a decrease in glutamate transport GLT-1 and an increase in extracellular glutamate were shown in ALS patients. Maybe that's why some transgenic animals expressing high levels of mutant human SOD1 in motor neurons do not develop the disease.

Hypothesis:

Glutamate, via the astrocytes, induces the motor neuron-specific degeneration.

Glutamate may fit into active site channel and be oxidized at the active site. The products of above reactions such as formyl or glutamyl radicals, which in turn may participate in longer range oxidative reactions. (Identification of substrates whose oxidation is catalyzed by mutant CuZnSOD in motor neurons may also provide clues.) (Formation of radicals may also alter NF

network.) In addition to glutamate, the mutations at various sites in SOD1 may open the loops that form the superoxide-binding pocket and expose the active site to reaction with other oxidants.

↓

Dismutase activity, peroxidase activity, hydroxyl radical generating activity, or the activity of nitration of tyrosine of SOD1 is altered.

↓

Increase glutamate levels and decrease/inactivate excitatory amino acid transporters (EAATs) or inappropriate activation of glutamate receptors

↓

Mutant SOD1 expression in several cell types contributes to motor neuron loss.

↓

The onset of disease and the degree of loss of motor neurons depend on mutant type.

One can see disorganization of neurofilaments, axonal strangulation, aggregation of protein and increase in intracellular calcium.

(related *in vivo* experiments are shown in above pages)

I focus on glutamate because it is the main excitatory neurotransmitter in the central nervous system. The synaptic activity of glutamate is normally terminated by reuptake of the neurotransmitter by excitatory amino acid transporters (EAATs), predominantly the EAAT1 and EAAT2 proteins on perisynaptic astrocytes.

Exp5. Quantitation of SOD1 and EAATs in blood and spinal cord.

At pre-muscle weakness stage (PMW), a rapid declining stage (RD), a slow declining stage (SD), and a paralysis stage (Para), blood samples from tail vein bleeds were solubilized. Ventral horn of cervical spinal cord is also dissected. Western blots are probed with anti-SOD1, anti-GLT-1 and anti-actin Abs. Also, immunohistochemical analyses can be done. Muscle, brain and spinal cord are removed from animals. Immunostaining is performed with antibodies to neurofilament, glutamate transporter GLT, and SOD1.

Exp6. *in vitro* experiments.

This hypothesis also needs *in vitro* experiments to demonstrate the mutant SOD1 could induce glutamate excitotoxic neuronal injury. In the cell culture, one can use non-neuronal cells such as astrocytes and several neuron cell types, such as neuroblastoma, glioma cells. Then cells are transfected with plasmid containing human wtSOD1 or human SOD1 mutants. One may use antobodies to neurofilament, glutamate transporter GLT, and SOD1 for western blot analysis. MnSOD, CAT, GPx activities and intracellular ROS can also be measured. Thus, one can have the intact information about antioxidant enzyme activities in SOD1 mutant-transfected cells.

Summary

Proposed mechanisms of ALS such as oxidative injury, peroxynitrite toxicity, cytoskeletal disorganization, glutamate excitotoxicity, disrupted calcium homeostasis, SOD1 aggregation, carbonyl stress, but there are some puzzling results. I think we should do *in vivo* experiments and find the proper h/m ratio that reflects the onset of disease. Onset of disease could be measured by motor function by measuring the grip strength of animals. I think astrocytes may be involved in the first signs of damage and induce motor neuron cell death. I focus on glutamate because it is the main excitatory neurotransmitter in the central nervous system. I think we should do *in vitro* experiments and quantitate SOD1 and EAATs in spinal cord, compare the difference between non-neuron cells and neuron cells, use antibodies to GLT, SOD1, neurofilament, and intracellular ROS.

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