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Amyotrophic Lateral Sclerosis: Unfolding the Toxicity of the Misfolded

Review

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Amyotrophic lateral sclerosis (ALS) is one of the most common adult-onset neurogenerative diseases, having a prevalence of ~5 per 100,000 individuals. This human disease, first described by Charcot in 1869, is characterized by the selective degeneration of motor neurons, the large nerve cells connecting the brain to the spinal cord and from the spinal cord to muscles, that control muscle movement. The loss of motor neurons leads to progressive atrophy of skeletal muscles. ALS is a relentless disease that manifests as progressive decline in muscular function resulting in eventual paralysis, speech deficits and, ultimately, death due to respiratory failure in the majority of ALS patients within 2 to 5 years of clinical onset. The weakness, which typically begins focally and propagates, is usually associated with the degeneration of both lower motor neurons in the brainstem and spinal cord, and upper motor neurons in the cerebral cortex. Approximately 10% of ALS patients are familial cases. The majority of ALS cases are sporadic (90%) with no known genetic component. While current evidence suggests that multiple genetic and environmental factors may be implicated in ALS pathogenesis, both sporadic and familial ALS cases share common pathological features such as the presence of abnormal neurofilamentous accumulations in degenerating motor neurons.

The discovery of missense mutations in the gene coding for the Cu/Zn superoxide dismutase 1 (SOD1) eight years ago in subsets of familial cases provided much hope for quick development of therapies, and it directed most ALS research on elucidating the mechanism of SOD1-mediated disease. Yet, understanding the toxicity of SOD1 mutants has been surprisingly challenging. Important efforts have also been devoted to clarifying the role of neurofilaments in ALS pathogenesis. Again, studies with transgenic mice yielded complex results. Here, I shall review the current hypotheses on mechanisms of ALS disease with emphasis on toxicity of SOD1 mutants and on cytoskeletal abnormalities.

Genes and Risk Factors for ALS

As shown in Table 1, little is known about the genetic defects that cause or predispose to ALS. To date, the only proven causes of ALS are missense mutations in the *SOD1* gene occurring in ~20% of familial ALS cases (Rosen et al., 1993; Cudkowicz et al., 1997). Over 70 mutations have been discovered spanning all exons of the *SOD1* gene. Recently, a genetic linkage study has identified another locus for a subtype of ALS with frontotemporal dementia (FTD) that maps to chromosome 9q21-q22 (Hosler et al., 2000). Three loci for juvenile-onset ALS have also been mapped. Two recessively inherited loci map to chromosomes 2q and 15q whereas one dominant juvenile-onset locus maps to chromosome 9q34 (Hentati et al., 1994, 1998; Chance et al., 1998). However, the genes responsible for these familial ALS-FTD and juvenile ALS cases have not yet been identified.

In a small number of sporadic ALS patients (~1% of cases), codon deletions or insertion in the KSP repeat motif of the neurofilament *NF-H* gene have been identified (for review see Cleveland, 1999). No such NF-H mutants have been detected in over 1000 control DNA samples. The combined data suggest that NF-H variants may represent risk factors for ALS disease.

For the vast majority of ALS cases, the factors triggering focal initiation and then spreading of motor neuron degeneration in sporadic ALS remain to be elucidated. Various hypotheses have been suggested as potential contributors of disease such as oxidative damage, excitotoxicity, mitochondrial defects, and autoimmunity but these could be secondary to the neurodegeneration process. The "virus hypothesis" as primary cause of ALS is now being revisited in light of a recent report of enterovirus (EV) nucleic acids detected by reverse transcriptase-PCR in the spinal cord of a high percentage of patients with sporadic ALS (Berger et al., 2000). More studies are needed to confirm these results and to demonstrate that EV sequences can play a causal role in ALS development.

Toxicity of SOD1 Mutants: Aberrant Copper-Mediated Catalysis?

SOD1 is an abundant and ubiquituously expressed protein. In view of its normal function in catalyzing the conversion of superoxide anions to hydrogen peroxide (Figure 1), it was initially thought that the toxicity of different SOD1 mutants could result from decreased free-radical scavenging activity. However, this idea was not supported by measurement of enzymatic activities and by transgenic mouse studies. Different SOD1 mutations show a remarkable degree of variation with respect to enzymatic activity, polypeptide half-life, and resistance to proteolysis, and these variables did not correlate with age of onset or rapidity of human disease progression. Several lines of transgenic mice expressing various SOD1 mutants have been generated, and they exhibit many pathological changes that occur in human ALS. Transgenic mice expressing mutants $SOD1^{G93A}$ or $SOD1^{G37R}$ developed motor neuron disease despite elevation in SOD1 activity levels (Cleveland, 1999). In addition, SOD1 knockout mice did not develop motor neuron disease (Reaume et al., 1996). Therefore, the conclusion from the combined results was that the mutations in SOD1 provoke a gain of new toxic properties.

Many studies have focused on aberrant copper-medi-

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Table 1. Gene/Loci Associated with Human ALS

Gene/Loci	Comments	References
Adult onset		
SOD1	Located on chromosome 21; Mostly missense mutations responsible for ~20% familial ALS	Rosen et al., 1993
NF-H	Located on chromosome 22; codon deletions in KSP repeats found in ~1% cases of sporadic ALS	Figlewicz et al., 1994; Al-Chalabi et al., 1999
EAAT2	RNA processing errors Mutation in one ALS case	Lin et al., 1998; Aoki et al., 1998
Cytochrome c oxidase	Mitochondrial DNA microdeletion in one ALS case	Borthwick et al., 1999
Ch 9q21–q22	ALS with frontotemporal dementia	Hosler et al., 2000
Juvenile onset		
Ch 2q33–q35	Autosomal recessive juvenile ALS	Hentati et al., 1994
Ch15q15–q24	Autosomal recessive juvenile ALS	Hentati et al., 1998
Ch 9q34	Autosomal dominant juvenile ALS	Chance et al., 1998

ated catalysis as potential sources of toxicity. One proposal is that misfolding of SOD1 induced by mutations would allow the access of abnormal substrates such as peroxynitrite to the catalytic site leading to the nitration of tyrosine residues (Beckman et al., 1993) (Figure 1). This is consistent with the increased levels of free 3-nitrotyrosine detected in the spinal cord of human ALS patients (Beal et al., 1997) and in mouse models of ALS (Bruijn et al., 1997). Nonetheless, there is no evidence of increased levels of nitrotyrosine bound to proteins in ALS patients or in mutant SOD1 mice as compared to controls (Bruijn et al., 1997; Strong et al., 1998). Another hypothesis suggests that SOD1 mutants have enhanced ability to use hydrogen peroxide as substrate to generate toxic hydroxyl radicals that can damage celullar targets including DNA, protein, and lipid membranes (Wiedau-Pazos et al., 1996) (Figure 1). In agreement with this proposal was evidence of elevated hydroxyl radical-like activity in mice expressing mutant SOD1 (Bogdanov et al., 1998) and of oxidative damage in neuronal tissue of sporadic and familial ALS (Ferrante et al., 1997).

However, the peroxynitrite or peroxidase activity hypotheses have been challenged by results of experiments manipulating the SOD1 activity in a mouse model of ALS. Bruijn et al. (1998) generated SOD1^{G85R} mice either in a SOD1 knockout background or in a transgenic background overexpressing wild-type SOD1. The absence of wild-type SOD1 in the SOD1^{G85R} mutants would be predicted to increase superoxide levels and its product peroxynitrite while the elevation of normal SOD1 activity would do the opposite (Figure 1). A similar argument can be applied for the peroxidase activity hypothesis. Yet, neither the elimination of endogenous SOD1 nor addition of wild-type SOD1 affected disease progression in SOD1^{G85R} mice. Thus, these results are inconsistent with mechanisms of disease involving superoxide-mediated oxidative damage.

SOD1 is a homodimeric protein and each subunit binds one zinc and one copper atom. Improper metal binding has been proposed as a mechanism by which mutations could exert their toxic gain of function. One subject of controversy is the possibility that mutant SOD1 subunits could fail to bind the zinc atom allowing rapid reduction of mutant SOD1 to the Cu^{+} form (Estevez et al., 1999). This reduced form of the enzyme could catalyze the backward reaction converting oxygen to superoxide, which could combine with nitric oxide to produce peroxynitrite. The toxicity of zinc depletion in SOD1 was demonstrated by the introduction via liposome fusion of purified, zinc-depleted SOD1 into cultured motor neurons. In this experimental paradigm, the wild-type SOD1 and mutant SOD1 lacking zinc were equally toxic to cultured neurons. However, the in vivo relevance of this hypothesis has been questioned due to results from transgenic mice (Williamson et al., 2000). If this model were right, a decrease in nitric oxide (NO) production would attenuate disease. Yet, the disruption of neuronal nitric oxide synthase (nNOS) did not increase survival of SOD1^{G93A} mice (Facchinetti et al., 1999). Nonetheless, the possibility exists that inducible NOS (iNOS) is being used as an alternative source for nitric oxide since immunoreactivity for iNOS has been detected in

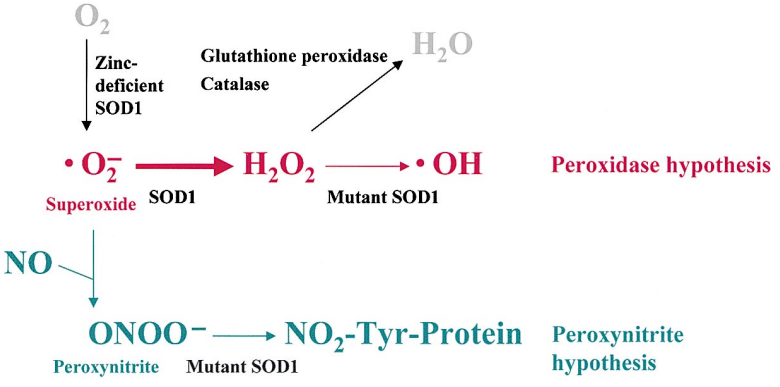

Figure 1. Proposed Copper-Mediated Oxidative Mechanisms to Explain the Toxicity of Mutant SOD1 (see text)

glial cells of mutant SOD1 mice (Almer et al., 1999). Another observation against the zinc-deficient SOD1 hypothesis is that overexpression of neurofilament proteins, which are abundant proteins capable of competing with zinc-deficient SOD1 for binding zinc, did not exacerbate disease in mice models of ALS but rather conferred protection (Couillard-Després et al., 1998, 2000; Kong and Xu, 2000).

A more definitive test for the hypothesis regarding zinc-deficient SOD1 will come from future studies that examine the requirement for copper in toxicity of mutant SOD1. The delivery of copper to SOD1 is mediated by a cytosolic protein called the copper chaperone for SOD1 (CCS). Wong et al. (2000a) have recently generated CCS knockout mice that exhibit considerable reduction in copper incorporation and SOD1 activity. The CCS null mice are viable and sensitive to paraquat, a herbicide that generates superoxide anion radicals in vivo. The generation of CCS null mice expressing mutant SOD1, which is now in progress (P. Wong, personal communication), should prove whether or not copper-mediated oxidative reactions are central to pathogenesis ALS linked to SOD1 mutations.

Toxic Protein Aggregates?

The most promising hypothesis is that the toxicity of mutant SOD1 results from the propensity of misfolded protein mutants to aggregate into cytoplasmic inclusion bodies. Intracellular SOD1 aggregates are formed in cultured motor neurons after microinjection of mutant but not wild-type SOD1 cDNAs (Durham et al., 1997). Aggregates immunoreactive to SOD1 were also detected in motor neurons and astrocytes of mice expressing mutant SOD1 as well as in human ALS cases linked to SOD1 (Bruijn et al., 1998). A recent study using $SOD1^{G93A}$ mice showed that the aggregation of SOD1 into high molecular weight, insoluble protein complexes (IPCs) is an early event in the pathogenic mechanism (Johnston et al., 2000). The SOD IPCs are detectable several months before appearance of inclusion bodies and pathology. Transfection studies in cultured cells suggest that SOD1 IPCs are sequestered into inclusion bodies resembling aggresomes. Aggresomes can be formed from a variety of mutant cytosolic proteins through retrograde transport on microtubules. Because such aggregates are likely substrates for dynein-mediated transport, it is possible that an increasing burden of IPCs could disrupt microtubule-dependent axonal transport of other substrates needed for neuronal viability, such as growth factors. This mechanism is compatible with evidence of defects in slow axonal transport, and of altered levels of the kinesin motor protein and of a regulator of fast transport of motor neurons in mice expressing mutant SOD1 (Cleveland, 1999; Dupuis et al., 2000). Other mechanisms may also contribute to toxicity of SOD1 aggregates. Noxious effects could also result from the cosequestering of essential cellular components. In addition, an abundance of intracellular aggregates could provoke neurodegeneration by overwhelming the capacity of the protein folding chaperones and/or of ubiquitin proteosome pathway to degrade important cellular regulatory factors.

Mitochondrial Defects

Studies in mice showed that the level of mutant SOD1 expression modulates the age of onset of symptoms but has little effect on the rate of progression of disease after onset. Interestingly, the onset of disease in mutant SOD1 mice correlates with a sudden increase in the number of vacuoles representing dilated mitochondria (Kong and Xu, 1998). This suggests that the toxicity of mutant SOD1 may be mediated by damage to mitochondria in motor neurons. Further evidence for a role of mitochondrial dysfunction was provided from the finding that a partial deficiency of manganese superoxide dismutase 2, a mitochondrial enzyme, exacerbated disease in $SOD1^{G93A}$ mice (Andreassen et al., 2000).

Mitochondrial pathology is also present in central nervous system tissue from human ALS cases. There is a report of selective decrease in the activity of the mitochondrial DNA-encoded enzyme cytochrome c oxidase in human spinal cord motor neurons (Borthwick et al., 1999). Oxidative damage to mitochondrial DNA leading to the accumulation of mitochondrial DNA mutations as well as other mitochondrial damages could be important mechanisms contributing to the selective loss of motor neurons in ALS. Chronic mitochondrial inhibition with malonate in cultured cells showed that motor neurons are particularly vulnerable to mitochondrial inhibition (Kaal et al., 2000).

Mitochondria dysfunction or damaged mitochondria can produce excess superoxide ion, release cytochrome c into the cytoplasm which activates caspase-3, and affect Ca^{2+} homeostasis. In motor neurons of $SOD1^{G93A}$ mice, there is evidence of impaired calcium homeostasis and this may explain in part the selective vulnerability of neuronal subtypes to degeneration (Siklos et al., 1998). Unlike oculomotor neurons that are spared in these mice as well as in human ALS, spinal motor neurons have fewer calcium binding proteins, such as parvalbumin, making them more prone to degeneration resulting from changes in cytosolic Ca^{2+} levels.

Apoptotic Death

The emerging evidence suggests that the mechanism of neuronal death in human ALS occurs through programmed cell death, i.e., apoptosis. This is supported by the demonstration of DNA fragmentation, as determined by in situ end labeling of DNA strand breaks, and increased immunoreactivity for proapoptotic Bax protein in selectively vulnerable CNS regions of sporadic cases of ALS (Martin, 1999). In addition, prostate apoptosis response 4 (Par-4), a protein induced in prostate cancer cells and in neuronal aopotosis, was found to be increased in spinal cord motor neurons in ALS patients (Pedersen et al., 2000). There is also compelling evidence that motor neuron death involves apoptosis in disease caused by SOD1 mutations. Transfection studies with cultured neuronal cells demonstrated the proapoptotic effect of mutant SOD1 (Durham et al., 1997). Moreover, hallmarks of apoptotic death, i.e., DNA fragmentation, caspase activation, and altered expression of Bcl-2 members, were found in the spinal cord of lines of mice expressing mutant SOD1 (Spooren and Hengerer, 2000). The members of the Bcl-2 family that have been widely implicated in the regulation of cell

death have been examined in transgenic SOD1^{G93A} mice. In asymptomatic SOD1^{G93A} mice, expression of the anti-apoptotic Bcl-2 and Bcl-xL and proapoptotic Bad and Bax was similar to normal mice (Vukosavic et al., 1999). However, in symptomatic SOD1^{G93A} mice, there was reduced expression of Bcl-2 and Bcl-xL whereas expression of Bad and Bax was increased. Additional support for a role of Bcl-2 in ALS is provided from the report that overexpression of Bcl-2 transgenes delays onset of disease and mortality in SOD1^{G93A} mice by over one month (Kostic et al., 1997). There is evidence that mutant SOD1 can activate p53, a nuclear phosphoprotein protein that may play a causative role in apoptosis. However, the targeted disruption of p53 did not affect disease progression and mortality in these ALS SOD1^{G93A} mice (Kuntz et al., 2000).

Members of the caspase family, which are cysteine proteases with aspartate specificity, are also central components in the apoptotic death pathway. Basically, the caspases are synthesized as pro-enzymes that are cleaved at specific aspartate residues. Upon activation, the caspases cleave other intracellular targets including other caspases resulting in an amplified cell death cascade. Although one group failed to detect sign of apoptosis in SOD1^{G93A} mice using in situ detection for nicked DNA and immunostaining for caspase-3 (Migheli et al., 1999), other groups reported caspase-1 and caspase-3 activation in motor neurons and astrocytes using three mouse models of ALS, the SOD1^{G93A}, SOD1^{G37R}, and SOD1^{G85R} mice (Pasinelli et al., 1998, 2000; Li et al., 2000). In addition, expression of a dominant inhibitory caspase-1 (Friedlander et al., 1997) or intraventricular administration of a caspase inhibitor (Li et al., 2000) slowed down disease progression in SOD1^{G93A} mice.

Interestingly, a recent study demonstrates a sequential cascade of caspase activation during disease progression (Pasinelli et al., 2000). Surprisingly, unlike apoptosis observed in the context of development where cell death occurs rapidly after initial caspase activation, caspase-1 is activated in ALS mice months before caspase-3 activation and prior to any evidence of motor neuron death. Caspase-3 activation occurs at the time of onset of motor axon loss and appearance of apoptotic death. It is noteworthy that caspase activation is also very prominent within astrocytes of mutant SOD1 mice (Li et al., 2000; Pasinelli et al., 2000), raising the possibility that caspase-1 activation may contribute to an inflammatory pathway causing astrocytosis that perhaps may contribute to motor neuron damage.

Excitotoxicity

Glutamate-induced excitotoxicity is another potential contributor to ALS pathogenesis. Depolarization of the neuronal membrane after activation of neuronal glutamate receptors activates voltage-dependent Ca^{2+} channels, allowing Ca^{2+} entry into the cell. Thus, excess activation of neuronal glutamate receptors can cause cell death via alterations in cytosolic free Ca^{2+} homeostasis. For spinal motor neurons, rapid recovery of synaptic glutamate is accomplished by the glutamate transporter EAAT2 present in astrocytes. Loss of EAAT2 transporter could lead to increased extracellular concentrations of glutamate and excitotoxic degeneration of motor neurons. The excitotoxicity hypothesis is supported by the observation that the majority of sporadic ALS cases (~65%) have a reduction in the astroglial glutamate transporter EAAT2 in motor cortex and spinal cord (Rothstein et al., 1995). The decreased EAAT2 levels in ALS may be due at least in part to abundance of aberrant EAAT2 mRNA species resulting from RNA processing error. A report by Lin et al. (1998) described multiple abnormalities of EAAT2 mRNAs in a subset of sporadic ALS patients including intron retention and exon skipping. Proteins translated from these mRNAs could be rapidly degraded and/or produce dominant negative effects, resulting in loss of EAAT2 protein and activity. However, a caveat to this potential mechanism is provided by a study that showed aberrant EAAT2 transcripts to be equally present in ALS patients and controls. (Meyer et al., 1999). The glutamate-induced excitotoxicity resulting from the loss of astroglial EAAT2 is most likely a secondary effect of the disease process in ALS. Germline mutations in the *EAAT2* gene are very rare and they do not explain the existence of variant EAAT2 mRNAs in ALS. Only one sporadic ALS case was identified with an *EAAT2* gene variant that affects N-linked glycosylation and glutamate clearance capacity (Aoki et al., 1998; Trotti et al., 2000).

In disease caused by SOD1 mutations, oxidative damage has been suggested to be another mechanism by which the glutamate transporter EAAT2 can be inactivated. This is supported by the observation that oxidative reactions triggered by hydrogen peroxide and catalyzed by the SOD1 A4V and I113T mutants but not wild-type SOD1 inactivated the glutamate transporter EAAT2 (Trotti et al., 1999). Alternatively, toxic properties of mutant SOD1 aggregates could provoke general astrocytic dysfunction affecting levels of EAAT2. During disease progression in SOD1^{G85R} mice, there is an increase of SOD1 inclusions in astrocytes correlating with a decrease in the EAAT2 glutamate transporter (Cleveland, 1999). Yet, the initial damage caused by mutant SOD1 probably occurs in motor neurons. At initial stage of disease in SOD1^{G93A} mice, expression of mutant SOD1 occurs at high levels in motor neurons but not in astrocytes (Levine et al., 1999). Moreover, in SOD1^{G93A} mice, significant increases in astrogliosis were not observed until after the onset of massive mitochondrial vacuolization in motor neurons and the beginning of clinical symptoms (Levine et al., 1999).

To test the importance of glial expression of mutant SOD1 in pathogenesis, Gong et al. (2000) generated transgenic mice bearing a mutant SOD1^{G86R} gene under the control of GFAP promoter. The restricted expression of mutant to glial cells was not sufficient to cause motor neuron disease. Therefore, while astrocytic dysfunction may contribute to degeneration processes, expression of mutant SOD1 in other cell types, most likely neurons, is essential to trigger motor neuron disease.

Neurofilament Inclusions: Detrimental or Protective Functions?

A pathological hallmark of both sporadic and familial ALS, including cases linked to SOD1 mutations, is the presence of abnormal intermediate filaments (IF) accumulations in the perikaryon and axon of motor neurons

(Hirano et al., 1984; Rouleau et al., 1996). In addition, genetic mutations in the neurofilament *NF-H* gene have been found in a small number of ALS cases (for review see Cleveland, 1999). Neurofilaments, which are the major type of IFs in motor neurons, are made by the copolymerization of neurofilament light (NF-L, 61 kDa), medium (NF-M, 90 kDa), and heavy (NF-H, 115 kDa) subunits. Various factors may account for the formation of neurofilament inclusions in human ALS including deregulation of gene expression or posttranslational modifications, and neurofilament gene mutations.

In situ hybridization studies revealed considerable reduction in levels of NF-L mRNA in degenerating spinal motor neurons of ALS cases (Wong et al., 2000b). Moreover, the decrease in mRNA levels is selective for NF-L. No significant changes were detected in NF-M and NF-H mRNA levels of ALS patients (Wong et al., 2000b). Because of its requirement for proper neurofilament assembly and transport, a lack of NF-L can provoke accumulations of NF-M and NF-H proteins in the cell bodies of motor neurons (Williamson et al., 1998). There is also evidence that disorganized filaments and intracellular transport defects could result from aberrant posttranslational protein modifications. Of particular relevance to ALS is the recent finding that excitotoxicity can induce neurofilament side-arm hyperphosphorylation in neurons and a slowing of neurofilament transport in cultured neurons (Ackerley et al., 2000). This phosphorylation may occur via members of the MAPK family including p42/p44MAPK and SAPKs. Other posttranslational protein modifications may also be involved in disorganization of neurofilaments. The presence of advanced glycation end products in neurofilament inclusions is associated with ALS. In addition, the NF-L protein is very susceptible to peroxynitrite-mediated nitration. However, although neurofilament inclusions in ALS are stained with anti-nitrotyrosine antibodies, the extent of nitration of tyrosine residues in the NF-L protein from the spinal cord of sporadic ALS cases did not differ from age-matched controls (Strong et al., 1998).

For some time, the neurofilament accumulations in ALS were widely viewed as a marker of neuronal dysfunction, perhaps reflecting axonal transport defects. However, recent transgenic mouse studies suggested that neurofilament accumulations could play a pathogenic role. The overexpression in mice of any of the three wild-type neurofilament subunits alone can induce the formation of perikaryal neurofilament accumulations. It is noteworthy that such neurofilament inclusions in cell bodies are relatively well tolerated by motor neurons and do not lead to massive death of motor neurons. For example, high-level expression of human NF-H proteins caused large perikaryal neurofilament inclusions resulting in atrophy of motor axons and altered axonal conductances (Kriz et al., 2000). The NF-H overexpression provoked severe motor dysfunction but did not cause motor neuron death even in two-year-old mice (Beaulieu et al., 2000). Remarkably, the motor neuron disease in NF-H transgenic mice was rescued by coexpressing NF-L subunits in a dose-dependent fashion (Meier et al., 1999), emphasizing the importance of subunit stoichiometry for proper neurofilament assembly and transport. The results would be consistent with a requirement for heterodimerization of NF-L to NF-M or NF-H subunits to achieve efficient translocation into the axonal compartment.

Proof that neurofilament abnormalities can provoke the death of motor neurons came from the expression of an assembly-disrupting NF-L mutant with a Leu to Pro substitution near the end of the conserved rod domain (Lee et al., 1994). To clarify the mechanism of cell death induced by this mutant NF-L, these transgenic mice were bred to mice overexpressing Bcl-2. Elevated levels of Bcl-2 did not protect the large motor neurons from mutant neurofilament-mediated death (Houseweart and Cleveland, 1999). While the mechanism of toxicity of mutant NF-L is not fully understood, it may be related in part to the formation of abnormal neurofilament inclusions in axons that may cause a strangulation of axonal transport. This is in contrast to the perikaryal location of inclusions induced by overexpression of wild-type neurofilament proteins. No such NF-L mutations were found in human ALS but a mutation in the rod domain of the *NF-L* gene has recently been reported in a family with Charcot-Marie-Tooth disease type 2 further demonstrating that neurofilament abnormalities could be a primary cause of neurodegeneration (Mersiyanova et al., 2000).

The presence of abnormal neurofilament inclusions in familial ALS caused by SOD1 mutations and in mice expressing mutant SOD1 supported the possibility that neurofilaments may act as toxic intermediates in the disease. To test this idea, mice expressing mutant SOD1 in NF-L knockout background (Williamson et al., 1998) or in a transgenic NF-H-β-galactosidase background (Eyer et al., 1998) were generated. NF-H-β-galactosidase is a fusion protein that leads to the trapping of neurofilaments in neuronal perikarya. Because motor neuron disease still occurred in both of these mutant SOD1 mice strains lacking axonal neurofilaments, one might conclude that axonal neurofilaments are not required for mutant SOD1-mediated disease (see Table 2). However, the absence of NF-L did lead to ~15% extension of life span in $SOD1^{G85R}$ mice. Even more surprising was the observation that the overexpression of wild-type human NF-H or to a lower extent mouse NF-H proteins, which raises perikaryal neurofilament content and lowers axonal levels, extended the longevity of mutant SOD1 mice by 65% and 15%, respectively (Couillard-Després et al., 1998; Kong and Xu, 2000).

These experiments failed to resolve whether the slowing of disease was a consequence of the depletion of neurofilaments in motor axons or of the accumulation of neurofilament proteins in the cell bodies of motor neurons. To further address this issue and to assess the potential role of axonal caliber as a vulnerability factor, $SOD1^{G37R}$ mice were generated in a context of one disrupted allele for each neurofilament gene. This allowed a reduction of neurofilament content and caliber of motor axons without altering the normal subunit stoichiometry and morphological distribution of neurofilaments (Nguyen et al., 2000). A 40% decrease in the content of intact neurofilaments did not extend the life span of $SOD1^{G37R}$ mice, and motor axons with reduced calibers remained equally vulnerable to degeneration. Therefore, the slowing of disease in mutant SOD1 mice lacking NF-L or overexpressing neurofilament proteins seems to be the result of perikaryal increase in neurofila-

Table 2. Effects of Changes in Neurofilament Expression on SOD1 Mutant Mice

	Life Span of Mutant SOD1 Mice	Neurofilament Proteins in Motor Neurons		
		Perikaryon	Axon	References
Knockout				
NF-L$^{-/-}$	Increased by 15% (40 days) (SOD1^{G85R})	Slight increase of NF-H and NF-M	Scarcity	Williamson et al., 1998
Triple$^{+/-}$ NF-L;NF-M;NF-H	No changes (SOD1^{G37R})	Normal	40% reduction	Nguyen et al., 2000
Transgene overexpression				
NF-H/β-galactosidase	No change (SOD1^{G37R})	Huge accumulation of neurofilaments	Scarcity	Eyer et al., 1998
Human NF-H	Increased by 65% (2–5 months) (SOD1^{G37R})	Large accumulation of neurofilaments	Substantial reduction	Couillard-Després et al., 1998
Human NF-L	No change (SOD1^{G37R})	Slight increase in NF-L	~50% increase	Couillard-Després et al., 2000
Mouse NF-L	Increased by 15% (40 days) (SOD1^{G93A})	Slight increase of neurofilaments	Increase	Kong and Xu, 2000
Mouse NF-H	Increased by 15% (40 days) (SOD1^{G93A})	Large accumulation of neurofilaments	Substantial reduction	Kong and Xu, 2000

ment proteins rather than a depletion of axonal neurofilament content. To date, overexpressing human NF-H has been the most effective approach to increase the longevity of mutant SOD1 mice but more work is needed to clarify the protective mechanism. Appealing possibilities are that perikaryal neurofilament accumulations may protect the cell by binding excess Ca^{2+} or by acting as a sink for oxidative damage and/or toxic posttranslational modifications. It remains to be explained why expression of NF-H-β-galactosidase had no net benefits on disease. While it cannot be excluded that this nonphysiological fusion protein could alter normal neurofilament properties, the exceedingly high levels of mutant SOD1 in mouse lines used for these studies could perhaps override the protective effects of perikaryal neurofilaments.

Peripherin Inclusions

Peripherin is another type of IF protein that is detected together with neurofilament proteins in the majority of axonal inclusion bodies, called spheroids, in motor neurons of ALS patients (Corbo and Hays, 1992). In the adult, the expression of peripherin is normally restricted to spinal motor neurons, peripheral sensory neurons and autonomic nerves. Transgenic mice overexpressing peripherin 4- to 7-fold in motor neurons developed a late onset motor neuron disease (Beaulieu et al., 1999). The peripherin-mediated disease, which is dramatically accelerated by a deficiency of NF-L (Beaulieu et al., 1999, 2000), is characterized by the presence of abundant IF inclusion bodies in motor axons containing peripherin together with NF-M and NF-H proteins. Evidence from transfection experiments suggests that a lack of NF-L promotes interaction of peripherin with NF-M and NF-H proteins to produce disorganized IF structures (Beaulieu et al., 1999). Unlike other types of neuronal IF proteins, the sustained overexpression of wild-type peripherin in mice caused the selective death of motor neurons during aging. The sequestration of organelles such as mitochondria within the peripherin inclusions and the axonal localization of these IF inclusions may contribute to neurotoxicity (Beaulieu et al., 2000). Such peripherin inclusions, as described above for neurofilament inclusions, could impair axonal transport (Cleveland, 1999). Since peripherin gene expression can be enhanced several fold by injury or by inflammatory cytokines, formation of peripherin inclusions may be part of a general response of motor neurons to noxious stress and chronic inflammation. Mutant SOD1 mice also develop axonal peripherin inclusions at early stage of disease (Beaulieu et al., 1999), and they should be useful in future studies to assess the potential contribution of peripherin to disease.

In summary, cytoskeletal abnormalities may be viewed as important risk factors in ALS. However, the combined studies of transgenic mouse models indicate that different types of IF inclusions may have disparate effects on neuronal function (Figure 2). The IF misorganization caused by mutant NF-L proteins or by sustained overexpression of peripherin is cytoxic and this may be related in part to the axonal localization of IF aggregates. Conversely, large accumulations of neurofilaments in perikarya that were induced by overexpression of wild-type NF-H proteins conferred remarkable protection against toxicity of mutant SOD1.

Therapeutic Interventions in Mice

Various approaches to therapy have been tested in mutant SOD1 mice and have been found to slow disease progression (Table 3). Riluzole, an inhibitor of synaptic glutamate release, is so far the only approved drug for treatment of human ALS. In SOD1^{G93A} mice, riluzole prolonged survival modestly by 10–15 days without affecting disease onset (Gurney et al., 1996). This suggests an excitotoxicity involvement after disease onset that can be partly alleviated by inhibitors of glutamate release. The best pharmacological treatments in mice to date have been with intracerebroventricular administration of N-Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), a broad caspase inhibitor (Li et al., 2000), and with the addition of creatine, a compound believed to improve mitochondrial function, to drinking water (Klivenyi et al., 1999).

Other therapeutic targets have been revealed through modulation of gene expression in mice. As mentioned above, the highest documented effect on longevity of

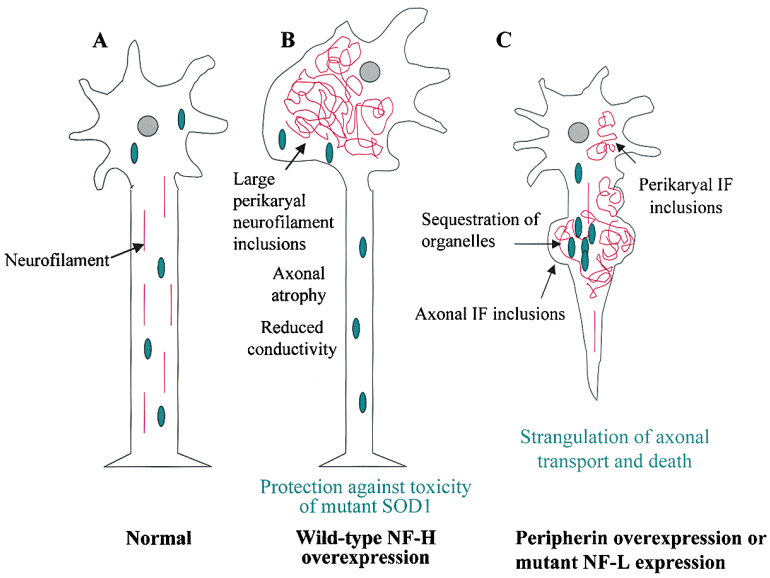

Figure 2. Different Types of IF Inclusions with Disparate Properties

(A) Normal motor protein. (B) The overexpression of wild-type NF-H proteins in mice induces the formation of large neurofilament accumulations in the cell body of motor neurons. Such perikaryal swellings do not cause cell death and they even confer protection in disease caused by mutant SOD1. (C) The sustained overexpression of peripherin transgenes caused motor neuron death in aging or earlier in a context of NF-L deficiency. The disease is characterized by the presence of abundant IF inclusions in motor axons that could impede axonal transport. Similar axonal IF accumulations occurred in motor axons of transgenic mice expressing mutant NF-L. However, motor neuron loss occurs soon after birth in mice expressing mutant NF-L, unlike peripherin transgenic mice that develop a late-onset motor neuron disease.

mutant SOD1 (~5 months) was obtained by overexpressing a human NF-H transgene (Couillard-Després et al., 1998) (see Table 2). Bcl-2 transgene overexpression extended the survival of SOD1^{G93A} mice by 5–6 weeks (Kostic et al., 1997). However, the intraspinal injection of recombinant adeno-associated virus (AAV) coding for bcl-2 in SOD1^{G93A} resulted in increased survival of motor neurons but was not sufficient to prolong the survival of SOD1^{G93A} mice (Azzouz et al., 2000). Nonetheless, these results support the feasibility of using AAVs as a gene transfer approach aiming to enhance survival of spinal cord motor neurons.

Remarkably, the intravenous injection of human umbilical cord blood mononuclear cells into SOD1^{G93A} mice after irradiation was able to substantially increase the life span (Ende et al., 2000). How this procedure provides benefit is not settled but the results suggest that cells of hematopoietic origin could influence disease progression.

Autoimmunity

A hypothesis of potential relevance to sporadic ALS pathogenesis suggests that an autoimmune response is responsible for initiating motor neuron degeneration. Support for this proposal first came from studies indicating the presence of antibodies to L-type voltage-gated Ca^{2+} channels in some ALS patients. These immunoglobulins from ALS patients are capable of inducing transient increases in intracellular calcium and death of cultured motor neurons (Colom et al., 1997).

Anti-Fas antibodies were also detected in sera from 26% of patients with sporadic ALS (Yi et al., 2000), a finding compatible with involvement of an immune mechanism. In mixed cultures of rat embryonic brain

Table 3. Therapeutic and Genetic Interventions in Mutant SOD1 mice

Protective Effects	Mutant Mouse	Increase in Life Span	References
Treatments			
Riluzole (inhibits glutamate release)	SOD1^{G93A}	10–15 days	Gurney et al., 1996
Creatine (enhances energy reserves)	SOD1^{G93A}	30 days	Klivenyi et al., 1999
zVAD-fmk (inhibits caspases)	SOD1^{G93A}	27 days	Li et al., 2000
Irradiation plus human umbilical cord blood	SOD1^{G93A}	10–50 days	Ende et al., 2000
Transgene overexpression			
Bcl-2	SOD1^{G93A}	30–35 days	Kostic et al., 1997
Dominant inhibitor of Caspase-1	SOD1^{G93A}	27 days	Friedlander et al., 1997
Gene Knockout			
nNOS$^{-/-}$	SOD1^{G93A}	No change	Facchinetti et al., 1999
p53	SOD1^{G93A}	No change	Kuntz et al., 2000
Gene therapy			
AAV/bcl-2	SOD1^{G93A}	No change, Increase survival of motor neurons	Azzouz et al., 2000

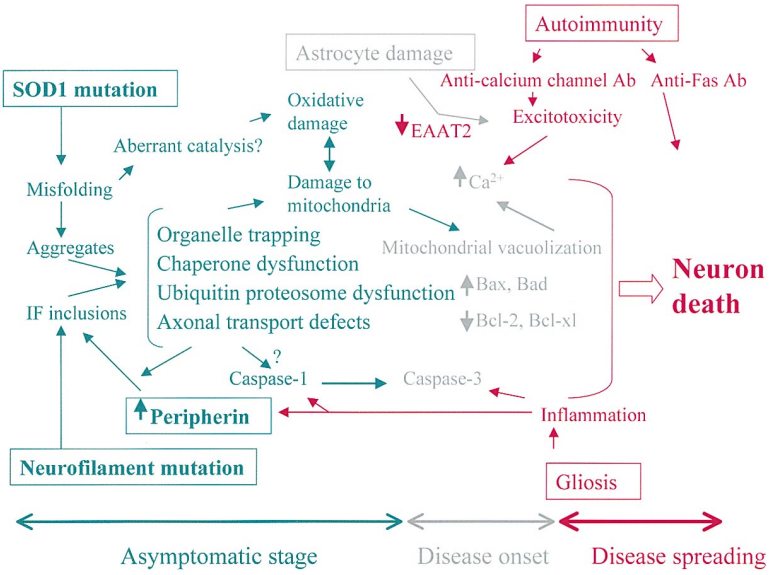

Figure 3. A Complex Pathway to Motor Neuron Death

Evidence, based mostly on studies with mice expressing mutant SOD1, suggests that multiple mechanisms contribute to ALS pathogenesis.

and spinal cord cells, ALS sera induced the apoptosis of a subpopulation of neurons identified as motor neurons. Fas is a member of the tumor necrosis factor family and the clustering of Fas by Fas ligand can activate a signaling pathway for apoptosis. To what extent these pathways contribute to mechanisms of motor neuron death in ALS merits further investigation even though an immune mechanism could represent a response secondary to neurodegeneration rather than a primary cause of disease.

Future Directions

Evidence to date suggests that the pathway to motor neuron death in ALS is complex and involves multiple cascades of events including formation of protein aggregates, oxidative damage, mitochondrial defects, alterations in calcium homeostasis, caspase activation, and changes in levels of Bcl-2 (Figure 3). Intracytoplasmic aggregates, formed by mutant SOD1 or IF proteins, emerged as important factors of toxicity that may interfere perhaps with intracellular transport and proteosome ubiquitin functions. Protein aggregates and damage occur in both neurons and astrocytes, but the degree to which gliosis contributes to motor neuron disease is not established. Excitotoxicity due to astrocyte dysfunction and inflammatory processes from microglia activation are additional factors that may contribute to the spreading of the neurodegenerative process.

The current knowledge of pathogenic mechanisms of ALS is mostly based on studies with mutant SOD1, which is responsible for only ~2% of all ALS cases. For the vast majority of ALS patients, the primary causes of disease are unknown. Thus, there is a need to identify new genes associated with familial forms of ALS. Another promising approach will be to identify genes whose mutation in mice produce ALS-like conditions. Mutagenesis in mice using the chemical mutagen ethylnitrosourea is now being used for large-scale screening of genes and pathways involved in various phenotypes. In addition, the existing mouse models of ALS provide an opportunity to search for modifier genes associated with different genetic backgrounds that can alter the onset or progression of the disease.

Currently, there is no drug available to cure motor neuron disease in human ALS and in mouse models. Nonetheless, in light of current knowledge of disease mechanism, antiapoptotic drugs such as caspase inhibitors and perhaps anti-inflammatory agents could offer potential treatment. Other strategies might include a search for agents that can prevent the abnormal aggregation of mutant SOD1 or the formation of peripherin/neurofilament inclusion bodies. As more associated genes are discovered, new therapeutic approaches could potentially be derived. Gene therapy approaches involving the use of recombinant viruses offer a promising strategy for the delivery of genes to enhance motor neuron survival and repair. The next few years will also provide some perspective on whether neural stem cells with the potential to differentiate into appropriate cells can be used to replace motor neurons or to repair damaged neurons via delivery of growth factors. In view of the complexity of the disease, a combination of different therapies acting in synergy will probably be needed for effective ALS treatment.

New technologies will likely drive future progress. For instance, the use of cDNA microarray technologies can be applied to compare the gene expression profile from sporadic and familial ALS cases, including cases linked to SOD1 mutations. This will help to determine to what extent the two forms of the disease share similar degenerative processes, a crucial issue for clinical intervention. Other questions related to SOD1-mediated disease need to be explored. It is not fully resolved how misfolded SOD1 mutants can trigger by aggregation death pathways selectively in subsets of motor neurons. The molecular mechanisms in various cell types that contribute to the onset and rapid spreading of motor neuron death are not completely understood. In this regard, profiling the differential gene expression in various tissues during disease progression in existing mouse models of ALS should provide a better understanding of the

molecular events involved in pathogenesis. Hopefully, further insights into the causes and molecular pathways associated with pathogenesis will lead to the development of effective therapeutic approaches that will stop the devastating course of this disease.

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