

Original articles

Amyotrophic lateral sclerosis : pathogenesis

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Introduction

Most patients with amyotrophic lateral sclerosis (ALS) have the sporadic form of this disease. In 5 to 10% of cases, ALS is a familial disorder, usually being transmitted as an autosomal dominant trait. In about 1 out of 5 of such families, mutations in the *SOD1* gene appear to cause this neurodegeneration (Rosen *et al.*, 1993). The discovery of these mutations resulted in the generation of transgenic mice, which overexpress mutant and wild-type human *SOD1*. This mouse develops motor neuron loss very much alike that observed in human ALS. This animal model has meant a breakthrough to our understanding of the pathogenesis of ALS.

***SOD1* mutations and familial ALS**

The *SOD1* gene is located on chromosome 21 and consists of 5 exons, which encode a peptide of 153 aminoacids, the superoxide dismutase type 1 (SOD1). It incorporates zinc and copper, and functions as a homodimer. It converts superoxide anions ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2), which is then further metabolized by glutathion peroxidase and catalase (Fridovich, 1986).

More than 70 *SOD1* mutations have been identified in ALS. Certain mutations are associated with a particular phenotype (Cudkowicz *et al.*, 1997 ; Juneja *et al.*, 1997). Some mutations are associated with early onset (L38V, G37S), while others are associated with particularly short (A4V) or relatively long (D100G) disease duration. A predominantly lower motor neuron presentation is found in patients with the A4V mutation.

Within the same family, onset and duration of disease may vary as well. This suggests that factors other than *SOD1*, genetic or environmental in nature, modify the phenotypic expression of this mutant (mt) *SOD1*-associated condition. Evidence for such modifying factors also comes from the study of the D90A mutation, which can behave

both as recessive and dominant trait. In Scandinavian patients, ALS is seen only in individuals homozygous for the D90A mutation (Andersen *et al.*, 1995), while non-Scandinavian individuals get ALS, even when they are heterozygous for this mutation (Robberecht *et al.*, 1997). Evidence suggests that a genetic component most probably is involved (al-Chalabi *et al.*, 1998). The identification of this factor may be of interest to understand the pathogenesis of ALS and may give rise to new treatment strategies.

The mechanism of mt*SOD1*-induced neuronal death

The detrimental effect of *SOD1* mutations most probably arises from an unexpected new function of the mutated enzyme, and not from a loss of normal dismutase function. All mutations identified so far are missense mutations or induce a truncated protein which misses only the final stretch of aminoacids, suggesting that the bigger part of the molecule needs to be present to cause disease. Furthermore, several mutations (among which the D90A mutation) leave the dismutase activity of the enzyme intact. In addition, mt*SOD1* transgenic mice develop motor neuron loss in spite of having normal endogenous *SOD1* activity, and mice in which the *SOD1* gene has been knocked out do not develop a motor neuron disease. Rather, the mutated enzyme seems to gain a cytotoxic function (Cleveland, 1999). It remains unclear what this function actually represents (Fig.1). According to one hypothesis, the mutations may affect the protein's conformation so that substrates other than $O_2^{\cdot-}$ gain access to the copper atom in the active center of the mutant molecule. Two possible substrates have been suggested : H_2O_2 and peroxy-nitrite ($ONOO^-$). MtSOD1 has indeed been found to have enhanced peroxidase activity. By converting H_2O_2 into toxic radicals, it becomes a source of reactive oxygen species (ROS) rather than a scavenger, but this is by no means certain (Wiedau-Pazos *et al.*, 1996, Yim *et al.*, 1996 ; Singh *et al.*, 1998). ROS-generating activity by mtSOD1 has been demonstrated in PC12 cells transduced with

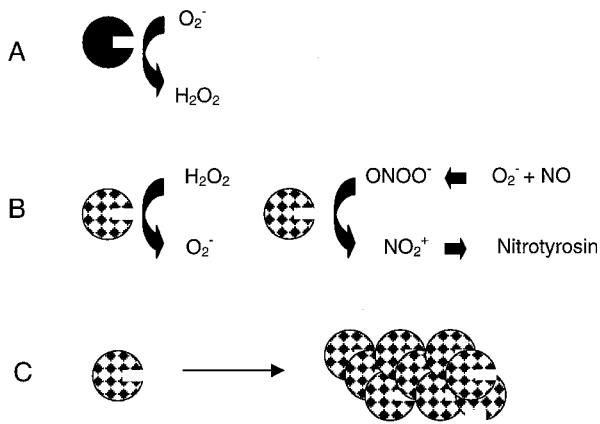


FIG. 1. — Mechanisms of mutant SOD1-induced toxicity. A. Wild type SOD1 converts superoxide anions into hydrogen peroxide. B. “Abnormal” substrates such as hydrogen peroxide or peroxynitrite may gain access to mutant SOD1, and result in the generation of toxic free radicals. C. Alternatively, mutant SOD1 may form aggregations in the cell and thus be toxic.

mtSOD1 (Ghadge *et al.*, 1997). Furthermore, several groups were able to demonstrate enhanced free radical production in brain or spinal cord of mtSOD1 mice (Liu *et al.*, 1998; Bogdanov *et al.*, 1998, Liu *et al.*, 1999).

On the other hand, it has suggested that the mtSOD1 becomes more accessible for $ONOO^-$, from which it generates nitronium ions (Beckman *et al.*, 1993). Nitronium ions may oxidatively damage proteins through nitrosylation. The affinity of the mutated protein for zinc has been found to be reduced, which has been claimed to explain its' enhanced nitronium-generating activity (Crow *et al.*, 1997; Estevez *et al.*, 2000). Nitrotyrosines have indeed been found in spinal cord from mtSOD1 overexpressing mice and in post mortem tissue from mtSOD1-associated familial ALS patients (Bruijn *et al.*, 1997; Beal *et al.*, 1997; Ferrante *et al.*, 1997). Interestingly, similar increased staining for nitrotyrosines has also been observed in sporadic ALS spinal cord (Abe *et al.*, 1995). The significance of this finding remains uncertain, however, as the nitrotyrosins found appear to be non-protein bound.

Disappointingly, changing the cellular content of O_2^- (by manipulating the cellular wtSOD1 level) did not affect the onset or course of the motor neuron disease of these mice (Bruijn *et al.*, 1998). This result indicates that our understanding of the biochemical changes of mtSOD1 is far from complete.

A second hypothesis suggests that the mutant enzyme has an abnormal tendency to aggregate, and that the aggregated proteins are toxic to the cell. Inclusions have indeed been observed in neurons and astrocytes of mtSOD1 mice (Bruijn *et al.*, 1997), and neuronal inclusions are well characterized in human mtSOD1-associated familial ALS (Shibata *et al.*, 1996; Ince *et al.*, 1998). In addition, mtSOD1 overexpressed in cultured motor

neurons forms aggregates (Durham *et al.*, 1997; Roy *et al.*, 1998). As for other neurodegenerative diseases associated with intraneuronal inclusions, the mechanism of aggregate-induced cell death remains uncertain.

Although the mechanism through which SOD1 mutations induce cell death is still unresolved, oxidative damage to motor neurons is widely accepted to be involved (Robberecht and de Jong, 2000). In several postmortem studies on human mtSOD1-associated familial ALS and on mtSOD1 transgenic mice, evidence of oxidatively damaged lipids, proteins and DNA has been found. Staining for 8-hydroxy-2'-deoxyguanosine (indicating oxidation-induced damage to nucleic acids), increased levels of carbonyl (a measure of oxidative damage to proteins), and increased immunocytochemical staining of malondialdehyde-modified proteins (a marker for oxidative damage to lipids) have been found in motor neurons of familial and sporadic ALS patients, and of mtSOD1 transgenic mice (for review see Robberecht and de Jong, 2000). Furthermore, cell lines overexpressing a mtSOD1 (Rabizadeh *et al.*, 1995) and primary cultures of nigral neurons from mtSOD1 transgenic mice (Mena *et al.*, 1997) are known to show enhanced sensitivity to oxidative stress, and primary fibroblast cultures from both familial and sporadic ALS patients were found to be more sensitive to H_2O_2 and 3-morpholinopyridone (Aguirre *et al.*, 1998).

Oxidative stress : linked to excitotoxicity ?

Although still largely circumstantial, evidence is growing that glutamate-induced excitotoxicity plays a role in the pathogenesis of ALS, and that oxidative stress induced by mtSOD1 and glutamate-induced cell death may be linked. In pathological studies (Gurney *et al.*, 1994; Dalcanto and Gurney, 1994; Wong *et al.*, 1995; Kong and Xu, 1998) mitochondria are the first, or at least among the first, targets for the deleterious effects of the mtSOD1. This will result in a decrease of ATP production, increased production of free radicals, and decreased function of ATP-requiring ionic pumps maintaining the electrochemical gradients across neuronal plasma membranes (Fig. 2). Calcium (Ca^{2+}) that enters the cell through stimulation of glutamate receptors, is cleared insufficiently and cytosolic Ca^{2+} concentrations will increase. This will initiate a variety of enzymatic processes leading to cell death. Final proof for this hypothesis remains to be provided. However, increased cytosolic Ca^{2+} concentrations have been observed in SH-SY5Y cells transfected with mtSOD1 (Carri *et al.*, 1997) and in peripheral blood lymphocytes from patients with sporadic ALS (Curti *et al.*, 1996). Furthermore, small vacuoles filled with Ca^{2+} have been observed in spinal motor neurons of

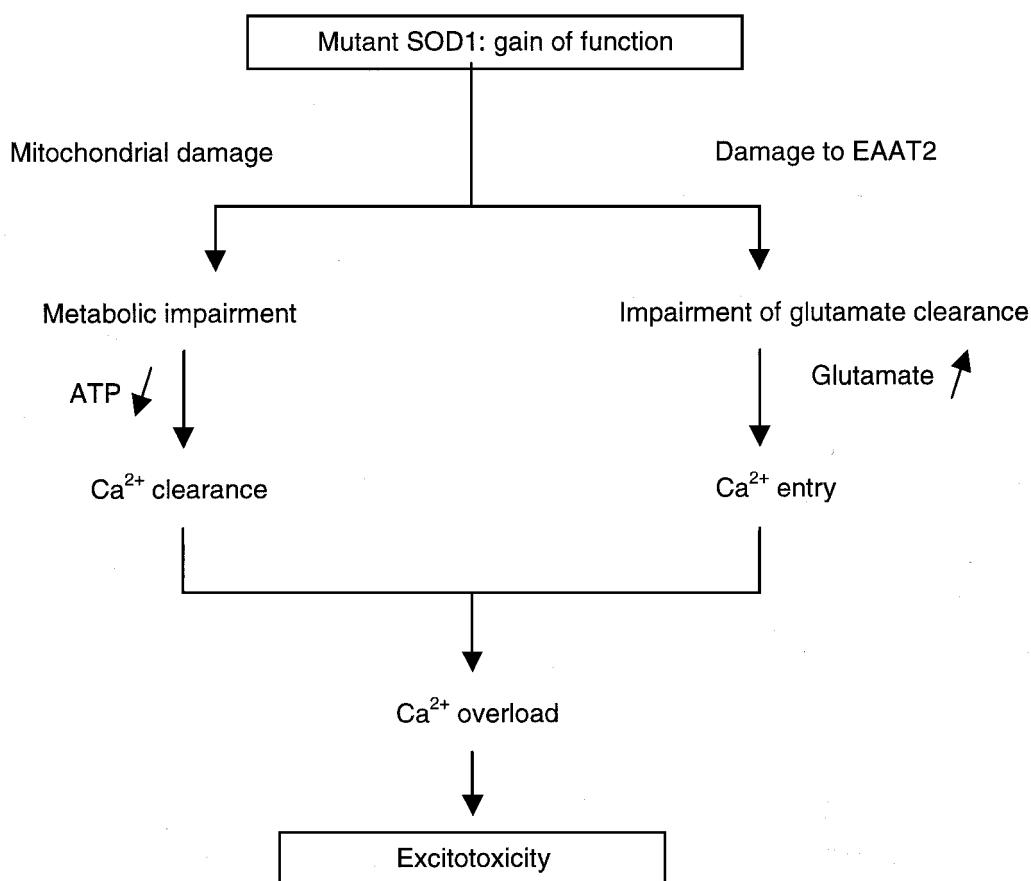


FIG. 2. — Hypothesized link between mutant SOD1 toxicity and excitotoxicity.

mtSOD1 mice (Siklos *et al.*, 1996) and increased intracellular Ca^{2+} has been observed in the motor terminals of patients with sporadic ALS (Siklos *et al.*, 1998).

A second mechanism linking excitotoxicity and oxidative stress has been proposed (Fig. 2): loss of EAAT2 has been observed in ALS postmortem tissue (Rothstein *et al.*, 1995) and in the spinal cord of mtSOD1 mice, most probably due to oxidative damage induced by the mtSOD1 (Trotti *et al.*, 1999). This suggests that mtSOD1-induced damage may induce excessive glutamergic stimulation due to insufficient clearance of glutamate from the extracellular space by the glial glutamate transporter EAAT2. It should also be mentioned that the formation of aggregates of mtSOD1 observed in cultured motor neurons injected with mtSOD1, seems to be dependent upon Ca^{2+} entry induced by glutamate stimulation (Durham *et al.*, 1997), providing a further link between these two mechanisms.

Sporadic ALS: a similar mechanism of neuronal loss?

Several of the pathogenic mechanisms mentioned above are thought to play a role not only in familial but also in sporadic ALS. The loss of the glial transporter protein, EAAT2, is well documented in sporadic ALS (Rothstein *et al.*, 1995)

and may result in excessive extracellular glutamate levels as mentioned earlier. The modest but definite effect of riluzole on ALS survival (Bensimon *et al.*, 1994; Lacomblez *et al.*, 1996) is sometimes considered as an argument in favor of the involvement of glutamate in the pathogenesis of ALS. However, as riluzole has multiple sites of action, and as we do not know how it exerts its effect in ALS, this argument remains controversial.

In tissue of patients with sporadic ALS, evidence for oxidation-induced damage to DNA, lipids, and proteins has been found, similar to that found in mutant SOD1-associated familial ALS, as has been discussed above. It therefore is widely accepted that in sporadic ALS, oxidative stress plays a role in the pathway leading to neuronal death. The cause for this oxidative stress remains unknown. A non-hereditary abnormality of SOD1 has been hypothesized (Bredesen *et al.*, 1997). However, no abnormalities in the SOD1 gene in sporadic ALS have been found and the activity of SOD1 in red blood cells and motor cortex of sporadic ALS patients is normal (Bowling *et al.*, 1993; Robberecht *et al.*, 1994; Shaw *et al.*, 1997).

Selective vulnerability

The selective vulnerability of motor neurons in mtSOD1-related familial ALS remains poorly

explained. Motor neurons certainly express high levels of SOD1, which could render them more susceptible to the cytotoxic effect of the mutated enzyme. Alternatively, motor neurons are large cells with long axons which are probably very sensitive to mitochondrial changes and energy failure. The process of mutant *SOD1*-induced sensitivity to excitotoxicity, may also contribute to the selectivity of motor neuron death in ALS. Indeed, motor neurons are sensitive to glutamate-induced excitotoxicity, due to the presence of calcium-permeable AMPA type glutamate receptors on their surface (Carriedo *et al.*, 1998 ; Vandenberghe *et al.*, 2000). This receptor allows unusually large amounts of Ca^{2+} to enter the motor neuron upon AMPA stimulation, resulting in excitotoxic cell death, most probably due to the high number of AMPA-receptors on the motor neuron's surface and not their unusual composition or desensitization characteristics (Vandenberghe *et al.*, 2000)

Another factor contributing to the vulnerability of motor neurons may be the poor cytosolic calcium-buffering capacity of motor neurons, as parvalbumin and calbindin D_{28k} appear to be absent from their cytosol. These proteins are thought to protect cells from excitotoxicity through their calcium-binding properties. It appears that motor neurons devoid of these proteins are the ones to degenerate in ALS, as demonstrated by pathological studies (Ince *et al.*, 1993 ; Alexianu *et al.*, 1994).

Other genes involved in the pathogenesis of motor neuron degeneration

In a small number of sporadic ALS patients, mutations have been found in the repetitive tail (KSP) domain of the gene encoding the heavy neurofilament subunit (Figuelewicz *et al.*, 1994 ; Tomkins *et al.*, 1998 ; al-Chalabi *et al.*, 1999). This is particularly interesting, as manipulating the genes of neurofilaments in transgenic mice overexpressing human mutant *SOD1*, significantly affects disease onset or duration of the disease (Julien *et al.*, 1998). Abnormalities of neurofilaments have been described in motor neurons of ALS patients, both with the sporadic and familial form of the disease. All these elements suggests that neurofilaments may play a pivotal role in the pathogenesis of the sporadic form of ALS.

Several other forms of ALS have been linked to chromosomal regions, but the underlying genes await identification. On chromosome 9q34, linkage has been established with a form of juvenile autosomal dominant familial ALS, characterised by distal atrophy and weakness with upper motor neuron involvement becoming apparent only late in the disease (Chance *et al.*, 1998). Autosomal recessive juvenile onset familial ALS is genetically and clinically heterogenous. Type 1 has been linked to chromosome 15q15-q22 (Hentati *et al.*, 1998).

Type 3 is characterized by predominant upper motor neuron involvement and has been linked to chromosome 2q33-q35 (Hentati *et al.*, 1994). Surprisingly, a locus for a dominant form of ALS has been identified on chromosome Xp11-q12 (Hong *et al.*, 1998). Motor neuron loss can also be seen as part of the dementia-parkinsonism-amyotrophy complex, which has been linked to chromosome 17q 21-22. The tau gene is located in this region, and mutations in it have been associated with familial frontotemporal dementia. However, in most families in which ALS and (frontal type) dementia occurs, no tau mutations are present (Cole and Siddique, 1999).

Several case reports have suggested other candidate genes for ALS : single ALS patients with a mutation in the gene encoding the cytochrome C oxidase subunit I (Comi *et al.*, 1998) and the APEX nuclease gene have been reported (Olkowski *et al.*, 1998).

Genetic factors most probably contribute to the pathogenesis of sporadic ALS. Two groups have found that the $\epsilon 4$ allele is significantly associated with bulbar onset ALS (Moullard *et al.*, 1996 ; al-Chalabi *et al.*, 1996), but several reports have not been able to confirm this. Polymorphisms of the APEX nuclease gene have been suggested to be associated with ALS (Hayward *et al.*, 1999) and an association between ALS and CYP2D6 (B) alleles (Siddons *et al.*, 1996), and between lower motor neuron disease and SMN mutations (Moullard *et al.*, 1998) have been reported. These results await confirmation in larger populations.

Modern genetics now allow to approach the genetic component of a sporadically occurring disease at the molecular level. The identification of such risk genes will greatly contribute to our understanding of the pathogenesis of this disease. Hopefully the identification of such factors will allow to develop a preventive strategy or to develop means of modifying the disease.

Conclusion

The discovery of *SOD1* mutations in familial ALS has meant a breakthrough in ALS research. The mechanism of almost selective motor neuronal death induced by mtSOD1 is likely to involve oxidative stress and/or abnormal protein aggregation. The identification of other genes underlying familial ALS and of genes rendering individuals susceptible to "sporadic" ALS, is eagerly awaited. Glutamate-induced excitotoxicity to motor neurons may play a pathogenic role in both familial and sporadic ALS, as failure of cellular energy metabolism from whatever primary cause, may render the motor neuron vulnerable to the Ca^{2+} load in the cytosol induced by excitatory stimulation. As such, the pathogenic mechanism of ALS may also be at

play in other neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease.

Hopefully, the results of the successful molecular and cellular studies reviewed here will soon be translated into an effective treatment for patients suffering from this dramatic disease.

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