State of the art and the dark side of amyotrophic lateral sclerosis

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Abstract
Amyotrophic lateral sclerosis (ALS) is a disorder that involves the degeneration of motor neurons, muscle atrophy, and paralysis. In a few familiar forms of ALS, mutations in the superoxide dismutase-1 (SOD1) gene have been held responsible for the degeneration of motor neurons. Nevertheless, after the discovery of the SOD1 mutations, no consensus has emerged as to which cells, tissues, and pathways are primarily implicated in the pathogenic events that lead to ALS. Ubiquitous overexpression of mutant SOD1 in transgenic animals recapitulates the pathological features of ALS. However, the toxicity of mutant SOD1 is not necessarily limited to the central nervous system. Views about ALS pathogenesis are now enriched by the recent discovery of mutations in a pair of DNA/RNA-binding proteins called TDP-43 and FUS/TLS as causes of familial and sporadic forms of ALS. Although the steps that lead to the pathological state are well defined, several fundamental issues are still controversial: are the motor neurons the first direct targets of ALS; and what is the contribution of non-neuronal cells, if any, to the pathogenesis of ALS? The state of the art of ALS pathogenesis and the open questions are discussed in this review.

Key words: Amyotrophic lateral sclerosis; Neurodegenerative disease; Muscle wasting; Oxidative stress; Excitotoxicity; Protein aggregation; Mitochondrial dysfunction; Insulin-like growth factor 1

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INTRODUCTION
Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that mainly affects pyramidal neurons in the motor cortex and lower motor neurons that originate in the brainstem and spinal cord. The disease was first described in 1869 by the French neurobiologist and physician Charcot et al[1] who linked the symptoms of ALS to a group of nerves specifically affected by the disease; the motor neurons that originate in the spinal cord. The name of the disease reflects the different tissue compartments that are severely affected. In particular, “amyotrophic” refers to the atrophy of muscle fibers and loss of muscle mass; “lateral” refers to the nerve tracks that run down both sides of the spinal cord, where many neurons affected by ALS are found; and “sclerosis” refers to the scar tissue that remains following degeneration of the nerves.

The most typical feature of this progressive lethal disease is the degeneration of cortical, bulbar and spinal motor neurons, except for the neurons that control the bladder, and the oculomotor neurons[2]. This leads to muscle weakness, fasciculations, muscle atrophy, speech...
and swallowing disabilities, progressive paralysis, and death caused by respiratory failure.

ALS is epidemiologically classified into two forms: sporadic (90%-95%) and familial (5%-10%)\(^3\). Among the familial cases, approximately 20% are caused by dominantly inherited mutations in the Cu/Zn superoxide dismutase-1 (SOD1) protein\(^6\). Although the major part of these mutations are missense, deletions and insertions have been observed at the level of the C-terminal region of the protein\(^6\). The best function of the metalloenzyme SOD1 is to convert superoxide, a toxic by-product of mitochondrial oxidative phosphorylation, to water or hydrogen peroxide. Initially, it has been suggested that mutation in the SOD1 gene that lead to a decrease in the protein enzymatic activity (loss of function hypothesis). However, subsequent studies have clarified that mutant SOD1 possesses a neurotoxic property (gain of function hypothesis) that is responsible for the pathogenic mechanism of the disease\(^7\).\(^8\). Indeed, the finding that overexpression of mutant SOD1 in transgenic mice recapitulates several clinical feature of ALS disease, even in the presence of endogenous mouse SOD1, has led to the conclusion that the disease results from a toxic gain of function\(^9\).\(^10\). In addition to SOD1 mutations, other gene defects have been reported to cause ALS\(^3\). In particular, mutations in more than 50 different human genes are implicated in the pathogenesis of motor neuron cell death\(^1\)\(^1\).

Among these, mutation of synaptotobrevin/vesicle-associated membrane protein-associated protein B (VAPB) gene causes familial forms of motor neuron diseases, including a rare, slowly progressing form of ALS (ALS8), typical severe ALS with rapid progression, as well as a late-onset form of spinal muscular atrophy\(^11\).\(^12\)-\(^14\). Several functions have been ascribed to VAPB proteins, including membrane trafficking, cytoskeleton association, and membrane docking interactions for cytoplasmic factors.

More recently, Fasana et al\(^15\) have demonstrated that ALS-linked VAPB mutation causes dramatic endoplasmic reticulum (ER) restructuring that might underlie its pathogenicity in motor neurons. In addition, Langou et al\(^16\) have provided evidence that ER stress and impaired homeostatic regulation of calcium are implicated in the death process. It has also been demonstrated that VAPB-P56S transgenic mice develop cytoplasmic accumulations of TDP-43, a DNA/RNA-binding protein, which suggests a link between abnormal VAPB-P56S function and TDP-43 mis-localization\(^17\).

The research field of ALS has been improved by the recent discovery of two new mutations in the DNA/RNA-binding proteins TDP-43 and FUS/TLS, which have triggered new interest in ALS pathogenesis\(^18\)-\(^21\). However, even in these cases, where a well-defined mutation has been linked to the disease, a clear correlation between the genetic defect and the pathophysiology of the disease has not yet been disclosed. Notably, alteration in TDP-43 sub-localization is also associated with inclusion body myositis\(^22\), which suggests that this nucleic-acid-binding protein plays a pathologic role in skeletal muscle.

Although most efforts have been aimed at defining the potential genes and pathways associated with motor neuron degeneration and to understand ALS pathogenesis, no consensus has yet emerged as to the primary toxicity of SOD1 mutations. The primary causes of ALS are therefore still unknown and no effective and decisive treatments are available.

**PATHOGENIC MECHANISMS OF ALS**

Several pathogenic mechanisms have been proposed to account for ALS, including glutamate-induced excitotoxicity, oxidative stress, protein aggregation, and mitochondrial dysfunction (Figure 1).

**Glutamate-induced excitotoxicity**

Several lines of evidence implicate glutamatergic toxicity as a contributory factor in the neuronal injury in ALS\(^23\). Excessive stimulation of glutamate receptors causes excitotoxicity. Under physiological conditions, excitotoxicity is prevented by rapid binding and clearance of synapse-released glutamate by high-affinity, Na\(^+\)-dependent glutamate transporters. The excitatory amino acid transporters (EAATs) play a crucial role in the regulation of extracellular glutamate concentration, which stimulates the reuptake of glutamate and therefore maintains a
physiological concentration, of about 1-3 μmol/L, in the synaptic cleft[24]. Five different subtypes of EAAT (1-5) have been described and they are differentially distributed throughout the brain[25]. Notably, EAAT2 is the major glutamate transporter and is widely distributed in the human central nervous system. The selective localization of EAAT2 in glial cells has implications for its excitotoxic mechanisms.

How does glutamate induces excitotoxicity? It has been demonstrated that glutamate transport is diminished in sporadic and familial ALS patients, due to a selective loss of the astroglial glutamate transporter, EAAT2, in the motor cortex and spinal cord[25]. Alterations in this protein might interfere with the normal clearance, which allows glutamate to remain in the environment and continue to activate the receptors. Once activated, the receptors cause calcium influx that cells are not able to buffer because of an insufficient number of calcium-binding proteins. Altered concentration of calcium activates apoptotic pathways, which leads to motor neuron death and degeneration.

In addition, alteration in the antioxidant enzyme SOD1 induces oxidative damage to the intracellular C-terminal part of EAAT2, which results in decreased glutamate transport[26]. Although glutamate-induced excitotoxicity represents a pathogenic event of ALS, it remains to be determined whether these changes represent a primary defect that is responsible for motor neuron degeneration, or are the result of ALS.

Oxidative stress and ALS

The observation that approximately 20% of familial ALS is caused by mutations in the SOD1 gene has placed oxidative stress as one of the potential pathogenic events that are associated with the disease. Several lines of evidence suggest a primary role of oxidative stress in the pathogenesis of ALS, both in neurons and in muscle where antioxidant enzyme activity is altered[27]. To date more than 150 mutations have been described for the SOD1 gene, most of which cause dominantly inherited disease[28]. Mutations in SOD1 that impair its functions can lead to increased oxidative damage, which promotes activation of apoptotic pathways. Mutant SOD1 causes mitochondrial alteration as membrane depolarization, decreased activity of respiratory complexes and cytochrome c release[29,30]. Of note, the role of oxidative stress in tissue homeostasis is complex. It is clear that transiently increased levels of oxidative stress might reflect a potentially health-promoting process, whereas uncontrolled accumulation of oxidative stress might have pathological implications[31]. Additional work is therefore necessary to understand and define precisely whether the manipulation of the redox balance represents a useful approach in the design of therapeutic strategies for neuromuscular diseases.

Protein aggregation and ALS

One of the characteristic features of ALS is the occurrence of extra- or intracellular fibrillar aggregates that contain misfolded proteins with β-sheet conformation[32,33]. Biochemical studies have demonstrated that mutant SOD1 protein tends to be misfolded or forms aggregates that in turn trigger a toxic cascade that leads to neuronal degeneration. More recently, it has been demonstrated that TDP-43 spontaneously forms aggregates that bear a remarkable ultrastructural similarity to TDP-43 deposits in degenerating neurons of ALS patients[34]. The C-terminal domain of TDP-43 is crucial for spontaneous aggregation. Several ALS-linked TDP-43 mutations within this domain (Q331K, M337V, Q343R, N345K, R361S, and N390D) increase the number of TDP-43 aggregates and promote toxicity in vivo[35]. Under most circumstances, cells activate the protein chaperones and the ubiquitin-proteasome system to maintain protein quality control. Normally, cells are capable of handling mutant proteins sufficiently to prevent them from exerting toxic effects and/or being sequestered into inclusions[36]. However, under circumstances of increased physiological or environmental stress, the ubiquitin-proteasome system can become overloaded and impaired. This results in engulfment of the cells, which become defective in the disposal of altered macromolecules and more prone to damage.

Mitochondrial dysfunction and ALS

Several lines of evidence have shown alteration in mitochondria associated with ALS[36]. Mitochondria represent a primary site of intracellular production of reactive oxygen species, and hence a major source of oxidative stress that, in turn, impairs the normal function of mitochondria[37]. Despite numerous reports demonstrating mitochondrial abnormalities associated with ALS, the role of mitochondrial dysfunction in disease onset and progression remains unknown. It has been suggested that mutant SOD1 causes dysfunction and structural damage of mitochondria in human patients and mouse models of ALS[38].

A proposed mechanism suggests that mutant SOD1 is imported into mitochondria[39,40,41], which causes direct damage of the organelle and activation of cell death[42]. The mechanisms that regulate SOD1 mitochondrial import involve the redox state of the cell, the intracellular distribution of the copper chaperone for SOD1, and the folding of SOD1[42]. Indeed, overexpression of copper chaperone for SOD1 increases mitochondrial localization of mutant SOD1, which in turn causes early loss of mitochondrial function and disease progression[40].

Mitochondrial dysfunction, which appears early in the course of ALS pathology, does not seem to be restricted to motor neurons, and it is present in other tissues, particularly skeletal muscle[43].

ALS: IS IT JUST A MOTOR NEURON DISEASE?

Although most efforts have been aimed at defining the potential genes and mechanisms that are associated
with motor neuron degeneration and to understand ALS pathogenesis, no consensus has yet emerged about which cells, tissues and pathways are directly affected by mutant SOD1. Several lines of evidence have suggested that the neurodegenerative action of mutant SOD1 genes operate through a dominant paracrine activity that emanates from non-neuronal tissues.

The obvious loss of motor neurons in the spinal cord initially focused attention on how mutant SOD1 might act within motor neurons to provoke neuronal degeneration and death. However, the mutant gene products are expressed widely, which raises the possibility that the toxicity might result from the action of mutant SOD1 protein in non-neuronal cells. This notion is supported by recent experimental evidence.

Notably, restriction of SOD1 mutant expression selectively to postnatal motor neurons fails to produce detectable signs of pathology or motor neuron disease. More recently, Jaarsma et al have demonstrated that transgenic mice in which mutant SOD1 was largely restricted to neurons, under the transcriptional control of Thy1.2 promoter, developed disease only at an old age. However, the disease progressed slowly without reaching the same degree of paralysis compared to the classical animal model of ALS in which the same mutant SOD1 gene is ubiquitously expressed. This suggests that other cell types are involved in ALS-associated neurodegeneration. In fact, analysis of chimeras that are generated between wild-type and SOD1 mutant mouse embryonic cells has revealed that wild-type non-neuronal cells in adult chimeric animals extend the survival of SOD1 mutant motor neurons. This suggests that the neurodegenerative action of mutant SOD1 operates through a dominant paracrine activity that emanates from non-neuronal cells.

In particular, it has been demonstrated that, diminishing the SOD1 mutant levels in microglia has little effect on the early disease phase, but markedly slows later disease progression. These results suggest that mutant SOD1 in motor neurons affect disease onset, whereas mutant SOD1 in microglia contributes to the propagation of disease at a late stage.

Notably, astrocyte activity normally increases at later stages of ALS disease and is concomitant with motor neuron degeneration, which suggests that astrocytes act as deadly neighbors that exacerbate motor neuron damage. Indeed, co-cultured motor neurons are less likely to survive when they are on astrocytes that express mutant SOD1, or exposed to astrocyte-conditioned medium, than on astrocytes that express normal SOD1.

Although mutant SOD1 is also expressed by muscle, it is not clear whether its presence in skeletal muscle directly contributes to any pathological sign of ALS. This issue has been recently investigated by work in our laboratory, which has demonstrated that muscle selective expression of SOD1 mutation causes pathological alterations and induces pre-symptomatic sign of ALS. Our data might explain previous findings that have shown how the ubiquitous expression of SOD1 in transgenic mice causes first muscle atrophy, which is later followed by alteration of the neuromuscular junction (NMJ), retrograde axonal degeneration, and lastly, motor neuron death. This retrograde and progressive (muscle-to-NMJ-to-nerve) sequential pattern of degeneration suggests the possibility that certain muscle abnormalities indeed precede motor neuron death rather than result from it.

These studies formally prove that: (1) muscle atrophy is not necessarily determined by motor neuron degeneration or activity, but it is causally linked to the toxic gain of function of SOD1 expression; and (2) skeletal muscle is a direct contributor of ALS pathogenesis. Our study, however, seems apparently in contrast with that reported by Miller et al who found that mutant SOD1 does not cause toxicity by its action within the muscle, which suggests that muscle is not a primary target for non-cell-autonomous toxicity in familial ALS. In these experiments, the authors found that the partial reduction of mutant SOD1 within muscle, using either a lentivirus that encodes a siRNA directed against mutant SOD1 or muscle selective SOD1 mutant gene excision, does not affect disease. The apparent discrepancy between the two studies can be explained considering that: (1) partial suppression of mutant SOD1 accumulation within muscle is not sufficient to delay the progression of the disease in SOD1G93A mice, which suggests that residual expression of SOD1G93A mutant gene is able to maintain a pathological muscle phenotype; and (2) low expression levels of MLC/SOD1G93A are sufficient to cause muscle atrophy and alteration in functional performance of the soleus muscle.

Moreover other studies support the evidence that skeletal muscle is a primary target of mutant SOD1 toxicity in mice. Wong and Martin have reported that skeletal-muscle-restricted expression of human mutant SOD1 gene causes motor neuron degeneration in old transgenic mice. Dupuis et al have reported that muscle selective alterations in mitochondrial function might initiate NMJ destruction, which is followed by distal axonopathy, astrocystosis in the spinal cord, and mild motor neuron loss. Moreover, Zhou et al have reported that alterations in the potential of mitochondrial inner membrane of fiber segments near NMJs occur in young SOD1G93A mice prior to disease onset.

All the above suggests that skeletal muscle is an important candidate to consider as a primary target of the toxicity that results from mutations in the SOD1 gene, and that the effective connection between muscle and nerve is crucial to the capacity of both partners to survive and function adequately throughout life. Of note, skeletal muscle is also a source of signals that influence neuron survival, axonal growth and maintenance of synaptic connections. Indeed, development in the absence of skeletal muscle results in the sequential ablation of motor neurons from the spinal cord to the brain. Thus, muscle clearly plays an important role in providing...
guidance and cues to the developing motor neurons, and in providing trophic support to maintain motor neuron and axon function\textsuperscript{[54]}. In absence of this trophic support, muscle can have a negative impact on the nervous system, and therefore, can contribute to the alteration in the functional connection between muscle and nerve.

Among growth factors, insulin-like growth factor 1 (IGF-1) has been implicated in anabolism of muscle and nerve tissues, which induces muscle hypertrophy and promotes neuronal survival\textsuperscript{[55,56]}. The therapeutic potential of IGF-1 in ALS was underscored by injection of SOD1\textsuperscript{G93A} mouse muscle with an adeno-associated virus that carried an IGF-1 gene, and by a transgenic approach in which the local form of IGF-1 was selectively expressed in skeletal muscle\textsuperscript{[57,58]}. The two approaches have demonstrated that muscle IGF-1 expression counteracts the symptoms of ALS and reduces components of catabolism, which activates satellite cells and markers of muscle regeneration\textsuperscript{[59,60]}. However, the use of IGF-1 in ALS patients has previously produced conflicting results and a new study has reported no benefit in either survival or functional scale\textsuperscript{[61]}. The reason for these discrepant results could reside in the different delivery approaches used in human and mouse models and/or in the different isoforms of IGF-1 used in the these studies.

MICRONORNA AND ALS

Recent studies have also uncovered profound and unexpected roles for a family of small regulatory RNAs, known as microRNAs (miRNAs) (or miR), in the control of cell proliferation, differentiation and development, and in the pathogenesis of several diseases.

miRNAs are endogenous, approximately 22 nucleotides long, and inhibit translation or promote mRNA degradation by annealing to complementary sequences in the 3\textsuperscript{′} untranslated regions of specific target miRNAs\textsuperscript{[62]}. miRNA expression profiles are highly dynamic during embryonic development and in adulthood. Mis-expression of miRNAs can perturb embryogenesis, organogenesis, tissue homeostasis and the cell cycle\textsuperscript{[63]}. Several miRNAs are expressed in the nervous system where they play a pivotal role in neuronal differentiation, synaptogenesis and plasticity\textsuperscript{[64]}, and deregulation of miRNAs can have profound effects on neuronal physiology and pathology\textsuperscript{[65]}. Although it is known that deregulation of specific miRNA-dependent regulatory circuitries correlates with the initiation and progression of several neurological disorders, the underlying mechanisms of these phenomena are still not fully understood. Both TDP-43 and FUS proteins, mutations of which can be involved in ALS pathogenesis, have been shown to interact specifically with the Drosha protein\textsuperscript{[66]}, thus suggesting that they are involved in the regulation of miRNA expression by modulating the activity of this processing enzyme.

In addition, several miRNAs, such as miR 1, miR 133, miR 214, miR 181 and miR 206, are specifically expressed in skeletal muscles\textsuperscript{[67]}. It has been reported recently that miR-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mouse models\textsuperscript{[68]}. In particular, it has been demonstrated that loss of miR-206 does not affect disease onset, but does accelerate disease progression and atrophy of skeletal muscle, which leads to kyphosis, paralysis and death\textsuperscript{[69]}. The mechanism by which miR-206 promotes a partially successful compensatory response to denervation involves the histone deacetylase 4 (HDAC4), which normally inhibits nerve re-innervation by blocking the expression of fibroblast growth factor binding protein 1 (FGFBP1)\textsuperscript{[70]}. Thus, miR-206 blocking the activity of HDAC4 guarantees the activation of FGFBP1 expression, which promotes the maintenance of NMJ integrity and plasticity. This evidence further reinforces the hypothesis that muscle-derived factors can promote a functional nerve-muscle interaction, which delays motor neuron degeneration.

CONCLUSION

Although there have been significant advances in understanding the biology of ALS, no consensus has emerged as to which cells, tissues and pathways are directly affected by mutant SOD1. Several pathways have been implicated in disease pathogenesis, including glutamate-mediated excitotoxicity, mitochondrial dysfunction, neuro-inflammation, apoptosis, oxidative stress, protein aggregation, and aberrant axonal transport (Figure 1). The results reviewed in this manuscript support the re-definition of ALS as a multi-systemic disease in which alterations in structural, physiological and metabolic parameters in different cell types (muscle, motor neurons, and glia) act synergistically to exacerbate the disease. Multi-interventional approaches, including novel methods to intercept the damage and to deliver molecules to vulnerable cells, have recently been shown to be effective\textsuperscript{[80]}. Thus, new avenues for promising therapeutic approaches can be derived from multidrug treatments and/or the delivery of growth factors by viral vectors, in combination with exercise and/or dietary regimes\textsuperscript{[81]}. From a clinical point of view, the most powerful future approach would be to target motor neurons, non-motor neuronal cells and skeletal muscle.

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