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Forum Editorial

Amyotrophic Lateral Sclerosis: Mechanisms and Countermeasures

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Amyotrophic lateral sclerosis (ALS) is currently an area of intense research interest. More than 130 years have passed since its first clinical description, and 15 years since the discovery of the first gene linked to the familiar form for the disease, coding for Cu, Zn superoxide dismutase (SOD1), which led to the design of a variety of experimental models including transgenic rodents overexpressing ALS-typical SOD1. Our knowledge of the molecular mechanisms operating in ALS has benefited from those models, and reasons exist to believe that advances in therapy are to be expected in the near future.

ALS is the most common adult-onset motoneuronal disease; invariably fatal in absence of any effective therapy, it affects six to eight people per 100,000. About one tenth of patients carry inheritable defects, and they are therefore diagnosed as affected by familial ALS (fALS). Our knowledge of the genes involved in fALS is still incomplete. Whereas association with mutations in *sod1* is definitely proven, linkage to variants of other genes is still to be ascertained beyond doubt. What is clear is that, despite some heterogeneity in symptoms, age at onset, and progression, the more frequent sporadic ALS (sALS) and fALS patients share common general features such as progressive muscle waste due to motor neuron loss, progressive paralysis, respiratory failure, and ultimately, death. From studies in the animal model, it is also quite clear that at least three distinct phases exist in the disease: asymptomatic (but alterations at cell and molecular level are already operating), onset (early symptoms are clinically detectable, and a number of motor neurons are already lost), and progression (overt motor impairment). The existence of these phases is due to a differential and possibly time-dependent contribution of various, but partially overlapping, factors (4, 10).

ALS is often described as a *multifactorial* disease, in which oxidative stress, mitochondrial damage, excitotoxicity, protein aggregation, and impairment of axonal transport are involved, and a *multisystem* disease, in which damage in motor neurons in the ventral horn of the spinal cord (and possibly other, cortical neurons) is intertwined with damage to non-neuronal cells (astrocytes, microglia, muscle cells). As it appears from recent studies, among the reasons we have not been able to devise a satisfactory therapy, we have to consider that the interplay among the different actors in this dramatic disease is so complex that it can hardly be modeled efficiently in preclinical studies and that interception of a single pathway of toxicity cannot be significantly effective in halting the progression. For instance, it is now ascertained that mutant SOD1 (mutSOD1) tends to form inclusions in the spinal cord of SOD1-linked fALS patients and in animal models of this disease. In line with a leading hypothesis, that postulates that the mutant protein acquires toxic properties that are independent of its normal physiologic function, mutSOD1s are or become unstable and misfold to form high-molecular-weight aggregates that are selectively toxic to motor neurons. These inclusions consist of SOD1-rich fibrils, and the high degree of correlation between the formation of fibrillar mutSOD1 aggregates and motor neuron degeneration suggests that these phenomena are somehow dependent (9). However, the build-up of amyloid fibrils may represent a mechanism by which the soluble oligomeric species are sequestered into less harmful, insoluble forms. This hypothesis is supported, for instance, by the observation that soluble misfolded mutSOD1s are enriched in spinal cords very early and throughout life in murine ALS models (24).

A distinct, but related, possibility is that small aggregates of mutSOD1 are toxic when localized into specific cell compartments. All mutSOD1s and, to a lesser extent, wild-type SOD1 tend to associate with mitochondria (14, 18), and oligomerization of mutSOD1 in mitochondria of motoneuronal cells prompts mitochondrial damage and cell toxicity (11).

Mutant SOD1 may also interact with components of the mitochondrial transport machinery leading to dysfunctional mitochondrial dynamics. In a recently proposed model, defects in anterograde transport preclude the arrival of healthy

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mitochondria to distal regions of the axon, whereas impaired retrograde transport of aged and dysfunctional mitochondria fails to move them toward the cell body, either to fuse with upcoming healthy mitochondria or to be degraded by autophagy (17). Motor axons, but not sensory axons, from asymptomatic mutSOD1 G93A rats show accumulation of mitochondria in discrete clusters, enriched in mutSOD1, and located at the axonal cortex at regular intervals. These clusters are also immunoreactive to nitrotyrosine and surrounded by diffuse cytochrome *c* immunoreactivity, whereas ubiquitin colocalizes with clusters only at late states of the disease (21). The ultimate consequence of this scenario is the accumulation of damaged mitochondria in motor neuron terminals, not only causing local energy depletion and Ca^{2+} imbalance, but also triggering synaptic dysfunction and loss and the activation of apoptotic pathways (17). Interestingly, this view is in line with early observations (20) of significantly increased calcium and increased mitochondrial volume in the motor nerve terminals of muscle biopsies from patients with sporadic ALS at diagnosis, compared with denervating neuropathies and other controls. This phenomenon seems, therefore, not restricted to the presence of mutant SOD1.

But what else happens to the great majority of patients, who do not carry mutSOD1? The question has been addressed in several studies. For instance, a role for metal deprivation in the toxicity of mutSOD1 has been proposed, on the ground that loss of zinc from SOD1 results in the remaining copper in the protein becoming extremely toxic to motor neurons in culture by a mechanism requiring nitric oxide (12). This theory proposes that the mutations do not directly confer the toxic gain-of-function to SOD1 but rather increase the susceptibility of the enzyme to lose zinc. The zinc-deficient SOD1 is more accessible, more redox reactive, and a better catalyst of tyrosine nitration, and indeed, increased levels of 3-nitrotyrosine in the central nervous system have been found in patients with sALS and in the mouse model of fALS (2, 8). Although experiments in transgenic mice have yielded contradictory evidence to the zinc-deficient hypothesis, and mutSOD1s have a modestly reduced affinity for zinc, wild-type SOD1 can be induced to lose zinc *in vitro* by dialysis at slightly acidic pH. This zinc-deficient hypothesis offers an explanation of how wild-type SOD1 can be toxic in sALS and non-mutSOD1 fALS patients and predicts that a therapeutic agent directed at zinc-deficient mutSOD1 could be even more effective in treating sporadic ALS patients (22).

With a similar theoretic approach, that wild-type SOD1 may be involved in the sporadic form of this disease (9), abnormally folded SOD1-containing species with a molecular weight that is roughly similar to that of dimeric SOD1 have been isolated from the spinals cords of sALS patients (16). An intriguing possibility is that wild-type SOD1 may behave like mutSOD1 under certain environmental conditions, such as fortuitous oxidative stress. Like mutSOD1, oxidized wild-type SOD1 can be conjugated with polyubiquitin and can interact with Hsp70 and with chromogranin B in motoneuronal cultured cells treated with hydrogen peroxide. Furthermore, exposure to oxidized wild-type SOD1 induces death in neuronal cultured cells in a dose-dependent fashion, similar to exposure to mutSOD1s (13).

Emerging evidence indicates that defects in the genes regulating NADPH oxidases may account for at least some forms of ALS associated with enhanced redox stress. NADPH oxidases are multi-subunit enzyme complexes that generate ROS and control proinflammatory signaling pathways through redox-dependent control of certain cytokine receptor activation. In a novel view (7), Engelhardt's group suggests that this proinflammatory process is mediated by the endosomal signaling through the activation of NADPH oxidases (specifically Nox1 and Nox2) by the small GTPase Rac1. In particular, Nox2 seems to play a major role in ROS production and microglia activation, and its deletion induces a remarkable increase in the survival index of SOD1-mutant mice. Activation of Nox2-mediated inflammation seems, therefore, to contribute mainly to the progression of the disease by increasing the redox burden from microglia.

Interestingly, several other genes that are known to be associated with the familial form, like ALS2 and SOD1, or the sporadic form of the disease, appear to converge on Rac1 regulation (7). Hence, the mechanisms by which defects in these proteins lead to ALS may overlap.

That redox stress plays an important role in ALS pathology is also demonstrated by the high levels of protein carbonyl and nitrotyrosine modifications, both markers of oxidative protein damage, found in the spinal cords of ALS patients and mouse models of familial ALS (1, 2, 8). Among the proteins that can be inactivated by the oxidative or nitrosative stress is the glial glutamate transporter EAAT2. A role for the dysregulation of glutamate homeostasis in both sALS and fALS has been proposed, based on the established vulnerability of motor neurons to glutamate excitotoxicity and on increased plasma levels of glutamate, decreased glutamate uptake, and decreased expression levels of EAAT2 documented in ALS patients (15). In a chronic neurodegenerative disease such as ALS, however, it is not clear whether these dysfunctions in the glutamate-transport system contribute to the pathogenesis or whether they are more a secondary event consequential to primary pathologic insults.

Nonetheless, it is obvious that because influx of Ca^{2+} mediated by glutamate is buffered by the endoplasmic reticulum and the mitochondria, failure of one or both these buffering systems (for instance, because of toxic SOD1 aggregates) would make motor neurons highly vulnerable.

Future Directions

As discussed in this issue, different pathways of toxicity are strictly intertwined and most probably cooperate in the pathogenesis of ALS. However, more experimentation is required to define precisely the timing of activation of those pathways and to devise new strategies for the treatment of ALS. As far as patients affected by SOD1-linked familial ALS are concerned, one possible approach could be to prevent the toxicity of the mutant protein by reducing its level in vulnerable cells. Prevention of mutSOD1 aggregation, for instance, might be accomplished in SOD1-linked fALS nonpharmacologically, by targeted RNA interference in affected tissues (10). As suggested from studies in the mouse model, gene silencing of mutSOD1 seems now feasible through direct nerve injection of recombinant adenoviral vectors that efficiently transfer transgenes into motor neurons. Even in this case, more studies to enhance the RNAi therapeutic efficacy are needed both to find new vectors for long-term transgene expression and to avoid preexisting immunity against human retroviruses (23). Nonetheless, with persistent efforts, we

hope that clinical application of RNAi therapy for ALS may be found in the near future.

For the majority of sporadic ALS patients, a key problem lies in the fact that in most cases, the diagnosis takes more than 1 year after the appearance of symptoms. In this light, finding reliable disease biomarkers that may both shorten the time for diagnosis and identify pathways implicated in the progression of the disease is crucial to define a potential effective therapy in symptomatic patients. In a recent study analyzing peripheral blood mononuclear cells from sporadic ALS patients and a rat model of mutSOD1-linked fALS, a few specific overnitrated proteins were individuated (19), suggesting that an increased level of nitrated proteins is not restricted to the spinal cord and that those nitrated proteins are promising candidate biomarkers for early diagnosis of both sALS and fALS. Although further studies are needed to extend and validate these observations and to design a proper set of antibodies to be used in daily clinical practice, this finding may provide a useful tool also for monitoring the efficacy of therapies and distinguishing between responder and nonresponder patients.

Overall, available evidence suggests that mechanisms that affect cellular redox stress either directly through mitochondrial alterations or indirectly through inflammatory processes may provide a unifying explanation for the pathogenesis of this disease.

However, results from clinical trials for ALS that are based on antioxidant strategies have so far yielded negative results, even if some of the potential therapeutics were effective in animal models. The reasons for such failures may be different. One possibility is that the mechanisms involved in the motor neuron damage may change during the progression of the disease, and such complexity should be considered in terms of a time-adjusted multidrug approach (5, 6). Another reason lies in the emerging heterogeneity of the disease in terms of genetic, biochemical, and clinical features (3). Most of the past trials have addressed potential treatments in ALS patients, considering them as belonging to a homogeneous population. Because several genes and a range of environmental influences have been suggested as possible risk factors to develop ALS, it would be interesting to define a distinct pathologic profile in specific individuals or groups of patients with ALS based on genetic and biomarkers assays. This should enable us to design more-specific therapeutic strategies with higher potential efficacy.

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