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PATHOGENESIS OF AMYOTROPHIC LATERAL SCLEROSIS

Magdalena Kuźma-Kozakiewicz
Department of Neurology, Medical University of Warsaw, Warsaw, Poland

Amyotrophic lateral sclerosis is a devastating neurodegenerative disease affecting both upper and lower motor neuron. Despite extensive research the primary cause of the disease has not been indentified and the causative treatment is lacking. The present article describes mechanisms involved in the disease development and progression, including oxidative stress, excitotoxicity, mitochondrial dysfunction, protein aggregation, RNA processing, alterations of cytoskeleton functions and axonal transport, glial cell involvement and programmed cell death. ***Biomed Rev* 2011; 22: 7-14.**

Key words: apoptosis, axonal transport, glial cells, glutamate excitoxicity, mitochondrial dysfunction, oxidative stress, superoxide dismutase

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting up to 500 000 people worldwide. It is caused by a selective and progressive loss of upper motor neurons of the corticospinal and corticobulbar tracts and lower motor neurons localized in the brain stem and anterior horns of the spinal cord. At present ALS is an incurable disease leading to respiratory insufficiency and death within three years from onset. The symptoms usually start in the fifth decade but they may become apparent before the age of thirty or over the age of 70. The onset is either bulbar or spinal. In the bulbar form, the main symptoms include dysarthria and dysphagia. The limb symptoms start with asymmetric muscle paresis and wasting either in distal or, less frequently, in proximal muscles. Both forms end up involving all voluntary muscles with the exception of sphincters. There is no sensory involvement. The disease progression varies between individuals. In approximately 20% of patients the survival exceeds 5 years and only in about 10%, more than 10 years (1). There is no causative treatment. A vast majority of ALS cases are sporadic (sporadic ALS, SALS). Only approximately 10% of cases are inherited, mainly as an autosomal dominant trait (familial ALS, FALS). Up to 23% of FALS and 7% of SALS cases are due to mutations in the *SOD1* gene (2). Eight percent of FALS and over 1% of SALS cases are caused by mutations in *TARDBP* and *FUS/TLS,* two recently discovered genes encoding for proteins involved in RNA processing (3). Other genetic factors among which the proteins alsin, progranulin, angiogenin, vascular endothelial cell growth factor, vesicle-associated membrane protein and

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Correspondence: Magdalena Kuźma-Kozakiewicz, MD, PhD, Department of Neurology, Medical University of Warsaw, Banacha 1a, PL- 02-097 Warsaw, Poland. Tel.: +48 22 5992 857; Fax: +48 22 5991 857;
E-mail: magdalena.kuzma@wum.edu.pl

senataxin are less frequent.

The present article highlights recent advances in our understanding of molecular mechanisms responsible for ALS.

OXIDATIVE STRESS

Radical oxygen species (ROS) are reactive forms of oxygen containing one unpaired electron. They can easily peroxidize organic and inorganic compounds changing their structural and functional properties. Lipid peroxidation may influence cell membrane permeability. Oxidation of proteins leads to alteration of their enzymatic activity and/or conformation. When reacting with nucleic acids, ROS may lead to mutagenesis. Radical oxygen species are synthesized in reactions of respiratory chain or beta-oxidation. In physiological conditions they are efficiently neutralized by a number of enzymatic and non-enzymatic cell defense mechanisms. However, the imbalance between their production and removal leads to oxidative stress. CuZn superoxide dismutase (SOD1) is a free radical scavenging enzyme, catalyzing the reaction of peroxidation of oxygen peroxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) and oxygen: $O_2^{\bullet-} + O_2^{\bullet-} + H_2 \rightarrow H_2O_2 + O_2$.The discovery of mutation in the *SOD1* gene in FALS in 1993 directed the studies on ALS pathogenesis toward the role of oxidative stress (4).

It was primarily suggested that mutations in *SOD1* decreased cell capability of neutralizing ROS. However, in many cases of *SOD1* mutations there were no changes in SOD1 enzymatic activity. Transgenic mice harboring FALS-linked *SOD1* mutations demonstrate normal or enhanced enzymatic activity (5). Moreover, it was proved that transgenic mice either knocked-out for *SOD1* or over-expressing the wild type SOD1 (wtSOD1) did not develop motor neuron disease (MND) (6). Interestingly enough, transgenic mice harboring human mutated SOD1 develop MND despite normal expression of wtSOD1 (7). It was therefore postulated that mutations in *SOD1* gene induce a toxic gain of function of the encoded protein (8). Hundred fifty seven ALS-linked mutations in the *SOD1* have been reported to date (for complete list please refer to http://www.alsod.org, http:// alsod1.iop.kcl.ac.uk/reports/mutations). Currently proposed mechanism of mutated SOD1-induced neurodegeneration include alteration of substrate specificity and instability of mutated protein (9). Nevertheless, nearly 20 years after the discovery, the exact mechanism in which the mutations lead to selective death of motor neurons remains unknown.

Despite the lack of influence of decreased SOD1 activity on the clinical course of ALS, several studies have shown signs of alteration of ROS scavenging mechanisms in MND (reviewed in 10). Increased concentration of carbonyl groups (products of protein peroxidation), malone dialdehyde (products of lipids peroxidation), and 8-OHdG (nucleic acids) was found in cortex and/or spinal cord of patients who died in course of SALS (10,11). There was an increase of 3-nitrotyrozine (products of tyrosine peroxidation) concentration in anterior horns of the spinal cord in both SALS and FALS patients. High level of 8-OHdG and hydroxynonenal (lipid peroxidation product) was observed in CSF of patients suffering from ALS. The presence of peroxidation products of proteins, lipids and nucleic acids was also shown in mouse transgenic models of MND both at pre symptomatic and symptomatic stage (10). Antioxidant treatment was able to delay disease onset and progression in SOD1-transgenic mice, but all completed clinical trials in ALS patients showed no clinical efficacy of antioxidants (1).

EXCITOTOXICITY

A report on increased glutamate concentration in CSF of ALS patients directed the pathogenetic studies toward excitatory amino acids (12). Exposition of cultured motor neurons to glutamate or aspartate was shown to induce cell death by apoptosis or necrosis, dependent on the amino acid concentration (13,14). In physiological conditions, activation of glutaminergic receptors, mainly N-methyl-D-aspartate (NMDA) receptors, leads to opening of post-synaptic calcium channels and the intracellular influx of Ca^{+2}. Given the role of calcium ions in regulation of cellular growth, differentiation and synaptic activity, maintaining the calcium homeostasis is vital. For this reason, an excessive activation of glutaminergic receptors due to pathologically increased glutamate concentration may alter calcium homeostasis and lead to cell death. The main protein responsible for a reduction of glutamate concentration in the synaptic cleft is a glial transporter of excitatory amino acids (EAAT2). It is a Na^{+}-dependent protein localized on the surface of glial cells, involved in presynaptic uptake of the glutamate. EAAT2 is particularly vulnerable to oxidative stress (15). Excessive influx of calcium ions due to activation of glutaminergic receptors leads to increased mitochondrial production of ROS, which alter EAAT2 function and increase excitotoxicity. Defects in EAAT2 have been found in 80% of SALS patients (16). Decrease of EAAT2 concentration was observed in brains of patients who died in course of ALS. In transgenic models of the disease, reduced EAAT2 expression correlated with increased concentration of glutamate in the nervous system (17). Riluzole, an inhibitor of NMDA receptors, is at present

the only drug for ALS approved by Food and Drug Administration in USA and by European Medicines Agency. Although its accurate mechanism of action remains unclear, it inhibits glutamate release from presynaptic membrane, increases its extracellular uptake and stabilizes voltage-gated sodium channels in inactive state (18). An 18-month treatment with riluzole prolongs patients' survival by approximately 7% (19).

Until 20 years ago, the NMDA receptor was the only glutamate receptor known to be Ca^{2+}-permeable. It is now well established that the ionotropic alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor of glutamate is densely distributed in the mammalian brain and is involved in mediating fast excitatory synaptic transmission, motor neurons showing selective vulnerability to activation of AMPA receptors; expression of modified human AMPA receptor in transgenic animals induces MND (20). The permeability of AMPA receptors for calcium ions depends on their subunit structure (21). Increased expression of GluR3, one of the AMPA receptor subunits, in motor neurons carrying *SOD1* mutations increases their susceptibility to kainate-induced excitotoxicity. The toxicity results from facilitation of the Ca2+ influx (22). Antisense oligonucleotides therapy against *GluR3* was able to delay motor impairment and extend survival of transgenic mice with *SOD1* mutation (23). Human trials have not been performed.

Noteworthy, recent data suggests a crosstalk between glutamate receptors and brain-derived neurotrophic factor (BDNF) in modulating synaptic functions (24,25, also see 20-22). Such a BDNF-NMDA/ AMPA receptor signaling might be pursued in the pathogenesis and therapy of ALS.

MITOCHONDRIAL DYSFUNCTION

Post mortem studies in ALS revealed the presence of abnormal mitochondria localized under the sarcolemma, in synaptic terminals and in anterior horn cells (26). Muscle biopsies performed in ALS showed big mitochondria with increased calcium concentration (26). There were also reports on impaired activity of complex I and IV of the respiratory chain (encoded by mitochondrial DNA) in skeletal muscles and spinal cord of ALS patients (27). In transgenic models of ALS with human *SOD1* mutation, aggregates of abnormal mitochondria with dilated external membrane were found within the motor neurons. They resulted from membrane detachment, which occurred after aggregation of mutated SOD1 in the intermembrane space of mitochondria. The presence of these structures early in course of the disease, when the loss of motor neurons is not yet accompanied by clinical symptoms, points at an active role of mitochondria in ALS pathogenesis (28). Currently, there are 2 clinical studies with the use of mitochondrial protection agents, which may show some promise in the treatment of ALS. Among them there are tamoxifen, which binds to the mitochondrial permeability transition pore, and dexpramipexol, which reduces the ROS production and improves mitochondrial function (1).

PROTEIN AGGREGATES

Although protein aggregates are a hallmark of neurodegeneration, there is still no agreement on their exact role. By binding proteins necessary for cell survival, the protein aggregates may lead to cell degeneration. On the other hand, they may protect the cell from toxic products by their direct binding. And finally, as by-products of pathological processes they can simply be markers of degeneration. There are three types of cellular inclusions typically found in ALS. They are hyaline conglomerate inclusions (HCI), Bunina bodies and ubiquinated inclusions (UBI) (29). Hyaline conglomerate inclusions are big inclusions of phosphorylated and non-phosphorylated neurofilaments and random proteins or cellular organelles. Since they were found in a number of neurodegenerative diseases and control tissue, they are considered unspecific derivatives of various pathological processes. Bunina bodies are small eosinophilic inclusions found in cell bodies of motor neurons localized in the spinal cord of 80–100% SALS cases. They are positive for cystatin C. Similar structures were found in physiological aging but not in other neurodegenerative diseases. Most characteristic aggregates present nearly in all cases of SALS are ubiquinated inclusions. They were found in motor neurons of brain stem and anterior horns of the spinal cord. They present a variety of shapes from fibrillary, through skein-like to compact ('Lewy-like') inclusions. Until 2007 the main protein compound of UBI was unknown. They were not reactive to antibodies against tau protein, neurofilaments, alpha-synuclein or cystatin C, the elements characteristic for neurodegenerative precesses (30). In 2007 Mackenzie and coworkers (31) reported that UBI were positive for transactive response DNA-binding protein (TDP-43), previously described in frontotemporal lobe degeneration (FTLD) (32). Most interestingly, TDP-43 was found in cases of SALS or FALS but not in FALS with mutation in *SOD1* gene indicating a different pathological entity of these two conditions (33). It was further identified in some cases of Alzheimer's and Parkinson disease, inclusion

body miositis and myopathy with rimmed vacuoles (34). In neurodegenerative diseases it did not however colocalize with tau and synuclein inclusions. Within the group of FALS, TDP-43 inclusions were also found in cases with *ANG* and *TARDBP* but not *FUS/TLS* mutations. TDP-43 is a 414-aminoacid protein encoded by *TARDBP* gen. In physiological conditions it is localized in the nucleus. However, no wt TDP-43 protein was found within the nuclei of the neuronal cells from patients affected by FTLD or ALS (35). In these cases all the pool of intracellular TDP-43 accumulated in the cytoplasm. It was subjected to hyperphosphorylation, ubiquitination and cleavage to generate C-terminal fragments. The cytoplasmic redistribution of TDP-43 was found to be an early event (36). Following this discovery, mutations in *TARDBP* were found responsible for 4% of FALS and1% of SALS cases (33). Till now 38 disease-causing mutations were identified.

RNA PROCESSING

Since *TARDBP* contains two RNA-recognition motifs, TDP-43 is a RNA- and, to a lesser extent, a DNA-binding protein. It attaches to a TG-rich fragment of RNA within the promoter of HIV-1 gene to stop its transcription (37). By binding to a 3'-UTR sequence encoding for human neurofilament light chain (hNFL1), it stabilizes the transcript and helps maintain the correct neurofilament stoichiometry (34). The plausible role of RNA processing in pathogenesis of ALS was reinforced by identification of disease causing mutations in *FUS/TLS* gene (38). It encodes for another RNA-binding protein called fused in sarcoma/translated in liposarcoma (FUS/TLS). FUS/TLS is a 526-aminoacid protein encoded by 15 exons. It was shown to bind both RNA and DNA, and, like TDP-43, function in diverse processes including transcription, alternative splicing and microRNA processing (33). The mutant form of FUS/TLS accumulates in cytoplasm of neurons and glial cells although the nuclear cleavage of FUS/TLS in ALS is less spectacular. No ubiquitination and phosphorilation of the protein was ever reported. Beside ALS, FUS/TLS forms neuronal intranuclear inclusions in poliglutamine diseases such as Huntington disease and spinocerebellar ataxias (39). It does not however form aggregates in FALS with *TARDBP* mutations.

Thirty mutations of *FUS/TLS* were found in ALS, being responsible for 4% of FALS and rare cases of SALS. Several *in vitro* studies performed recently shaded more light on the role of TDP-43 and FUS/TLS in RNA processing (33).

ALTERATIONS OF CYTOSKELETON FUNCTIONS AND AXONAL TRANSPORT

Among other factors, neural cell homeostasis strongly depends on axonal transport. It provides cell with neurotrophic factors, carries signal proteins, cell organelles, cleaved proteins and membrane fragments. Human motor neurons may be 5000 fold larger than average cells and their axon length may exceed 1 meter (40). For this reason, the efficient axonal transport within motor neurons is particularly important. It depends on interactions between cytoskeleton and motor proteins. The cytoskeleton is composed of microtubules (MT) and MT-associated proteins, actin filaments/actin-associated proteins, and intermediate filaments, neurofilaments being a subtype of the latter. Microtubules are composed of assembled (polymerized) tubulin heterodimers. In course of their formation, the tubulin dimers are preferentially added to the plus end of the MT assuring its growth from the cell body towards the periphery (41). The transport of cargoes by MT-dependent ATP-associated motor proteins, kinesin and dynein, is baseds on MT's structural polarity. Actin filaments are localized mainly in the cell cortex thus not directly involved in neuronal transport. Neurofilaments, run along the axons and their main role is to control axonal caliber. Since the speed of signal conduction depends on axonal diameter, the neurofilaments are particularly abundant in large-diameter axons (42). Kinesins transport synaptic vesicles, membrane constituents and mitochondria from the cell body towards the plus end of the microtubules localized in the cell periphery. On the contrary, neurotrophic factors, exogenous substances and waste membrane fragments use the retrograde transport mediated by the denein/dynactin complex (43).

Several mutations of genes encoding for motor proteins have been found in motor neuron diseases. Hereditary spastic paraplegia is caused by mutations of kinesin heavy chain (*KIF3A*) (44). ALS with vocal cord paralysis was linked to mutation in the p150 subunit of the dynactin (45) Transgenic mice harbouring this mutation develop progressing loss of motor neurons (46). Mice overexpressing dynamitin, a dynactin inhibiting protein also develop MND (47). Mutation of the dynein heavy chain produces large fibers sensory neuropathy leading to secondary motor neuron loss in transgenic animals (48,49). The impairment of axonal transport was observed in preclinical stage of MND in transgenic mice with human *SOD1* mutation. It was postulated that accumulation of SOD1 aggregates in cells with already impaired transport might enhance motor neuron distress causing cell death in the mechanism of axonal strangulation (50).

GLIAL CELLS INVOLVEMENT

Expression of mutated SOD1 exclusively in motor neurons or in glial cells does not induce MND in transgenic models of the disease (51). Studies with the use of transgenic chimers expressing mutated human SOD1 or wtSOD1 in different population of cells within the same organism provided more evidence on the involvement of glial cells in the pathogenesis of ALS. It was found that expression of mutated SOD1 in some cells of the CNS induced the animals death in time inversely proportional to the number of cells harboring the mutation. Moreover, not all motor neurons harboring the mutation underwent degeneration. When mutated SOD1 was expressed in all motor neurons and only in a proportion of glial cells, the neuronal death would depend on the number of non-neuronal cells harboring the mutation (52).

The results of these studies show that the loss of motor neurons depends on the expression of mutated SOD1 in non-neuronal cells and the microenvironment they create. The expression of mutated SOD1 in glial cells is thus indispensible but still insufficient to induce MND.

Another issue is the plausible toxicity of the microglia in MND. Microglial cells expressing mutant human SOD1 reduce survival of motor neurons derived from human neural stem cells (53). The toxicity is alleviated in the presence of stem cell-derived astrocytes. On the other hand, IgG immune complexes or proinflammatory lipopolisacharides isolated from ALS patients are capable of inducing microglia activation, generation of ROS and release of glutamate, what induces toxicity towards primary motor neurons (54,55). The over-activated microglia is even able to render otherwise neuroprotective astrocytes dysfunctional and toxic to motor neurons (56). Anti-inflammatory treatment provides neuroprotection in the culture (57). The toxicity of spinal cord microenvironment may therefore be an important obstacle in implementation of stem cell-bases treatment strategies in ALS (1).

PROGRAMMED CELL DEATH

Although the morphological features of programmed cell death (apoptosis) were observed in some motor neurons of transgenic ALS model with *SOD1* mutation, the involvement of apoptosis in ALS is controversial (58,59). In humans, the *post mortem* studies in the spinal cord allowed to identify three stages of motor neurons death. In the first stage, called chromatolisis, there was cell edema, loss of Nissl substance and a translocation of the nucleus to the cell periphery. The second stage started with the loss of cell processes followed by cytoplasm homogenization and chromatin shrinkage. The last, apoptotic, step ended up with reduction of cell volume with round or fusiform shape formation. There was however no nucleus fragmentation typical for apoptotic cell death (60).

Even if the cell death in ALS does not occur in a way typical for classic apoptosis, several intracellular changes consistent with those observed in apoptosis have been found in motor neurons cell line NSC34, transgenic models of ALS/MND and ALS in humans (reviewed in 58). The up-regulation of caspase 9 expression was found in NSC34 cells cultured in the presence of mutated SOD1. Trophic deprivation resulted in additional activation of effector caspases 3 and 6 in the same model. Activation of caspase 1 and 3 was also observed in the spinal cord of transgenic mice in course of the disease. In another study, the activation of caspase 9 and 7 was accompanied by translocation of proapoptotic Bax protein from cytoplasm to mitochondria with cytochrom c release. Also in human *post mortem* studies there was an increase of caspase 1 and 9 expression in the spinal cord as well as increased activity of caspase 3 in anterior horns of the spinal cord and motor but not sensory cortex in ALS (61). In transgenic ALS model with *SOD1* mutation, there a decreased concentration of antiapoptotic Bcl-e and Bcl-xL proteins and increased expression of proapoptotic Bad and Bax proteins was observed in the spinal cord of symptomatic animals (59). The changes in human tissues are less spectacular. Immunohistochemical studies did not show differences between the reactivity of Bcl-2 and Bax proteins in ALS motor cortex and spinal cord compared to control. However in ALS, the proapoptotic Bax proteins was enriched in mitochondria compared to cytosol in anterior horn cells and motor cortex compared to the sensory cortex of the same patients (60).

CONCLUSION

Amyotrophic lateral sclerosis is a disorder of complex pathogenesis. The role of oxidative stress and mitochondrial dysfunction is supported by the usual middle age symptom onset. Like in physiological aging, at this time-point the antioxidant mechanisms are no longer thoroughly efficient. Since a high percentage of oxygen is used in mitochondria, these organelles are also the main source of ROS. These can increase the glutamate release or decrease its retrograde uptake by EAAT2. The NMDA-dependent influx of calcium ions induces the synthesis of nitric oxide (NO) within the cell, what may in turn lead to nitration-dependent impairment of neurofilaments

phosphorylation. The protein aggregates formed in this process lead to impairment of axonal transport and motor neuron death in the mechanism of axonal strangulation. The imbalanced calcium homeostasis may activate the mitochondrial pathway of apoptosis. The toxic environment created by the glial cells might further decrease the efficacy of reparatory mechanisms. Although the recent discovery of the involvement of DNA/RNA binding proteins in pathogenesis of ALS shaded more light on the pathogenesis of ALS without *SOD1* mutations, it did not yet allow identifying the trigger point for the motor neuron death in this lethal disease.

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