Therapy for Amyotrophic Lateral Sclerosis

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Contributor: Kiyoung Kim

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a progressive neurodegenerative disease characterized by the selective loss of motor neurons in the spinal cord and motor cortex. Muscle weakness and degradation at 2–5 years after the onset of symptoms can result in fatal muscle dystrophy, paralysis, and death. Most ALS cases (90–95%) occur sporadically with no clearly related risk factors, while approximately 5–10% of ALS cases are considered to be hereditary, attributed to various mutations in specific genes.

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1. Overview

Amyotrophic lateral sclerosis (ALS) is a rare neurological disorder that affects the motor neurons responsible for regulating muscle movement. However, the molecular pathogenic mechanisms of ALS remain poorly understood. A deficiency in the antioxidant tripeptide glutathione (GSH) in the nervous system appears to be involved in several neurodegenerative diseases characterized by the loss of neuronal cells. Impaired antioxidant defense systems, and the accumulation of oxidative damage due to increased dysfunction in GSH homeostasis are known to be involved in the development and progression of ALS. Aberrant GSH metabolism and redox status following oxidative damage are also associated with various cellular organelles, including the mitochondria and nucleus, and are crucial factors in neuronal toxicity induced by ALS. In this review, we provide an overview of the implications of imbalanced GSH homeostasis and its molecular characteristics in various experimental models of ALS.

2. Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a progressive neurodegenerative disease characterized by the selective loss of motor neurons in the spinal cord and motor cortex [1][2]. Muscle weakness and degradation at 2–5 years after the onset of symptoms can result in fatal muscle dystrophy, paralysis, and death [3][4]. Most ALS cases (90–95%) occur sporadically with no clearly related risk factors, while approximately 5–10% of ALS cases are considered to be hereditary, attributed to various mutations in specific genes [1]. Pathogenic mutations in genes encoding *Cu/Zn-superoxide dismutase 1* (*SOD1*), *chromosome 9 open reading frame 72* (*C9orf72*), *optineurin* (*OPTN*), *p97/valosin-containing protein* (*VCP*), *TAR DNA-binding protein* (*TDP-43*), *fused in sarcoma* (*FUS*), *Ewing sarcoma breakpoint region 1* (*EWSR1*), and *TATA box-binding protein-associated factor 15* (*TAF15*), have been linked to the sporadic and familial forms of ALS [5][6][7][8][9]. There are only two FDA-approved drugs for ALS, and both extend patient lifespan by a few months [10][11]. Therefore, there is an urgent demand for the development of therapeutics for ALS.

SOD1, the first discovered ALS-linked gene, codes for an important enzyme in the defense mechanism against oxidative stress. Mutations in the *SOD1* gene account for approximately 20% of familial ALS cases [12]. Previous studies have suggested that mutant SOD1 forms insoluble aggregates in the mitochondria and induces mitochondrial defects that lead to cell death [13]. Recently, dominant mutations in specific genes coding for different RNA-binding proteins with prion-like domains, including *TDP-43*, *FUS*, *EWSR1*, and *TAF15*, have been found in sporadic and familial ALS cases [14][15][16][17]. Several studies have discovered mutations and functions of RNA-binding proteins in the pathogenesis of ALS. The expression of wild-type or mutant human TDP-43 in mice has been found to result in the degeneration of motor neurons [18]. There is evidence of ALS onset as a result of the presence of cytoplasmic inclusions in TDP-43 mutants in familial and sporadic ALS patients [3]. Furthermore, FUS is involved in RNA metabolism, including RNA processing, splicing, transport, and translation, by regulating cellular localization and degradation [9][15]. FUS is also localized in the nucleus and has been identified as a component of cytoplasmic inclusions in patients with familial ALS [19]. ALS-linked mutations in FUS lead to the mislocalization of FUS to the cytoplasm from the nucleus [20][21]. A recent study found a missense mutation in the *EWSR1* gene in patients with ALS [17]. Similar to other FET proteins, the mutant EWSR1 protein is mislocalized in the cytoplasm of the spinal cord [22]. The overexpression of EWSR1, namely EWSR1^{G511A} and EWSR1^{P552L}, in neurons leads to neuronal dysfunction in *Drosophila* [17]. In addition, mutations in the *TAF15* gene have been implicated in the pathogenesis of familial and sporadic ALS, and the TAF15 protein also forms prion-like aggregates in the cytoplasm of spinal cord neurons of rats, leading to increased neuronal toxicity [16][23][24]. The property of cytoplasmic aggregate formation in several ALS-linked proteins has been suggested to play a critical role in the development and progression of ALS. Therefore, the clearance and degradation of cytoplasmic aggregates in motor neurons could be a therapeutic strategy for ALS. However, despite efforts by several research groups, the pathogenic mechanisms underlying neurodegeneration through gene mutations and the screening of potential therapeutics remain poorly understood.

Oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) and the antioxidant system, which removes oxidative damage in cells and neurons. Therefore, maintaining this balance is critical in the pathogenesis of neurodegenerative diseases, including ALS. Numerous pathological studies have found evidence of increased oxidative stress in ALS. Previous studies have revealed increased oxidative damage to proteins in the tissue of ALS patients postmortem. Increased levels of protein carbonyl were observed in the spinal cord and motor cortex of patients with sporadic ALS [25]. Furthermore, several biomarkers for antioxidant defense and ROS damage are altered in the peripheral tissues and cerebrospinal fluid of patients with ALS [26][27]. The activity and expression of SOD, catalase, glutathione reductase (GR), and glutathione transferase (GST) are also reduced in the cerebrospinal fluid or peripheral blood mononuclear cells of patients with familial or sporadic ALS [28][29][30]. The reduced form of glutathione (GSH), a tripeptide, is well-known to non-enzymatically react with ROS to scavenge free radicals. A reduction in the GSH/oxidized glutathione (GSSG) ratio and GSH levels in cerebrospinal fluid has also been observed in patients with ALS [31]. Thus, the antioxidant defense mechanism against oxidative stress, which includes reactive species scavengers, is an important system in ALS, and the dysregulation of GSH homeostasis is believed to contribute to the development and progression of ALS. Although uncertainties remain regarding the mechanisms underlying the association between GSH and the dysfunction of

neuronal cells and the specific functions of GSH that are critical for inducing neuronal toxicity, such as ROS production and protein aggregation in ALS pathogenesis, dysfunctional GSH metabolism, GSH-related enzymatic systems, such as GR and GST, and an imbalanced redox status are increasingly postulated to be crucial to the development and progression of ALS.

3. The Roles of GSH as an Antioxidant in the Nervous System

GSH is found at different levels in all cells. It accounts for approximately 95% of the total non-protein thiol groups in cells and is ubiquitously distributed in the body. High levels of GSH have been found in the nervous system [32]. Many studies have suggested that an intensive metabolic exchange of GSH occurs between astrocytes and neurons, whose interactions appear to be critical for neuronal GSH homeostasis and the protection of neurons in the brain against ROS and oxidative damage.

GSH Synthesis and Cellular Distribution in Neurons

GSH is a tripeptide composed of glutamate, cysteine, and glycine. It is the most abundant thiol molecule found in tissues, including the brain, with a concentration of approximately 1–10 mM in the latter compared to approximately 2–3 mM in neurons, which is higher than that in the blood or cerebrospinal fluid (approximately 4 μM) [33][34][35][36]. GSH is synthesized in the cytosol by consecutive ATP-dependent reactions catalyzed by two enzymes, namely γ-glutamate-cysteine ligase (γ-GCL) and GSH synthetase (GS) [37][38]. γ-GCL mediates the first step of GSH synthesis, an ATP-dependent enzymatic process, with glutamate and cysteine to form the dipeptide, γ-glutamyl cysteine. γ-GCL is composed of a catalytic subunit (GCLC) and a modulatory subunit (GCLM), and is a rate-limiting enzyme in GSH synthesis [39]. Conditional *GCLC* knockout mice in whole neuronal cells displayed GSH depletion and neuronal cell death [40]. *GCLM* knockout mice exhibited reduced GSH levels and abnormal behavior phenotypes [41]. GS in the last step of GSH synthesis mediates the formation of GSH, which combines with glycine to form γ-glutamyl cysteine [42].

GSH is oxidized to glutathione disulfide (GSSG) by the reaction of glutathione peroxidase (GPx) coupled with the reduction of hydrogen oxide or hydroperoxides. GSH is regenerated from GSSG by GR, which uses NADPH as an electron donor [43]. Therefore, the reaction catalyzed by GPx and GR mediates GSH recycling. GSH is consumed by the extracellular release of GSH from cells via the generation of GSH-conjugates in the cytosol or oxidation to form GSSG. These processes induce a reduction in intracellular GSH. Thus, to maintain the intracellular levels of GSH, the synthesis, and recycling of GSH and the inhibition of GSH release must be induced. The depletion or redox imbalance of GSH (decreased GSH/GSSG ratio) has been reported to be involved in various neuronal dysfunction and neurodegenerative diseases, including autism, Alzheimer's disease, Parkinson's disease, and Huntington's disease [44][45][46][47].

Although GSH is synthesized exclusively in the cytosol, it is present in the most important cellular organelles, namely the mitochondria, endoplasmic reticulum (ER), peroxisome, and nucleus [48]. These results suggest that

GSH has specific functions in different cell compartments. Mitochondrial GSH represents 10–15% of the cellular GSH [49]. As the mitochondria lack the enzymes involved in de novo GSH synthesis, the maintenance of mitochondrial GSH levels depends on its uptake from the cytosol via carrier-mediated transporter systems. Many studies have shown that mitochondrial protection systems against free radicals and ROS, such as GSH, are critical for protecting neuronal cells from oxidative stress in the mitochondria. Moreover, mitochondrial GSH is important for protecting the organelles from ROS generated via the oxidative phosphorylation system. Muyderman et al. found that the selective depletion of mitochondrial GSH in astrocytes significantly increased hydrogen peroxide-induced apoptotic cell death and provided evidence for the crucial role played by mitochondrial GSH in preserving cell viability [50]. Wüllner et al. investigated the effects of acute GSH depletion and reduced mitochondrial GSH in relation to mitochondrial dysfunction in the cerebellar granule neurons of rats [51]. They found that the depletion of neuronal mitochondrial GSH led to a significant increase in ROS production and cell death in the nervous system. Furthermore, Wilkins et al. investigated the mechanism of mitochondrial GSH transport in the brain [52]. They showed that the dysfunction of mitochondrial GSH transporters, such as dicarboxylate and 2-oxoglutarate carriers, could result in an increased susceptibility of neurons to oxidative stress. These results demonstrate that the maintenance of mitochondrial GSH via sustained mitochondrial GSH transport is critical for protecting neurons from oxidative stress. Feng et al. also showed a reduction in mitochondrial GSH in the brain and spinal cord of *GCLC*-deficient mice [40]. Therefore, reduced mitochondrial GSH levels might be associated with mitochondrial defects in the nervous system.

The cytosol in cells is maintained in a reduced state to stabilize the free thiol groups. However, the ER environment is more oxidized than the cytosol to promote disulfide bond formation [48][53]. The redox environment in the ER influences the activity of various enzymes, including protein disulfide isomerase (PDI), which is responsible for the formation of disulfide bonds [53]. Previous studies have reported that the maintenance of redox status by regulating the GSH:GSSG ratio in the ER is considerably more oxidized than that in the cytosol [54]. Measuring the GSH levels in the ER revealed that the GSH:GSSG ratio in the ER is between 1:1 and 3:1 [55]. Furthermore, several studies have suggested the role of ER GSH in the formation of protein disulfide bonds; GSH is suggested to act as a net reductant in the ER by maintaining ER oxidoreductases, including PDI and endoplasmic reticulum oxidation 1 (ERO1) in a reduced state or by directly reducing non-native disulfide bonds in folding proteins [56][57][58]. Tsunoda et al. revealed that the selective depletion of ER GSH by expressing a cytosolic GSH-degrading enzyme, ChaC1, in the ER did not alter protein folding or ER stress response [59]. Although this study suggests the existence of an alternative electron donor that maintains the redox status in the ER, this result does not exclude the importance of the role of GSH in protein folding in the ER. However, direct evidence for the role of ER GSH in neuronal cells has yet to be found. However, as misfolded and aggregated proteins in the ER are one of the causes of neurodegenerative diseases, there is a need to determine the precise role of GSH in the ER of neurons.

GSH is present in the peroxisome—it is transported from the cytosol to the peroxisome by diffusion across the peroxisomal membrane [60][61]. Furthermore, GSSG is thought to be exported to the cytosol through a peroxisomal glutathione transporter, Opt2, and is subsequently reduced to GSH by cytosolic GR in an NADPH-dependent manner [62]. Catalase, peroxidase, and GSH are major components of the peroxisomal antioxidant system. GSH

peroxidase (GPx) in the peroxisome requires GSH as a cellular reductant to reduce hydrogen peroxide to water [63].

GSH also plays a critical role in the nucleus. Previous studies have shown that the distribution of GSH to the nucleus is a critical factor in cell proliferation [64]. In plants, GSH depletion blocks the transition from G1 to S phase in the cell cycle of the root [65]. Moreover, GSH recruitment into the nucleus can regulate chromatin structure and condensation, which controls gene expression [66]. Therefore, the recruitment and translocation of GSH from the cytosol to the nucleus during the cell cycle has a great influence on cellular redox homeostasis and gene expression. Jeong et al. investigated GSH levels in living mammalian cells using a fluorescent real-time thiol tracer (FreSHtracer) [67]. They found that GSH levels were markedly higher in the nucleus than in the cytosol, and GSH was required for the maintenance of stem cell functions. Miller et al. also investigated the precise localization of GSH in the mouse central nervous system and found that GSH is synthesized in neurons and diffuses into the nucleus to protect DNA from oxidative stress [68]. However, the molecular functions of GSH in the nucleus of neurons and the mechanisms of GSH transport to the nucleus in neurons are not yet clearly understood and remain a topic of debate.

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