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Protein Aggregates in Pathological Inclusions of Amyotrophic Lateral Sclerosis

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that is characterized by a progressive loss of upper and/or lower motor neurons (Bruijn et al., 2004). Dysfunction and death of these neurons lead to muscle weakness, atrophy and spasticity. A fatal event for the majority of patients is a failure of the respiratory muscles, which generally occurs within one to five years of disease onset. The typical age of onset is between 50 and 60 years, and the prevalence rate is 5 – 10 cases per 100,000 populations (de Belleroche et al., 1996). No effective cures for this disease are currently available, and the pathomechanism still remains controversial. The majority of ALS cases have no genetic component (sporadic ALS, sALS), while about 10 % are inherited in a dominant manner (familial ALS, fALS).

Historically, ALS has been described by Charcot and Joffroy in 1869 (Charcot & Joffroy, 1869), and linkage analysis of fALS families was performed in 1991, by which the genetic locus was identified to be linked to chromosome 21q (Siddique et al., 1991). In 1993, Rosen et al. (Rosen et al., 1993) and Deng et al. (Deng et al., 1993) have found that mutations in the Cu,Zn-superoxide dismutase (SOD1) gene, which lies on chromosome 21q, are associated with fALS. Because SOD1-related fALS exhibited several clinicopathological similarities to sALS, various animal models including rodents, worms and flies have been constructed, in which mutant forms of SOD1 are expressed. Using these models, furthermore, various drugs have been continuingly tested to cure or alleviate ALS. In 2001, ALS2 (or called alsin) has been also identified as a new gene associated with a rare, recessively inherited and slowly progressed juvenile onset form of ALS, which is, however, significantly different from the disease phenotypes of sALS (Hadano et al., 2001; Yang et al., 2001). Accordingly, studies on mutant SOD1 have served as a "gold standard" for a long time and provided valuable insight into molecular pathomechanisms of ALS.

Recent progress on genetic analysis has fuelled the identification of other genes responsible for fALS: for example, **T**AR **D**NA-binding **p**rotein-43 (TDP-43) gene reported in 2008 (Gitcho et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008), **Fu**sed in **S**arcoma (FUS) gene in 2009 (Kwiatkowski et al., 2009; Vance et al., 2009). Each of TDP-43 and FUS mutations describes approximately 4 % of total fALS cases, which is a smaller number than that of SOD1 mutations (~20 % of total fALS cases). Unlike SOD1, however, TDP-43 and/or FUS pathologies are observed in many of sALS patients (Deng et al., 2011b; Mackenzie et al., 2007) and also in other neurodegenerative diseases (Lagier-

Tourenne et al., 2010); much attention has thus been increasingly paid on TDP-43/FUS genes. FALS cases with SOD1/TDP-43/FUS mutations exhibit distinct disease phenotypes such as site and age of onset (Millecamps et al., 2010), and the pathomechanism of fALS might be dependent upon which of the genes is mutated. Common to all ALS cases, nonetheless, inclusions can be found in motor neurons of the affected spinal cord; therefore, molecular characterization of such pathological inclusions will be important in understanding the ALS diseases.

2. Protein aggregates in pathological inclusions

Inclusions have been found in many neurodegenerative diseases and are formed by abnormal accumulation of protein aggregates (Ross & Poirier, 2004). Two types of protein aggregates generally constitute pathological inclusions: amorphous and fibrillar aggregates. Amorphous aggregates are assembled from unstructured protein molecules, while well-ordered protein aggregates often exhibit fibrillar morphologies. Among such fibrillar protein aggregates, amyloid constitutes an important structural entity; the amyloid-like aggregate *in vitro* and *in vivo* can be characterized by its fibrillar morphology with widths of approx. 10 nm (Soto, 2003). In a molecular level, amyloid is rich in β-sheet structures, which are maintained by hydrogen-bonding interactions between main chains of constituent proteins; therefore, all proteins seem to possess innate propensities of forming a common amyloid structure (Dobson, 2003). Fibrillar aggregates of amyloid-β peptides, which constitute the senile plaques in the Alzheimer disease patients, are the most famous example of amyloid-like aggregates (Serpell & Smith, 2000). It, however, remains controversial whether the pathological protein aggregates in ALS adopt amyloid-like structures. In all cases of ALS, two types of inclusions characterize the surviving motor neurons in a spinal cord; one is called skein-like inclusions, which are immunostained by anti-ubiquitin antibody, and the other, Bunina body, is eosinophilic but ubiquitin-negative (Piao et al., 2003). Pathogenic proteins such as SOD1, TDP-43 and FUS constitute the skein-like inclusions, while Bunina bodies have been shown to be immunoreactive for cystatin C and transferrin (Okamoto et al., 2008). In this chapter, therefore, I will overview recent studies on the *in vitro* and *in vivo* aggregates of ALS-pathogenic proteins, SOD1, TDP-43 and FUS as follows.

2.1 SOD1-positive inclusions in ALS patients

SOD1 is a copper and zinc-containing protein with 153 amino acids, and its physiological function is to detoxify superoxide radical by its dismutation into oxygen and hydrogen peroxide (McCord & Fridovich, 1969). As of now, approximately 150 pathogenic mutations have been reported (http://alsod.iop.kcl.ac.uk), and any preference is not found for the site and type of mutations.

A subset of these pathogenic mutations affects SOD1 activity, while some mutant SOD1s fully retain the catalytic activity (Hayward et al., 2002). Also, transgenic mice expressing mutant SOD1 reproduce disease-like symptoms (Turner & Talbot, 2008), which are absent in SOD1-knockout mice (Reaume et al., 1996). It has, therefore, been considered that pathogenic mutations in SOD1 cause fALS not through loss of an SOD1 physiological function but by gain of new toxic properties. One of such toxic properties proposed so far includes mutation-induced aggregation of SOD1 proteins, while it remains controversial

whether SOD1 aggregation is really toxic or even protective. Neuronal hyaline inclusions in the spinal cord are pathological hallmark of mutant SOD1-linked fALS (Shibata et al., 1996b), and those inclusions are ultrastructurally composed of the granule-coated fibrils with a diameter of 15 – 25 nm that can be labeled by anti-SOD1 antibodies (Kato et al., 2000). Such fibrillar morphologies of protein aggregates are reminiscent of the amyloid formation observed in many neurodegenerative diseases (Ross & Poirier, 2004). Pathological inclusions containing mutant SOD1 seem, however, not fulfill some of the criteria for identification as amyloid; that is, neither Congo Red staining with apple-green birefringence nor Thioflavin T/S staining with significant enhancement of fluorescence has been observed in the fALS patient with $SOD1^{A4V}$ (Kerman et al., 2010). Given that these staining methods for amyloid diagnosis have not been routinely performed in the tissue sections from fALS patients with SOD1 mutations, more investigations will be required on the tissue samples of fALS patients with several different types of SOD1 mutations.

Until recently, involvement of wild-type SOD1 ($SOD1^{WT}$) in pathogenesis of sALS had been considered to be limited (Shibata et al., 1994; Shibata et al., 1996a; Watanabe et al., 2001). Indeed, an antibody called SEDI (**S**OD1-**e**xposed-**d**imer-**i**nterface) that only labels monomer/misfolded forms of SOD1 were failed to immunostain the motor neurons from sALS patients (Liu et al., 2009). Nonetheless, novel antibodies raised against 4 – 20, 57 – 72, and 131 – 153 of an SOD1 amino acid sequence were found to specifically detect unfolded SOD1 and also immunostain inclusions in motor neurons of the spinal cord from all the 29 sporadic and 8 familial ALS patients (Forsberg et al., 2010). Furthermore, motor neurons in the spinal cord of sALS patients (four out of nine cases) are found to be immunoreactive to another monoclonal antibody called C4F6, which specifically recognizes the misfolded conformation of SOD1 (Bosco et al., 2010). The staining patterns of C4F6 in sALS are, however, diffusive, and solubility of $SOD1^{WT}$ is also not different between sALS patients and controls. Pathological $SOD1^{WT}$ in sALS would, therefore, be relatively soluble, compared to mutant SOD1 within the inclusions in fALS patients. Although it appears that SOD1 is not involved in fALS cases without SOD1 mutations (Bosco et al., 2010), more prevailing roles of SOD1 proteins in the pathogenesis of ALS have been recently considered.

2.1.1 SOD1 aggregates in mouse models

Transgenic mice expressing human SOD1 with several fALS-associated mutations have been published, and phenotypes in each model mouse have been concisely summarized in (Turner & Talbot, 2008). These transgenic mice well reproduce the formation of ubiquitin-positive SOD1 inclusions, which is the pathological hallmark of SOD1-related fALS diseases (Watanabe et al., 2001). Biochemical analysis on the inclusions has further suggested the ubiquitination of mutant SOD1, based upon which the molecular pathogenesis of ALS has been proposed to include the inhibition of intracellular ubiquitin-proteasome system clogged by insoluble SOD1 aggregates (Basso et al., 2006). Retardation of proteasomal activity thus decelerates degradation of misfolded mutant SOD1, which further promotes the SOD1 aggregation. Recent mass analysis on inclusions in $SOD1^{G93A}$, $SOD1^{G37R}$, and $SOD1^{H46R/H48Q}$ transgenic mice has, however, not supported any direct modifications on SOD1, and ubiquitinated mutant SOD1 may be a minor constituent of inclusions (Shaw et al., 2008). Immunohistochemical analysis has also shown that the inclusions in transgenic mice are immunostained with antibodies to a copper chaperone for SOD1 (see below) and

several heat shock proteins such as Hsp70; however, the results are not always consistent with those in human fALS cases (Watanabe et al., 2001).

It is also important to note that amounts of inclusions in the transgenic mice depend upon the type of mutations in the SOD1 transgene. For example, transgenic mice expressing $SOD1^{G37R}$ and $SOD1^{G93A}$ exhibit prominent vacuolation of mitochondria with less amounts of inclusions; in contrast, significant amounts of inclusions are produced in $SOD1^{G85R}$ and $SOD1^{H46R}$ mice (Turner & Talbot, 2008). While such mutation-dependency of inclusion formation remains to be more characterized, H46R and G85R mutations notably reduce the affinity of SOD1 for copper/zinc ions (Hayward et al., 2002). In contrast, enzymatic activity of SOD1 is almost fully retained by G37R and G93A mutations, suggesting little effects on metal affinity/binding (Hayward et al., 2002). It is thus possible that SOD1 aggregation is facilitated by dissociation of metal ions from SOD1 (*vide infra*).

Ultrastructurally, both filamentous and amorphous aggregates that are immunoreactive to SOD1 and ubiquitin constitute the pathological inclusions in the transgenic mice. Unlike pathological inclusions in human fALS cases ($SOD1^{A4V}$) (Kerman et al., 2010), Thioflavin S does stain inclusions formed in $SOD1^{G37R}$ and $SOD1^{G93A}$ mice, suggesting the formation of amyloid-like protein aggregates (Furukawa et al., 2008; Wang et al., 2002). The apparent discrepancy in Thioflavin S staining between human and mouse needs to be described, but it remains unknown if this is due to the difference in the types of mutations (A4V in human vs. G37R/G93A in mouse). It is notable that SOD1 inclusions in $SOD1^{L126X}$ mice are not stained with Thioflavin S (Wang et al., 2005); therefore, tinctorial properties of SOD1 aggregates in pathological inclusions would depend upon the types of SOD1 mutations.

Disulfide cross-linking between SOD1 molecules is also an important feature of SOD1 aggregates in transgenic mice (Furukawa et al., 2006). A natively folded SOD1 possesses a highly conserved "intra"-molecular disulfide bond, but, in contrast, abnormal disulfide linkages "between" SOD1 molecules are detected in inclusions of the transgenic mice. Accumulation of disulfide-linked SOD1 aggregates occurs primarily in the later stages of the disease, concurrent with the appearance of rapidly progressing symptoms (Karch et al., 2009). Furthermore, the conserved intramolecular disulfide is found to be reduced in most of mutant SOD1 molecules constituting inclusions (Karch et al., 2009). Abnormalities in the thiol-disulfide chemistry of SOD1 would thus facilitate aggregation of mutant SOD1 proteins. These results of SOD1 transgenic mouse models strongly suggest the important roles of post-translational maturation of SOD1 polypeptides in the formation of pathological aggregates, which can be well reproduced by the following *in vitro* experiments.

2.1.2 SOD1 aggregates *in vitro*

Intracellular maturation of SOD1 requires several post-translational processes including copper and zinc binding and formation of an intramolecular disulfide bond (Furukawa & O'Halloran, 2006). These processes confer the significantly high structural stability to an SOD1 protein to the extent that the melting temperature of active SOD1 is around 90 ^{o}C (Forman & Fridovich, 1973). Many of ALS mutations have been shown to reduce the melting temperature of copper and/or zinc-bound form of SOD1 (Rodriguez et al., 2002), which is, however, still high enough to retain its structure at the physiological temperature (~37 ^{o}C). In general, aggregation associates with drastic conformational changes of protein molecules (Dobson, 2003); therefore, high structural (thermal) stability of fully mature SOD1 even in

the presence of ALS mutations might not fit to the general aggregation mechanism. In contrast, immature forms of SOD1 have been shown to exhibit significantly reduced thermostability; the melting temperature of the most immature SOD1 (*i.e.* a metal-unbound (apo) and disulfide-reduced form ($SOD1^{SH}$)) is as low as 43 ^{o}C (Furukawa & O'Halloran, 2005). Very interestingly, furthermore, apo-$SOD1^{SH}$ with ALS mutations (A4V and G93A) starts to melt at the temperature below 37 ^{o}C, suggesting protein unfolding/misfolding under physiological conditions (Furukawa & O'Halloran, 2005). Although it has been claimed that apo-SOD1s are not universally destabilized by the ALS-causing mutations (Rodriguez et al., 2005), the majority of ALS mutations (18 out of 20 mutations they examined) have been shown to indeed decrease melting temperature of apo-SOD1 to some degrees. It is, therefore, possible that mutation-induced destabilization of SOD1 structures plays critical roles in the formation of pathological inclusions found in SOD1-related fALS.

2.1.3 A key role of a disulfide reduction in SOD1 aggregation

Indeed, I have shown for the first time that the SOD1 polypeptide without any post-translational modifications (*i.e.* apo-$SOD1^{SH}$) exhibits the highest propensities for the formation of amyloid-like fibrillar aggregates *in vitro* (Furukawa et al., 2008). Under physiological solution conditions (such as salt concentration and solution pH), *in vitro* aggregation of SOD1 is completely inhibited by addition of Zn^{2+} ion and/or introduction of an intramolecular disulfide bond. SOD1 is a very rare cytosolic protein that keeps a disulfide bond for its enzymatic activity (Furukawa et al., 2004). As described below, the intramolecular disulfide bond in SOD1 is introduced by its copper chaperone in the cell (Furukawa et al., 2004). Without this disulfide, SOD1 favors a monomeric state; but, once the disulfide forms, SOD1 adopts a dimeric structure (Arnesano et al., 2004). Dimerization of SOD1 appears to protect its disulfide from reduction by burying it in the dimer interface. In most of the mutant SOD1 proteins, however, presumably due to perturbation in the native tertiary structure, the affinity for copper and/or zinc ion is reduced (Hayward et al., 2002), and the susceptibility to a disulfide reduction is increased (Tiwari & Hayward, 2003). Given the reducing environment of the cytosol with high metal-chelating capacity (Rae et al., 1999), therefore, a molecular pathomechanism of SOD1-related fALS would involve increased concentration of aggregation-prone apo-$SOD1^{SH}$ state in neurons. Consistently, insoluble mutant SOD1 purified from inclusions in the transgenic mice appear to have no metal ions bound (Lelie et al., 2011). Although aggregation propensities of apo-$SOD1^{SH}$ were not consistently increased by all pathogenic mutations, the intracellular population of aggregation-prone apo-$SOD1^{SH}$ state is considered to increase by the fALS-causing mutations.

Consistent with these *in vitro* observations, intracellular formation of SOD1 aggregates has been shown to be inhibited by co-expression of a copper chaperone for SOD1 (CCS) in cultured cells (Furukawa et al., 2008). CCS is a metallochaperone that specifically loads a copper ion and introduces a disulfide bond into apo-$SOD1^{SH}$ (Culotta et al., 1997; Furukawa et al., 2004). Intracellular concentration of aggregation-prone apo-$SOD1^{SH}$ will thus be effectively reduced by CCS. Unlike the cultured cells, however, $SOD1^{G93A}$ transgenic mice exhibited accelerated progression of disease-like phenotypes with shortening of mean survival from 242 to 36 days upon overexpression of CCS (Son et al., 2007). In particular, the double transgenic mice presented severe mitochondrial pathologies. CCS has been known to regulate the mitochondrial import of SOD1 (Field et al., 2003; Kawamata & Manfredi, 2008);

therefore, in CCS/SOD1^{G93A} double transgenic mice, CCS overexpression would overload mitochondria with toxic SOD1^{G93A}. Because of such a short survival, it remains unclear whether CCS possesses a preventive role in SOD1 aggregation in rodent models. If CCS effectively protects SOD1 from aggregation by facilitating the post-translational maturation, knockout of the *CCS* gene is expected to increase the population of apo-SOD1^{SH} state and then aggravate the inclusion pathologies in ALS-model mice. Again, however, this is not the case; there are virtually no effects on the disease onset/progression, when the *CCS* gene is knocked out from SOD1^{G37R}, SOD1^{G85R}, and SOD1^{G93A} transgenic mice (Subramaniam et al., 2002). Notably, a significant level of SOD1 activity is confirmed in CCS-knockout mice, and this is in sharp contrast to the observations in *S. cerevisiae*, where SOD1 activity is completely vanished upon genetic removal of the *CCS* gene (Culotta et al., 1997). A CCS-independent pathway(s) of SOD1 activation has, therefore, been recently proposed in mammals, and the presence of a Pro residue in an SOD1 C-terminal region determines the dependency of its enzymatic activation on CCS (Leitch et al., 2009). It, therefore, remains difficult to test the potentially protecting role of CCS in SOD1 aggregation in animal models. Interestingly, *C.elegans* has no *CCS* gene and activates SOD1 in a CCS-independent mechanism (Jensen & Culotta, 2005). Worms may thus be able to use as a model to examine effects of fALS mutations on a CCS-independent pathway of SOD1 activation.

In vitro aggregates prepared from SOD1^{SH} have been recently found to exhibit mutation-dependent structural polymorphism (Furukawa et al., 2010). Three regions in an SOD1 amino acid sequence are resistant to be proteolyzed by proteases in the fibrillar SOD1^{SH} aggregates. These three regions can be regarded as building blocks in an SOD1 aggregate structure, and non-native interactions of these building blocks constitute a core in SOD1 aggregate. FALS-causing mutations are found to modulate non-native interactions of those three building blocks, leading to the formation of discrete core structures among SOD1 aggregates with different mutations. Such a mutation-dependent core structure, furthermore, determines morphological as well as biochemical properties of SOD1 aggregate, which may describe distinct disease phenotypes in patients with different mutations in SOD1 (Wang et al., 2008).

2.1.4 Aggregation of SOD1 with an intact disulfide under destabilizing conditions

Notably, apo-SOD1 with an intramolecular disulfide bond (apo-SOD1$^{S\text{-}S}$) is considered to be partially unfolded under physiological temperature, given the melting temperature of around 40 – 50 ^{o}C (Furukawa & O'Halloran, 2005). While partial unfolding of apo-SOD1$^{S\text{-}S}$ could induce misfolding and aggregation, 10 μM apo-SOD1$^{S\text{-}S}$ without free cysteines (C6S and C111S mutations) was not aggregated even with agitation in a physiological buffer (100 mM Na-Pi, 100 mM NaCl, 5 mM EDTA, pH 7.0) for at least 72 hours (Furukawa et al., 2008). In the same conditions, I have also confirmed no aggregation of wild-type apo-SOD1$^{S\text{-}S}$ (with intact free cysteines, unpublished). Several other groups have nonetheless reported the aggregation of SOD1$^{S\text{-}S}$, which will be summarized below.

One of the conditions for triggering aggregation of non-reduced SOD1 is the addition of a chaotropic agent, guanidine hydrochloride (GdnHCl). Valentine and colleagues have performed agitation of 50 μM apo-SOD1$^{S\text{-}S}$ with Teflon balls in a buffer (10 mM K-Pi, pH 7.4) containing 1 M GdnHCl and found the formation of Thioflavin-T (ThT)-positive aggregates after approximately 40 hours (Chattopadhyay et al., 2008; Oztug Durer et al., 2009). These apo-SOD1$^{S\text{-}S}$ aggregates possess fibrillar morphologies, and its formation is accelerated

when the solution pH becomes acidic (pH 3.0, 4.0, 5.0). Interestingly, aggregation of apo-$SOD1^{S\text{-}S}$ was accelerated by addition of sub-stoichiometric amounts of soluble apo-$SOD1^{SH}$, suggesting that apo-$SOD1^{SH}$ can initiate aggregation of apo-$SOD1^{S\text{-}S}$ by acting as nuclei. Thus, SOD1 with a disulfide bond would be aggregation-incompetent, but, once the disulfide is somehow reduced in a small fraction of $SOD1^{S\text{-}S}$ proteins during the *in vitro* aggregation reaction, the resultant $SOD1^{SH}$ might convert the residual $SOD1^{S\text{-}S}$ into aggregation-competent conformations. Chia et al. have also shown that aggregation of 10 μM $SOD1^{S\text{-}S}$ in the presence of 0.5 M GdnHCl at pH 4.0 is accelerated by addition of spinal cord homogenates from transgenic mice expressing $SOD1^{G93A}$ (120 days old) (Chia et al., 2010). Such a seeded aggregation was not observed by adding spinal cord homogenates of the non-transgenic littermates. Given that $SOD1^{G93A}$ mice contain pathological inclusions at 120 days of age (Gurney et al., 1994), the *in vitro* $SOD1^{S\text{-}S}$ aggregation is seeded by either pathological SOD1 aggregates, soluble $SOD1^{SH}$ or both.

Another aggregation-inducing condition is the addition of trifluoroethanol (TFE). TFE has been considered to stabilize secondary structures but disrupt the native tertiary structure of various intact proteins (Buck, 1998). Consistent with the high thermostability, a holo-form of $SOD1^{S\text{-}S}$ remains soluble in the presence of up to 80 % TFE; in contrast, apo-$SOD1^{S\text{-}S}$ readily forms visible aggregate in the presence of 15 % TFE (Stathopulos et al., 2003). Stathopulos et al. have further proposed the correlation of the melting temperature with the amount of TFE required for aggregation by using several fALS-causing mutant apo-$SOD1^{S\text{-}S}$ proteins (Stathopulos et al., 2003). Morphologies of TFE-induced $SOD1^{S\text{-}S}$ aggregates are fibrillar, but those aggregates exhibit 2- to 3-fold ThT fluorescence enhancement and small spectral changes upon addition of Congo Red. These tinctorial changes may be comparable to those in pathological SOD1 aggregates to the extent that inclusions in fALS patients are usually not stained by both Thioflavin S and Congo Red (Kato et al., 2000). Given that the typical amyloid aggregates lead to ~1000-fold increase in the intensity of ThT fluorescence and red-shift a spectrum of Congo Red (Klunk et al., 1999; LeVine, 1999), TFE-induced $SOD1^{S\text{-}S}$ aggregates will not be categorized as a typical amyloid.

It is also interesting to note that TFE-induced SOD1 aggregates act as seeds to trigger aggregation of endogenous SOD1 in cultured cells. Münch et al. have prepared the *in vitro* aggregates of $SOD1^{H46R}$ by adding 20 % TFE, although the thiol-disulfide status in the mutant SOD1 was not specified (Munch et al., 2011). These *in vitro* aggregates were found to penetrate inside neuronal cells, *Neuro2a*, by macropinocytosis, rapidly exit the macropinocytic compartment, and then nucleate aggregation of the transiently overexpressed $SOD1^{H46R}$-GFP in the cytosol. Intracellular $SOD1^{WT}$-GFP was not aggregated by exogenously added $SOD1^{H46R}$ aggregates. Importantly, the intracellularly seeded aggregates have been found to transfer from cell to cell; thereby, the phenotypes can be "infected" among cells. Such a seeded infection of protein aggregates is reminiscent of the prion phenomena and has been reported in several other pathogenic proteins for neurodegenerative diseases such as α-synuclein (Luk et al., 2009), Tau (Clavaguera et al., 2009), polyglutamine (Ren et al., 2009), and TDP-43 (Furukawa et al., 2011). Although it has not been confirmed if ALS is infectious, the seeding properties of TFE-induced aggregates may describe the propagation of pathological changes with the progression of diseases.

Metal-catalyzed oxidation of Zn-deficient SOD1 also leads to the protein aggregation (Rakhit et al., 2002; Rakhit et al., 2004). $CuCl_2$ with ascorbic acid generates reactive oxygen species, which then oxidize histidine residues in SOD1. This is consistent with the fact that

the aggregation is significantly retarded at pH < 5.5. While SOD1 exists as a dimer, the oxidized SOD1 dissociates into monomers and then forms non-amyloid aggregates with amorphous and fibrillar morphologies. The oxidation-induced aggregation does not occur when SOD1 is in a holo state. Zinc-binding affinity of SOD1 has been known to decrease with fALS mutations (Hayward et al., 2002); therefore, mutant SOD1 is more susceptible to aggregation through the metal-catalyzed oxidation than the wild-type protein.

Incubation time will be another key factor to induce the aggregation of a mature SOD1 (*i.e.* a fully metallated SOD1 with an intramolecular disulfide bond). Usually, the aggregation kinetics of proteins has been monitored for at most 3 – 5 days, where either fully mature or even partially mature SOD1 does not aggregate in a physiological buffer without any chaotropic reagents. Nonetheless, Hwang et al. have extended the incubation time up to more than 300 hours (> 10 days) and found the fibrillar aggregation of fully mature SOD1 (with C6A/C111S mutations) under physiological conditions (~300 μM proteins, pH 7.8, 37 ^{o}C) (Hwang et al., 2010). The SOD1 aggregates after a prolonged incubation did not show apple-green birefringence upon binding Congo Red nor strong enhancement of ThT fluorescence, consistent with properties of inclusions in SOD1-related fALS patients (Kato et al., 2000). It remains unknown if SOD1 retains metal ions even in the aggregated state, it is possible that such a long incubation of SOD1 proteins somehow leads to the partial loss and/or the altered binding geometries of metal ions.

In summary, SOD1 can adopt theoretically 44 types of modified states when metal binding, disulfide formation and dimerization are taken into account (Furukawa & O'Halloran, 2006). Many papers point out the strengths of the SOD1 aggregation model for ALS; however, as mentioned above, there is still no consensus on which state of SOD1 is responsible for aggregation observed in fALS cases. Researchers including myself have thus continuingly pursued a mechanism describing why more than 100 ALS-causing mutations in SOD1 commonly facilitate the SOD1 aggregation process.

2.2 TDP-43-positive inclusions in ALS patients

TDP-43 is a DNA/RNA binding protein with 414 amino acids and contains two RNA recognition motifs (RRM1 and RRM2) and a C-terminal auxiliary region (Ayala et al., 2005). As of now, more than 40 mutations have been identified in the TDP-43 gene as being pathogenic, and most of the mutations are localized in the C-terminal region (http://alsod.iop.kcl.ac.uk). One of physiological functions of TDP-43 is to regulate an alternative splicing of several gene transcripts (Ayala et al., 2008a; Buratti & Baralle, 2001); usually, TDP-43 is localized at the nucleus but is also known to shuttle between nucleus and cytoplasm (Ayala et al., 2008b). Under pathological conditions, in contrast, TDP-43 is cleared from the nucleus and is mislocalized at the cytoplasm, where the ubiquitin- and TDP-43-positive inclusions are observed (Arai et al., 2006; Neumann et al., 2006). Formation of TDP-43 inclusions has been confirmed in sALS and SOD1-negative fALS but not in SOD1-linked fALS (Mackenzie et al., 2007). Actually, before identification of pathogenic mutations in the TDP-43 gene, proteomic analysis of ubiquitin-positive inclusions in sALS patients has revealed TDP-43 as a major component of inclusions (Arai et al., 2006; Neumann et al., 2006). TDP-43 immunoreactive inclusions have also been observed in many other neurodegenerative diseases such as frontotemporal lobar degeneration (FTLD), Huntington disease, and Alzheimer disease, which recently leads to a new disease category called TDP-43 proteinopathies (Geser et al., 2009).

In pathological inclusions, TDP-43 is abnormally hyper-phosphorylated and cleaved to generate C-terminal fragments (Arai et al., 2006; Neumann et al., 2006). Pathological TDP-43 is also distinct from its normal counterpart because it exhibits decreased solubility in a buffer containing a detergent, Sarkosyl. Ultrastructurally, inclusions observed in TDP-43 proteinopathies are characterized by bundles of straight fibrils with 10 – 20 nm diameter that are immunostained by anti-TDP-43 antibodies (Lin & Dickson, 2008). Similar to SOD1-positive inclusions, however, TDP-43 inclusions are also not stained by Thioflavin S and Congo Red (Kerman et al., 2010), implying less amyloid characters. Interestingly, the C-terminal fragments are enriched in the cytoplasmic inclusions in brain of ALS patients, but in the spinal cord, inclusions are composed of full-length TDP-43 (Igaz et al., 2008). Furthermore, Hasegawa et al. have found the immunoblot distinction of TDP-43 among different TDP-43 proteinopathies (Hasegawa et al., 2008); for example, Sarkosyl-insoluble fractions of ALS and FTLD brains exhibit different electrophoretic band patterns of the C-terminal fragments of phosphorylated TDP-43 in the Western blots. Depending upon the clinicopathological subtypes of TDP-43 proteinopathies, multiple pathways can thus be considered for the formation of TDP-43 inclusions; however, molecular mechanisms of truncation and phosphorylation in TDP-43 remain unknown.

2.2.1 TDP-43 aggregates in mouse models

Homozygous disruption of the TDP-43 gene is embryonic lethal in mice (Kraemer et al., 2010), and post-natal deletion of the TDP-43 gene by utilizing a Cre recombinase also produces lethality albeit without any ALS-like symptoms (Chiang et al., 2010). Expression of wild-type human TDP-43 has also been reported to be toxic in mice in a dose-dependent manner; indeed, TDP-43 transgenic mice exhibit a wide variety of motor dysfunctions, which appears to depend upon the promoter regulating the expression of the transgene (Da Cruz & Cleveland, 2011). More toxic effects of ALS-causing mutations (A315T and M337V examined so far) in the TDP-43 transgene has not been established yet. Surprisingly, any of the transgenic mice expressing wild-type and mutant TDP-43 have not reproduced the formation of ubiquitin- and TDP-43-positive inclusions. When human TDP-43 with A315T mutation is expressed in mice under the control of mouse prion promoter (Wegorzewska et al., 2009), the mice develop gait abnormality with an average survival of about 150 days, and ubiquitin-positive inclusions are observed in specific neuronal populations including spinal motor neurons. Despite this, those ubiquitin-positive inclusions are not immunostained with anti-TDP-43 antibodies, and very limited amounts of C-terminally truncated TDP-43 are confirmed. Furthermore, mutant TDP-43 exhibits similar solubility in a Sarkosyl-containing buffer to that of mouse endogenous wild-type TDP-43. Although truncation as well as insolubilization of TDP-43 characterizes the TDP-43 proteinopathies, both of these pathological processes may hence not be required for neurodegeneration.

In contrast, Wils et al. have constructed a mouse expressing wild-type human TDP-43 under the control of a neuronal murine Thy-1 promoter and found a dose-dependent degeneration of cortical and spinal motor neurons (Wils et al., 2010). Immunohistochemical analysis has further confirmed the formation of ubiquitin-positive inclusions, which are stained by an anti-TDP-43 antibody and also an antibody recognizing Ser409/410-phosphorylated TDP-43. Abnormal phosphorylation on TDP-43 is thus reproduced in this model mouse; furthermore, the C-terminal truncation of human TDP-43 is observed albeit much less amounts than that in ALS patients. Despite this, human TDP-43 in the affected mice remains

soluble in a Sarkosyl-containing buffer, showing that the pathological processes of TDP-43 are not completely reproduced in the transgenic mouse model.
Transgenic rats expressing human wild-type and mutant (M337V) TDP-43 have also been made (Zhou et al., 2010). Soon after the birth, TDP-43^{M337V} transgenic rats become paralyzed at 20 - 30 days and die at postnatal ages; in contrast, TDP-43^{WT} transgenic rats exhibit no paralysis by the age of 200 days. Mutation-specific toxicity of TDP-43 has thus been reproduced in these rat transgenic models, but TDP-43 inclusions are rarely detected and present only in the cortex of paralyzed TDP-43^{M337V} transgenic rats. A very faint amount of truncated TDP-43 is detected, and phosphorylated TDP-43 is accumulated at the cytoplasm of spinal motor neurons. These molecular changes of TDP-43 are, however, confirmed in both TDP-43^{WT} and TDP-43^{M337V} transgenic rats, implying little roles of truncation and phosphorylation in expressing the mutant-specific toxicity of TDP-43. Accordingly, it still remains to be established in the rodent models how mutant TDP-43 exerts its toxicity and is involved in the inclusion formation under pathological conditions.

2.2.2 TDP-43 aggregates *in vitro*

Bacterially expressed TDP-43 normally forms insoluble inclusion bodies, which hampers biochemical characterization of TDP-43 proteins. Johnson et al. have nonetheless succeeded to obtain soluble full-length 6 x His-tagged TDP-43 by using a cold shock expression system in *E.coli* (Johnson et al., 2009) Agitation of 3 μM full-length TDP-43 in 40 mM HEPES/150 mM KCl/20 mM $MgCl_2$/1 mM DTT, pH 7.4 at 25 ^oC increases solution turbidity within an hour, supporting the high aggregation propensities of TDP-43. A TDP-43 truncate that is devoid of the C-terminal auxiliary domain does not increase its solution turbidity, suggesting an important role of the C-terminal domain in the aggregation *in vitro*. Aggregates of full-length TDP-43 exhibit both filament-like and thread-like morphologies but did not react with the amyloid-diagnostic dyes, Congo Red and ThT. A subset of fALS-linked mutations (M337V, Q331K) slightly facilitates the aggregation kinetics of full-length TDP-43. A high propensity for fibrillation has been also shown for the synthetic peptide fragment of a TDP-43 C-terminal region (Gly 287- Met 322) (Chen et al., 2010). Fibrillar aggregates of the C-terminal peptide did not increase the intensity of Thioflavin T fluorescence. Interestingly, an ALS-causing mutation, G294A, but not A315T renders the fibrillar aggregates ThT-positive. While fibrils of all C-terminal peptides (wild-type, A315T, G294A) possess β-sheet rich structures, ALS mutations would affect the biochemical/structural properties of TDP-43 aggregates.
I have recently reported that bacterially expressed full-length TDP-43 is resolubilized, purified in the presence of GdnHCl, and then refolded by dilution of GdnHCl (Furukawa et al., 2011). Such refolded TDP-43 proteins retain the physiological DNA binding function but forms fibrillar aggregates by agitation at 37 ^oC in 100 mM Na-Pi/100 mM NaCl/5 mM EDTA/5 mM DTT/10 % glycerol, pH 8.0. A C-terminal half of TDP-43 assumes a core in the fibrillar aggregates and reproduces the fibrillation propensities of full-length TDP-43 proteins. These *in vitro* TDP-43 fibrils are insoluble in a Sarkosyl-containing buffer, which is a consistent feature with the pathological inclusions. A seeding activity is also a notable feature of TDP-43 fibrils *in vitro*, where pre-formed fibrils (or called "seeds") function as a structural template to facilitate the recruitment of soluble proteins into insoluble fibrils. This seeding reaction has been found to also occur inside the cultured cells by transducing the cells with *in vitro* TDP-43 fibrils; thereby, the formation of Sarkosyl-insoluble and ubiquitinated TDP-43 inclusions is well

reproduced in the cell. This is notable because simple overexpression of TDP-43 in the cultured cells has never generated the Sarkosyl-insoluble inclusions. It remains controversial whether the aggregation of TDP-43 is a cause or a result of the disease; however, as recently proposed in the other neurodegenerative diseases (Aguzzi & Rajendran, 2009; Brundin et al., 2010), a seeding activity of TDP-43 proteins may contribute to the propagation of pathological changes with the progression of diseases.

All recent *in vitro* studies on TDP-43 proteins have revealed its high propensities for aggregation, which are provided by the C-terminal auxiliary domain. Given that most of the fALS-causing mutations are located at this domain, the mutational alteration in the aggregation propensities of TDP-43 might be a part of the ALS pathomechanism. More *in vitro* experiments will, however, be required to reveal if the aggregation reactions of TDP-43 are affected by mutation, truncation, and phosphorylation.

2.3 FUS-positive inclusions in ALS patients

FUS was initially identified as the N-terminus of FUS-CHOP (CCAAT/enhancer binding protein homologous protein), a fusion oncoprotein expressed in human myxoid liposarcoma with the t(12;16) chromosomal translocation (Crozat et al., 1993). Like TDP-43, FUS is a DNA/RNA binding protein with 526 amino acids and comprised of multiple domains as follows (from N-terminal to C-terminal); a Q/G/S/Y-rich domain, a G-rich domain, an RNA-recognition motif (RRM), an R/G-rich domain, a Zn-finger motif, and a region containing a nuclear localization signal (NLS) (Dormann et al., 2010; Iko et al., 2004). Under physiological conditions, FUS has been proposed to be involved in transcription regulation (Uranishi et al., 2001), RNA splicing (Yang et al., 1998), and RNA transport including nucleo-cytoplasmic shuttling (Zinszner et al., 1997).

Late in 2007, which was before identification of pathological mutations in the *FUS* gene, FUS protein was found as one of major proteins recruited into neuronal intranuclear inclusions in patients of Huntington disease (Doi et al., 2008). In this neurodegenerative disease, a polyglutamine tract in a protein called huntingtin (HTT) is abnormally expanded, leading to fibrillar aggregation of mutant HTT in affected neurons (Zoghbi & Orr, 2000). FUS is sequestered by fibrillar HTT aggregates and then becomes insoluble and possibly dysfunctional (Doi et al., 2008). Loss of physiological functions of FUS would, therefore, contribute to neuronal cell death in Huntington's disease (Doi et al., 2008) as well as other polyglutamine diseases (Doi et al., 2010).

Then, fALS-causing mutations in the *FUS* gene have been identified in 2009 (Kwiatkowski et al., 2009; Vance et al., 2009), and, as of now, at least 40 mutations have been reported, most of which are localized at a G-rich domain and a C-terminal NLS-containing region (http://alsod.iop.kcl.ac.uk). Although neuropathological analysis of fALS patients with *FUS* mutations has been still limited, cytoplasmic mislocalization of nuclear FUS protein in motor neurons is a major pathological hallmark. Indeed, as shown by a recent study (Dormann et al., 2010), fALS-causing mutations at the C-terminal region of FUS result in the functional impairment of the NLS, facilitating the cytoplasmic mislocalization of mutant FUS. In FUS-related fALS, FUS-immunoreactive cytoplasmic inclusions are observed, which have been recently found to exhibit mutation-dependent heterogeneity (Mackenzie et al., 2011). For example, P525L mutation in FUS associates with a relatively early onset (twenties) of ALS, where round FUS-immunoreactive neuronal cytoplasmic inclusions are found. In contrast, late-onset (forties to sixties) ALS cases are linked to R521C mutation in FUS and

have tangle-like FUS-immunoreative neuronal and glial cytoplasmic inclusions. Furthermore, it has been reported that FUS-immunoreactive inclusions are observed in spinall spinal anterior horn neurons in all sporadic and familial ALS cases tested, except for those with SOD1 mutations (Deng et al., 2011b). Although mutations in FUS account for only a small fraction of fALS and sALS cases, FUS proteins may be a common component of cytosolic inclusions in non-SOD1 ALS. In motor neurons of patients with juvenile ALS, FUS has been shown to form filamentous aggregates with a diameter of 15 – 20 nm, which are often associated with small granules (Baumer et al., 2010; Huang et al., 2011). Staining with Thioflavin T/S and Congo Red has not, however, been performed yet on the inclusions of FUS-linked ALS. It also remains unknown if pathological FUS decreases its solubility or is modified/truncated in inclusions.

2.3.1 FUS aggregates in a rat model

As of now, there is no mouse model of FUS-linked ALS, but a transgenic rat expressing wild-type or ALS-causing mutant (R521C) FUS has been published (Huang et al., 2011). Only the mutant FUS transgenic rats developed paralysis at an early age (1 – 2 mo) with a significant loss of neurons in the frontal cortex and dentate gyrus. These pathological changes are not observed in age-matched wild-type FUS transgenic rats, although, at the advanced age (> 1 yr), wild-type FUS transgenic rats display a deficit in spatial learning and memory with a moderate loss of neurons in the frontal cortex and dentate gyrus. Immunohistochemical analysis of the cortex and spinal cord has shown the appearance of ubiquitin-positive inclusions at the paralysis stages of both wild-type and mutant FUS rats; however, the inclusions are not immunostained with anti-FUS antibodies. Given that several different anti-FUS antibodies show distinct immunoreactivities toward FUS-containing inclusions in sALS cases (Deng et al., 2011b), more detailed investigations will be necessary to characterize the possible aggregation of FUS forming pathological inclusions.

2.3.2 FUS aggregates *in vitro*

There is only one paper published on the aggregation reaction of purified FUS proteins (Sun et al., 2011); Sun et al. have prepared GST-fused FUS proteins intervened with a TEV protease site and found that the cleavage of 2.5 – 5 μM GST-FUS with a TEV protease produces full-length FUS in 100 mM Tris/200 mM trehalose/0.5 mM EDTA/20 mM glutathione, pH 8.0, and starts aggregation without a lag-time at 22° C in the absence of agitation. The resultant *in vitro* aggregates of FUS do not increase the intensity of ThT fluorescence and are completely soluble in an SDS-containing buffer. They have further examined the aggregation reactions of several truncated FUS proteins and shown that the N-terminal region of FUS (1 – 422) is enough to reproduce the aggregation behavior of full-length FUS. Aggregates of both full-length FUS and truncated FUS (1 – 422) are fibrillar in the morphologies, which resemble to the FUS inclusions in the ALS patients. No effects of fALS-causing mutations (H517Q, R521H, R521C) are observed on the *in vitro* fibrillation kinetics of full-length FUS proteins.

3. Conclusion

In this chapter, recent progress has been reviewed on aggregation mechanisms of ALS pathogenic proteins, SOD1, TDP-43 and FUS. Common to all these three proteins,

structural/biochemical characters of aggregates *in vitro* are much dependent upon experimental conditions, and it remains obscure which of aggregates *in vitro* reproduces the pathological inclusions in patients. In particular, post-translational processes such as metallation, disulfide formation, phosphorylation, and truncation appear to affect the aggregation pathway(s) of the pathogenic proteins. In future, therefore, it will become more important to correlate any abnormalities in these post-translational modifications with pathogenicity of ALS.

Very recently, mutations in another gene, optineurin (OPTN), have been linked to fALS cases, and hyaline inclusions in the anterior horn cells of spinal cord were immunoreactive for OPTN in patients with OPTN mutation (E478G) (Maruyama et al., 2010). Furthermore, albeit controversial, skein-like inclusions in all the sALS and non-SOD1 fALS have been reported to be immunostained with an anti-OPTN antibody (Deng et al., 2011a). Aggregation of an OPTN protein would thus be of relevance to describe the pathomechanism of both sporadic and familial ALS.

In spite of recent efforts to identify the causative genes for fALS, most of the cases are still genetically unidentified (Da Cruz & Cleveland, 2011). Given that the skein-like inclusions in the spinal anterior horn cells are characteristic of ALS, proteomic analysis of those inclusions will help to identify as-yet-unknown proteins pathogenic for ALS. In addition, the component analysis of skein-like inclusions will help to describe the common pathomechanism of sporadic and familial ALS cases.

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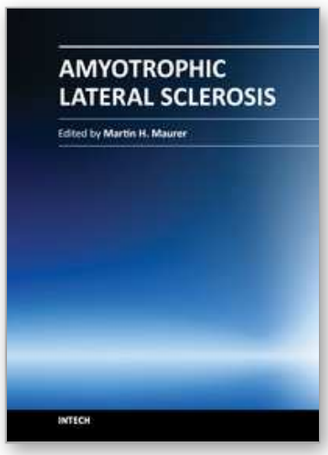

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Though considerable amount of research, both pre-clinical and clinical, has been conducted during recent years, Amyotrophic Lateral Sclerosis (ALS) remains one of the mysterious diseases of the 21st century. Great efforts have been made to develop pathophysiological models and to clarify the underlying pathology, and with novel instruments in genetics and transgenic techniques, the aim for finding a durable cure comes into scope. On the other hand, most pharmacological trials failed to show a benefit for ALS patients. In this book, the reader will find a compilation of state-of-the-art reviews about the etiology, epidemiology, and pathophysiology of ALS, the molecular basis of disease progression and clinical manifestations, the genetics familial ALS, as well as novel diagnostic criteria in the field of electrophysiology. An overview over all relevant pharmacological trials in ALS patients is also included, while the book concludes with a discussion on current advances and future trends in ALS research.

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