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ABSTRACT

Protein quality control in the endoplasmic reticulum (ER) is an elaborate process conserved from yeast to mammals, ensuring that only newly synthesized proteins with correct conformations in the ER are sorted further into the secretory pathway. It is well known that high-mannose type *N*-glycans are involved in protein-folding events. In the quality control process, proteins that fail to achieve proper folding or proper assembly are degraded in a process known as ER-associated degradation (ERAD). The ERAD pathway comprises multiple steps including substrate recognition and targeting to the retro-translocation machinery, retrotranslocation from the ER into the cytosol, and proteasomal degradation through ubiquitination. Recent studies have documented the important roles of sugar-recognition (lectin-type) molecules for trimmed high-mannose type *N*-glycans and glycosidases in the ERAD pathways in both ER and cytosol. In this review, we discuss a fundamental system that monitors glycoprotein folding in the ER and the unique roles of the sugar-recognizing ubiquitin ligase and peptide:*N*-glycanase (PNGase) in the cytosolic ERAD pathway.

1. Introduction

Approximately one-third of all proteins in eukaryotic cells are targeted to the secretory pathway, and the first compartment encountered by these proteins is the endoplasmic reticulum (ER) [1–3]. The nascent proteins enter the ER in unfolded states through the Sec61 translocon complex [4] and are folded or assembled in the ER through a process that involves various molecular chaperones and folding enzymes. In yeast, the Ssh1p complex has also been identified as a translocon channel in the ER [5,6]. Simultaneously, co- and post-translational protein modifications, such as glycosylation, disulfide-bond formation, and glycosylphosphatidylinositol (GPI)-anchor formation, occur in the ER. Although the ER contains high concentrations of molecular chaperones and folding-assisted enzymes, all newly synthesized proteins do not mature correctly. The ER provides a protein quality control system that monitors protein-folding state and consequently only correctly folded proteins are delivered to downstream compartments (Golgi, endo-lysosomes, and plasma membrane) or secretion [7].

Most proteins in the secretory pathway are glycosylated. Whereas highly diverse oligosaccharides attached to mature proteins are implicated in many extracellular processes, there is evidence that partially trimmed intermediates of high-mannose type *N*-glycans are involved in promoting protein folding, quality control, trafficking, and sorting of glycoprotein [8–10].

The oligosaccharyl transferase catalyzes the transfer of triglucosyl high-mannose oligosaccharide, $Glc_3Man_9GlcNAc_2$, from dolichol pyrophosphate to the asparagine residue in Asn-X-Ser/Thr motifs on the nascent polypeptide chains entering the ER lumen (Fig. 1A). After coupling to the polypeptide, the *N*-linked glycan is trimmed by glucosidase I, glucosidase II, and ER mannosidase I. Monoglucosylated high-mannose oligosaccharide, $Glc_1Man_9GlcNAc_2$, is associated with lectin-type molecular chaperones calnexin or calreticulin (Fig. 1B) [8–10]. The ER houses the deglucosylation–reglucosylation cycle, which consists of glucosydase II and UDP-glucose/glycoprotein glucosyltransferase (UGGT) [11]. These functionally opposite ER enzymes mediate the on/off-cycle in the calnexin–calreticulin system. The repeated cycles of deglucosylation–reglucosylation might be prolonged until the glycoprotein reaches its native conformation [9,12,13]. It is postulated that glycoproteins are allowed to move to the Golgi complex once they achieve correct folding during a certain period of mannose trimming in the ER. Correctly folded glycoproteins are transported from the ER to the Golgi complex through loading onto the transport vesicles. The 53-kDa membrane protein of the ER-Golgi intermediate compartment (ERGIC-53), vesicular integral protein of 36 kDa (VIP36), and VIP36-like (VIPL) have been identified as putative transmembrane cargo receptors, which constitute the family of leguminous type (L-type) lectins [14–17]. These cargo receptors share structural similarities in their lectin domains but exhibit distinct sugar-binding specificities and affinities, which can be finely tuned by sensing pH of each organella [18]. After the correctly folded or assembled glycoproteins move to the Golgi complex, the *N*-glycans receive further trimming of mannose residues and addition of terminal glycosylation to produce diverse *N*-glycan structures [19].

In the ER quality control system, improperly folded proteins or incompletely assembled oligomers are retained in the ER, and are finally retrotranslocated into the cytosol, upon persistence of misfolding,

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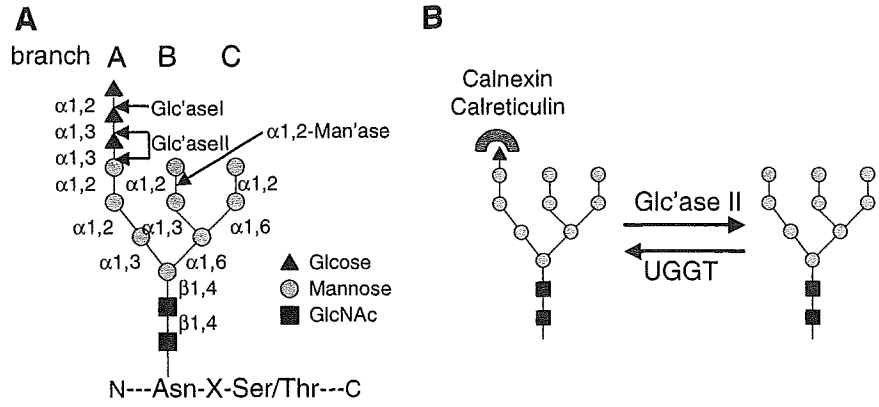

Fig. 1. (A) Composition and structure of $Glc_3Man_9GlcNAc_2$. Linkages are shown and the three *N*-glycan branches are labeled A, B and C. The sites of action of various glycosidases are shown. (B) Lectin-type molecular chaperones calnexin or calreticulin associate with the glucose residue on $Glc_1Man_9GlcNAc_2$. The ER provides a complete calnexin–calreticulin system with deglucosylation–reglucosylation enzymes. Repeated rounds of reglucosylation lead to prolonged association of lectins with the misfolded protein until the glycoprotein reaches its native conformation. Glc'aseI (glucosidase I), Glc'aseII (glucosidase II), α1, 2-Man'ase (α1, 2-mannosidase) and UGGT (UDP-glucose/glycoprotein glucosyltransferase).

where they are degraded by the ubiquitin–proteasome system. This disposal process of folding-defective proteins is called ER-associated degradation (ERAD) [20,21]. It remains elusive how ERAD substrates are selected from proteins that are or might be properly folded. However, recent progress has established that a specific trimming status of *N*-glycan is a signal for the recognition of ERAD substrates. The targeting process as well as the ERAD substrate recognition are mediated by several lectin-type molecules and mannosidases [10,22–24]. It has been shown that the retrotranslocated glycoproteins are ubiquitinated and then deglycosylated by peptide:*N*-glycanase (PNGase) before they are subjected to proteasomal degradation in the cytosol, indicating that *N*-glycosylated proteins are present in the cytosol [25,26]. Indeed, glycoprotein-specific F-box proteins, which are substrate recognition components in the SCF (Skp1-Cullin1-F-box protein) complex type E3 ubiquitin ligase, are found in the cytosol of mammals [27]. This review presents various lectin-type molecules and glycan-modifying enzymes involved in the individual steps of ERAD, which are substrates for recognition, targeting, retrotranslocation, ubiquitination, and degradation by the proteasome (Fig. 2).

2. Substrate recognition and targeting in the ER

2.1. Trimming status of mannose residues on N-glycan as a signal for substrate recognition in ERAD

When the improperly folded or incompletely assembled proteins fail to restore their functional states, they are degraded through the ERAD pathway. The structures of high-mannose type *N*-glycans seem to be important for ERAD in both mammal and yeast. In all eukaryotes, the common structure core glycan, $Glc_3Man_9GlcNAc_2$, is first attached to nascent proteins, then three glucose residues on the A branch are removed by glucosidase I and II (Fig. 1A). Truncation of terminal α1, 2-linked mannose residue on the B branch of high-mannose oligosaccharide is carried out by the ER α1, 2-mannosidase Mns1p in yeast, or the ER α-mannosidase I in mammal. In mammals, the ER α-mannosidase can occasionally remove additional α1, 2-linked mannose residues and generate $Man_{5-7}GlcNAc_2$ as well as $Man_8GlcNAc_2$ structures [28–31]. The finding that this mannose trimming reaction by the ER α-mannosidase is slower than glucosidase I and II reaction led to the concept of the mannose-timer hypothesis [32,33]. Selective inhibition of ER α-mannosidase I with kifnensin and 1-deoxymannojirimycin or Mns1p gene disruption stabilizes misfolded glycoproteins [34–36]. Moreover, overexpression of ER α-mannosidase I accelerates glycan trimming and degradation of misfolded glycoprotein substrates [30]. These observations indicate that the ER α-mannosidase product $Man_8GlcNAc_2$ seems to serve as a degradation signal for ERAD and suggest the presence of lection-like molecule that recognizes $Man_8GlcNAc_2$ or further trimmed structures.

Two types of lectin-like molecules involved in selection and targeting of aberrant proteins for the ERAD pathway were discovered recently. The first is the α-mannosidase-like lectin, EDEM1 and Htm1p/Mnl1p, in mammal and yeast, respectively [37–39]. Although yeast has no paralogue of Htm1p/Mnl1p, two novel EDEM1 paralogues, EDEM2 and EDEM3, have been reported in mammal [40–42]. The other type of lectin-like molecules is a protein that contains mannose 6-phosphate receptor homology (MRH) domain, Yos9p in yeast [43–46], and OS-9 and XTP3-B/ERlectin in mammal [47,48]. These two types of lectin-like molecules are required for degradation of misfolded glycoproteins, but the nature of the glycan degradation signal(s) that is (are) recognized by these lectin-like molecules remains unclear. Recent studies concluded that Htm1p generates a *N*-glycan signal for glycoprotein degradation, which is recognized by Yos9p as described below [49,50].

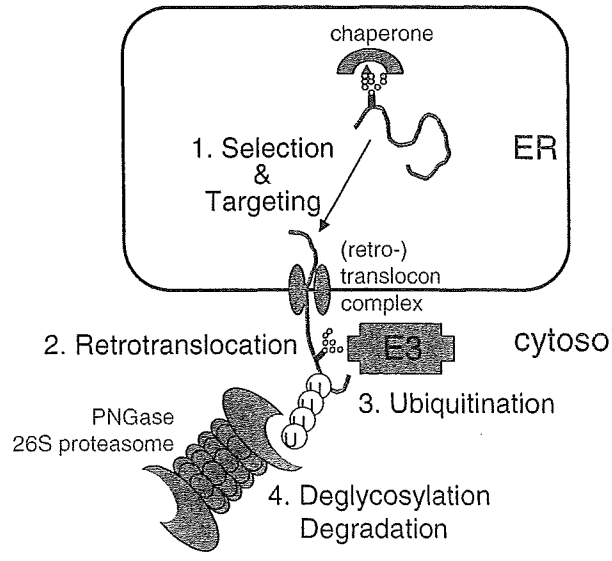

Fig. 2. The ERAD pathway. In step 1, misfolded glycoproteins are recognized by chaperone molecules or enzymes and targeted to the (retro-)translocon complex. In step 2, polypeptides are retrotranslocated from the ER into cytosol through the channel. In step 3, polyubiquitination occurs when the polypeptide chain becomes accessible in the cytosol. In step 4, polypeptides modified by the polyubiquitin chain are degraded by the proteasome. De-ubiquitinating enzymes remove the polyubiquitin chain and deglycosylation by PNGase might also be required for efficient degradation.

2.2. EDEMs in mammal and Htm1p/Mnl1p in yeast

EDEM1 (for ER degradation enhancing α-mannosidase-like protein) is a type II ER membrane integral protein that has all the signature motifs of class I α1, 2-mannosidases (glycosylhydrolase family 47) but no detectable enzyme activity as a processing α-mannosidase *in vivo* or *in vitro* [38]. In some cell types, EDEM1 is expressed as a soluble luminal protein after efficient cleavage of the signal sequence [38,41]. The gene expression of EDEM1 is up-regulated by ER stress, and overexpression of EDEM1 accelerates the turnover of the null Hong Kong variant of α1-antitrypsin (NHK), an ERAD substrate glycoprotein [38,51]. It has been reported that EDEM1 and calnexin interact with each other and thereby facilitate the transfer of misfolded glycoproteins from the calnexin–calreticulin cycle to the ERAD pathway [51,52]. In yeast, disruption of EDEM orthologue Htm1p/Mnl1p gene delays the degradation of aberrant glycoproteins but not non-glycosylated ERAD substrates [37,39]. Furthermore, EDEM1 associates with derlin-2 and derlin-3, which are retrotranslocation channel candidates [53]. On the other hand, Htm1p/Mnl1p has been implicated in the turnover of ERAD-L pathway of which ERAD substrates have lesions in luminal domain [54]. These observations suggest that EDEM1 and Htm1p/Mnl1p are probably lectins involved in the selection or targeting of misfolded glycoproteins for ERAD, but no direct evidence has been provided yet for interaction between high-mannose type glycans and these lectins. However, EDEM1 has chaperone-like activity that accelerates ERAD by preventing the formation of disulfide-bonded dimers [55] or covalent aggregation [56] containing misfolded glycoproteins.

In mammals, two paralogues, EDEM2 and EDEM3, are identified as soluble ER glycoproteins that accelerate the degradation of misfolded glycoproteins [40–42]. ER mannosidase I, EDEMs and Htm1p/Mnl1p have significant sequence identity in the mannosidase homology region but they differ in the remaining domains (Fig. 3A). Interestingly, EDEMs are inducible proteins whose intraluminal concentrations are increased upon stress-induced activation of the transcription factor Xbp1, a mediator of unfolded protein response (UPR) [41]. RNA interference directed against EDEM proteins [51,57] or inactivation of the Ire1/Xbp1 pathway of UPR results in sub-optimal disposal of misfolded glycoproteins [58], prevents protein folding and reduces secretory capacity [59].

EDEM3 was recently reported to have mannosidase activity *in vivo*; overexpression of this protein results in mannose trimming to $Man_7GlcNAc_2$ and $Man_6GlcNAc_2$ [42]. Surprisingly, overexpression of EDEM1 can also accelerate the removal of a terminal mannose residue from the A branch [56]. These observations suggest that the EDEM family proteins carry α-mannosidase activities involved in the extraction of misfolded glycoproteins from the calnexin–calreticulin cycle rather than acting as lectin [56]. Furthermore, in yeast, overexpression of Htm1p/Mnl1p also produces $Man_7GlcNAc_2$ glycan. By using various mutant strains, Clerc et al. [50] defined the function of Htm1p/Mnl1p as a α1, 2-specific exomannosidase, which generates the $Man_7GlcNAc_2$ glycan with a terminal α1, 6-linked mannose residue. This terminal α1, 6-linked mannose residue produced by Htm1p/Mnl1p is recognized by Yos9p as the glycan signal for destruction of glycoproteins through the ERAD pathway [49].

2.3. OS-9 and XTP3-B in mammal and Yos9p in yeast

Yos9p (yeast osteosarcoma 9) was discovered in a genetic screening to identify components that participate in glycoprotein turnover [43]. Yos9p is a glycoprotein containing an N-terminal signal sequence, a MRH domain, and a C-terminal HDEL peptide that retains it in the ER lumen (Fig. 3B).

Studies using yeast indicate that proteins are targeted to the appropriate ERAD pathway based on the site of the misfolded lesion [54,60,61]. Substrate proteins with lesions in the cytoplasmic, luminal, and membrane-spanning domains follow ERAD-C, ERAD-L, and ERAD-M pathways, respectively. Two ER-associated E3 ubiquitin ligases, Hrd1p/Der3p complex and Doa10p, are involved in the ERAD pathway in yeast [62–64]. ERAD-L substrates that include membrane and soluble proteins with luminal lesions use Hrd1p/Der3p complex, while ERAD-C substrates use a Doa10p-containing complex. It seems that ERAD-M substrates also use Hrd1p/Der3p complex. It was reported that Yos9p plays a critical role in the recognition and/or targeting to ERAD of soluble and membrane bound proteins by using pathway-specific substrates [44,45]. Moreover, Yos9p is recruited into a protein complex containing the Hrd1p/Der3p and the luminal chaperone Kar2p through association with Hrd3p, a luminally-exposed transmembrane tetratricopeptide repeat (TPR)-containing protein known to associate with Hrd1p [62–64]. Thus, Yos9p is a critical component of ERAD-L pathway [46].

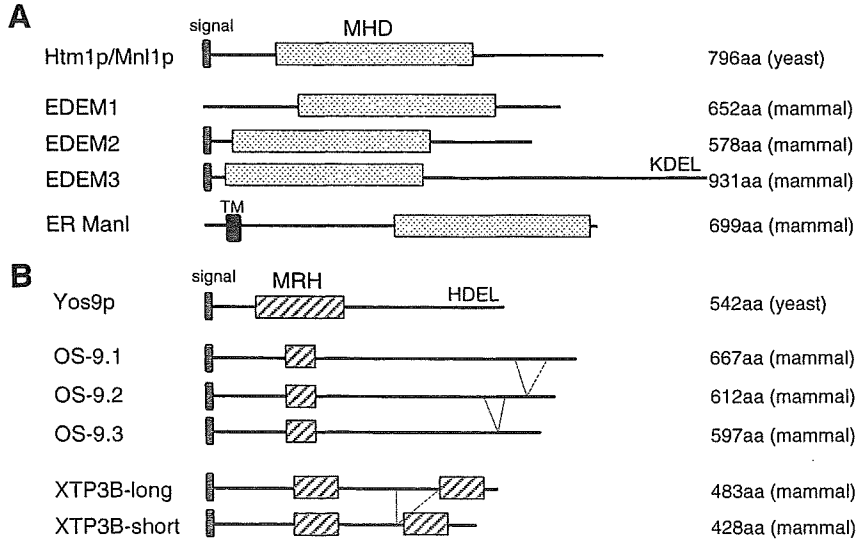

Fig. 3. The domain structure of Htm1p/Mnl1p and Yos9p protein families. (A) Yeast Htm1p/Mnl1p and its mammalian orthologues EDEM family. The mannosidase homology domain (MHD), signal sequence, C-terminal KDEL (ER retention signal), and transmembrane domain (TM) are shown. (B) The mannose 6-phosphate receptor homology (MRH) domain containing proteins. Conserved MRH domains, signal sequence, and C-terminal HDEL (ER retention signal) are shown. Alternative splicing generates variant forms; i.e., the regions depicted by dashed lines are deleted.

Disruption of Yos9p abrogates the degradation of the glycosylated but not the non-glycosylated forms of CPY*, a typical ERAD substrate [43–46]. Furthermore, Yos9p mutations that replace conserved residues in the carbohydrate-binding domain, reduce the rate of misfolded glycoprotein degradation, suggesting that Yos9p acts through the binding of glycans [44,46]. Yos9p can discriminate between mutant CPY* and wild-type CPY, and perhaps to bind glycoproteins carrying $Man_8GlcNAc_2$ and $Man_5GlcNAc_2$ glycans. Recently, Quan et al. [49] characterized the glycan-binding specificities of recombinant Yos9p by using frontal affinity chromatography and found that it recognizes glycans containing terminal α1, 6-linked mannose residues. Other Htm1p/Mnl1p and Yos9p studies showed that Htm1p/Mnl1p acts upstream of Yos9p to mediate the generation of glycans containing terminal α1, 6-linked mannose residues as the glycan signal for ERAD (Fig. 4).

In mammal, two Yos9p orthologues, OS-9 and XTP3-B/Erlection, which contain one and two MRH domains, respectively, were recently characterized as ER-resident luminal glycoproteins [47,48]. OS-9 has two or three transcriptional variants and their depletion causes significant impairment of the glycosylated ERAD substrate NHK degradation [47]. In comparison, knockdown of XTP3-B has no effect on NHK clearance [47], but overexpression of XTP3-B retards the degradation of NHK [48]. XTP3-B also has two transcriptional variants and only the long isoform strongly inhibits glycan-less mutant NHK-QQQ [48]. The mutant form transthyretin, a non-glycosylated serum protein, is also bound to XTP3-B but not OS-9 [47]. Although no interaction between OS-9 and XTP-B is found, OS-9 interacts with GRP94, the ER-resident Hsp90 paralogue. Knockdown of GRP94 stabilizes NHK, indicating that GRP94 is required for degradation of ERAD substrate [47]. Thus, OS-9 and XTP3-B are both glycoproteins containing MRH domain(s) but each has distinct substrate recognition mode and associates.

Both proteins, except for the short form of XTP3-B, associate with Hrd1 ubiquitin ligase complex through SEL1L (suppressor of lin-12-like protein, the mammalian Hrd3p orthologue), a multiply glycosylated type I ER membrane protein. Christianson et al. [47] proposed that the MRH domains of OS-9 and GRP94 are required for interaction with SEL1L but not substrates. The conclusion that mannose binding is used to target quality control sorting receptors to the ER membrane ubiquitin ligase complex rather than for substrate recognition is in conflict with Yos9p studies from yeast. However, very recent study by Hosokawa et al. revealed that OS-9 recognizes *N*-glycans on NHK but not SEL1L and that the lectin activity of OS-9 is required to enhance glycoprotein ERAD [65]. The OS-9 MRH domain specifically binds to *N*-glycans lacking the terminal mannose from the C branch generated by some mannosidase not determined. Further studies are required for a better understanding of the complete mechanism of selection and targeting of ERAD substrates.

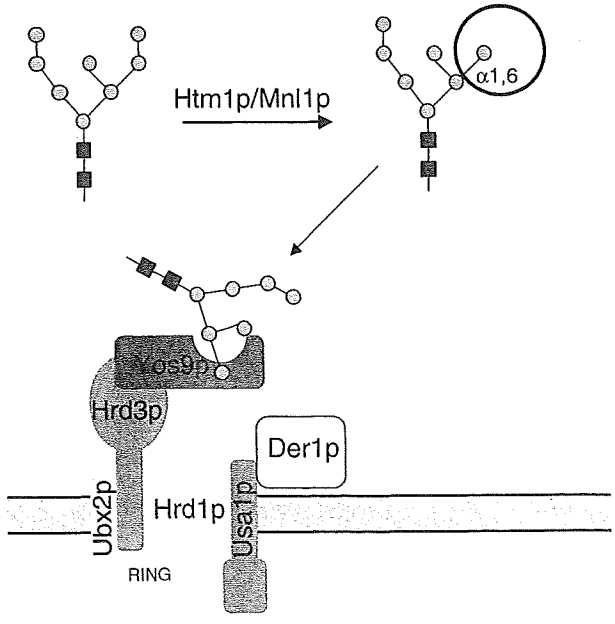

Fig. 4. Model of dual recognition of glycoprotein substrates in yeast ERAD pathway [49]. Htm1p/Mnl1p has α1,2-specific exomannosidase activity for high-mannose type *N*-glycans and produces the terminal α1, 6-linked mannosyl residue (marked with circle), which is recognized by Yos9p in the components of the Hrd1p ubiquitin ligase complex (see text for details).

3. Ubiquitination and proteasome degradation in the cytosol

In the ERAD pathway, protein ubiquitination participates in both protein extraction from the ER into the cytosol and subsequent proteasomal degradation [20]. Protein ubiquitination requires the action of an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. In this ubiquitin pathway, E3 is responsible for the selection of target proteins [66]. In yeast, RING-finger type E3 ubiquitin ligases, Hrd1p and Doa10p, have been implicated in the degradation of every studied ubiquitinated ERAD substrate [54,63]. Mammalian orthologues of Hrd1p and Doa10p have been identified [67,68], but the repertoire of E3s involved in the ERAD pathway is greatly expanded in mammals [21,22]. Since bulky *N*-glycans on proteins can impair their entry into the interior of the cylinder-shaped proteasome, *N*-glycans are postulated to preclude proteasomal degradation of ubiquitinated substrates. PNGase is a cytosolic deglycosylation enzyme and the reaction catalyzed by this enzyme is considered important for subsequent proteasomal degradation [69]. In this section, we describe the glycoprotein-specific E3s and PNGase.

3.1. Fbs proteins

E3s constitute a diverse family of proteins or protein complexes. One of the best-characterized families of E3s is the SCF complex, consisting of common components, Skp1, Cullin1, and Roc1/Rbx1, and a variable component F-box protein (Fig. 5A). The F-box protein consists of an F-box domain that binds to Skp1 and a C-terminal substrate-binding domain. Human and mouse genomes encode 69 and 74 F-box proteins respectively, and a large number of F-box proteins function in the specific ubiquitination of a wide range of substrates [70]. Fbs1 (*F-box protein that recognizes sugar chain 1*) is a glycoprotein-specific F-box protein, which is identified as a novel member of the ERAD-linked E3 component in mammal [71]. In addition to Fbs1, at least four F-box proteins with high homology to the C-terminal substrate-binding domain have been reported in human [72,73]. These F-box proteins cluster into two groups as demonstrated by phylogenetic analysis (refer to TreeFam in Sanger institute): one group is composed of Fbs1/Fbg1/Fbx2/NFB42, Fbs2/Fbg2/Fbx6b and Fbg3/Fbx44, and the other of Fbg4/Fbx17 and Fbg5/Fbx27 (Fig. 5B). In the human and mouse genomes, these two groups of genes are located separately, but the genes in each group are arranged in tandem with very short intergenic regions [72]. These Fbs family proteins are conserved in mammals and *C. elegans* has two hypothetical F-box proteins (C14B1.3 and T01E8.4), which show significant homology to this family proteins [72].

The expression of Fbs1, Fbg4, and Fbg5 is tissue-specific, but that of Fbs2 and Fbg3 is relatively ubiquitous. Among the Fbs family members, Fbs1 can bind strongly to the high-mannose type glycans, while Fbs2 and Fbg5 bind them more weakly, but no sugar-binding activities have so far been detected for Fbg3 and Fbg4 [27,71,74,75]. To gain further insight into Fbs family proteins, identification of endogenous substrates of Fbg3 and Fbg4 will be essential.

3.1.1. Function of Fbs1

Fbs1 can bind to proteins modified with high-mannose type *N*-glycans in the ER, while the physical association between Fbs1 and its

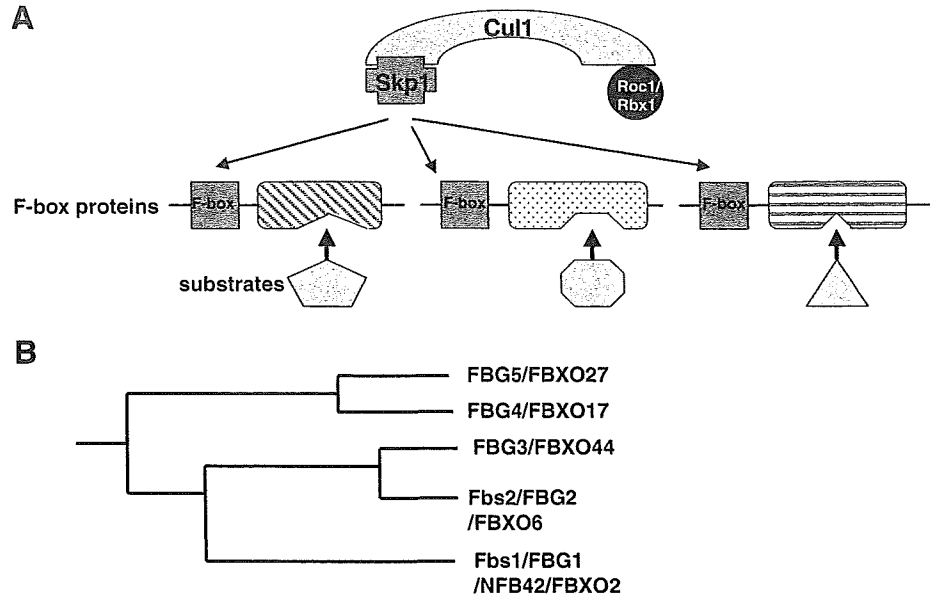

Fig. 5. (A) Structure of SCF complex. In this type E3, cullin1 (Cul1) functions as a molecular scaffold that simultaneously interacts at the amino terminus with the crucial adaptor subunit, Skp1 and at the carboxyl terminus with a RING-finger protein, Roc1/Rbx1, and a specific E2. Skp1 binds to one of many F-box proteins through the F-box domain. (B) Phylogenetic tree of Fbs protein family [73].

substrates, such as pre-integrin β1, is detected in the cytosol in the presence of the proteasome inhibitor. In addition, overexpression of the dominant-negative form of Fbs1 lacking the F-box domain is associated with inhibition of the degradation of ERAD substrates, thus suggesting that SCF^{Fbs1} is involved in the ERAD pathway [71]. Fbs1 is expressed in all major areas of the brain [76]. Indeed, it has been reported that SHP substrate-1 (SHPS-1), a transmembrane glycoprotein abundantly present in the central nervous system [77], and NR1, a subunit of N-methyl-D-aspartate (NMDA)-type glutamate receptors that plays a central role in neuronal development and information storage in the brain, are degraded through the ERAD pathway, and such degradation is mediated by SCF^{Fbs1} [78].

A recent study reported that Fbs1 is essential in inner-ear homeostasis [79]. Orthologues of Fbs1 and Skp1 have been identified in the guinea pig as abundant proteins in the organ of Corti, the sensory organ of the cochlea, where they are called OCP1 and OCP2, respectively [80]. Furthermore, mice with targeted deletion of Fbs1 develop age-related hearing loss with cochlear degeneration and high cochlear levels of the inner-ear gap junction protein, connexin26, which interacts with OCP1 [81], have been described in Fbs1-deficient mice [79].

Although Fbs1 can form the SCF^{Fbs1} complex as an E3 [82], the major part of Fbs1 is present as Fbs1–Skp1 heterodimer or Fbs1 monomer but not in SCF^{Fbs1} [83]. The restricted presence of Fbs1 as SCF^{Fbs1} complex is found in the fraction bound to the ER membrane and the SCF^{Fbs1} complex is associated with p97/VCP, a cytosolic ATPase member of the AAA ATPase family [84]. It has been suggested that the p97–Ufd1–Npl4 complex is required for extraction of misfolded proteins from the ER into the cytosol [85]. Moreover, extraction of the substrate of SCF^{Fbs1} seems to be dependent on the ATPase activity of p97, suggesting that SCF^{Fbs1} is positioned in the ER membrane in such a way that it can ubiquitinate substrates immediately after retrotranslocation to the cytosol [84]. Recent data suggest that the inefficient SCF complex formation of Fbs1 is due to the short linker sequence between its F-box domain and the substrate-binding domain [83]. Fbs1 can prevent the aggregation of glycoproteins *in vitro* and *in vivo*. Since the Fbs1 protein is abundant in neuronal tissues, it is likely that Fbs1 assists in the clearance of aberrant glycoproteins in neuronal cells by suppressing aggregation formation, independent of the E3 activity [83]. Hence, Fbs1 has an additional function as a unique chaperone for these proteins.

3.1.2. Mechanism of N-glycan recognition by Fbs1

Fbs1 and Fbs2 interact with glycoproteins containing high-mannose type *N*-glycans. Pull-down analysis using various oligosaccharides revealed that $Man_{3-9}GlcNAc_2$ is required for efficient Fbs1 binding, while modifications of mannose residues by other saccharides and deletion of an inner GlcNAc residue diminished its binding, suggesting that it specifically recognizes the inner chitobiose structure (GlcNAc–GlcNAc) in the *N*-glycan [74]. X-ray crystallographic and nuclear magnetic resonance (NMR) data indicate that the sugar-binding domain of Fbs1 is composed of a 10-stranded β-sandwich with two α-helices, and binds the $Man_3GlcNAc_2$ portion of oligosaccharides through the loops connecting the β-strands (Fig. 6A) [86,87]. In general, the internal chitobiose of *N*-glycans in native glycoproteins is shielded by the amino acid residues surrounding the glycosylation site, and therefore is unlikely to make contact with Fbs1. The finding that Fbs1 and Fbs2 interact more efficiently with denatured glycoproteins than with native ones led to the hypothesis that the innermost position of *N*-glycans becomes exposed upon protein denaturation and is used as a signal by unfolded glycoproteins to be recognized by these E3s (Fig. 6B) [84]. Thermodynamic analysis of interactions between synthetic *N*-glycan and Fbs1 has revealed that $Man_3GlcNAc_2$ has a substantially stronger affinity than high-mannose type glycans $Man_{8-9}GlcNAc_2$, probably because the outer mannose residues cause steric repulsion [88]. This is consistent with trimming status mannose residues on *N*-glycans of ERAD substrates prior to emergence into the cytosol [28–31].

3.2. PNGase

PNGase was originally thought to contribute to the ERAD pathway because deglycosylation of glycoproteins should increase the efficiency of proteasomal degradation of the substrate [69]. Indeed, the results of RNA interference (RNAi) and inhibitor studies show the involvement of PNGase in the ERAD pathway [69,89], and degradation of a ricin toxin A-chain mutant mediated by ERAD pathway is dependent on PNG1 [90].

PNGase belongs to the transglutaminase (TGase) superfamily and its catalytic triad consisted of Cys-His-Asp is essential for enzyme activity [91–93]. The gene encoding the cytoplasmic PNGase was first identified in yeast as PNG1, and is conserved throughout eukaryotes [93]. While yeast PNG1 possesses a core catalytic domain, PNG1 orthologues in

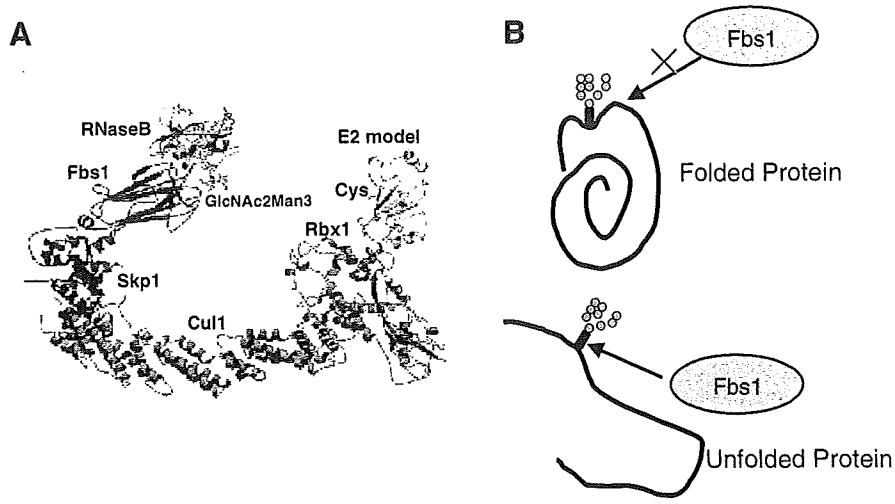

Fig. 6. (A) Structural model of SCF^{Fbs1}–RNaseB–E2 complex constructed based on the crystal structures of Fbs1–Skp1, Skp1–Cul1–Rbx1m, the E2 subunit of the c-Cbl-UbcH7 structure, and the sugar-binding domain of Fbs1–RNaseB structure [81]. (B) A hypothetical model depicting the molecular mechanism of substrate recognition by Fbs1. The intramolecular interaction of the innermost GlcNAc with the polypeptide moiety generally hampers the binding of Fbs1 to chitobiose portions of glycoproteins as a result of steric hindrance in their native states. Fbs1 recognizes the innermost position of *N*-glycans as a signal for unfolded glycoproteins.

higher eukaryotes possess additional domains flanking the core domain (Fig. 7).

Yeast PNG1 closely associates with the 26S proteasome through interaction with the DNA-repair protein Rad23p [94]. Mammalian PNGase also bind to HR23A/HR23B, Rad23p orthologues [95]. Rad23p or HR23 contains an N-terminal Ubl domain, which interacts with the proteasome, and two UBA domains, which interact with ubiquitin, suggesting its shuttling role of substrate to the proteasome [96]. It is well known that Rad23p also binds to Rad4p (XPC protein) and participates in the DNA-repair events [97]. Interestingly, the Rad4p-binding domain of Rad23p is also responsible for interaction with the core domain of PNGase [98]. Based on the crystallographic data of yeast PNG1–Rad23p complex bound to a PNGase irreversible inhibitor, Z-VAD-fmk, PNG1 comprises three domains, a catalytic domain, a Zn-binding domain, and a Rad23-binding domain. The active site of PNGase is located in a deep cleft, which is sufficiently wide to accommodate unfolded polypeptides but not open for native glycoproteins, thus explaining the specificity of PNGase for denatured substrates [99].

PNG1 orthologues in higher eukaryotes carry additional domains. In mammalian PNGase, the N-terminal PUB (Peptide: *N*-glycanase/UBA or UBX-containing proteins) domain, a novel protein–protein interaction site [100], and the C-terminal mannose-binding domain, which presumably contributes to the oligosaccharide-binding specificity of PNGase [101] are found in addition to the core domain. The activity of the truncated PNGase mutants lacking the C-terminal domain is significantly reduced, suggesting that the presence of the domain may accelerate substrate turnover. Interestingly, the crystal structure of the C-terminal domain of mouse PNGase reveals that the β-sandwich architecture resembles the sugar-binding domain of Fbs1 and accommodates the Manα1–6(Manα1–3)Manα1–6 moiety of *N*-glycans in a saddle-shaped depression at one end distal to the N-and C-termini of this domain [101]. Therefore, the C-terminal domain seems to capture the mannosyl branches of high-mannose type *N*-glycans, leaving their sugar-peptide junction oriented toward the active site, and enhances the catalytic activity.

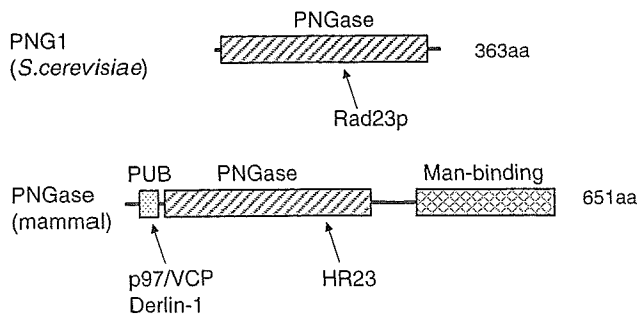

Fig. 7. The domain structure of yeast PNG1 and mammalian PNGase. The interacting proteins for N-terminal PUB domain and catalytic core domain (PNGase) are shown. The PNGase domain comprises three domains, a catalytic domain, a Zn-binding domain, and a Rad23–binding domain.

The N-terminal PUB domain is thought to be involved in the ubiquitin-related pathway based on the engagement of this domain in interactions with Derlin-1 and p97/VCP [102–104]. The ERAD machinery is likely to form an efficient glycoprotein degradation complex for ubiquitination, deglycosylation, and proteasomal degradation, especially in mammals, because PNGase is associated with ERAD substrate extraction machinery (p97/VCP or derlin-1) through the PUB domain and the proteasome through the binding of its core domain with HR23.

It is not clear whether deglycosylation precedes, follows, or occurs simultaneously with ubiquitination. To recognize glycoprotein substrates by SCF^{Fbs1} or SCF^{Fbs2}, it is necessary that these glycoprotein-specific E3s ubiquitinate the substrates prior to deglycosylation by PNGase.

4. Perspectives

The protein quality control in the ER, which exploits *N*-glycans as tags for incompletely folded proteins, is conserved from yeast to mammals. Since *N*-glycan in the ER represents a conserved structure in all eukaryotic cells, different from diverse *N*-glycans expressed on the surface of cells, it is appropriate to provide a common role for *N*-glycans in protein quality control of many different proteins. The repertoire of *N*-glycan recognition molecules and enzymes, however, are diverse and complex in higher eukaryotes compared with yeast. In assisting protein folding as the first stage of quality control in the ER, the calnexin/calreticulin cycle is incomplete in *S. cerevisiae* because reglucosylation enzyme UGGT has not been found. Correspondingly, in the second stage of glycoprotein quality control in the ERAD pathway, higher eukaryotes have acquired diversified system.

First, multiple mammalian orthologues of Htm1p/Mnl1p or Yos9p exist, such as EDEM1, 2, and 3, and OS-9 and XTP3-B. In yeast, the refined model of Htm1p/Mnl1p as an α-mannosidase produces terminal α1, 6-linked mannose residues, as a targeting signal for degradation, which is recognized by Yos9p. However, the mechanisms involved in the

recognition of the unfolded status of ERAD substrates by the Htm1p/Mnl1p are not fully understood. All EDEMs, as well as the ER α-mannosidase I, accelerate ERAD of misfolded glycoproteins such as NHK, but *in vivo* differences in their substrate-specificities and enzymatic activities remain poorly understood. Both OS-9 and XTP3-B possess alternative splicing variants and their substrate recognition mechanisms and associates are likely different.

Second, mammalian PNGase possesses N-terminal PUB domain in addition to the core domain, which is responsible for catalytic activity and association with 26S proteasome thorough HR23. The PUB domain contributes to the recruitment of the PNGase-26S proteasome complex to the ER through the ERAD machinery on the ER such as p97 or Derlin-1. On the other hand, yeast PNG1 fails to associate with p97, but not the Rad23p-proteasome, because it lacks the PUB domain. In the conserved ERAD pathway, why are there differences between yeast and mammals?

Third, there are several ERAD-E3 variants in mammals. In addition to Hrd1 and Doa10, gp78, CHIP, Parkin, RNF5/RMA1, and $SCF^{Fbs1,2}$ have been characterized as the ERAD E3s in mammals [21]. As described in this review, Fbs proteins recognize the exposed innermost chitobiose moiety in aberrant glycoproteins as a tag for incompletely folded proteins, but the recognition systems of Fbs proteins and C-terminal domain of PNGase have not been found in yeast. Interestingly, the C-terminal mannose-binding domain of PNGase appears in *C. elegans* PNGase1 [25], and the orthologues of Fbs proteins are also found in *C. elegans* gene although the *N*-glycan-binding activities have not been reported in this species [72]. With regard to Fbs1, the major part of the protein is not present as SCF^{Fbs1} E3 complex, suggesting that Fbs1 plays other roles beside ERAD E3. For example, several *N*-glycosylated proteins are found in the cytosol (for review, see [26]). It is possible that Fbs1 regulates such glycoprotein in the brain as a chaperone molecule.

In the conserved ERAD pathway, how can we explain the presence of so many complex ERAD system in mammals than yeast? And what functions do they serve? Answers to these questions require further studies with the aim to provide a better insight into the molecular mechanisms of mammalian ERAD.

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筋萎縮性側索硬化症(ALS)の治療戦略

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1 はじめに

神経変性疾患は，特定の神経系が選択的に変性・細胞死を生じる疾患の総称である．神経変性疾患の神経病理学的な特徴は，神経細胞及び非神経細胞の内外に認められる脳内のタンパク質凝集物(封入体)である．そのうち筋萎縮性側索硬化症(ALS)は，上位及び下位運動ニューロンが選択的に変性していく疾患である．40～70歳代で発症し，平均発症年齢は約65歳である．通常発症後四肢及び球麻痺が進行性の経過をたどり，3～5年で呼吸不全などで死亡することが多い．ALSの約90%は孤発性，約10%は家族性である.[1] 高次脳機能など，その他の神経系には目立った症状を認めず，運動神経が選択的に侵され，患者の苦痛が大きいこと，症状の重篤さにも関わらず有効な治療がないことより，治療方法の開発が精力的に行われてきた．

1993年に，家族性ALSの一部はCu/Zn superoxide dismutase(SOD 1)の変異による[1]ことが発見された．その後の研究で，家族性ALSの約20%がSOD 1変異によるとされたが，最近の学会報告では日本では家族性ALSの50%前後がSOD 1変異による．また，孤発性ALSの数%がSOD 1変異によることも明らかになった.[2] 変異SOD 1は活性を残存しているものもあり，その活性低下と臨床経過とは相関せず，変異SOD 1タンパク質の毒性による運動神経変性と考えられる．

さらに最近になって，ALS患者剖検脳脊髄運動神経細胞のタンパク質凝集物の主要構成成分がtransactivation responsive element(TAR) DNA-binding protein of 43 kDa(TDP-43)であることが判明した.[3,4] 驚くべきことに，その後の遺伝学的解析によって，家族性及びごく一部の一見孤発性にみえるALSが，TDP-43遺伝子の変異によって発症することも次々と報告された.[5]

本稿では，SOD 1及びTDP-43の関与するALSの治療戦略について概説する．

2 ALSの病態仮説

SOD 1関連ALS

ALSの病態モデルとして，変異SOD 1トランスジェニックマウスによる研究が精力的に行われ，様々な病態仮説が提唱されている．酸化ストレス，グルタミン酸による興奮毒性，炎症性機序，ミトコンドリア異常，軸索輸送障害，小胞体ストレス，ユビキチン・プロテアソームシステムやオートファジーなどのタンパク質品質管理機構の破綻，凝集タンパク質による細胞毒性などである．

また変異SOD 1トランスジェニックマウスモデルの解析により，変異SOD 1が神経細胞に対して毒性を発揮するだけでなく(自律性神経細胞毒性)，アストロサイトやミクログリアといった非ニューロン細胞内の変異SOD 1タンパク質発現が，ニューロンの変性を促進するという，非自律性神経細胞毒性機序も報告されている.[6,7]

しかし変異SOD 1患者及びモデルマウスでは，一部報告[8]を除いて細胞内TDP-43タンパク質凝集がみられず，核移行性の変化も原則的にはみられない[9]ため，孤発性ALSのモデルとしては問題点が指摘されている．

TDP-43関連ALS

近年発表されたTDP-43の病態仮説については，さらに他の機序が提唱されている．ALS患者剖検組織においてはTDP-43のリン酸化,[3,10] カスパーゼによる切断[11]がみられ，TDP-43に対する免疫組織染色では核の染色性が失われ細胞質に移行する.[3,4] TDP-43はRNA結合ドメインを持ち，種々のRNA及びタンパク質に結合して，核内において

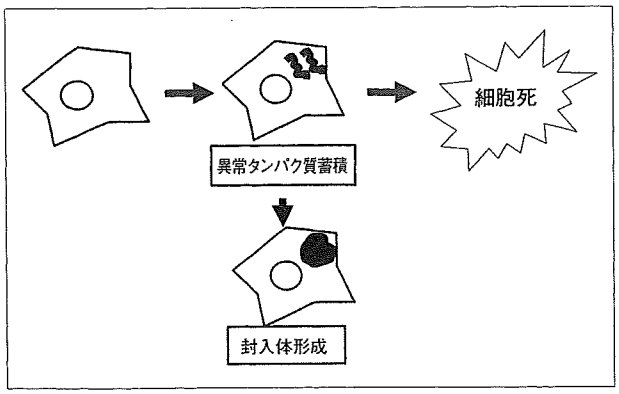

図1　異常タンパク質による細胞毒性

異常タンパク質が蓄積すると，それを分解するだけでなく，封入体に凝集して無毒化する．しかし，細胞の処理能力を超えるタンパク質が蓄積すると，細胞死を引き起こす．

mRNA のスプライシングなどを行っている.[12] そのため，細胞内の修飾された TDP-43 の核移行が低下することにより核内の TDP-43 の機能低下を来たし，毒性を発揮するという機能喪失モデルも提唱されている．

他の神経変性疾患同様，変異タンパク質が毒性を獲得して神経細胞障害，細胞死を起こす可能性も考えられる(gain of toxic function モデル)(図1)．細胞内では，異常タンパク質を分解したり，無毒な封入体に凝集させることで対応していくと考えられるが，その防御機構を超えて異常タンパク質が蓄積すると細胞毒性を発揮して細胞死を引き起こすものと考えられる．

3 ALS 治療開発の現状

先述のように，ALS では変異 SOD 1 トランスジェニックマウスが家族性 ALS の病態モデルとして確立しており，その動物モデルによる治療法開発が行われている．例えば，酸化ストレスに対し抗酸化剤であるビタミンEや，ミトコンドリア機能改善目的にコエンザイム Q_{10} で運動神経症状の進行を遅延し生存期間を延長し，有効性が認められた.[13] また，シクロオキシゲナーゼを抑制し抗炎症作用を有するミノサイクリンなども有効性が認められた.[13]

また，アストロサイトのグルタミン酸トランスポーター1の発現を上昇させる既存薬剤のハイスループット・スクリーニングを行い，第3世代セフェム系抗生物質であるセフトリアキソンが発見され,[14] ヒトへの臨床試験も行われている．

他に，神経栄養因子による神経保護を目指した治療も検討されている．インスリン様増殖因子(IGF-1)などが有効性を認めた.[13]

そのほか様々な治療アプローチがなされてきたが，いずれの薬剤も臨床試験で明らかな有効性を証明できず，現在 ALS に対し有効性が証明された薬剤はグルタミン酸遊離阻害や興奮性アミノ酸受容体との非競合的阻害，電位依存性 Na^+ チャネル阻害等の作用を有するリルゾールのみである．しかしリルゾールによる延命効果は約3か月に留まり，筋力・運動機能の改善は望めず，その効果は限定的である.[15]

4 SOD 1 タンパク質量制御による家族性 ALS の治療法

変異 SOD 1 トランスジェニックマウスでは，トランスジーンのコピー数が多いほど表現型が重篤である.[16] また，変異 SOD 1 トランスジェニックラットでは，トランスジーンのコピー数の多いラットのみが ALS を発症し，コピー数の少ないラットでは発症しない.[17] したがって，変異 SOD 1 に関連した ALS では，変異 SOD 1 の量を減らすことが治療につながる可能性がある．

実際の実験治療として，RNA 干渉や antisense oligonucleotide を用いてタンパク質発現量を低下させることで病態を改善した，という報告がある．RNA 干渉を用いた実験[18]では，変異 SOD 1 mRNA に相補的な siRNA を変異 SOD 1 マウスにかけ合わせたダブルトランスジェニックマウスで，その生存期間が著明に延長したという報告がある．また，antisense oligonucleotide を脳室内に投与し，変異 SOD 1 タンパク質の細胞内産生抑制を行ったマウスでも表現型の改善が見られる.[19]

さらに，lox 配列で変異 *SOD1* 遺伝子を挟み SOD 1 本来のプロモーターで変異 SOD 1 を発現して，通常の変異 SOD 1 マウスと同様に ALS を発症するトランスジェニックマウスを組織特異的 Cre

発現マウスとかけ合わせることによって，運動ニューロンでのみ変異SOD1発現を低下させるとALSの発症が遅延し，ミクログリアでのみ変異SOD1発現を低下させると疾患の進行を遅らせたという報告がある.[6] 同様の手法で，アストロサイト内変異SOD1発現低下により疾患の進行を遅らせたという報告もある.[7]

他の神経変性疾患モデルでも，原因タンパク質発現量を減少させることで治療につながる可能性が示唆されている．ハンチントン病はハンチンチン(Htt)というタンパク質のポリグルタミン(polyQ)が異常に増えることで，そのタンパク質の凝集，神経細胞変性が認められる疾患であり，polyQ Httを過剰発現するトランスジェニックマウスはハンチントン病のモデルとして知られている．そのマウスのpolyQ発現量を，Tet-Offシステムを用いて後天的に減少させると，発症を抑えられたという報告がある.[20] また，アルツハイマー病モデルである変異タウトランスジェニックマウスでも，同様のシステムで発現量を抑えることで，記憶機能の改善及び神経細胞変性の抑制が観察されている.[21]

以上から，我々はSOD1の転写を抑制してSOD1タンパク質量を減少する低分子を，低分子化合物・既存薬ライブラリのハイスループット・スクリーニング・システム(図2)を構築して，家族性ALS治療薬スクリーニングを行っている．我々はSOD1の本来のプロモーターの支配下にレポーター遺伝子としてルシフェラーゼを発現するコンストラクトを構築した．

化合物がプロモーターに作用し転写を抑制すれば，ルシフェラーゼの発現量が低下しルシフェラーゼの基質から産生される蛍光物質の量が低下する．アストロサイトが非自律性神経細胞死，疾患の進行に関連することから，ヒトアストロサイト由来の細胞株を使用している．また，ルシフェラーゼ反応基質を96-ウェル・プレート上で自動分注後吸光度を測定する装置を用いて測定している．このような方法でこれまでに9,600種類の化合物をスクリーニングし，蛍光物質の産生を減少させる，すなわちSOD1の転写を抑制する化合物を177種類見いだしており，これからさらにALSモデル細胞やALSモデル動物での効果を確認して，臨床的に有用な化合物を絞り込んでいく予定である．低分子化合物は，大量生産が可能であり，安価で安定した供給を行うことが可能となる．また，既存薬を用いればヒトへの使用における安全性も既に確認されており，速やかな臨床への応用も可能となる．我々の開発した方法は，ALS治療開発における新たなアプローチの1つとなるものと考える．

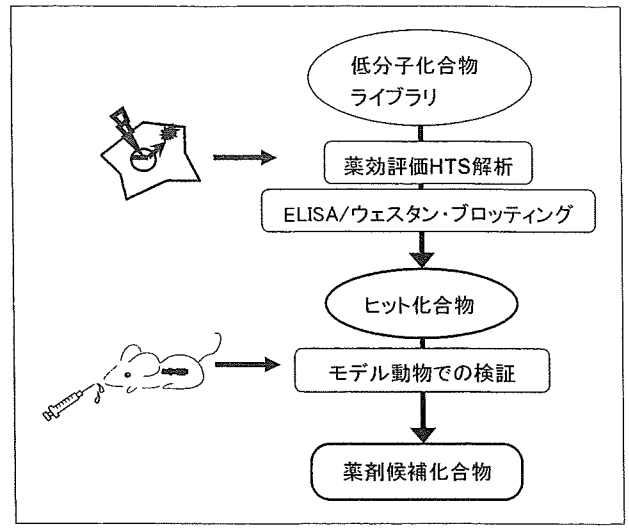

図2 我々のALS治療開発戦略の概要

SOD1プロモータ下にルシフェラーゼを発現するコンストラクトを導入した，ヒトアストロサイト由来細胞株を用いて，SOD1転写を抑制する化合物をスクリーニングする．ELISAやウェスタン・ブロッティングでSOD1タンパク質量を特異的に減少する化合物を抽出する．変異SOD1トランスジェニックマウスでその効果を確認する．

5 SOD1タンパク質を減少させることによる問題点

SOD1は細胞内に発生したsuperoxide radicalsを過酸化水素に分解する酵素であり，抗酸化ストレス酵素の1つに挙げられる．したがって，SOD1を減少させることは，酸化ストレス反応に対する脆弱性を来たす可能性がある．

SOD1ノックアウトマウスの解析によると，脊髄運動神経軸索を切断した後の神経細胞死がSOD1非ノックアウトマウスに比して多く見られる.[22] ほかに，神経筋接合部の形成不全や軸索変性が，高齢のSOD1ノックアウトマウスで見られるという報告もある.[23] したがって，SOD1を大きく低下させることは脊髄運動神経変性を来たす可能性

がある．

ただし，SOD 1ノックアウトマウスの寿命は非ノックアウトマウスに比べ著変が見られない．[24] さらに，SOD 1ヘテロノックアウトマウスはノックアウトマウスに比して軸索切断による神経細胞死の程度が軽度である．[24] また，他のアンチオキシダントによる代償も期待できるため，SOD 1を特異的部分的に減少させることが，重篤な副作用を来たさずに，治療効果を得るために重要である．

さらに，コピー数が多い野生型 *SOD1* 遺伝子のトランスジェニックマウスでも軸索変性やミトコンドリアの変性，脊髄運動ニューロンの減少が，高齢になれば出現することが報告されている．[24] 変異型SOD 1と野生型SOD 1のダブルトランスジェニックマウスは，野生型SOD 1量に応じて進行が速くなるとの報告[24]もあり，野生型SOD 1細胞内産生も制御する試みは相乗的に有効である可能性がある．

先述のように，他のアプローチで明らかな有効性を認めた治療がほとんどないこともあり，原因となる異常タンパク質を直接減少させるアプローチが有望と考えられることから，SOD 1タンパク質量を減少させる有用性は，部分的特異的に行うことができれば，そのリスクよりも大きく，根本的な治療につながると考えられる．

6 孤発性ALSに対する治療的アプローチ

先述のように，孤発性ALSではTDP-43タンパクの細胞質内封入体を認め，核における染色性が失われている．核移行シグナルを欠損させた *TDP-43* 遺伝子ないし，易凝集性の高いC末端 *TDP-43* 遺伝子にGFPを付して導入したTDP-43凝集細胞モデルを用いて，タンパク質凝集を抑制させる薬剤が報告されている．[25]

ただし，TDP-43に関連したALSの病態生理はまだ未解明の部分が多く，今後TDP-43の生理的機能及び変異TDP-43の神経細胞に対する影響の詳細な解析がまたれる．

7 おわりに

ALS，特に変異SOD 1関連ALSの病態に沿った治療戦略について概説した．精力的な研究がなされてはいるが，未だ病勢を決定的に改善する薬剤は開発されていない．この難病に対する治療法が早く発見され，多くの患者が治療される日が来ることを望む．

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を計算する。浸透圧ギャップは血漿浸透圧実測値と計算値（上記の間で提示された式より計算）との差で、通常10mOsm/kgH_2O以下である。これが10mOsm/kgH_2O以上を示す場合には、マンニトール、エタノール、メタノール、エチレングリコール、トルエンなど、外因性浸透圧物質の増加が考えられる。

血漿膠質浸透圧

再び収的巨大な粒子であるコロイド。分子が懸濁液を形成しているようにコロモ状態でも、浸透圧を形成する。

この浸透圧を膠質浸透圧（colloid osmotic pressureまたはoncotic pressure）と呼ぶ。膠質浸透圧は、一定容積中に含まれるコロイド粒子の数が多いほど大きくなる。血漿浸透圧と異なり、氷点降下法では測定できない。臨床的には、血漿蛋白質の約6割を占めるアルブミン1g/dlにつき5・5mmHg、4・5g/dlで約25mmHgの膠質浸透圧を示す。これは血漿全膠質浸透圧の8～9割に相当し、間質から血管に向かう力である。

ネフローゼ症候群や肝硬変のように血漿アルブミン濃度が減少する病態では、血漿膠質浸透圧が低下し血管内から間質に水の移動が生じ、浮腫が出現することになる。

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◇◇◇回答◇◇◇

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（熊本県 N）

パーキンソン病の神経細胞移植治療

Q 神経細胞を移植したパーキンソン病患者の場合、健康なはずの移植された細胞にパーキンソン病の病理所見が出現することが報告された（Nature Medicine, 2008）。以下について。

(1)移植手術はパーキンソン病の根本治療法となりうるのか。

(2)近年、神経新生（neurogenesis）の研究が盛んであるが、この研究はパーキンソン病の新しい治療ターゲットとして有望なのか。

A Nature Medicineの5月号に掲載されたスウェーデンとアメリカとカナダの3グループからの論文に基づいたご質問である[1)-3)]。パーキンソン病の病理を特徴づけるのは、α-シヌクレインの凝集であるレビー小体である。上記3論文の結果をまとめると、胎児の黒質ドーパミン神経細胞を移植されて4～16年を経た計8例のパーキンソン病患者剖検例を調べたところ、驚くべきことに移植後10年以上を経た3例で移植片中の本来は健康なドーパミン神経にレビー小体が形成されていることが見出された。これはレビー小体病理がパーキンソン病患者組織から移植片に〝伝播〟するという、これまで神経変性疾患ではプリオン病でしか知られていなかったメカニズムが、パーキンソン病でも働くことを示したことになる。

〝伝播〟が生じるメカニズムに関してはまだ分からないが、移植後10年以内の5例の移植片ではレビー小体形成は見られなかったことから、少なくとも〝伝播〟には10年は必要なことが推察される。また、1例では移植後14年を経ても移植片にレビー小体形成が見られていない。この例とレビー小体ができた例との違いの一つは、前者ではミクログリアの活性化が乏しかったことで、レビー小体〝伝播〟への炎症の関与をうかがわせる。

その他、酸化的ストレス、興奮毒性、神経栄養因子の欠乏などがメカニズムとして提唱されており、今後その解明はパーキンソン病の病態解明の上できわめて重要な課題となるであろう[4)]。

ただ、気をつけなければならないのは、レビー小体の形成が細胞の機能や生存に与える影響がまったく不明なことである。レビー小体は、それ自体が異常構造として細胞毒性を持つという考えから、レビー小体は毒性を持つ異常蛋白質を封じ込めるために作られるものであり、細胞保護的に働いてい

るというものまで様々な説があり、決着がついていない。移植片にレビー小体ができるからといって、その細胞が障害されるとは限らないのである。

また、移植後10年以上を経ないとレビー小体形成は見られないことから、たとえレビー小体に毒性があったとしても10年以内の効果は十分望みうることになる。胎児黒質移植自体は2件の二重盲検試験で有意な症状の改善効果がなく、不随意運動が生じるなどの副作用があることが示され、現在行われていないが、広く移植治療の一つとしての方法論の改善で有用な段階療法になる可能性は秘めている。

そのような意味で、今後も新たな治療の選択肢として研究を続けるべきと思われる。

神経新生の治療に関しても同様の理由で、Nature Medicineの論文の結果が、治療法そのものを否定することにはならない。動物実験ではあるが、海馬における神経新生が抗うつ薬の効果発現に必須であることが示された[5]。これは、神経新生治療がパーキンソン病を含めた様々な神経疾患でも有用であることを強く示唆している。

パーキンソン病でも黒質ドーパミン神経の新生を示唆する報告があり、期待が持たれる[6]。ただし、一方でレビー小体が移植細胞、新生した細胞に生じた場合、それが細胞機能に与える影響を正確に評価する研究も進め、移植治療、神経新生治療の可能性と限界を明らかにすることが、これらの治療を将来的に現実のものとする上で重要であろう。

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◇◇◇回答◇◇◇

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高橋良輔

赤痢アメーバ症の診断と治療

Q 赤痢アメーバ症の診断と治療について、以下を。

(1)糞便検体の採取方法（顕微鏡検査用、培養検査用）。

(2)血液検査用の検体採取方法（検査名、検査結果の判読）。

(3)治療方法、治療期間。

(4)治療効果の確認方法。

（東京都　M）

A 糞便の顕微鏡検査で赤痢アメーバの嚢子あるいは栄養型に一致する形態を確認しても、赤痢アメーバ（*Entamoeba histolytica*）と報告してはならない。赤痢アメーバないしは非病原性のアメーバ（*E.histolytica*/*E.dispar*, 以下Eh/Edと略）と報告すべきである。赤痢アメーバ症を疑わせる症状がある場合、Eh/Edの検出をもって治療適応となる。健康人から検出されたEh/Edは治療適応とならない[1]。

(1)結果報告書に赤痢アメーバと記述できるのは、赤痢アメーバに特異的な検査を実施した場合のみである。赤痢アメーバの検査は国立感染症研究所あるいは地方衛生研究所で実施可能である[2]。検体採取法はそちらの指示に従っていただきたい。

自らの手で確認したい場合は、赤痢アメーバ抗原検出キットであるE. HISTOLYTICA II（関東科学から購入可）を入手すればよい。ただし、これは正式な検査法として認められていない。このため、法律上「診断に用いてはならない」ことになっている。

(2)アメーバ性肝膿瘍では、必ずしも糞便中に赤痢アメーバが検出できるとは限らない。血中抗赤痢アメーバ抗体の検出をもって、抗アメーバ薬の適否を判断する場合が多い。詳細は、参考文献をご覧いただきたい[2]。

(3)治療方法

第1選択薬はメトロニダゾールの経口投与である。海外の文献では2250mg／日の記述もみられるが、日本人には、1500mg分3、10日間で十分である[3]。なお、

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Commentary

Edaravone in ALS

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Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by progressive and relatively selective degeneration of upper and lower motor neurons. Patients suffer from atrophy and paralysis of systemic voluntary muscles including respiratory muscles, leading to respiratory failure and subsequent death 3–5 years after the disease onset. Effective therapy for ALS that ameliorates its clinical course is still not known (Mitchell and Borasio, 2007).

Although ALS usually develops sporadically, 5 to 10% of cases are familial and hereditary. Twenty percent of familial ALS (FALS) are caused by mutations in the *copper and zinc-dependent superoxide dismutase* (*SOD1*) gene, which was first reported in 1993 (Rosen et al., 1993). Mutant SOD1 brought a breakthrough to this field, since mutant SOD1 transgenic mice recapitulate the clinical symptoms and pathological findings of human FALS (Gurney et al., 1994). Mutant SOD1 transgenic mouse models provided invaluable tools for testing effective drugs which extend their lifespan. Up to now, more than 20 drugs have been claimed to be effective in the therapy of mouse ALS.

A big problem, however, is arising: none of these drugs have yet to be shown to be effective as well in human sporadic ALS (SALS) patients (Benatar, 2007). Why? A couple of explanations are conceivable. First, mutant SOD1 transgenic mice may not be a good model for human sporadic ALS cases despite their apparent similarities. Indeed, mutant SOD1 associated FALS and SALS exhibit different microscopic neuropathology. The former is characterized by Lewy body-like inclusion containing mutant SOD1, whereas for the latter skein-like or round inclusions containing TDP-43. Since TDP-43 is implicated in the pathogenesis of SALS as well as in a subgroup of FALS, developing a new ALS mouse model based on TDP-43 could solve these problems in the future (Neumann et al., 2007). A second possible explanation is that the most therapies in mouse models are initiated prior to disease onset, which is impossible in human patients until presymptomatic diagnosis for ALS becomes available. Thirdly, whether drug dosage and bioavailablity comparable to mouse experiments are replicated in human trials remains unclear.

An alternative explanation is the difference in the design of mouse experimental therapies and human clinical trials. Randomized controlled trials, which are designed to eliminate numerous confounding factors including observation biases, are standard in human clinical trials. In contrast, mouse experiments are generally not performed as rigorously as human trials, increasing risks of producing "false positive" results (Benatar, 2007).

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a free radical scavenger that has been approved in Japan since 2001 as a therapeutic agent to reduce neuronal damage caused by acute ischemic stroke (Yoshida et al., 2006). Edaravone eliminates lipid peroxide and hydroxyl radicals by transferring an electron to the radical, thereby ameliorating the ischemic neuronal damage. Oxidative stress is implicated as one of the pathogenetic mechanisms for ALS (Barber et al., 2006). Moreover, a small-sized open trial of edaravone suggested that edaravone is safe and may delay the progression of functional motor disturbances in ALS patients (Yoshino and Kimura, 2006). Thus. edaravone is a promising therapeutic agent for human motor neuron diseases including ALS.

In a previous issue, Ito et al. reported an experimental therapy of a mutant SOD1 mouse model using edaravone (Ito et al., 2008). Taking the problems associated with the therapeutic experimental design in mouse experiments, they carefully optimized the dosage of edaravone so that the pharmacokinetic profile after intraperitoneal injection became comparable to that in human patients. Moreover, they started treatment only after the disease onset, similar to human ALS treatment. Furthermore, they used only female mice for analysis considering the gender difference in lifespan and randomized blind analyses were adopted for all the behavioral as well as pathological observations. This methodological rigorousness has never been considered seriously in previous experimental therapies of mutant SOD1 ALS mouse models, most of which have failed to be replicated in human patients.

Edaravone significantly slowed the motor function decline as assessed by multiple behavioral tests such as rotarod tests. However, the lifespan of edaravone-treated mice were not significantly higher

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than those of control mice, suggesting that edaravone may improve the motor function of the ALS mice without apparent lifespan expanding effects (Ito et al., 2008). This uncoupling in the mechanisms underlying motor function and lifespan further implies that pathways causing motor function decline are not necessarily the ones causing eventual death, usually by respiratory muscle failure. That said, it would be possible to identify drugs that can improve the quality of life in ALS patients without affecting lifespan, which seems to be an easier goal compared with identifying lifespan-extending drugs for ALS. Moreover, it was clinically important that edaravone was effective even when administered after the disease onset. On the other hand, it would be intriguing to administer edaravone to ALS mice at their presymptomatic stage to understand how the point at which edaravone is used during the course of disease affects its outcome.

It is noted that high-dose edaravone treatment leads to a decrease of mutant SOD1 accumulation in the spinal cord. Since administration of edaravone resulted in a marked decrease of 3-nitrotyrosine/tyrosine ratio, a marker of oxidative stress, suppression of oxidative stress is likely to be upstream of the inhibition of aggregate formation (Kabashi and Durham, 2006; Valentine and Hart, 2003). It has long been debated how oxidative stress is induced by SOD1 mutations (Barber et al., 2006). Reduced enzymatic activity of SOD1 and generation of peroxynitrite due to aberrant copper chemistry have been proposed as plausible mechanism explaining "gain of toxic function" of mutant SOD1 (Beckman et al., 1993; Deng et al., 1993; Robberecht et al., 1994). However, the fact that a subgroup of SOD1 mutants retains full enzymatic activity and that H46R and H48Q mutants which completely lose binding sites for copper still cause ALS suggests that mechanisms unrelated to SOD1 activity may also be involved (Borchelt et al., 1994; Valentine et al., 2005; Wang et al., 2003). It has been shown that mutant SOD1 overexpression in a neuronal cell line leads to transcriptional repression of antioxidant proteins by reducing the level of transcriptional factor NRF2 (Kirby et al., 2005). It would be intriguing to investigate whether edaravone affects the level of NRF2 when administered to ALS mice.

Another interesting unresolved question is which cells are the targets of edaravone. Recently, it has been shown that motor neuron death in mutant SOD1 ALS mouse models is non-cell autonomous (Boillee et al., 2006; Yamanaka et al., 2008). In other words, mutant SOD1-expressing astroglial or miroglial cells promote motor neuron death. In this context, edarovone may decrease the aggregates in non-neuronal glial cells, resulting in amelioration of neurodegeneration. These questions should be addressed in further analysis in the future.

A recent systematic review of randomized controlled trials of antioxidant therapies against ALS including vitamin E and acetylcysteine has shown that there is no substantial evidence to support their clinical use (Orrell et al., 2008). However, the evidence for the beneficial effects of edaravone on human ALS patients awaits the publication of the results of a phase III clinical trial of ALS, currently ongoing in Japan (http://www.als.net/research/studies/tdfAnimalStudyList.asp).

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Nicotinic Receptor Stimulation Protects Nigral Dopaminergic Neurons in Rotenone-induced Parkinson's Disease Models

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Parkinson's disease (PD) is the second most common neurodegenerative disease and is characterized by dopaminergic (DA) neuronal cell loss in the substantia nigra. Although the entire pathogenesis of PD is still unclear, both environmental and genetic factors contribute to neurodegeneration. Epidemiologic studies show that prevalence of PD is lower in smokers than in nonsmokers,. Nicotine, a releaser of dopamine from DA neurons, is one of the candidates of antiparkinson agents in tobacco. To assess the protective effect of nicotine against rotenone-induced DA neuronal cell toxicity, we examined the neuroprotective effects of nicotine in rotenone-induced PD models in vivo and in vitro. We observed that simultaneous subcutaneous administration of nicotine inhibited both motor deficits and DA neuronal cell loss in the substantia nigra of rotenone-treated mice. Next, we analyzed the molecular mechanisms of DA neuroprotective effect of nicotine against rotenone-induced toxicity with primary DA neuronal culture. We found that DA neuroprotective effects of nicotine were inhibited by dihydro-β-erythroidine (DHβE), α-bungarotoxin (αBuTx), and/or PI3K-Akt/PKB (protein serine/threonine kinase B) inhibitors, demonstrating that rotenone-toxicity on DA neurons are inhibited via activation of α4β2 or α7 nAChRs-PI3K-Akt/PKB pathway or pathways. These results suggest that the rotenone mouse model may be useful for assessing candidate antiparkinson agents, and that nAChR (nicotinic acetylcholine receptor) stimulation can protect DA neurons against degeneration.

Key words: Parkinson's disease; rotenone; nicotine; dopaminergic neuron; neuroprotection

Parkinson's disease (PD) is the second most common progressive neurodegenerative disorder. It is characterized by relatively selective degeneration of dopaminergic neurons in the substantia nigra and loss of dopamine in the striatum resulting in resting tremor, rigidity, bradykinesia and postural instability (Dunnett and Björklund, 1999; Shimohama et al., 2003). Although the pathogenesis of PD is still unclear, it is thought that both environmental and genetic factors cause neurodegeneration. Rural residency, pesticides and intrinsic toxic agents were reported as environmental risk factors for sporadic PD. Recent studies revealed several mutations in familial PD genes such as α-synuclein, parkin, PINK1, LRRK2 (leucine-rich repeat kinase 2), DJ-1, and UCH-L1 (ubiquitin C-terminal hydrolase-L1) (Schapira, 2006). Epidemiological studies suggest that the use of pesticides increases the risk of PD, possibly via reduced activity of complex I in the mitochondrial respiratory chain in the substantia nigra and result in the pathogenesis of PD (Parker et al., 1989; Mann et al., 1992; Mizuno et al., 1998). 6-hydroxydopamine (6-OHDA), a H_2O_2 pro-oxidant and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a mitochondrial complex I inhibitor, have been widely used to produce toxin models of sporadic PD. Chronic exposure to rotenone, a nature-derived pesticide, could be a more

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