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[Review]

Possible Application of Electron Spin Resonance to Monitoring of Prion Diseases and Hypotheses on Oxidative Action and Propagation of Copper-bound Infectious Protein

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Hypotheses on oxidative actions of copper-bound form of prion proteins (PrP) in neurotransmitter-rich neuronal tissues and possible systemic propagation mechanism of pathogenic form of PrP are proposed. In addition, possible use of electron spin resonance spectroscopy for monitoring of copper-bound PrP signals and the presence of redox-active form of PrP are proposed.

Key words: Copper-bound prion protein (銅結合プリオン蛋白質), Electron spin resonance spectroscopy (電子スピン共鳴法), Oxidative action (酸化反応), Redox regulation (酸化還元制御)

Introduction

Prion protein (PrP) from the brains of animals with transmissible spongiform encephalopathies (TSE) is present as partially protease-resistant form (PrP^{res}) while that in uninfected brains presents as fully sensitive form (PrP^{sen}) (e.g., Jeffrey *et al.*, 2000). In general, PrP^{sen} represents the intrinsic cellular PrP (PrP^{C}) and PrPres represents the infectious scrapie isoform of PrP (PrP^{Sc}).

The deposition of abnormal protein fibrils is a prominent pathological feature of many different "protein conformational" diseases, including some important neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, motor neuron disease and "prion" dementias (Tabner *et al.*, 2001). In the cases of β-amyloid which accumulates in the brain in Alzheimer's disease and of α-synuclein accumulating in Lewy bodies in Parkinson's disease, there are good evidences that the toxic mechanism involves the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\cdot}$), supporting the view that one of the fundamental molecular mechanisms underlying the pathogenesis of cell death in neurodegenerative diseases could be the direct production of ROS during formation of the abnormal protein aggregates (Tabner *et al.*, 2001).

Roles for PrP in Oxidative Damage: Controversial Views

Recently, evidence of oxidative stress in scrapie, the archetype disease of the TSE, which leads to neuronal degeneration, has been accumulated and oxidative stress has been proposed to play an important role in the pathogenesis of other several neurodegenerative disorders

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(Kim *et al.*, 2001). To date, anti-oxidative roles of PrP^C are well recognized. It has been shown that *E. coli* cells expressing PrP sequence (octapeptide repeats region) acquired the resistance to copper and copper-dependent oxidative damages indicating that PrPC possibly contributes to protection of cells from free copper ion-catalyzed generation of ROS such as HO$^\bullet$ (Shiraishi *et al.*, 2000). According to Wong *et al.* (2001), copper-bound PrPC shows a superoxide dismutase (SOD)-like activity in vitro, and its expression passively contributes to the cellular response to oxidative damages to the cells. Sauer *et al.* (1999) has shown in tumor spheroids that an increase in ROS level stimulates the production of PrP^C, in addition to other ROS scavenging enzymes such as Cu, Zn-SOD and catalase, while ROS-lowering treatments effectively down-regulates the expression of PrP^C. This implies that PrP^C expression in tumor spheroids is finely regulated by the internal redox state to meet the requirement to protect cells from ROS. However, a drastic depress in SOD-like activity has been shown in the affinity-purified total PrP preparation isolated from scrapie-infected mouse brains suggesting that prion disease results in denature of PrP thus no longer active in ROS removal (Wong *et al.*, 2001).

Opazo *et al.* (2003) have propounded the importance of metals in neurobiology and proposed two opposing roles for copper-bound PrP as an anti-oxidant supporting the neuronal functions and as a pro-oxidant leading to neurodegenerative process. It is generally accepted that, like amyloid-β peptide, prion induces apoptotic cell death in neuronal tissues (Agostinho and Oliveira, 2003). PrP amino acid sequence 106–126 containing a copper-binding site forms the region likely contributing to apoptotic neurotoxicity since a synthetic highly fibrillogenic copper-binding peptide corresponding to PrP 106–126 has been shown to be highly toxic to neuronal cells by mimicking PrPSc (Belosi *et al.*, 2004; Agostinho and Oliveira, 2003). Since this process involves the generation of ROS, this case supports the role for a copper-binding region as a pro-oxidative catalyst (Agostinho and Oliveira, 2003).

Taken together, it is likely that prion disease represented by conversion of PrP^C to PrP^{Sc} results in loss of PrP's SOD-like activity and loss in Cu-uptake required for SOD production, leading to enhanced oxidative burst thus the cells could be readily killed by ROS. It is tempting to speculate by analogy from the nature of PrP (106–126) peptide active in PrP^{Sc}-like fibril formation and induction of ROS production, that fibrils formed from PrPSc likely plays a role as a prooxidant.

Copper-binding Sites on PrP

Importance of metalloproteins in neurobiology has been shown both as oxidant and antioxidant in neurodegenerative processes (Opazo *et al.*, 2003). Copper is an essential trace element but its redox reactivity leads to risks of damage to cells and tissues, especially in neurodegenerative diseases such as Menkes' and Wilson's disease occurring via disorders of copper metabolism, and Alzheimer's disease and 'prion' diseases, the conformational diseases, as reviewed elsewhere (see: Rossi *et al.*, 2004; Vassalo and Herms, *et al.*, 2003; Rotilio *et al.*, 2000; etc.). The likely roles for copper in induction of critical steps in the apoptotic pathways leading to neurodegeneration is outlined in the above reviews.

Recent studies have shown that PrPs form a group of copper-binding proteins (Aronof-Spacer *et al.*, 2000; Burns *et al.*, 2003). Human PrP has four copper-binding sites in the "octarepeats" region (PrP 60–91) in which amino acid sequence PHGGGWGQ appears four times and each repeat binds single Cu^{2+} at physiological neutral and basic range of pH (Bonomo *et al.*, 2000). The bovine PrP sequence contains six octarepeats

thus possessing at least six putative copper-binding sites with high affinity (Morante *et al.*, 2004; Brun *et al.*, 2004). In chicken, the copper-binding sites analogous to the octarepeats are known as hexa-repeats with each repeat consisting of HNPGYP and here again His residues play a key role in anchoring of copper (Stanzak *et al.*, 2004). Morante *et al.* (2004) showed that partial occupancy of copper on bovine PrP is manifested by binding of copper to PrP in the intermolecular or inter-octarepeat orientations while total occupancy of copper is manifested by intra-repeat binding of copper to the octarepeat region.

In vitro studies have shown that the actual least motif in the octarepeats necessarily required for binding of copper consists of 4 amino acids HGGG (Bonomo *et al.*, 2000) or 5 amino acids HGGGW (Burns *et al.*, 2002). Although there are additional Cu-binding sites on PrP such as amino acid regions 92–96 (GGGTH) (Burns *et al.*, 2003), 124–126 (KHM) (Belosi *et al.*, 2004) and 180–193 (VNITKQHTVTTTT) (Brown *et al.*, 2004), and all studies suggested that His residue in each region (or each repeat unit) plays a key role in anchoring the copper (Fig. 1). The binding of copper to octarepeats likely stimulates the endocytosis of PrP (Burns *et al.*, 2002) thus enabling the internalization and metabolism of copper required as a building block for many enzymes such as Cu, Zn-SOD (Klamt *et al.*, 2001). It has been shown that Cu^{2+} binding by PrP^{C} (but not by PrP^{Sc}) is likely required for internalization of copper into cytoplasm (Klamt *et al.*, 2001). Since many antioxidant enzymes depend on copper (e.g., Cu, Zn-SOD), their function could be affected in prion diseases (Klamt *et al.*, 2001; Wong *et al.*, 2001).

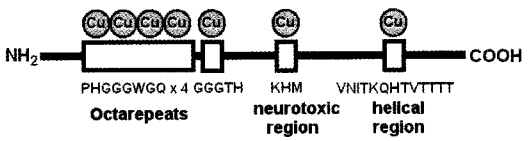

Fig. 1. Putative copper-binding sites on PrP. His residues play key role binding to copper-(II).

There is a question to be addressed if PrP^{Sc} formation results in modification of copper-metabolism. Since the fibril-forming PrP^{Sc} is no longer capable of copper internalization, despite of copper-binding activity, the whole organism likely becomes more sensitive to ROS injury during development of prion disease, leading to a progressive oxidative disruption of tissues and vital organs, especially central nervous system (Klamt *et al.*, 2001). However, Rachidi *et al.* (2003) presented an opposing evidence that level of copper delivery could not be altered by controlled expression of recombinant N-terminal Cu^{2+}-binding region of PrP in rabbit kidney epithelial cells (RK13), while , this study showed a PrP-dependent increase in binding of copper to cell surface.

Then what is the consequence of copper binding to PrP? Is binding of copper to PrP required for pathogenesis It has been shown that His residues in the octarepeats of PrP protein contribute to formation of oligomeric complex through binding to glycosaminoglycans at acidic conditions and more importantly the copper-bound octarepeats also contribute to formation of much more stable complex with glycosaminoglycans (Cu as a bridging element) and the copper-bridged glycosaminoglycan–PrP complexes are more resistant to proteinase action (Gonzalez-Iglesias *et al.*, 2002). Therefore copper-binding (Cu-bridge) partially takes place in formation of pathogenic form of PrP. If internalization of copper-bound PrP were not likely required for Cu delivery (Rachidi *et al.*, 2003), a likely role for Cu^{2+} may be development of PrP propagation (entry into uninfected cells) required for pathogenesis via Cu^{2+}-stimulated PrP endocytosis (Burns *et al.*, 2002).

There have been arguments if absorption of copper by PrP results in protection of cells from

copper-dependent oxidative damages or not? According to Shiraishi *et al.* (2000), a peptide corresponding to octarepeats binds and arrests copper at redox-inactive state and thus inhibits the copper-catalyzed oxidation of ascorbate and glutathione, and generation of HO· accompanying copper-dependent ascorbate oxidation. Therefore indicating that PrP can function to sequester copper ions in the redox inactive state, rendering copper-induced generation of ROS impossible. Controversially, some groups reported the role for tryptophan residues in octarepeats act in reduction of Cu(II) to Cu(I), thus maintaining the metal at redox active state (Ruiz *et al.*, 2000). Furthermore, in nature, does the formation of copper–PrP complex enhance the oxidative burst via an additional mechanism leading to cell death, or initiation of pathogenic PrP propagation through relay of conformational changes? Regarding this question, the present paper emphasizes the possible model that copper-bound form of PrP catalyzes the generation of ROS at speci-fic occasions in the neuronal tissues. The hypotheses and discussion are presented in the below sections.

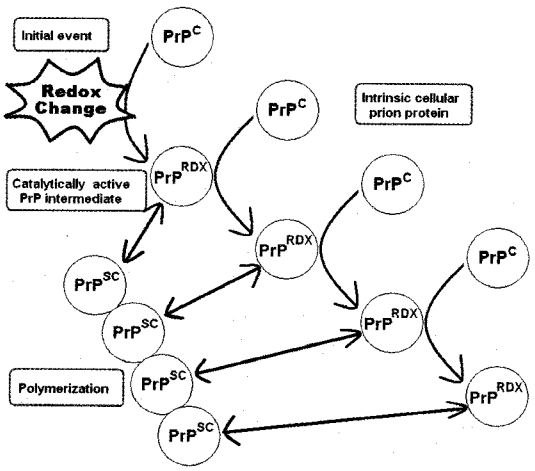

Fig. 2. Possible mechanism of PrP^{Sc} propagation involving redox activated intermediate PrP^{RDX} that auto-catalysis the conversion of PrP^{C} to PrP^{RDX}. Eventually PrP^{RDX} monomers are active in polymerization leading to formation of fibril of PrP^{Sc}. Since it has been demonstrated that the formation of PrP^{Sc} fibril is a reversible event, infectious redox intermediate could be released to the plasma, thus enabling the systemic propagation of PrP^{Sc} in the infected individuals of animal bodies.

Redox regulations

Interestingly, the amino-terminal part of PrP (23–98) contains amino acid residues susceptible to oxidation, such as His, Lys, Arg, and Pro, thus PrP is sensitive to oxidative changes such as ROS, and by this mechanism conformational changes in PrP may be readily induced by redox changes (Shiraishi and Nishikimi, 2002).

In addition to the above amino acid residues on PrP, methionine residues may be a target of oxidative changes. Recently, H_2O_2-dependent oxidation of methionine residues has been reported for recombinant SHa (29–231) prion protein (Requena *et al.*, 2004). The likely target residues include Met^{109}, Met^{112}, Met^{129} and Met^{134} and the susceptibility to oxidation of each Met residue was shown to be a function of accessibility to solvent. While copper-catalyzed oxidation leads to extensive aggregation of PrP, the H_2O_2-dependent Met oxidation on PrP leads only to a modest increase in β-sheet structure.

According to recently proposed model, there may be an intermediate structure of PrP between PrP^{C} and PrP^{Sc}, to be formed from PrP^{C} in response to cellular redox changes thus designated as PrP^{RDX} (Lee and Eisenberg, 2003). An *in vitro* study demonstrated that PrP^{RDX} of recombinant hamster PrP is catalytically active and capable of further conversion of PrP^{C} to PrP^{RDX} (Fig. 2). At the end, PrP^{RDX} accumulates and disulfide bonds are formed among each PrP^{RDX} molecule, and lastly PrP^{Sc} is formed. This attractive model successfully explained how pathogenic form of PrP could be propagated by the presence of an intermediate PrP. The first step required for initiation of PrP-autocatalytic

propagation of pathogenesis, is the generation of PrP^{RDX} possibly stimulated by redox events in the tissues.. According to Lundberg *et al.* (1997), three distinguishable species may be involved during conversion of α-helix to β-sheet structure of a PrP-derived peptide (spanning 3 out of 4 α-helical regions of PrP), namely monomeric species, fibrous species and a likely intermediate species (amorphous aggregate) when examined with electron microscopy. The amorphous aggregates were shown to be required for formation of fibrils, indicating that elongation growth of such aggregates occurs. It is tempting to speculate that this *in vitro* amorphous aggregate possibly corresponds to the early phase of PrP^{RDX}-dependent PrP polymerization or proteinase K-sensitive small fragments of PrP^{Sc} *in vivo*. Since sensitivity of the amorphous aggregates to proteinase treatment has not been tested to date, the structure and role for this third structure remains open. Further studies are required for testing the view.

In the following section, we discuss a possible contribution of PrP-catalyzed redox changes such as oxidation of catecholamines accompanying ROS production, in aid of PrP^{RDX} generation.

Does the Dopamine Oxidase-like Activity of Copper-bound PrP^{C} Protect or Damage the Cells?

According to earlier studies, administration of neurotoxic agent 6-hydroxydopamine which has been used as an experimental model to study the dopamine function in brain, into the brain results in destruction of nigrostriatal dopamine-containing neurons and as a consequence it triggers a profound behavioral deficits (Kumar *et al.*, 1995; Carder *et al.*, 1989; Zigmond *et al.*, 1987; Ungerstedt 1968). Auto-oxidation of catecholamines is known to trigger the oxidative burst, via formation of orthoquinone, $2H^+$, and $2e^-$, that result in reduction of molecular oxygen to superoxide anion radical ($O_2^{\cdot -}$) which allows further propagation of chain reactions leading to formation of H_2O_2 and hydroxyl radical ($HO^{\cdot}$), thus damaging the nerve cells (Kumar *et al.*, 1995; Cadet *et al.*, 1989; Jonsson, 1988; Pryor, 1986; Cohen, 1984). Sano *et al.* (1997) have shown that an inhibitor of catecholamine-degrading monoamine oxidase (MAO), deprenyl (selegiline) induces the progression of Alzheimer's disease indicating that removal of catecholamines by MAO protects the nerve cells from oxidative damages. Since any form of MAO has been shown to catalyze the oxidation of dopamine (Grimsby *et al.*, 1997), the dopamine oxidase-like activity of PrP (Shiraishi and Nishikimi, 2002) may have some impact mimicking the action of MAOs, by analogy.

On the other hand, Sandri *et al.* (1990) have proposed the danger of oxidative burst accompanying the MAO-catalyzed oxidation of catecholamines. Therefore, oxidative burst mediated by MAO may have some damaging impacts when the ROS removal by ROS-scavenging mechanisms is not sufficiently functioning.

In addition to MAOs, some metal-bound proteins mimicking the activity of MAO also participate in the catecholamine-dependent oxidative burst. It has been shown that phenylethylamine, a precursor of catecholamine neurotransmitters, induces the generation of ROS such as H_2O_2, $O_2^{\cdot -}$ and $HO^{\cdot}$ when reacted with yeast cellular enzymes similar to MAOs (Pinontoan *et al.*, 2002) and plant enzymes such as peroxidases and Cu-containing amine oxidase inhibitor-sensitive enzymes (Kawano *et al.*, 2000a, b; Kawano, 2003; Muto and Kawano, 2003). Hemoglobin also catalyzes the monoamine oxidation leading to production of ROS such as $O_2^{\cdot -}$ (Kawano *et al.*, 2002; Kawano and Hosoya, 2002), thus propounding the hindered dangers in use of hemoglobin-based molecules as artificial blood substitutes when circulated in the brain or around neuronal tissues despite the proposed applications (Alayash, 1999). By analogy to the

copper-bound enzymes such as Cu-amine oxidases from various organisms, the copper-bound form of PrP^{C} may possess a pseudo-amine oxidase activity. Actually it has been shown that PrP-derived peptide corresponding to amino acid sequence 23–98 of PrP catalyzes the oxidation of dopamine and ascorbate (Shiraishi and Nishikimi, 2002). This reaction may be the source of ROS as demonstrated with various metalloproteins as mentioned above.

Monitoring of the Dangers

In most experimental models, presence of PrP^{res} has been considered as the only reliable indicator of infectivity of prion disease (Jeffrey *et al.*, 2000; Safar *et al.*, 2005). Although pathogenic abnormal conformer of PrP, PrP^{Sc} is no longer soluble and thus not likely to be transmitted or migrate from the site of accumulation to other parts of the body, in fact, PrP^{Sc} with lower degree of polymerization is still soluble and sensitive to proteinase K treatment and thus it is hardly detectable with conventional immunological methods relying proteinase K sensitivity (Safar *et al.*, 2005). However, accumulation of proteinase K-insensitive fibrils is observable only after full development of pathogenesis in the prion disease-infected individuals and thus diagnosis in earlier steps of pathogen spread in side the bodies must be developed. In addition, conventional immunoassays for detection of PrP^{Sc} requiring proteolytic treatment are known to be accompanied with bulk of fluke positive signals when demonstrated with higher sensitivity and reversely, lowered fluke detection results in lowered sensitivity, and thus novel approaches with enhanced sensitivity and accuracy are required.

It has been shown in mice that PrP is produced in excess by scrapie-infected cells such as spleen follicular dendric cells where it is released into the extracellular space (Jeffrey *et al.*, 2000). Then it is likely that macrophages acquire the extracellularly released PrP and accumulate within lysosomes following phagocytosis of PrP (Jeffrey *et al.*, 2000). Such quantitative changes in total PrP in soluble fractions may be a good parameter for diagnosis of prion disease development. Total PrP detected in the soluble fraction obtained from prion disease-infected individuals may be the mixture of PrP^{C} and small fragments of PrP^{Sc} fibrils and both are likely sensitive to strong digestion by proteinase K (Jeffrey *et al.*, 2000). According to Safar *et al.* (2005), a novel approach for diagnosis of human prion disease has been developed in recent years, in which conformation-dependent immuno assay is employed. This approach allows the determination of both proteinase K-sensitive and resistant forms of PrP^{Sc} without the use of proteolytic digestion while all conventional immunoassays for PrP^{Sc} rely on limited proteolysis to eliminate PrP^{C}. As one of such sensitivity-enhanced diagnostic approaches we would like to propose the use of ESR in monitoring of prion diseases.

Electron Spin Resonance (ESR) Spectroscopy and PrP Diagnosis

ESR spin-trapping method can be applicable for monitoring of ROS production accompanying protein aggregation in neurodegenerative diseases (Tabner *et al.*, 2001). In in vitro studies, labeling of β-amyloid monomers with spin enables the kinetic analysis of protein polymerization using ESR (Lundberg *et al.*, 1997).

By detecting the copper-specific ESR signals, interaction between Cu(II) and PrP's N-terminal octapeptide repeats region (Aronoff-Spencer, 2000; Burns *et al.*, 2002, 2003; Bonomo *et al.*, 2000, 2005; Mentler *et al.*, 2005), neurotoxic central region (Gaggelli *et al.*, 2004; Jones *et al.*, 2004), and C-terminal region (Cereghetti *et al.*, 2001; Brown *et al.*, 2004) can be monitored with ESR. Copper incorporation by chicken prion hexapeptide repeat monitored by ESR was also reported (Stanczak *et al.*, 2004).

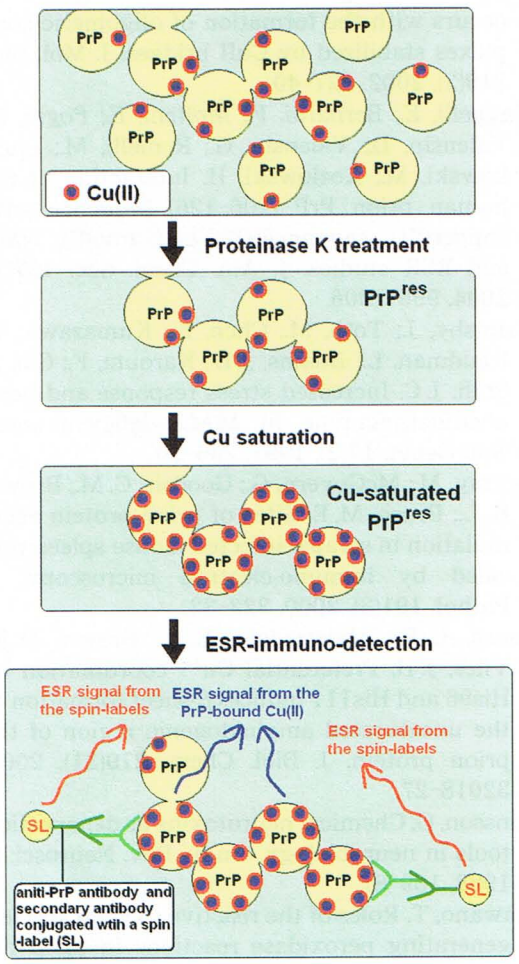

Fig. 3. Proposed use of PrP-targeted ESR-active immuno-probes in combination with Cu-dependent ESR monitoring. SL, spin labels.

Here we would like to propose the use of ESR in copper signal-based quantification of PrP in combination with the use of ESR-active immunoprobes targeting PrP for development of a novel conformation-dependent immunoassay. Anti-PrP antibody and spin label-conjugated secondary antibody could be used as the novel PrP-targeted ESR-active immuno-probes enabling the detection of PrP^{Sc} with high accuracy and high sensitivity, by detecting the PrP^{Sc}-dependent ESR signals and by confirmation with copper-specific ESR signals (Fig. 3). For this procedure, the samples must be exposed to proteolytic pretreatment followed by Cu(II)-loading incubation and washing with buffer, enhancing the copper-dependant ESR signal detection. To the resultant copper-saturated samples, ESR-active immuno-probes were added stepwisely. Then the immuno-labeled samples are ready for ESR analysis. This approach may enable the detection and confirmation of PrPSc signals at the same time.

Future Works

We are now planning the development of novel ESR-active immuno-probes for detection of oligomeric PrP^{Sc} and PrP^{RDX} by designing the PrP^{Sc}-specific and PrP^{RDX}-specific functional probes conjugated with secondary antibodies supposed to be used in combination with anti-PrP antibodies. For functional analysis the use of spin-trapping moieties on secondary antibodies sensitive to thiol radicals formed on PrP^{RDX} protein (Cys residues) or dopamine oxidase substrate oxidation may be applicable.

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