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Review article:

Role of PrPc Related to Apoptosis

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ABSTRACT

Prion diseases are transmissible neurodegenerative disorders that are invariably fatal in human and animals. Although the nature of the infectious agent and pathogenic mechanisms of prion diseases are not clear, it has been reported to be associated with aberrant metabolism of cellular prion protein (PrPc). In various reports, it has been postulated that PrPc may be involved in programmed cell death or apoptosis. Apoptosis of neuronal cells can also be induced in vitro by exposure to PrPsc or a neurotoxic peptide fragment corresponding to amino acids 106-126，118-135 of human prion protein (PrP106-126； PrP118-135). While the anti-apoptotic and anti-oxidative function of PrPc is regulated by not only OR (Octapeptide Region,amino acid residue 51-91) but also the N-terminal half of HR(Hydrophobic Region, amino acids residue 95-132). PrPc may participate in apoptosis through extrinsic pathway by binding to certain chaperon molecules or through induced by certain stresses like inflammatory factors. It can also by modulating Bcl-2/Bax, endogenous dismutase activity and calcium channel, control the activation and translocation of the mitochondria which leads to apoptosis involving certain effective molecules of caspase family. Meanwhile above biological events would be controlled by cAMP/APK, p38/MAPK, JNK, p53, NF-κB pathways et al. Further understanding of the apoptosis in PrPc have important implications for designing therapy of prion diseases, as well as for understanding pathogenic mechanisms operative in other neurodegenerative disorders and the role of prion in biology.

Keywords: cellular prion protein (PrPc); apoptosis; structure; pathways; regulation

INTRODUCTION

Apoptosis, or programmed cell death, is a major control mechanism by which cells die if DNA damage is not repaired (Lowe S and Wand Lin AW, 2000). Apoptosis is also important in controlling cell number and proliferation as part of normal development.

Prion diseases are transmissible neurodegenerative disorders that are invariably fatal in humans and animals. Although the nature of the infectious agent and pathogenic mechanisms of prion diseases are not clear, it has been reported that prion diseases may be associated with aberrant metabolism of cellular prion protein (PrPc)(Mastrangelo P and Westaway D,2001). In various reports, it has been postulated that PrPc may be involved in one or more of the following: neurotransmitter metabolism, cell adhesion, signal transduction, copper metabolism, antioxidant activity or programmed cell death(Kim BH et al., 2004).

Various evidences proved the involvement of apoptosis in the biological metabolism of PrPc. The analysis of chromatin structure in cases of human spongiform encephalopathies could show the possible involvement of apoptosis in neuronal cell death. Genomic DNA was purified from peripheral blood lymphocytes and from a biopsy of the brain cortex in a case of Creutzfeldt-Jakob disease. The DNA purified from the brain consisted of partially degraded DNA in internucleosomal-sized fragments, whereas the DNA from peripheral blood lymphocytes showed unbroken DNA of high molecular weight. These results are consistent with the possible activation of the apoptotic endonuclease and the internucleosomal fragmentation of DNA of the brain cortex in the patient affected by Creutzfeldt-Jakob disease, suggesting the involvement of apoptosis in neuronal cell death in human spongiform encephalopathy (Lucas M et al., 1997). Histological and electron microscopical studies have shown that cell death in prion disease occurs in form of apoptosis. Kim BH et al (Kim BH et al., 2004)established the cell lines stably expressing PrPc from PrP knockout (PrP(-/-)) neuronal cells and examined the role of PrPc under apoptosis and/or serum-deprived condition and found that PrP(-/-) cells were vulnerable to apoptotic cell death and that this vulnerability was rescued by the expression of PrPc. Apoptosis of neuronal cells can also be induced in vitro by exposure to PrPSc or a neurotoxic peptide fragment corresponding to amino acids 106-126，118-135 of human prion protein (PrP106-126；PrP118-135). Both in vitro and in vivo the toxicity of PrPsc and PrP fragments appears to depend on neuronal expression of PrPc and on microglial activation. Activated microglial cells release pro-inflammatory cytokines and reactive oxygen species. Cell culture experiments suggest an important role of microglia-mediated oxidative stress in the induction of neuronal cell death (Giese A and Kretzschmar HA, 2001). It’s reported recently that only prion-infected neuronal cells become apoptotic after mild inhibition of the proteasome, and this was strictly dependent upon sustained propagation of PrPsc. Whereas cells overexpressing PrPc developed cytosolic PrPc aggregates, this did not cause cell death. In contrast, only in prion-infected cells, mild proteasome impairment resulted in the formation of large cytosolic perinuclear aggresomes that contained PrPsc, heat shock chaperone 70, ubiquitin, proteasome subunits, and

vimentin. Similar structures were found in the brains of prion-infected mice. PrPsc aggresome formation was directly associated with activation of caspase 3 and 8, resulting in apoptosis(Kristiansen M et al.,2005). Abundant experiments in our lab also revealed that the vitro transfectant of AGS/PrPc was more resistant to serum deprivation induced apoptosis compared to that of control cell line AGS/pcDNA3.1B, suggesting PrPc expression, which was universal in gastric cell lines and presented high level in gastric carcinoma, was at least partly mediated though certain anti-apoptotic mechanism(Du J et al., 2005; Liang J et al., 2006). As for the structure of PrPc related to apoptosis, involved pathways and regulation of the apoptotic proteins were concluded below.

Structure of PrPc related to apoptosis

Cellular prion protein (PrPc), synthesized in endocytoplasmic reticulum and anchored on the extracellular face of the plasma membrane by a glycosylphosphatidyl-inositol moiety, its structure has not been determined directly for practical reasons. The human PrPc molecule contains a signal peptide (1–22), five octapeptide repeats (51–91), a highly conserved hydrophobic region (112–145), three peptide sequences responsible for -helix structure (H1, H2 and H3), and a signal sequence for GPI anchor (231–254)(Fig 1). PrP primary and tertiary structures are highly conserved in mammals, which suggest that this protein participates in fundamental biological processes, however, the role of PrP in the cell remains uncertain. Previous studies have showed the amino acids 106-126，118-135 of human prion protein (PrP106-126；PrP118-135), as well as the OR(Octapeptide Region,amino acid residue 51-91) , N-terminal half of HR(Hydrophobic Region, amino acids residue 95-132) involved in the apoptosis function.

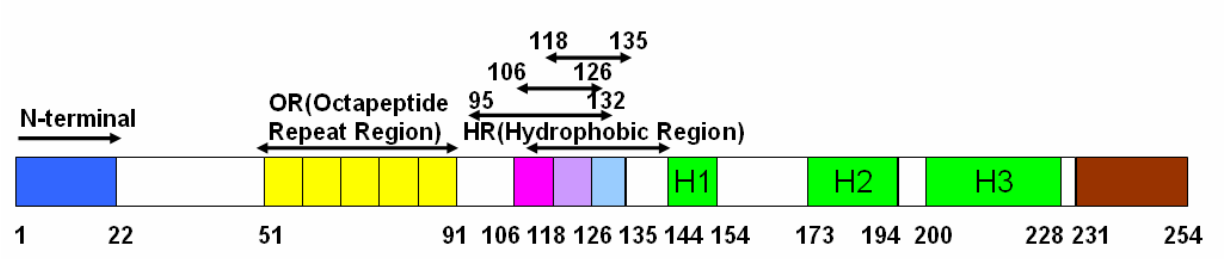

Figure 1: Representation of the domains of PrPc. The human PrPc molecule contains a signal peptide (1–22), five octapeptide repeats (51–91), a highly conserved hydrophobic region (112–145), three peptide sequences responsible for -helix structure (H1, H2 and H3), and a signal sequence for GPI anchor (231–254). Numbers below the boxes correspond to the amino acids in each domain. Double arrows indicate the domains of PrPc related to apoptosis, which is also represented by amino acid numbers in parentheses.

PrP 106-126

A synthetic peptide homologous to residues 106-126 of PrP (PrP 106-126) maintains many characteristics of PrPsc, that is, the ability to form amyloid fibrils and to induce apoptosis in neurons. In

vitro or vivo variant experiments have been carried out to examine the PrP106-126 induced apoptosis. Forloni G et al(Forloni G et al., 1993) first reported neuronal death resulting from chronic exposure of primary rat hippocampal cultures to micromolar concentrations of a peptide corresponding to residues 106-126 of the amino-acid sequence deduced from human PrP complementary DNA. DNA fragmentation of degenerating neurons indicated that cell death occurred by apoptosis. Then he used chronic (5-7 d) exposure to PrP106-126 fragment induced neuronal death by apoptosis model, as suggested by biochemical and morphological analysis, confirmed by ultrastructural examination the apoptotic mechanism(Forloni G et al., 1996). Kretzschmar HA et al(Kretzschmar HA et al., 1997) using the synthetic peptide of the prion protein (PrP106-126) showed that this peptide was toxic only to normal neurons whereas nerve cells derived from PrP knock-out (PrP0/0) mice were unaffected by this neurotoxic effect. Later the synthetic peptide PrP 106-126, obtained from the amyloidogenic region of the PrP, constituted a classic model system to study prion-induced neurodegeneration as it retained the ability to trigger cell death in neuronal cultures(O'Donovan CN et al., 2001; Ciesielski-Treska J et al., 2004; Bergstrom AL et al., 2005).

PrP 118-135

Prion protein fragment (118-135) displays membrane-destabilizing properties and is able to induce, in a nonfibrillar form, the fusion of unilamellar liposomes by which apoptosis could also be induced. Low concentrations of the PrP 118-135 peptide, in a nonfibrillar form, induces a time- and dose- dependent apoptotic cell death on rat cortical neurons, including caspase activation, DNA condensation, and fragmentation (Pillot T et al., 2000). Knock-out PrP gene mice exhibites similar sensitivity to the nonfibrillar PrP118-135-induced cell death and electrical perturbations, strongly suggesting that cell death occurs independently of PrP expression. Interestingly, a variant nonfusogenic PrP118-135 peptide has no effects on in vivo neuronal viability, suggesting that the PrP118-135-induced cell death is mediated by its membrane destabilizing properties(Chabry J et al., 2003).

However, PrP106-126 and PrP118-135 peptides may trigger different pathways leading to neuronal apoptosis. Sponne I et al(Sponne I et al., 2004) used humanin (HN), which was shown to protect neuronal cells from various insults involved in human neurodegenerative diseases, to modulate the apoptosis induced by the soluble PrP118-135 and PrP106-126 fragment. The effects of HN did not require a preincubation with the PrP118-135 fragment, strongly suggesting that these peptides rescue cells independently of a direct interaction with the prion peptide. By contrast HN had no effect on the fibrillar PrP106-126 peptide-induced cell death. This protective effect for neurons from PrP118-135-induced cell death strongly suggests that PrP118-135 and PrP106-126 peptides may trigger different pathways leading to neuronal apoptosis. However, the mechanism remains to be elucidated.

N-terminal octapeptide repeat region

Cellular prion protein PrPc contains two evolutionarily conserved domains among mammals: the octapeptide repeat region (OR; amino acid residue 51-90) which requires for selective binding to copper ions and the hydrophobic region (HR; amino acid residue 112-145). Both domains in N-terminal are vital to anti-apoptosis function. Some studies revealed that removal of the OR could enhance apoptosis and decrease SOD activity. Deletion of the N-terminal half of HR (amino acids residue 95-132) could abolish its ability to activate SOD and to prevent apoptosis, whereas that of the C-terminal half of HR (amino acids residue 124-146) has little if any effect on the anti-apoptotic activity and SOD activation(Sakudo A et al., 2005). These data are consistent with a model in which the anti-apoptotic and anti-oxidative function of PrPc was regulated by not only OR but also the N-terminal half of HR, suggesting copper binding and neuronal trafficking both contributes to PrPc functioning in vivo, hence provided clue to current hypotheses to explain Dpl/PrPc antagonism, by competitive ligand binding, plays a pro-apoptotic role (Drisaldi B et al., 2004; Sakudo A et al., 2005).

Pathways involved in PrPc and apoptosis

There are two major mechanisms of cell death-necrosis and apoptosis. Cells that are damaged by external injury undergo necrosis, while cells that are induced to commit programmed suicide because of internal or external stimuli undergo apoptosis. The first, referred to as the extrinsic or cytoplasmic pathway, is triggered through the Fas death receptor, a member of the tumor necrosis factor (TNF) receptor superfamily(Zapata JM et al., 2001). The second pathway is the intrinsic or mitochondrial pathway that when stimulated leads to the release of cytochrome-c from the mitochondria and activation of the death signal(Hockenbery D et al., 1990). Both pathways converge to a final common pathway involving the activation of a cascade of proteases called caspases that cleave regulatory and structural molecules, culminating in the death of the cell. The pathways are linked; overexpression of Bcl-2 in the intrinsic pathway may lead to the inhibition of extrinsic mediated apoptosis (Scaffidi C et al., 1998). Thus, any distinction between the two pathways would be simplistic.

Extrinsic or cytoplasmic pathway

This pathway comprises several protein members including the death receptors, the membrane-bound Fas ligand, the Fas complexes, the Fas-associated death domain, and caspases 8 and 10, which ultimately activate the rest of the downstream caspases leading to apoptosis. PrPc, the membrane protein, could bind to other proteins or suffer from stress like inflammatory factors(Veerhuis R et al., 2002) to block pro-apoptotic pathway. Binding proteins or chaperon molecules, first supposed by Pruisner as proteinX(Telling GC et al., 1995), and later proved to be LR/LRP, ST1, HSP and so on, summed in the chart by Kil Sun Lee et al (Kil Sun Lee et al., 2003), could induce signals to rescue cells from apoptosis . For example, amino acids 113-128 from PrPc and 230-245 from STI1 cell surface binding and pull-down experiments showed that

recombinant PrPc binds to cellular STI1, and co-immunoprecipitation assays strongly suggest that both proteins are associated in vivo. Moreover, PrPc interaction with either STI1 or peptid representing the ST1 binding domain could induce neuroprotective signals through a cAMP/PKA signalling pathway that rescue cells from apoptosis(Zanata SM et al., 2002).

The intrinsic pathway

Bcl-2/Bax

The bcl-2 gene was originally identified at the chromosomal breakpoint of the translocation of chromosome 18 to 14 in follicular non-Hodgkin lymphoma (NHL)(Tsujimoto Y et al., 1984).The Bcl-2 family, key regulators of apoptosis, includes proapoptotic members such as Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk, and antiapoptotic members such as Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1(Reed JC., 1994). Experiments in our laboratory showed by western blot that cells transfected with PrPc would down-regulate Bax and up-regulate Bcl-2, suggesting PrPc played an important role in anti-apoptosis pathways (Du J et al., 2005; Liang J et al., 2006).Analysis of interaction sites in homo- and heteromeric complexes containing Bcl-2 family members and the cellular prion protein showed that PrPc binds to the C-terminus of Bcl-2 but not Bax(Kurschner C and Morgan JI, 1996). Bax binding required almost the entire Bcl-2 molecule, while PrP associated with the carboxy terminus of Bcl-2 (amino acids 200-236). Antiapoptotic Bcl-2 members act as repressors of apoptosis by blocking the release of cytochrome-c, whereas proapoptotic members act as promoters. These effects are more dependent on the balance between Bcl-2 and Bax than on Bcl-2 quantity alone(Reed JC, 1998). PrPc may disrupt chains of Bcl-2 molecules at the homomeric association site in the transmembrane region to reduce the release of cytochrome-c.

SOD/ROS

The speculation that PrPc may be a superoxide dismutase (SOD) was perceived as particularly attractive in view of its multiple copper binding sites in N-terminal octapeptide repeat region, and it was suggested that amino-proximally truncated PrPc may depress endogenous dismutase activity(Akikazu Sakudoa et al., 2005). Compared apoptosis of Prnp(-/-) cells with that of Prnp(-/-) cells expressing the wild-type PrPc or PrPc lacking N-terminal octapeptide repeat region under serum-free conditions showed re-introduction of PrPc rescued cells from apoptosis, upregulated superoxide dismutase (SOD) activity, enhanced superoxide anion elimination, and inhibited caspase-3/9 activation. On the other hand, N-terminally truncated PrPc enhanced apoptosis accompanied by potentiation of superoxide production and caspase-3/9 activation due to inhibition of SOD(Sakudo A et al., 2003). Reactive oxygen species (ROS), prevented by an antioxidant system: low molecular mass antioxidants, enzymes regenerating the reduced forms of antioxidants and ROS-interacting enzymes such as SOD (Blokhina O et al., 2003), is produced in mitochondria and could control the expression of Bcl-2 family proteins by regulating their phosphorylation and ubiquitination (Li D et al., 2004). In vitro transfectant of

AGS/PrPc was observed to stimulate less ROS as compared to that of AGS/pcDNA3.1B by us (Liang J., 2006). These results suggest that PrPc protects cells from apoptosis via superoxide- and mitochondria-dependent pathways by upregulating SOD activity.

Calcium channel

Ca2+ signalling is involved in virtually all cellular processes: among the others, it controls cell survival, proliferation and death regulating a plethora of intracellular enzymes located in the cytoplasm, nucleus and organelles (Munaron L et al., 2004). Synthetic fragments of PrP106-126 has been shown to be toxic to neurons in culture and cell death occurs by apoptosis was observed to involve Ca2+ uptake through voltage-sensitive Ca2+ channels(Brown DR et al., 1997). Microfluorimetric analysis was applied to monitor intracellular calcium concentration of single cell and showed that PrP 106-126 caused a complete blockade of the increase in the cytosolic calcium levels induced by K+ depolarization. Electrophysiological studies demonstrated the L-type voltage-sensitive calcium channel blocker nicardipine could also induce apoptosis(Florio T et al., 1998; Thellung S et al., 2000). These data demonstrate that PrP106-126 may alter the activity of L-type voltage-sensitive calcium channels and control the release of cytochrome-c from the mitochondia to regulate cell apoptosis.

Following above several death signals, proapoptotic proteins undergo post translational modifications that include dephosphorylation and cleavage resulting in their activation and translocation to the mitochondria leading to apoptosis (Scorrano L and Korsmeyer S, 2003). In response to apoptotic stimuli, the outer mitochondrial membrane becomes permeable, leading to the release of cytochrome-c and second mitochondria-derived activator of caspase. Cytochrome-c, once released in the cytosol, interacts with Apaf-1, leading to the activation of caspase-9 proenzymes. Active caspase-9 then activates caspase-3, which subsequently activates the rest of the caspase cascade and leads to apoptosis. Activated caspases lead to the cleavage of nuclear lamin and breakdown of the nucleus through caspase-3(Reed JC, 1997).

The final pathway: Caspases

The final pathway that leads to execution of the death signal is the activation of a series of proteases termed caspases. Although not all caspases are involved in apoptosis, the caspases that have been well described are caspases-3, -6, -7, -8, and -9(Thornberry NA and Lazebnik Y, 1998; Mancini M et al., 1998). The intrinsic and extrinsic apoptotic pathways converge to caspase-3, which cleaves the inhibitor of the caspase-activated deoxyribonuclease, and the caspase-activated deoxyribonuclease becomes active leading to nuclear apoptosis. The upstream caspases that converge to caspase-3 are caspases-9 and -8 in the intrinsic and extrinsic pathways, respectively(Hachiya NS et al., 2005; Hachiya NS et al., 2005; Hetz C et al., 2005). The downstream caspases induce cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins, inhibitory subunits of endonucleaes (CIDE family), and finally, destruction of

"housekeeping" cellular functions (Fig 2). Caspases also affect cytoskeletal structure, cell cycle regulation, and signaling pathways, ultimately leading to the morphologic manifestations of apoptosis, such as DNA condensation and fragmentation, and membrane blebbing(Irene M et al., 2005).

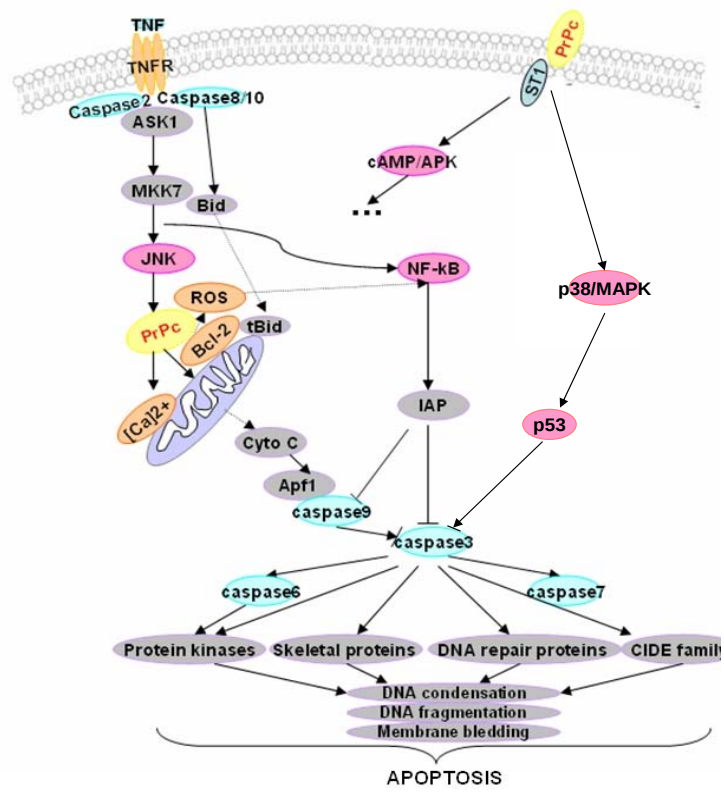

Figure 2: Pathways related to apoptosis in PrPc. PrPc may participate in apoptosis through extrinsic pathway by binding to certain chaperon molecules like ST1 or suffer from stress like inflammatory factors like TNF-α. It can also by modulating Bcl-2/Bax, endogenous dismutase activity and calcium channel, control the activation and translocation of the mitochondria which leads to apoptosis involving certain effective molecules of caspase family. Meanwhile above biological events would be controlled by cAMP/APK, p38/MAPK, JNK, p53, NF-κB pathways.

Regulation of the apoptotic proteins cAMP/PKA

PKA activation has been implicated previously in the regulation of apoptosis in some studies. In the retinal explanted from neonatal rats or mice kept in vitro for 24 h and anisomycin (ANI) induced apoptosis model, the peptide activated both cAMP/protein kinase A (PKA) and Erk pathways, and partially prevented cell death induced by ANI in explants from wild-type rodents, but not from PrPc-null mice. Neuroprotection was abolished by treatment with phosphatidylinositol-specific phospholipase C, with human peptide 106-126, with certain antibodies to PrPc or with a PKA inhibitor, but not with a MEK/Erk inhibitor. In contrast, antibodies to PrPc that increased cAMP also induced neuroprotection. Thus, engagement of PrPc transduces

neuroprotective signals through a cAMP/PKA-dependent pathway(Chiarini LB et al., 2002).

p38/MAPK

p38 MAPKs are members of the MAPK family that are activated by a variety of environmental stresses and inflammatory cytokines(Lewis TS et al., 1998). The p38 MAPkinase blockers, SB203580 and PD169316 were observed to prevent the apoptotic cell death evoked by PrP106-126 and western blot analysis revealed that the exposure of the cells to the peptide induced p38 phosphorylation in SH-SY5Y cell(Thellung S et al., 2002). Controversially, a recent report by Carimalo et al. (Carimalo J et al., 2005) observed MAPK p38 inhibitor SB203580 couldn't reduce PrP106-126 exposure to its aggregated form and couldn't reverse its induction to a massive neuronal death. The reason may lie in the different cell models they used.

JNK

Carimalo et al. (Carimalo et al., 2005) used immunocytochemistry showed a peak of phosphorylated c-Jun-N-terminal kinase (JNK) translocation into the nucleus after 8 h in the PrP106-126 induced neuronal cell death model, along with the activation of the nuclear c-Jun transcription factor. Both pharmacological inhibition of JNK by SP600125 and overexpression of a dominant negative form of c-Jun significantly reduced neuronal death in neuronal apoptosis induced by PrP106-126. Apoptosis was also studied after exposure of tg338 cortical neurons in primary culture to sheep scrapie agent by them. In this model, prion-induced neuronal apoptosis was found to increase gradually along with time, with 40% cell death after 2 weeks exposure. Immunocytochemical analysis showed early c-Jun activation after 7 days. They deduced that JNK-c-Jun pathway plays an important role in neuronal apoptosis induced by PrP106-126.

p53

p53 functions as a transcription factor regulating downstream genes important in cell cycle arrest, DNA repair, and apoptosis. After DNA damage, p53 holds the cell at a checkpoint until the damage is repaired. If the damage is irreversible, apoptosis is triggered Benchimol (Benchimol S, 2001). Results from several laboratories indicate that apoptosis via the p53 pathway is involved in prion disease pathogenesis. PrPc expression was found to enhance staurosporine-stimulated neuronal toxicity and DNA fragmentation, caspase 3-like activity and immunoreactivity, and p53 immunoreactivity and transcriptional activities(Paitel E, 2003). PrPc could control p53 at a post-transcriptional level and is reversed by Mdm2 transfection and p38 MAPK inhibitor. So it's proposed that endogenous cellular prion protein sensitizes cells to apoptotic stimuli through a p53-dependent caspase 3-mediated activation controlled by Mdm2 and p38 MAPK. Combined pharmacological, mutational and cell biology approaches also indicate that p53-dependent caspase 3 activation triggered by cellular prion is dependent on its endocytosis(Sunyach C and Checler F, 2005).

NF-κB

NF-κB is a nuclear transcription factor that regulates expression of a large

number of genes involved in the regulation of apoptosis, viral replication, tumorigenesis, inflammation, and many autoimmune diseases(Maldonado V et al., 1997) In its inactive form, NF-κB is sequestered in the cytoplasm, bound inhibitor proteins of the I-κB family. The various stimuli that activate NF-κB cause phosphorylation of I-κB, which is followed by its degradation. This results in exposure of the nuclear localization signals on NF-κB subunits and the subsequent translocation of the molecule to the nucleus. In the nucleus, NF-κB binds with the consensus sequence of various genes and thus activates their transcription. NF-κB has been shown to have both anti- and pro-apoptotic functions that may be determined by the nature of the death stimulus rather than by the origin of the tissue (Maldonado V et al., 2000). Under physiologic conditions, the activation of NF-κB induces resistance to apoptotic stimuli through the activation of many complex proteins including PrPc combining proteins. However, in response to certain stimuli, NF-κB activation may lead to induction of apoptosis. Activation by prion peptide PrP106-126 could induce a NF-κB-driven pro-inflammatory response in human monocyte-derived dendritic cells (Bacot SM et al., 2003). While other research revealed prion accumulation in astrocytes might activate NF-κB through the increase of ROS generation, and thus alterations in NF-κB-directed gene expression may contribute to both the neurodegeneration and proinflammatory responses (Kim JI et al.,1999).

CONCLUSION

Apoptosis, the important biological phenomena, plays a vital role in the biological function of PrPc. PrPc may participate in apoptosis through extrinsic pathway by binding to certain chaperon molecules or through induced by certain stresses like inflammatory factors. It can also by modulating Bcl-2/Bax, endogenous dismutase activity and calcium channel, control the activation and translocation of the mitochondria which leads to apoptosis involving certain effective molecules of caspase family. Meanwhile above biological events would be controlled by cAMP/APK, p38/MAPK, JNK, p53, NF-κB pathways et al. Further understanding of the apoptosis in PrPc have important implications for designing therapy of prion diseases, as well as for understanding pathogenic mechanisms operative in other neurodegenerative disorders and the role of prion in biology.

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