Studies on cell-autonomous mechanisms for neurodegeneration in prion disease

（プリオン病における神経細胞自律的変性機構に関する研究）

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Summary of the thesis

Contents

PREFACE

Prion disease is a group of fatal neurodegenerative disorders in both animals and humans [1, 2]. The pathological hallmarks of prion diseases include vacuolation in the neuropil, microglial activation, astrogliosis, synaptic loss, and specifically, accumulation of the pathogenic isoform of prion protein (PrP^{Sc}) in the central nervous system. PrP^{Sc}, a key component of the infectious agent, prions, is a product of self-seeding conformational conversion, or misfolding, of cellular isoform of PrP (PrP^{C}) encoded by the host gene *Prnp* [3-5]. The production of PrP^{Sc} is strongly associated with prion propagation and neurodegeneration in prion diseases; PrP-deficient mice are totally resistant to prion infection [6-8], brain tissue devoid of PrP was not impaired by exogenous PrP^{Sc} produced in PrP-expressing tissue grafts nearby [9], and the conditional knockout of the neuronal *Prnp* protected or even rescued mice from prion infection as well as neurodegeneration in spite of the presence of PrP^{Sc} in glial cells and extracellular parenchymal space [10, 11]. These facts suggest that not just the presence of PrP^{Sc} but intraneuronal prion propagation, i.e., misfolding of intrinsically neuronal PrP, is required for the development of prion diseases. However, we have less knowledge of the cellular and molecular mechanisms that account for the neurodegeneration in prion diseases. Since experiments did not fully support the loss of physiological function of PrP^{C} [12-16] and the gain of toxic function by misfolding into PrP^{Sc} [6-11] as the mechanisms of neurodegeneration,, there ought to be alteration or abnormal induction of cellular responses and abnormalities in PrP molecules in the prion pathogenesis.

It is still an open question that which cell types are primarily responsible for neurodegeneration in prion diseases. Several examinations on the roles of activated glial cells have been carried out [17-27], though it has not been concluded yet whether the contribution of activated glial cells is protective or harmful to prion-infected neurons. There is the importance of a specific analysis of prion-induced neuronal responses to discover whether the

neurodegeneration is consequent exclusively on neuron-autonomous mechanisms.

Primary neurons may serve as versatile *ex vivo* models [28-30], thus in my previous study primary cultures enriched in murine cerebral cortical neurons (CxN) and thalamic neurons (ThN) were established [31]. In this thesis, the study was extended with the use of the primary cultures to analyze neuron-autonomous events to elucidate the mechanisms of neurodegeneration in prion diseases. In Chapter I, I describe the induction of the early event in UPR as a neuron-autonomous response to the propagation of prions, and in Chapter II, I describe the transcriptome analysis of prion-infected neurons to evaluate any changes in neurons caused by the propagation of prions.

Chapter I

Conversion of PrP^{C} into PrP^{Sc} in neurons is one of the key pathophysiological events in prion diseases. However, the molecular mechanism of neurodegeneration in prion diseases has yet to be fully elucidated because of a lack of suitable experimental models for analyzing neuron-autonomous responses to prion infection. One of promising cellular responses that would be involved in the prion pathogenesis is called unfolded protein response (UPR) [59-61]. UPR is a cellular response to endoplasmic reticulum (ER) stress in order to deal with protein misfolding [53-56]. The involvement of a UPR pathway mediated by protein kinase R-like endoplasmic reticulum kinase (PERK) in the progress of prion disease has been demonstrated using mouse models [62, 63] so that I examined whether upregulation of the PERK pathway was neuron-autonomous or not. In the present study, I used neuron-enriched primary cultures of cortical and thalamic mouse neurons to analyze autonomous neuronal responses to prion infection. PrP^{Sc} levels in neurons increased over the time after prion infection; however, no obvious neuronal losses or neurite alterations were observed by immunocytochemistry. The prion-infected neurons were further analyzed for UPR-related molecules by immunoblot and RT-PCR. It showed an increasing tendency of phosphorylation of PERK in prion-infected neurons. Interestingly, a finer analysis of individual neurons by immunocytochemistry simultaneously for PrP^{Sc} and phosphorylated PERK (p-PERK) demonstrated a positive correlation between the number of PrP^{Sc} granular stains and p-PERK granular stains, in cortical neurons at 21 dpi. Although the phosphorylation of PERK was enhanced in prion-infected cortical neurons, there was no sign of subsequent translational repression of synaptic protein synthesis or activations of downstream UPR in the PERK-eIF2α pathway. These results suggest that PrP^{Sc} production in neurons induces ER stress in a neuron-autonomous manner; however, it does not fully activate UPR in prion-infected neurons. These findings provide insights into the autonomous neuronal responses to prion propagation and the involvement of neuron-non-autonomous factor(s) in the mechanisms of neurodegeneration in prion diseases.

Chapter II

Identification of cell biological processes/functions that are affected by prion propagation in neurons will facilitate to clarify the neuropathogenesis of prion diseases. To gain insight into the autonomous neuronal responses to prion propagation, I analyzed transcriptome of CxNs and ThNs cultures infected with prions. Over 2,000 differentially expressed genes were identified by comparison of gene expression profiles between mock- and prion-infected CxNs or ThNs at 14 and 21 dpi. A total of 108 differentially expressed genes were found in common across 2 or 3 experimental conditions based on neuronal type and days after inoculation. Of the 108 genes, 4 genes were previously reported as differentially expressed genes in prion disease, and 13 genes were associated with other neurodegenerative disorders accompanied by protein misfolding such as Alzheimer's disease (AD). Upregulation of *Ncstn* in prion-infected ThNs at both 14 and 21 dpi suggests the presence of neuron-autonomous response to prion propagation, which is also suggested to be involved in the development of AD [148]. Bioinformatic analyses of differentially expressed genes using Gene Ontology and Ingenuity Pathway Analysis (IPA) did not suggest any canonical pathways or biological functions that are directly related to the neuronal cell death and neurodegeneration. This interpretation implies that prion propagation in neurons would not totally activate neuron-autonomous processes that lead to neuronal death, which is consistent with the findings in the Chapter I. However, interestingly, IPA analysis suggested that alterations of some biological processes in prion-infected CxNs and ThNs, which are associated with cytoskeleton reorganization and lipid metabolism. Although obvious alterations in cell biological process were not suggested from the transcriptome of prion-infected neurons, information on comprehensive gene expression profiles of prion-infected neurons will be useful for understanding the mechanisms of neurodegeneration in prion diseases.

CONCLUSION

Conversion of PrP^{C} into PrP^{Sc} in neurons is one of the key pathophysiological events in prion diseases. A line of evidence indicates that microglia and astrocytes play important roles in the neuropathogenesis of prion diseases as non-autonomous neuronal cell death. Prion propagation in neurons itself is also believed to be a key event in the neuropathogenesis of prion diseases; however, genuine neuronal responses to prion propagation have yet to be fully elucidated because of a lack of suitable experimental models for analyzing neuron-autonomous responses to prion infection. To analyze neuron-autonomous events specifically, I optimized a neuron-enrichment protocol which was able to control astrocytic growth in cortical and thalamic neuronal cultures over a month. These primary neurons were susceptible to prion infection and the characteristics of PrP^{Sc} produced in the primary neurons resembled those observed in brains of prion-infected mice. Therefore, in my thesis, I extensively studied neuron-autonomous events to prion infection using the primary neuronal cultures infected with prions.

In Chapter I, autonomous neuronal responses to prion infection was analyzed using neuron-enriched primary cultures of mouse neurons. PrP^{Sc} levels in neurons increased over the time after prion infection; however, no obvious neuronal losses or neurite alterations were observed. Interestingly, a finer analysis of individual neurons co-stained with PrP^{Sc} and p-PERK, the early cellular response of the PERK-eIF2α pathway, demonstrated a positive correlation between the number of PrP^{Sc} granular stains and p-PERK granular stains, in cortical neurons at 21 dpi. Although the phosphorylation of PERK was enhanced in prion-infected cortical neurons, there was no sign of subsequent translational repression of synaptic protein synthesis or activations of downstream UPR in the PERK-eIF2α pathway. These results suggest that PrP^{Sc} production in neurons induces ER stress in a neuron-autonomous manner; however, it does not fully activate UPR in prion-infected neurons.

In Chapter II, I analyzed transcriptome of neuron-enriched primary CxNs and ThNs

infected with prions in order to gain insight into the autonomous neuronal responses to prion propagation. Bioinformatic analyses of differentially expressed genes using Gene Ontology and the IPA did not suggest any canonical pathways or biological functions that are directly related to the neuronal cell death and neurodegeneration. This interpretation implies that prion propagation in neurons would not totally activate neuron-autonomous processes that lead to neuronal death, which is consistent with the findings in the Chapter I. However, interestingly, IPA analysis suggested that alterations of some biological processes in prion-infected ThNs, which are associated with cytoskeleton reorganization and lipid metabolism. Although obvious alterations in cell biological process were not suggested from the transcriptome of prion-infected neurons, information on comprehensive gene expression profiles of prion-infected neurons will be useful for understanding the mechanisms of neurodegeneration in prion diseases.

In this study, I have tackled to the lack of our understanding of the neuron-autonomous mechanism of neurodegeneration in prion diseases. I disclosed that prion propagation itself induced the early event of ER stress in neurons; however, it does not fully activate UPR in prion-infected neurons. Transcriptome analyses could not expect any specific neuronal events that were affected by prion propagation. These facts suggest the presence of additional, non-neuronal factor(s) in the mechanism of neurodegeneration in prion diseases. Although further studies are required for a comprehensive understanding of the mechanisms of neurodegeneration in prion diseases, I believe that this study contributes to elucidation of neuropathogenesis of prion diseases and identification of potential targets for therapeutic intervention in near future.

REFERENCES

1. **Colby DW, Prusiner SB**. Prions. *Cold Spring Harb Perspect Biol* 2011;3(1):a006833.
2. **Collins SJ, Lawson VA, Masters CL**. Transmissible spongiform encephalopathies. *Lancet* 2004;363(9402):51-61.
3. **Wang K, Lee I, Carlson G, Hood L, Galas D**. Systems biology and the discovery of diagnostic biomarkers. *Dis Markers* 2010;28(4):199-207.
4. **Pan KM, Baldwin M, Nguyen J, Gasset M, Serban A et al.** Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci U S A* 1993;90(23):10962-10966.
5. **Prusiner SB**. Prions. *Proc Natl Acad Sci U S A* 1998;95(23):13363-13383.
6. **Bueler H, Aguzzi A, Sailer A, Greiner RA, Autenried P et al.** Mice devoid of PrP are resistant to scrapie. *Cell* 1993;73(7):1339-1347.
7. **Prusiner SB, Groth D, Serban A, Koehler R, Foster D et al.** Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies. *Proc Natl Acad Sci U S A* 1993;90(22):10608-10612.
8. **Manson JC, Clarke AR, McBride PA, McConnell I, Hope J**. PrP gene dosage determines the timing but not the final intensity or distribution of lesions in scrapie pathology. *Neurodegeneration* 1994;3(4):331-340.
9. **Brandner S, Isenmann S, Raeber A, Fischer M, Sailer A et al.** Normal host prion protein necessary for scrapie-induced neurotoxicity. *Nature* 1996;379(6563):339-343.
10. **Mallucci GR, White MD, Farmer M, Dickinson A, Khatun H et al.** Targeting cellular prion protein reverses early cognitive deficits and neurophysiological dysfunction in prion-infected mice. *Neuron* 2007;53(3):325-335.
11. **Mallucci G, Dickinson A, Linehan J, Klohn PC, Brandner S et al.** Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis. *Science* 2003;302(5646):871-874.
12. **Bueler H, Fischer M, Lang Y, Bluethmann H, Lipp HP et al.** Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature* 1992;356(6370):577-582.
13. **Manson JC, Clarke AR, Hooper ML, Aitchison L, McConnell I et al.** 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. *Mol Neurobiol* 1994;8(2-3):121-127.
14. **Richt JA, Kasinathan P, Hamir AN, Castilla J, Sathiyaseelan T et al.** Production of cattle lacking prion protein. *Nat Biotechnol* 2007;25(1):132-138.
15. **Benestad SL, Austbo L, Tranulis MA, Espenes A, Olsaker I**. Healthy goats naturally devoid of prion protein. *Vet Res* 2012;43:87.
16. **Mallucci GR, Ratte S, Asante EA, Linehan J, Gowland I et al.** Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. *Embo j* 2002;21(3):202-210.
17. **Falsig J, Julius C, Margalith I, Schwarz P, Heppner FL et al.** A versatile prion replication assay in organotypic brain slices. *Nat Neurosci* 2008;11(1):109-117.
18. **Hollister JR, Lee KS, Dorward DW, Baron GS**. Efficient uptake and dissemination of scrapie prion protein by astrocytes and fibroblasts from adult hamster brain. *PLoS One* 2015;10(1):e0115351.

19. **Krejciova Z, Alibhai J, Zhao C, Krencik R, Rzechorzek NM et al.** Human stem cell-derived astrocytes replicate human prions in a PRNP genotype-dependent manner. *J Exp Med* 2017;214(12):3481-3495.
20. **Victoria GS, Arkhipenko A, Zhu S, Syan S, Zurzolo C**. Astrocyte-to-neuron intercellular prion transfer is mediated by cell-cell contact. *Sci Rep* 2016;6:20762.
21. **Cronier S, Carimalo J, Schaeffer B, Jaumain E, Beringue V et al.** Endogenous prion protein conversion is required for prion-induced neuritic alterations and neuronal death. *Faseb j* 2012;26(9):3854-3861.
22. **Sakai K, Hasebe R, Takahashi Y, Song CH, Suzuki A et al.** Absence of CD14 delays progression of prion diseases accompanied by increased microglial activation. *J Virol* 2013;87(24):13433-13445.
23. **Hasebe R, Tanaka M, Suzuki A, Yamasaki T, Horiuchi M**. Complement factors alter the amount of PrP(Sc) in primary-cultured mouse cortical neurons associated with increased membrane permeability. *Virology* 2016;496:9-20.
24. **Carroll JA, Race B, Williams K, Striebel J, Chesebro B**. Microglia Are Critical in Host Defense against Prion Disease. *J Virol* 2018;92(15).
25. **Gomez-Nicola D, Fransen NL, Suzzi S, Perry VH**. Regulation of microglial proliferation during chronic neurodegeneration. *J Neurosci* 2013;33(6):2481-2493.
26. **Nakagaki T, Satoh K, Ishibashi D, Fuse T, Sano K et al.** FK506 reduces abnormal prion protein through the activation of autolysosomal degradation and prolongs survival in prion-infected mice. *Autophagy* 2013;9(9):1386-1394.
27. **Zhu C, Herrmann US, Falsig J, Abakumova I, Nuvolone M et al.** A neuroprotective role for microglia in prion diseases. *J Exp Med* 2016;213(6):1047-1059.
28. **Hannaoui S, Maatouk L, Privat N, Levavasseur E, Faucheux BA et al.** Prion propagation and toxicity occur in vitro with two-phase kinetics specific to strain and neuronal type. *J Virol* 2013;87(5):2535-2548.
29. **Cronier S, Laude H, Peyrin JM**. Prions can infect primary cultured neurons and astrocytes and promote neuronal cell death. *Proceedings of the National Academy of Sciences of the United States of America* 2004;101(33):12271-12276.
30. **Alleaume-Butaux A, Nicot S, Pietri M, Baudry A, Dakowski C et al.** Double-Edge Sword of Sustained ROCK Activation in Prion Diseases through Neuritogenesis Defects and Prion Accumulation. *PLoS Pathog* 2015;11(8):e1005073.
31. **Tanaka M, Fujiwara A, Suzuki A, Yamasaki T, Hasebe R et al.** Comparison of abnormal isoform of prion protein in prion-infected cell lines and primary-cultured neurons by PrPSc-specific immunostaining. *J Gen Virol* 2016;97(8):2030-2042.