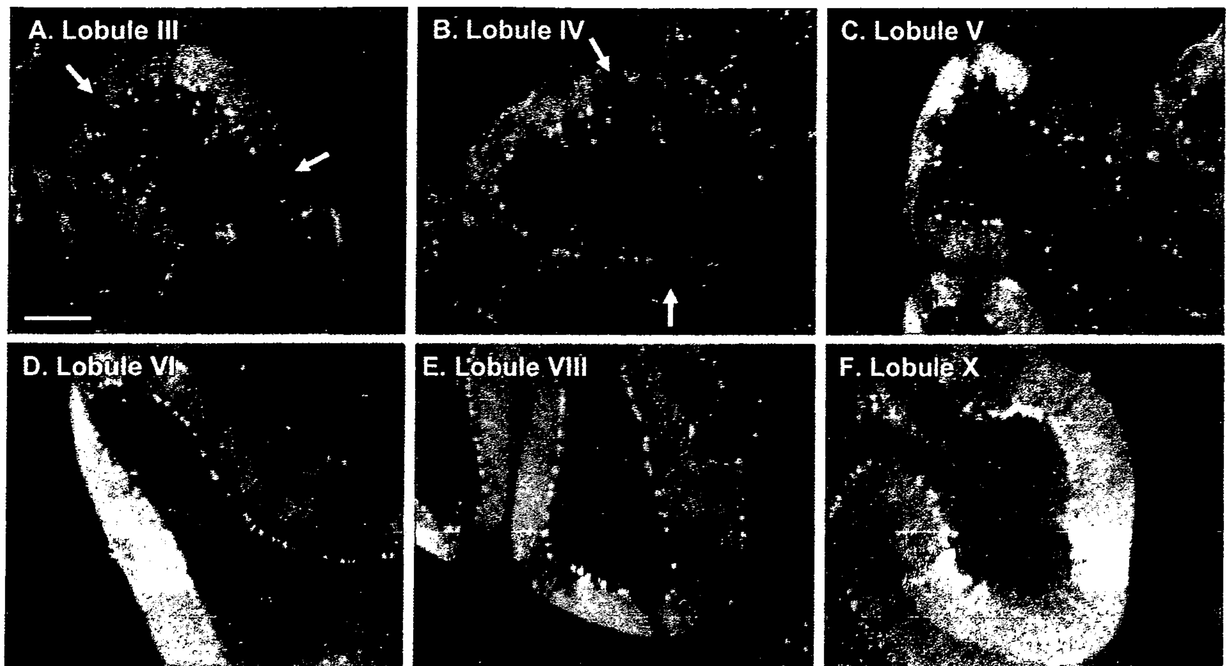

Figure 2. Degeneration of Purkinje cells in Tg(Dpl) mice is asymmetric. Brain sections from a group 2 mouse at 100 days of age were stained with anti-calbindin antibody to visualize cerebellar Purkinje cells. Representative images from lobules III (**A**), IV (**B**), V (**C**), VI (**D**), VIII (**E**), and X (**F**) are shown. At this stage, lobules III to V show severe Purkinje cell loss (**A–C**), lobules VI and VIII are less affected (**D** and **E**), and lobule X is spared (**F**). Purkinje cell loss was not uniform within a lobule; Purkinje cell-free regions showed reduced calbindin staining of the adjacent molecular layer due to the absence of Purkinje cell dendrites (**arrows** in **A** and **B**). Scale bar = 150 μm.

the δ2 glutamate receptor,[38] and from Tg(F35) mice, which show apoptosis of granule cells due to expression of an N-terminally truncated form of PrP.[22,39] Positive staining for TUNEL and activated caspase-3 was evident in degenerating Purkinje cells (0.1 to 1 per ×20 field) in *Lurcher* mice (Figure 5, C and G) and in degenerating granule cells (20 to 50 per ×20 field) in Tg(F35) mice (Figure 5, D and H).

Bax Deletion Does Not Alter Expression Levels of Dpl or Bak

Western blotting was used to analyze the levels of PrP, Dpl, Bax, and Bak in brain homogenates prepared from Tg(Dpl) mice (Figure 6). Bak, a proapoptotic member of the Bcl-2 family, is expressed as an alternatively spliced BH3-only isoform in postnatal neurons.[40] β-Actin was used as a loading control. Groups 1 to 4 mice all expressed Dpl at similar levels (lanes 1 to 4), but only groups 3 and 4 mice expressed PrP (lanes 3 and 4). Bax was present only in groups 1 and 3 mice (lanes 1 and 4), whereas Bak was present at similar levels in all mice (lanes 1 to 5). *Prn-p*$^{0/0}$ Bax$^{0/0}$ mice lacking the Dpl transgene did not express PrP, Dpl, or Bax (lane 5). Thus, the lack of effect of Bax deletion on the Tg(Dpl) phenotype is not because of compensatory up-regulation of Bak or Dpl. We also found that levels of Bcl-2 were similar in mice from groups 1 to 4 (not shown), suggesting that expression of antiapoptotic family members are not altered by Bax deletion.

Discussion

In this study, we have tested whether Bax plays a role in the neurodegenerative phenotype produced in

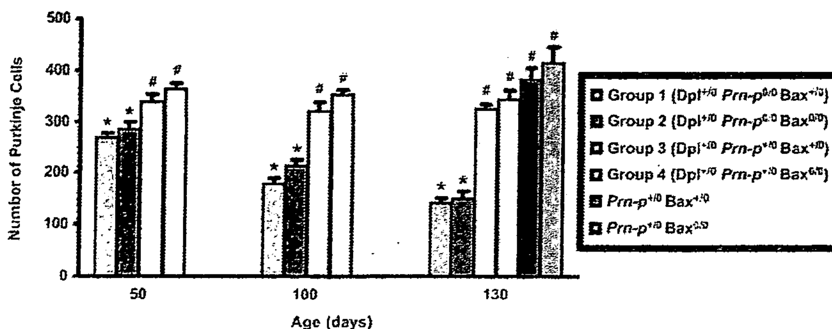

Figure 3. Quantitation of Purkinje cell loss in Tg(Dpl) mice. Purkinje cells were counted in six serial sections from lobule IV of the cerebellum from three mice of each of the indicated genotypes, as described in Materials and Methods. Bars represent the mean ± SD. Means for bars marked with an **asterisk** are statistically different ($P < 0.01$, Student's *t*-test) from means for bars marked with a **number sign**. Means for groups 1 and 2 are not statistically different from each other at 50, 100, or 130 days.

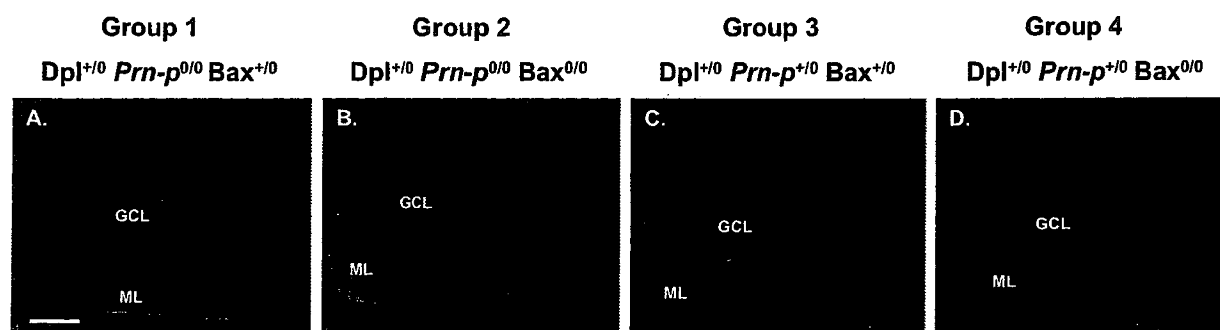

Figure 4. Bax deletion does not affect astrocytosis in the cerebellum of Tg(Dpl) mice. **A–D:** Brain sections from mice of the indicated genotypes at 130 days of age were stained with an antibody to glial fibrillary acidic protein. GCL, granule cell layer; ML, molecular layer. Scale bar = 100 μm.

transgenic mice by neuronal expression of the PrP paralog Dpl. Bax is a multidomain, proapoptotic member of the Bcl-2 family, which is known to play a major role in mitochondrially mediated apoptosis in the CNS, including in the cerebellum.[25,26,41] We found that deletion of both copies of the Bax gene had no effect on the development of clinical symptoms in Tg(Dpl) mice or on the characteristics of Purkinje cell degeneration seen in these animals.

Bax-Independent Purkinje Cell Death in Tg(Dpl) Mice

Why does Purkinje cell degeneration in Tg(Dpl) mice occur independently of Bax? Purkinje cells have been shown to express Bax,[42] which appears to be the only multidomain, proapoptotic regulator present in neurons.[40] Moreover, Bax has been demonstrated to play a role in developmental death of Purkinje cells. Thus, elimination of Bax partially rescues the apoptotic death of Purkinje cells that is thought to occur naturally during the embryonic and postnatal periods, causing as much as a 30% increase in total Purkinje cell number.[37] Therefore, Purkinje cells possess the molecular machinery required for execution of a Bax-dependent, mitochondrially mediated pathway of apoptosis.

Presumably, Dpl must be activating alternative non-Bax-dependent pathways in Purkinje cells. Current thinking suggests that cells die via three kinds of processes: apoptosis, autophagy, and necrosis.[27,43] Apoptosis can involve the intrinsic (mitochondrial) pathway, which is dependent on Bcl-2 family members such as Bax, or the

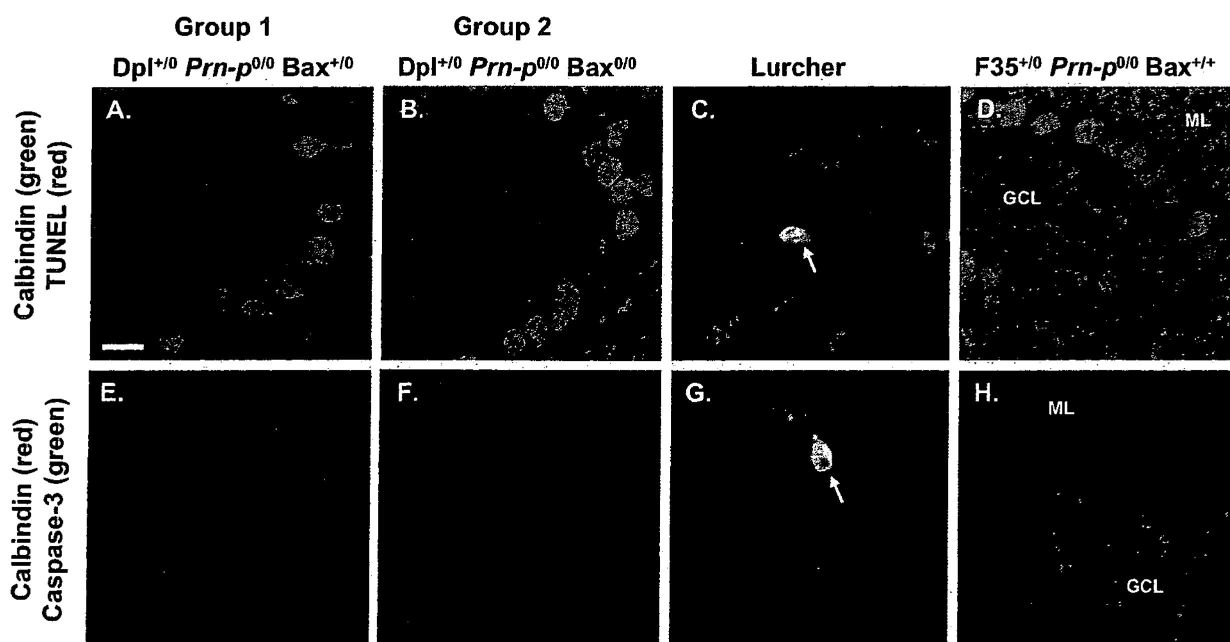

Figure 5. Purkinje cell death in Tg(Dpl) mice occurs independently of detectable DNA cleavage and caspase-3 activation. Brain sections were prepared from the following mice at 100 days of age: group 1 (**A** and **E**), group 2 (**B** and **F**), *Lurcher* (**C** and **G**), and Tg(F35) (**D** and **H**). Sections were stained for calbindin (green) and TUNEL (red) (**A–D**) or for calbindin (red) and activated caspase-3 (green) (**E–H**). A representative area from cerebellar lobule IV is shown for each brain. **Arrows** in **C** and **G** indicate Purkinje cells in *Lurcher* mice that are positive for TUNEL and activated caspase-3, respectively. In contrast, no Purkinje cells positive for these markers are seen in groups 1 or 2 mice (**A**, **B**, **E**, and **F**). Numerous granule cells stained by TUNEL and for activated caspase-3 are apparent in the granule cell layer of Tg(F35) mice (**D** and **H**). GCL, granule cell layer; ML, molecular layer. Scale bar = 20 μm.

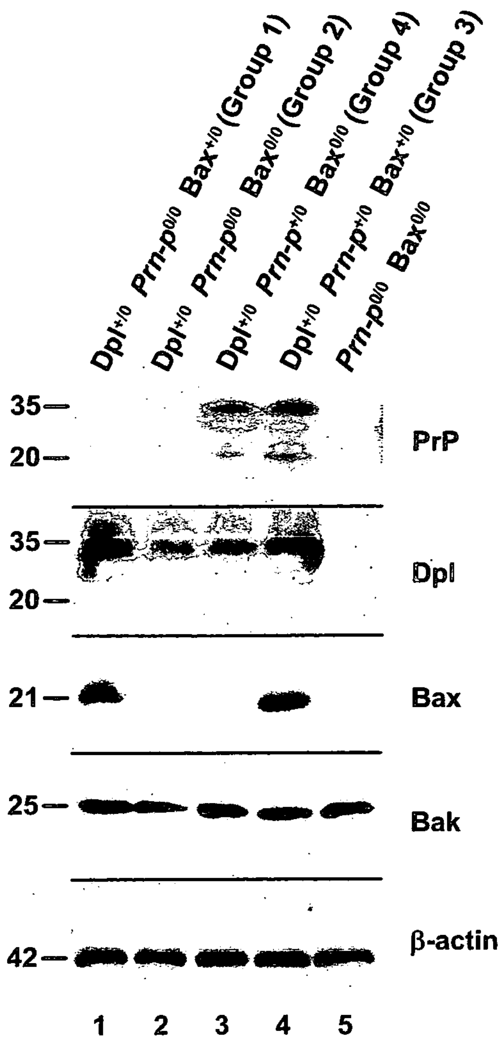

Figure 6. Bax deletion does not alter expression levels of Dpl or Bak. Brain homogenates from mice of the indicated genotypes were analyzed by Western blotting using antibodies directed against PrP, Dpl, Bax, Bak, or β-actin (as a loading control). Molecular size markers are given in kilodaltons.

extrinsic pathway, which is initiated by cell surface death receptors.[27] Thus, Dpl may be acting via an extrinsic apoptotic pathway, which in some cell types can induce death without amplification by the mitochondrial pathway.[44] Alternatively, Dpl may activate autophagic or necrotic mechanisms.

Evidence from mouse cerebellar mutants indicates that Purkinje cells possess several non-Bax-dependent pathways for neuronal death. For example, elimination of Bax does not prevent postnatal degeneration of Purkinje cells in *Lurcher* mice, which express a mutant form of the δ2 glutamate receptor.[45,46] Recent work has demonstrated that Purkinje cell death in *Lurcher* mice occurs via an autophagic process mediated by the protein Beclin-1.[47,48] Autophagy is an organelle engulfment process that is usually considered to be distinct from apoptosis.[43] Interestingly, however, dying Purkinje cells in *Lurcher* animals display caspase activation and DNA fragmentation, characteristics of apoptosis.[38,49] Elimination of Bax prevented caspase activation and DNA fragmentation, even though Purkinje cell death was not blocked.[46,50] Thus, it has been suggested that both autophagic and apoptotic processes play a role in Purkinje cell degeneration in *Lurcher* mice, as well as in other cerebellar mutants.[51]

Possible Nonapoptotic Neuronal Death in Tg(Dpl) Mice

Interestingly, we did not observe DNA cleavage or caspase-3 activation in Purkinje cells of Tg(Dpl) mice, suggesting the absence of apoptosis in these cells. Purkinje cells are known to contain pro-caspase-3.[38] In addition, caspase-3 activation and DNA fragmentation have been documented in these cells during developmentally regulated death, as well as in many cerebellar mouse mutants including *Lurcher*, *Purkinje cell degeneration (pcd)*, *Toppler*, *Woozy*, *Tambaleante*, and *Sticky*.[51,52] Thus, Purkinje cells are capable of undergoing caspase-mediated apoptotic death. Of course, it is possible that our failure to detect DNA cleavage and caspase-3 activation in Tg(Dpl) mice is attributable to the relatively small number of Purkinje cells present in the cerebellum (~220,000),[37] the extended time course over which cell loss occurred (>50 days), or the rapid engulfment and elimination of apoptotic cells. Apoptosis is much easier to appreciate in the granule cell layer, which contains a much larger number of cells (3×10^7).[37] Arguing against these possibilities, however, we monitored staining for TUNEL and activated caspase-3 throughout the whole cerebellum from 30 to 180 days, corresponding to the entire course of Purkinje cell degeneration. Because the loss of Purkinje cells occurs asymmetrically in different lobules of the cerebellum (Figure 2), our analysis is likely to have captured cells in several different stages of degeneration. As a positive control, we were able to capture small numbers of apoptotic Purkinje cells in the brains of *Lurcher* mice. If Purkinje cells in Tg(Dpl) mice are dying in the absence of caspase activation and DNA fragmentation, this would suggest the involvement of nonapoptotic processes, perhaps autophagic mechanisms as in *Lurcher* mice. Analysis of Tg(Dpl) brain tissue using markers for these alternative death pathways will help shed light on this possibility.

Comparison of Neuronal Degeneration Induced by Dpl and N-Terminally Deleted PrP

In a recently published study, we analyzed the effect of Bax elimination on the neurodegenerative phenotypes produced in Tg mice by expression of either of two N-terminally deleted forms of PrP, Δ32–134 or Δ105–125 (collectively referred to here as ΔPrP).[39] We found that deletion of Bax slowed but did not prevent clinical illness and development of neuropathology induced by PrP Δ32–134 and had no effect on neurodegeneration induced by PrP Δ105–125. We thus concluded that ΔPrP activates both Bax-dependent and Bax-independent

neurotoxic pathways, with the latter assuming a dominant role in the terminal stage of the disease. Because Dpl structurally resembles ΔPrP and because each of these proteins induces neurodegeneration that is suppressible by coexpression of wild-type PrP, it is likely that they activate related neurotoxic pathways. Indeed, we have postulated that these proteins interact with a common receptor that serves to transduce the toxic signal, with PrP Δ105–125 having the highest affinity for the receptor.[23] The Bax-independent mechanisms engaged by both Dpl and ΔPrP may represent the downstream elements of such a common cellular program.

In contrast to the Tg(NSE-Dpl) mice analyzed here, mice expressing PrP Δ32–134 and PrP Δ105–125 display neuronal degeneration with prominent apoptotic features, including caspase-3 activation and DNA fragmentation.[22,23,39] This discrepancy between the effects of Dpl and of ΔPrP may reflect the different neuronal populations that are impacted by the two kinds of proteins. Neuronal loss in Tg(ΔPrP) mice involves primarily cerebellar granule cells with sparing of Purkinje cells, whereas Tg(NSE-Dpl) mice show Purkinje cell loss with sparing of granule cells. It is thus possible that granule cells and Purkinje cells differ in how they read out the death signals initiated by Dpl and ΔPrP. The fact that granule cell death induced by PrP Δ32–134 includes a Bax-dependent component, whereas Dpl-induced death of Purkinje cells is entirely Bax-independent, may also reflect the presence of different cell death pathways in these two neuronal cell types. Indeed, there are prominent differences between granule and Purkinje cells in how they respond to several other kinds of death-inducing stimuli.[41]

The different pathologies observed in Tg(NSE-Dpl) and Tg(ΔPrP) mice are probably attributable to expression in different neuronal cell types, rather than to intrinsic differences in the neurotoxic activities of the two kinds of proteins. Dpl mRNA expression in Tg(NSE-Dpl) mice is detectable in Purkinje cells but not in granule cells,[29] consistent with other evidence that the NSE promoter is more active in Purkinje than in granule cells.[53,54] This restricted expression pattern presumably explains the Purkinje cell-specific pathology seen in Tg(NSE-Dpl) mice. However, when expression is driven by a *Prn-p* cosmid, Dpl causes TUNEL-positive apoptosis of granule cells as well as degeneration of Purkinje cells.[15] Conversely, expression of PrP Δ32–134 using the Purkinje cell-specific L7 promoter causes Purkinje cell degeneration.[55] It would be of interest to determine whether neuronal loss in the latter two lines of mice is Bax-independent, as it is in Tg(NSE-Dpl) animals, or whether it also includes a Bax-dependent component as in Tg(PrP Δ32–134) mice.

Conclusion

Taken together, our results highlight an important role for Bax-independent pathways in the neurotoxicity of Dpl and raise the possibility that Dpl activates nonapoptotic mechanisms of neuronal death in Purkinje cells. It will be important now to define further the relevant neurotoxic pathways, to identify the commonalities between the actions of Dpl and ΔPrP, and to determine how the activities of these proteins manifest themselves in different neuronal and non-neuronal cell types. It is likely that this information will provide important clues to the normal functions of Dpl and PrP^C and how subversion of PrP^C activity might contribute to prion-induced neurodegeneration.

Acknowledgments

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References

1. Aguzzi A, Polymenidou M: Mammalian prion biology: one century of evolving concepts. Cell 2004, 116:313–327
2. Prusiner SB: Prions. Proc Natl Acad Sci USA 1998, 95:13363–13383
3. Westergard L, Christensen HM, Harris DA: The cellular prion protein (PrP^C): its physiological function and role in disease. Biochim Biophys Acta 2007, [Epub ahead of print]
4. Zahn R, Liu A, Luhrs T, Riek R, von Schroetter C, Lopez Garcia F, Billeter M, Calzolai L, Wider G, Wüthrich K: NMR solution structure of the human prion protein. Proc Natl Acad Sci USA 2000, 97:145–150
5. Stewart RS, Drisaldi B, Harris DA: A transmembrane form of the prion protein contains an uncleaved signal peptide and is retained in the endoplasmic reticulum. Mol Biol Cell 2001, 12:881–889
6. Hegde RS, Mastrianni JA, Scott MR, Defea KA, Tremblay P, Torchia M, DeArmond SJ, Prusiner SB, Lingappa VR: A transmembrane form of the prion protein in neurodegenerative disease. Science 1998, 279:827–834
7. Narwa R, Harris DA: Prion proteins carrying pathogenic mutations are resistant to phospholipase cleavage of their glycolipid anchors. Biochemistry 1999, 38:8770–8777
8. Stahl N, Baldwin MA, Hecker R, Pan K-M, Burlingame AL, Prusiner SB: Glycosylinositol phospholipid anchors of the scrapie and cellular prion proteins contain sialic acid. Biochemistry 1992, 31:5043–5053
9. Moore RC, Lee IY, Silverman GL, Harrison PM, Strome R, Heinrich C, Karunaratne A, Pasternak SH, Chishti MA, Liang Y, Mastrangelo P, Wang K, Smit AF, Katamine S, Carlson GA, Cohen FE, Prusiner SB, Melton DW, Tremblay P, Hood LE, Westaway D: Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. J Mol Biol 1999, 292:797–817
10. Behrens A, Genoud N, Naumann H, Rulicke T, Janett F, Heppner FL, Ledermann B, Aguzzi A: Absence of the prion protein homologue Doppel causes male sterility. EMBO J 2002, 21:3652–3658
11. Paisley D, Banks S, Selfridge J, McLennan NF, Ritchie AM, McEwan C, Irvine DS, Saunders PT, Manson JC, Melton DW: Male infertility and DNA damage in Doppel knockout and prion protein/Doppel double-knockout mice. Am J Pathol 2004, 164:2279–2288
12. Rossi D, Cozzio A, Flechsig E, Klein MA, Rülicke T, Aguzzi A, Weissmann C: Onset of ataxia and Purkinje cell loss in PrP null mice inversely correlated with Dpl level in brain. EMBO J 2001, 20:694–702
13. Sakaguchi S, Katamine S, Nishida N, Moriuchi R, Shigematsu K, Sugimoto T, Nakatani A, Kataoka Y, Houtani T, Shirabe S, Okada H, Hasegawa S, Miyamoto T, Noda T: Loss of cerebellar Purkinje cells in aged mice homozygous for a disrupted PrP gene. Nature 1996, 380:528–531
14. Li A, Sakaguchi S, Atarashi R, Roy BC, Nakaoke R, Arima K, Okimura N, Kopacek J, Shigematsu K: Identification of a novel gene encoding a PrP-like protein expressed as chimeric transcripts fused to PrP exon 1/2 in ataxic mouse line with a disrupted PrP gene. Cell Mol Neurobiol 2000, 20:553–567
15. Moore RC, Mastrangelo P, Bouzamondo E, Heinrich C, Legname G, Prusiner SB, Hood L, Westaway D, DeArmond SJ, Tremblay P: Dop-

pel-induced cerebellar degeneration in transgenic mice. Proc Natl Acad Sci USA 2001, 98:15288–15293
16. Anderson L, Rossi D, Linehan J, Brandner S, Weissmann C: Transgene-driven expression of the Doppel protein in Purkinje cells causes Purkinje cell degeneration and motor impairment. Proc Natl Acad Sci USA 2004, 101:3644–3649
17. Nishida N, Tremblay P, Sugimoto T, Shigematsu K, Shirabe S, Petromilli C, Erpel SP, Nakaoke R, Atarashi R, Houtani T, Torchia M, Sakaguchi S, DeArmond SJ, Prusiner SB, Katamine S: A mouse prion protein transgene rescues mice deficient for the prion protein gene from Purkinje cell degeneration and demyelination. Lab Invest 1999, 79:689–697
18. Genoud N, Behrens A, Miele G, Robay D, Heppner FL, Freigang S, Aguzzi A: Disruption of Doppel prevents neurodegeneration in mice with extensive Prnp deletions. Proc Natl Acad Sci USA 2004, 101:4198–4203
19. Radovanovic I, Braun N, Giger OT, Mertz K, Miele G, Prinz M, Navarro B, Aguzzi A: Truncated prion protein and Doppel are myelinotoxic in the absence of oligodendrocytic PrP^C. J Neurosci 2005, 25: 4879–4888
20. Behrens A, Brandner S, Genoud N, Aguzzi A: Normal neurogenesis and scrapie pathogenesis in neural grafts lacking the prion protein homologue Doppel. EMBO Rep 2001, 2:347–352
21. Tuzi NL, Gall E, Melton D, Manson JC: Expression of doppel in the CNS of mice does not modulate transmissible spongiform encephalopathy disease. J Gen Virol 2002, 83:705–711
22. Shmerling D, Hegyi I, Fischer M, Blättler T, Brandner S, Götz J, Rülicke T, Flechsig E, Cozzio A, von Mering C, Hangartner C, Aguzzi A, Weissmann C: Expression of amino-terminally truncated PrP in the mouse leading to ataxia and specific cerebellar lesions. Cell 1998, 93:203–214
23. Li A, Christensen HM, Stewart LR, Roth KA, Chiesa R, Harris DA: Neonatal lethality in transgenic mice expressing prion protein with a deletion of residues 105–125. EMBO J 2007, 26:548–558
24. Baumann F, Tolnay M, Brabeck C, Pahnke J, Kloz U, Niemann HH, Heikenwalder M, Rülicke T, Bürkle A, Aguzzi A: Lethal recessive myelin toxicity of prion protein lacking its central domain. EMBO J 2007, 26:538–547
25. Yuan J, Yankner BA: Apoptosis in the nervous system. Nature 2000, 407:802–809
26. Akhtar RS, Ness JM, Roth KA: Bcl-2 family regulation of neuronal development and neurodegeneration. Biochim Biophys Acta 2004, 1644:189–203
27. Danial NN, Korsmeyer SJ: Cell death: critical control points. Cell 2004, 116:205–219
28. van Delft MF, Huang DC: How the Bcl-2 family of proteins interact to regulate apoptosis. Cell Res 2006, 16:203–213
29. Yamaguchi N, Sakaguchi S, Shigematsu K, Okimura N, Katamine S: Doppel-induced Purkinje cell death is stoichiometrically abrogated by prion protein. Biochem Biophys Res Commun 2004, 319: 1247–1252
30. Shindler KS, Latham CB, Roth KA: bax deficiency prevents the increased cell death of immature neurons in bcl-x-deficient mice. J Neurosci 1997, 17:3112–3119
31. Büeler H, Fischer M, Lang Y, Fluethmann H, Lipp H-P, DeArmond SJ, Prusiner SB, Aguet M, Weissmann C: Normal development and behavior of mice lacking the neuronal cell-surface PrP protein. Nature 1992, 356:577–582
32. Chiesa R, Piccardo P, Ghetti B, Harris DA: Neurological illness in transgenic mice expressing a prion protein with an insertional mutation. Neuron 1998, 21:1339–1351
33. Zanusso G, Liu D, Ferrari S, Hegyi I, Yin X, Aguzzi A, Hornemann S, Liemann S, Glockshuber R, Manson JC, Brown P, Petersen RB, Gambetti P, Sy MS: Prion protein expression in different species: analysis with a panel of new mAbs. Proc Natl Acad Sci USA 1998, 95:8812–8816
34. Quaglio E, Chiesa R, Harris DA: Copper converts the cellular prion protein into a protease-resistant species that is distinct from the scrapie isoform. J Biol Chem 2001, 276:11432–11438
35. Rossi F, Jankovski A, Sotelo C: Target neuron controls the integrity of afferent axon phenotype: a study on the Purkinje cell-climbing fiber system in cerebellar mutant mice. J Neurosci 1995, 15:2040–2056
36. Wassef M, Sotelo C, Cholley B, Brehier A, Thomasset M: Cerebellar mutations affecting the postnatal survival of Purkinje cells in the mouse disclose a longitudinal pattern of differentially sensitive cells. Dev Biol 1987, 124:379–389
37. Fan H, Favero M, Vogel MW: Elimination of Bax expression in mice increases cerebellar Purkinje cell numbers but not the number of granule cells. J Comp Neurol 2001, 436:82–91
38. Selimi F, Doughty M, Delhaye-Bouchaud N, Mariani J: Target-related and intrinsic neuronal death in Lurcher mutant mice are both mediated by caspase-3 activation. J Neurosci 2000, 20:992–1000
39. Li A, Barmada SJ, Roth KA, Harris DA: N-terminally deleted forms of the prion protein activate both Bax-dependent and Bax-independent neurotoxic pathways. J Neurosci 2007, 27:852–859
40. Uo T, Kinoshita Y, Morrison RS: Neurons exclusively express N-Bak, a BH3 domain-only Bak isoform that promotes neuronal apoptosis. J Biol Chem 2005, 280:9065–9073
41. Vogel MW: Cell death, Bcl-2, Bax, and the cerebellum. Cerebellum 2002, 1:277–287
42. Wüllner U, Weller M, Schulz JB, Krajewski S, Reed JC, Klockgether T: Bcl-2, Bax and Bcl-x expression in neuronal apoptosis: a study of mutant weaver and lurcher mice. Acta Neuropathol (Berl) 1998, 96:233–238
43. Yuan J, Lipinski M, Degterev A: Diversity in the mechanisms of neuronal cell death. Neuron 2003, 40:401–413
44. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME: Two CD95 (APO-1/Fas) signaling pathways. EMBO J 1998, 17:1675–1687
45. Selimi F, Vogel MW, Mariani J: Bax inactivation in Lurcher mutants rescues cerebellar granule cells but not Purkinje cells or inferior olivary neurons. J Neurosci 2000, 20:5339–5345
46. Doughty ML, De Jager PL, Korsmeyer SJ, Heintz N: Neurodegeneration in Lurcher mice occurs via multiple cell death pathways. J Neurosci 2000, 20:3687–3694
47. Selimi F, Lohof AM, Heitz S, Lalouette A, Jarvis CI, Bailly Y, Mariani J: Lurcher GRID2-induced death and depolarization can be dissociated in cerebellar Purkinje cells. Neuron 2003, 37:813–819
48. Yue Z, Horton A, Bravin M, DeJager PL, Selimi F, Heintz N: A novel protein complex linking the $\delta 2$ glutamate receptor and autophagy: implications for neurodegeneration in Lurcher mice. Neuron 2002, 35:921–933
49. Norman DJ, Feng L, Cheng SS, Gubbay J, Chan E, Heintz N: The lurcher gene induces apoptotic death in cerebellar Purkinje cells. Development 1995, 121:1183–1193
50. Selimi F, Campana A, Weitzman J, Vogel MW, Mariani J: Bax and p53 are differentially involved in the regulation of caspase-3 expression and activation during neurodegeneration in Lurcher mice. C R Acad Sci III 2000, 323:967–973
51. Dusart I, Guenet JL, Sotelo C: Purkinje cell death: differences between developmental cell death and neurodegenerative death in mutant mice. Cerebellum 2006, 5:163–173
52. Lee JW, Beebe K, Nangle LA, Jang J, Longo-Guess CM, Cook SA, Davisson MT, Sundberg JP, Schimmel P, Ackerman SL: Editing-defective tRNA synthetase causes protein misfolding and neurodegeneration. Nature 2006, 443:50–55
53. Martinou JC, Dubois-Dauphin M, Staple JK, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D, Catsicas S, Pietra C, Huarte J: Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron 1994, 13:1017–1030
54. Marangos PJ, Schmechel DE: Neuron specific enolase, a clinically useful marker for neurons and neuroendocrine cells. Annu Rev Neurosci 1987, 10:269–295
55. Flechsig E, Hegyi I, Leimeroth R, Zuniga A, Rossi D, Cozzio A, Schwarz P, Rulicke T, Gotz J, Aguzzi A, Weissmann C: Expression of truncated PrP targeted to Purkinje cells of PrP knockout mice causes Purkinje cell death and ataxia. EMBO J 2003, 22:3095–3101

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Editor-Communicated Paper

Serum Withdrawal-Induced Apoptosis in Zrchl Prion Protein (PrP) Gene-Deficient Neuronal Cell Line Is Suppressed by PrP, Independent of Doppel

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Abstract: **Previous studies have shown that cellular prion protein (PrP^C) plays anti-apoptotic and anti-oxidative role against cell death induced by serum-deprivation (SDP) in an immortalized prion protein gene-deficient neuronal cell line derived from Rikn prion protein (PrP) gene-deficient (*Prnp*$^{-/-}$) mice, which ectopically produce excess Doppel (Dpl) (PrP-like glycoprotein). To investigate whether PrP^C inhibits apoptotic neuronal cell death without Dpl, an immortalized cell line was established from the brain of Zrchl *Prnp*$^{-/-}$ mice, which do not show ectopic expression of Dpl. The results using a Zrchl neuronal *Prnp*$^{-/-}$ cell line (NpL2) showed that PrP^C potently inhibited SDP-induced apoptotic cell death. Furthermore, PrP^C expression enhanced the superoxide dismutase (SOD) activity in NpL2 cells. These results indicate that Dpl production did not affect anti-apoptotic and anti-oxidative functions of PrP, suggesting that PrP^C may be directly correlated with protection against oxidative stress.**

Key words: **Prion protein, Oxidative stress, Apoptosis, Doppel**

Scrapie and bovine spongiform encephalopathy (BSE) in animals and Creutzfeldt-Jakob disease (CJD) in humans are neurodegenerative disorders caused by the intracerebral accumulation of an abnormal prion protein (PrP) designated as PrP^{Sc} (19, 33). PrP^{Sc} is implicated as the pathogenic agent in these diseases. The cellular prion protein (PrP^C), a 30- to 33-kDa membrane glycoprotein anchored by a glycosylphosphatidylinositol moiety (2), is highly expressed in neurons (12) and glia cells (17).

To examine the function of PrP^C in the living system, several PrP-deficient (*Prnp*$^{-/-}$) mouse lines generated independently by different groups using a homologous recombination technique are designated accordingly as

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Abbreviations: BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; DMEM, Dulbecco's modified Eagle's medium; Dpl, Doppel; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; MAP-2, microtubule associated protein-2; OD, optical density; ORF, open-reading frame; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLL, poly-L-lysine; PMSF, phenylmethyl-sulfonyl fluoride; PrP, prion protein; PrP^C, cellular prion protein; PrP^{Sc}, abnormal prion protein; *Prnp*, prion protein gene; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay; ROS, reactive oxygen species; SDP, serum deprivation; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; SOD, superoxide dismutase; STI1, stress-inducible protein-1; SV40, simian virus 40; UV, ultraviolet; WT, wild-type.

ZrchI (8), ZrchII (22), Npu (14), Ngsk (23), Rcm0 (16) and Rikn (38). Initial studies generating two independent lines of mice (ZrchI, Npu) have suggested that these *Prnp*$^{-/-}$ mice are anatomically and behaviorally normal (8), although these *Prnp*$^{-/-}$ cultured neurons are more sensitive than wild-type (WT) neurons to oxidative stress *in vitro* (14). Further studies have demonstrated that copper-bound PrP^{C} elicits superoxide dismutase activity (7), suggesting that the loss of PrP^{C}-mediated anti-oxidant function may be involved in prion diseases (36).

There are other groups of *Prnp*$^{-/-}$ mice that apparently display neurological abnormalities. The recombinant mice (Ngsk, Rcm0, ZrchII, and Rikn) produce similar symptoms and indicate late-onset ataxia with Purkinje cell loss (23, 38). Ngsk, Rcm0, ZrchII and Rikn *Prnp*$^{-/-}$ mice were generated using different targeting cassettes previously used for non-phenotype mice. A detailed study of Ngsk, Rcm0 and Rikn *Prnp*$^{-/-}$ mice has shown that they ectopically express a novel protein known as Doppel (Dpl) (16, 30). The phenotype of these mice can be rescued by reintroduction of the PrP gene to abolish the abnormal phenotype (18).

Previously, we have reported that PrP^{C} prevents apoptotic cell death in *Prnp*$^{-/-}$ cell lines established from Rikn *Prnp*$^{-/-}$ mice (13, 31, 32). In addition, serum-deprived Rikn *Prnp*$^{-/-}$ mouse cell lines are more sensitive than WT cells to apoptosis. Transfection of a PrP^{C}- or Bcl-2-expressing construct into these cells furnishes protection from apoptosis induced by serum deprivation (SDP), thus abolishing the sensitive phenotype (13, 26).

Removal of serum from neuronal cultures induces intracellular oxidative stress (1); elucidation of an anti-apoptotic effect of PrP^{C} is warranted to clarify its protective effects against oxidative stress using Rikn *Prnp*$^{-/-}$ cell lines (31, 32). However, since these cell lines have excess Dpl expression, the involvement of Dpl in the mechanism of action could not be excluded, although the function of Dpl remains unknown. Therefore, the anti-oxidative property of PrP^{C} was analyzed to determine the mechanism of apoptosis in the presence or absence of PrP in Dpl-nonexpressing ZrchI *Prnp*$^{-/-}$ cells.

Data on apoptosis using ZrchI *Prnp*$^{-/-}$ mouse brain-derived cell lines were thus reassessed after SDP in the present study. The results suggest that PrP^{C} induced antioxidative conditions to prevent apoptosis *via* a mechanism independent of Dpl expression.

Materials and Methods

Mice. ZrchI *Prnp*$^{-/-}$ and Rikn *Prnp*$^{-/-}$ mice, respectively described by Büeler et al. (8) and Yokoyama et al. (38), were used in the experiments.

Reagents. Unless otherwise specified, chemical reagents of the highest grade were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and Wako Pure Chemical Industries (Osaka, Japan). The anti-PrP monoclonal antibody 6H4 was purchased from Prionics, Switzerland. Anti-Bax and anti-Bcl-x rabbit polyclonal antibodies were from Pharmingen (Oxford, U.K.). Anti-p53 rabbit polyclonal antibody was from Santa Cruz Biotechnology (Calif., U.S.A.). Anti-caspase-3 rabbit polyclonal antibody and anti-caspase-9 polyclonal antibody were from Cell Signaling Technology (Beverly, Mass., U.S.A.). HRP-conjugated rabbit-specific IgG (H+L) polyclonal antibody was from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). Alexafluor 546 anti-mouse IgG (H+L) goat polyclonal antibody was from Molecular Probes (Eugene, Oreg., U.S.A.).

Immortalization of neuronal cells. Hippocampal cells were isolated from the brain of embryonic day 14 mice using the standard techniques (9, 10). Tissues were dissected and the cells were dissociated in the presence of trypsin (0.25 mg/ml), and then resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FCS). The cells were plated on poly-L-lysine (PLL)-coated tissue culture plastic dishes at a 100 mg of hippocampus tissue/5 ml and allowed to settle. Monolayers of primary brain cells were then exposed to 10^5 plaque-forming units/ml of a ψ vector with 8 μg/ml polybrene to enhance viral infection, as described by Cepko (9). After incubation with retrovirus for 16 hr, the medium was replaced with neurobasal medium (NB) (Gibco BRL, Gaithersburg, Md., U.S.A.) and then complemented with B27 supplement (B27) (Gibco), glutamine, penicillin and streptomycin (NB/B27 medium) (37). Passages were repeated once per week for more than 1 month. Staining with microtubule associated protein-2 (MAP-2) antibodies was used to screen neuronal cell lines. Retrovirus vector was introduced as described above (9).

Cell lines. The ZrchI *Prnp*$^{-/-}$ neuronal cell line (NpL2) was grown in NB/B27 medium. HpL3-4 (13) was used for the Rikn *Prnp*$^{-/-}$ cell line. Cells were collected with mild trypsinization (0.25 mg/ml for 1 min) before confluence and resuspended in growth medium before being replated on PLL-coated dishes at a density of 1×10^5 cells/ml.

Culture media of these cell lines were replaced to

examine the SDP effect on NpL2. Cells were plated on culture dishes at 5×10^5 cells/dish in the growth medium. Two days later, cells were washed thrice with phosphate-buffered saline (PBS) before being placed in fresh media for indicated period.

Immunofluorescent staining. Cells grown on PLL-coated substrate were fixed in 4% paraformaldehyde for 10–20 min at room temperature before staining with PrP, MAP-2 and glial fibrillary acidic protein (GFAP). When staining Dpl, cells were fixed with 10% formalin. After rinsing in PBS, cells were incubated overnight at 4 C with a primary antibody. The cells were then rinsed again in PBS before being incubated with a secondary antibody for 60 min. Negative controls consisted of cells incubated in control serum or without any primary antibody. Primary antibodies included the anti-PrP monoclonal antibody 6H4, anti-Dpl rabbit sera (27, 30), anti-GFAP (Clontech, PaloAlto, Calif., U.S.A.) rabbit sera, anti-MAP2 (Sigma) monoclonal and anti-simian virus 40 (SV40) monoclonal antibodies, while secondary antibodies included the Alexa-conjugated anti-mouse IgG (Molecular Probes) and rhodamine-conjugated anti-rabbit IgG (Dako, Tokyo).

Establishing stable gene-transfer to cell lines. The recombinant constructs, such as pMSCVpuro-PrP, pMSCVpuro-Dpl and pMSCVpuro-EGFP (27), were transfected into NpL2 cells by the lipofectamine-mediated method (Gibco). These cells were selected after incubating for more than 3 days in a complete medium containing 1 μg/ml puromycin (Wako Chem. Co., Ltd.). The recombinant stable transfectants were named NpL2-PrP, NpL2-Dpl and NpL2-E, respectively. PrP and Dpl expression of *Prnp*-transfected lines were examined by immunofluorescence.

Detection of apoptosis. DNA fragmentation was detected by the DNA ladder assay (25). Harvested cells were prepared with lysis using TE buffer [10 mM Tris-HCl (pH 7.4), 10 mM EDTA (pH 8.0) and 0.5% Triton X-100] for 20 min on ice before centrifugation (12,000×*g*, 30 min, 4 C). The aqueous phase was incubated with 400 μg/ml DNase-free RNase A (Nippongene, Tokyo) for 1 hr at 37 C. Proteins were digested with 400 μg/ml of proteinase K for 1 hr at 37 C before DNA was precipitated in a solution of 0.4 M NaCl in 50% isopropanol overnight at −20 C. Precipitated DNA samples were resuspended in TE buffer and electrophoresed in 2.0% agarose gel. The gel was stained with ethidium bromide (0.5 μg/ml) for 10 min and destained with ultra-purified water for 10 min. DNA bands were visualized by an ultraviolet (UV) light transilluminator and photographed (Bio-Rad, Cambridge, Mass., U.S.A.).

Quantitative internucleosomal DNA fragmentation was determined by cell-death detection using enzyme-linked immunosorbent assay (ELISA) (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instruction. The assay determines the apoptosis index by detecting histone-associated DNA fragments (mono- and oligo-nucleosomes) generated from the apoptotic cells.

Assay for superoxide dismutase (SOD) activity. A SOD assay kit (WST Dojindo, Kumamoto, Japan) was used to measure the SOD activity as described previously (24, 25, 28, 29). Each protein extract (20 μg) was assayed and compared with 1 U of bovine erythrocyte Cu/Zn-SOD activity. The SOD activity was estimated using the standard curve of SOD activity *versus* absorbance.

Western blot analysis. Collected cells were pelleted with PBS and 10% FCS, before sonication at pH 7.4 in radio immunoprecipitation assay (RIPA) buffer [10 mM Tris-HCl containing 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS) and 0.15 M sodium chloride supplemented with 2 mM phenylmethyl-sulfonyl fluoride (PMSF)]. Protein concentrations were determined by the Bio-Rad DC assay and resolved by SDS-PAGE. Cell samples were prepared by resuspension in 2× SDS gel-loading buffer [90 mM Tris-HCl (pH 6.8), 10% mercaptoethanol, 2% SDS, 0.02% bromophenol blue and 20% glycerol] and each volume of protein was separated in 10–12% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Hybond-P; Amersham-Pharmacia Biotech, Piscataway, N.J., U.S.A.) for 60 min at 15 V. Nonspecific binding was blocked by 1-hr incubation at room temperature with Block Ace (Dainippon Pharmaceutical, Osaka, Japan). Membranes were incubated with specific primary antibodies in PBS containing 0.1% Tween-20 (PBS-T) and 10% Block Ace for 1 hr before further incubation with rabbit-specific HRP-conjugated secondary antibodies (diluted 1 : 5,000 in PBS-T) for 1 hr. Membranes were detected by the enhanced chemiluminescence (ECL) reagent (Amersham-Pharmacia Biotech) according to the manufacturer's instruction, and exposed to X-ray film (Amersham-Pharmacia Biotech) for measuremnts.

Reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was performed by the previous reported methods (13, 27, 30). Dpl cDNA was amplified by PCR using DPL-KOZAK-SF and DPL-STOP-SR primers (27), whereas cDNA of PrP, neurofilament light subunit (NF-L), neurofilament middle subunit (NF-M), or neurofilament heavy subunit (NF-H) was amplified using the previous described primers (13).

Statistical analysis. Statistical significance was veri-

fied using the unpaired Student's t-test. Differences where $P<0.05$ were considered to be statistically significant.

Results and Discussion

Among the various cell lines with different phenotypes established thus far, ZrchI $Prnp^{-/-}$ neuronal cell line (Fig. 1A) was analyzed. The immunoreactivity of nuclear SV40 large T antigen was visualized in the immortalized NpL2 cells, respectively (data not shown). Although the RT-PCR technique detected the expression of neither PrP mRNA nor Dpl mRNA in ZrchI $Prnp^{-/-}$ cell lines (Table 1), such was not the case in Dpl mRNA-expressing Rikn $Prnp^{-/-}$ cell lines.

NpL2 in medium NB/B27 manifested rapid cell proliferation and neuronal characteristics of being able to produce neuritis and varicosities (data not shown). In DMEM with 10% FCS medium, NpL2 cells showed enriched cytoplasm. By immunocytochemistry, NpL2 cells were stainable with a neuron-specific marker, MAP-2 (data not shown), but not with the glial cell marker, GFAP. These staining methods can therefore differentiate and categorize NpL2 cells as either neuronal glial cells or both. To further study the cellular activities of PrP and Dpl, NpL2 cells were transfected with a plasmid with an open-reading frame (ORF) containing the PrP or Dpl gene. Transfection of these plasmids into NpL2 cells resulted in PrP or Dpl expression (Fig. 1). In addition, Western blotting showed distinct positive production of PrP in NpL2-PrP (Fig. 1).

The remarkable feature of neuronal NpL2 cells was observed when the medium was replaced by serum-free DMEM medium. SDP induced morphological alterations within 1 hr after culture (extension of neurite-like structures). Within 4 days in serum-free media, almost all NpL2 cells died. Cells curled up, displayed cytoplasmic condensation followed by neurite disruption.

The rate of apoptotic cell death was analyzed with the DNA ladder assay to elucidate the relationship of PrP to apoptosis. SDP conducted in these series of NpL2 transfectants consistently induced DNA fragmentations in three transfectants (Fig. 2A) 24 hr after culture. The detectable sign of NpL2-PrP was apparently the weakest among all samples. These results indicate that a lack of PrP resulted in Dpl-nonexpressing neuronal cells sensitive to SDP-induced oxidative stress and apoptotic cell death. In addition, NpL2-E and NpL2-Dpl showed DNA fragmentations in NB without B27 at 24 hr after culture.

At 24 hr after medium replacement, DNA fragmentation was assessed by ELISA using monoclonal antibodies. Under apoptotic condition in DMEM, the fragmented DNA value in NpL2-E approximated 3.7-fold higher than that obtained in DMEM/FCS (Fig.2B). However, fragmented DNA values of NpL2-PrP in DMEM and in DMEM/FCS were not different. These results indicate that value of DNA fragmentation decreased with reintroduction of *Prnp*. Furthermore, in NB without B27, the fragmented DNA value of NpL2-E in B27-free NB approximated 6-fold higher than that obtained in NB/B27. B27 in the media apparently suppressed DNA fragmentation of NpL2-E.

Although the mechanism(s) remains unclear, many reports (1, 4, 6, 28, 29) have documented that Cu/Zn SOD delays neuronal death. The SOD activity of transfectants was therefore measured. The lysate of NpL2-PrP displayed PrP^{C} with enhanced SOD activity compared to NpL2-E cells (Fig. 2C), suggested that the anti-apoptotic effect of PrP^{C} might be directly correlated with the protection of cells against oxidative stress.

Next, changes of apoptosis-related protein production were analyzed by Western blotting. The expressions of p53, Bax, Bcl-x_L, caspase-9, and caspase-3

Table 1. The immunofluorescence and expression of mRNA of various markers in established cell lines

Sample	Mouse brain			Cell line	
Type	WT	ZrchI $Prnp^{-/-}$	Rikn $Prnp^{-/-}$	NpL2 (ZrchI)	HpL3-4 (Rikn)
PrP	+	−	−	−	−
Dpl	−	−	+	−	+
MAP-2*	+	+	+	+	−
GFAP*	−	+	+	−	−
NF-L	+	+	+	+	+
NF-M	+	+	+	+	−
NF-H	+	+	+	+	−

* Reactivity by immunofluorescence. No asterisk: RT-PCR.

Various markers were tested by immunofluorescence and RT-PCR to confirm the characteristics of established cell lines by previously reported methods (13). Brain samples of WT and ZrchI and Rikn $Prnp^{-/-}$ mice were pooled as a control. The protein production or mRNA expression was either positive (+) or negative (−).

genes were studied. Proteins were extracted from neuronal cell lines cultured in serum-free or serum-maintained medium for 0, 24, and 48 hr. Equivalent amounts of protein from NpL2-E and NpL2-PrP were electrophoresed on a 10–12% PAGE gel and blotted. Various proteins were detected, and the expression levels and/or activated forms were quantified (Fig. 3). In both NpL2-E and NpL2-PrP, changes of the steady state

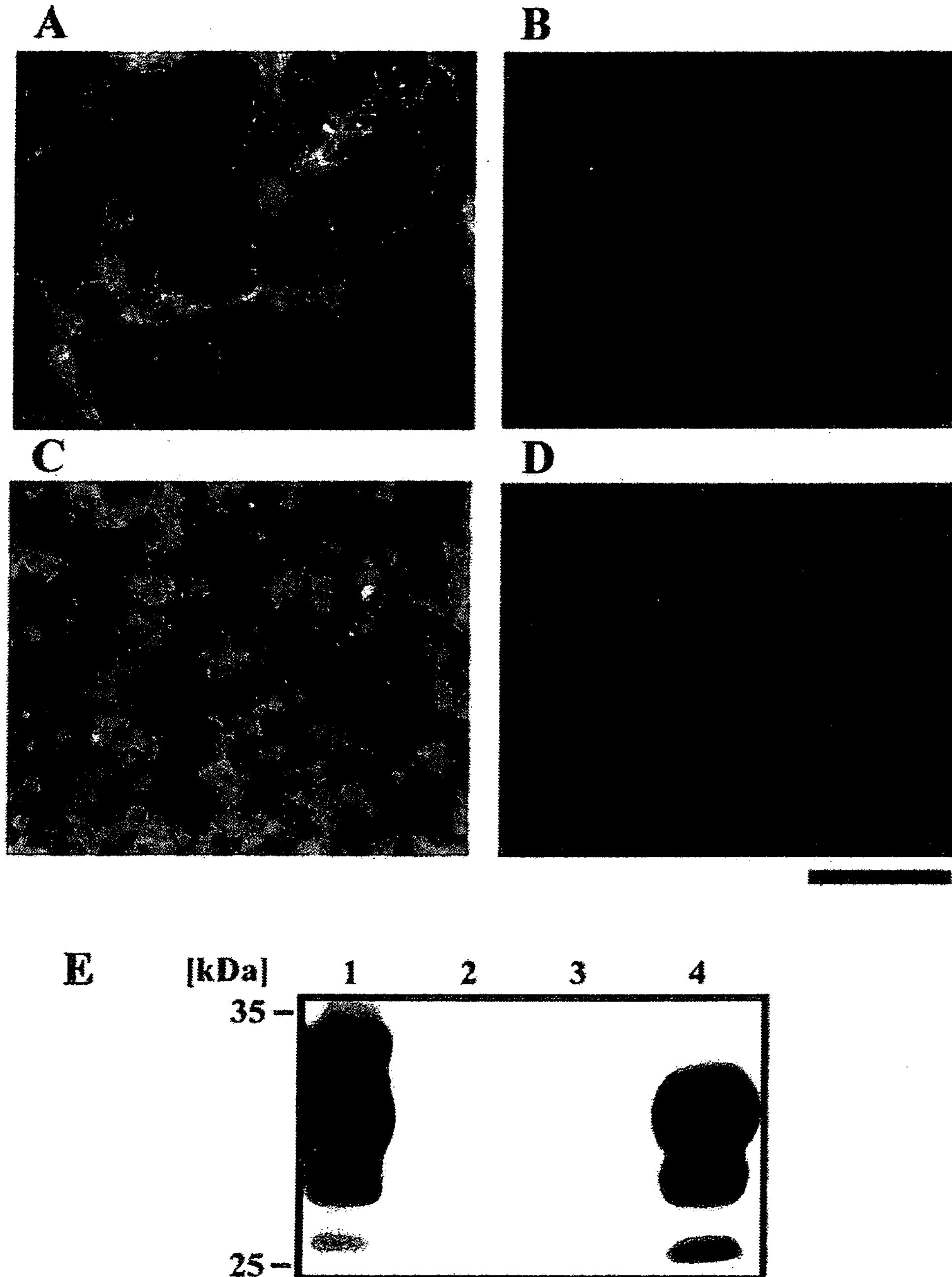

Fig. 1. Transfection of PrP or Dpl gene to NpL2 cells. Neuronal cell line, NpL2 cells transfected with modified pMSCV-puro construct expressing PrP or Dpl protein. To confirm the expression of PrP and Dpl in each transfectant, PrP and Dpl were stained with monoclonal antibodies against PrP and Dpl in NpL2-PrP (*A*) and NpL2-Dpl (*C*), respectively. NpL2-E samples were incubated with anti-PrP (*B*) and anti-Dpl (*D*) accordingly (NpL-E; Bar: 50 μm). (*E*) Western blotting analysis of transfectants was performed as described in "Materials and Methods." The WT mouse brain (lane 1) was used as a positive control. Lanes 2, 3 and 4 represent NpL2, NpL2-E and NpL2-PrP, respectively.

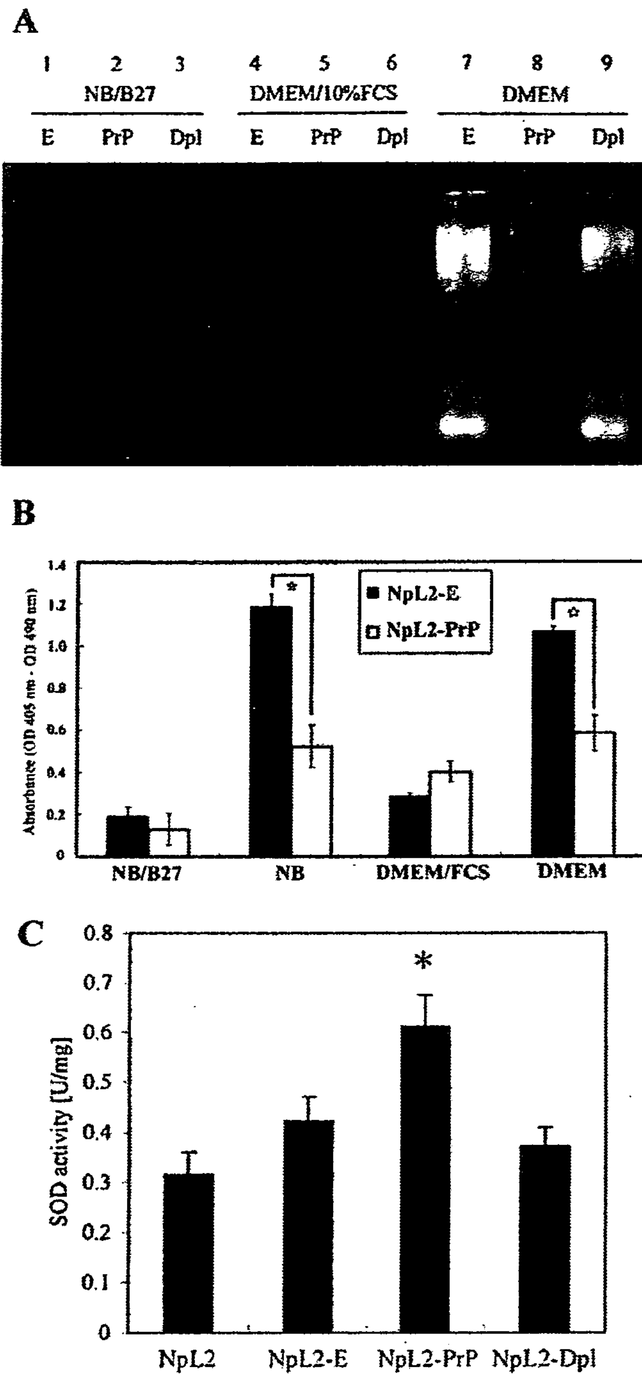

Fig. 2. Detection of DNA fragmentation in NpL2-E and NpL2-PrP cells. (*A*) DNA ladder patterns confirmed that apoptosis was induced by serum deprivation. DNA fragmentation assay showed that NpL2-PrP cells resisted apoptosis more than NpL2-E and NpL2-Dpl. Samples were collected 24 hr after changing the medium. Three transfectants in media NB/B27 (lanes 1–3), DMEM/10% FCS (lanes 4–6) and DMEM (lanes 7–9). (*B*) DNA fragmentation was assessed by the ELISA method using monoclonal antibodies 48 hr after replacement of media such as (NB/B27, NB, DMEM/FCS or DMEM). Values of optical density (OD) are indicated. Absorbance measured in triplicate is expressed as the mean ± standard error of the mean (SEM). Differences where $P<0.01$ (*) were considered statistically significant. These results suggest that the DNA fragmentation value was decreased by reintroduction of *Prnp*. (*C*) Studies on the effect of PrP^c in SOD activity demonstrated that the SOD level of NpL2-PrP cells was higher than those of the other transfectants. SOD activity was determined by a xanthine-based spectrophotomeric assay. Results are expressed as units/mg protein. Differences where $P<0.05$ (*) were statistically significant compared with NpL2-E cells.

levels of pre-apoptotic p53 and Bax were not observed in response to SDP. p53 activates *bax* to trigger apoptosis, implying that induction of apoptosis in *Prnp*$^{-/-}$ cells by SDP may be mediated by a certain p53-independent pathway(s). Bcl-x_L blocks the Bax-dependent release of cytochrome *c* and Bax expression (1, 11). SDP induced a marked reduction in the cellular level of anti-apoptotic Bcl-x_L protein (Fig. 3). In Western blotting of caspase 3 and 9, NpL2-E exhibited the active forms of caspase-3 (16k) and caspase-9 (35k), respectively (Fig. 3). However, HpL2-PrP did not show the active form of either caspase-3 or -9 (Fig. 3). These results suggest that mitochondria play a central role in SDP-induced apoptosis of these cells.

We have previously demonstrated that a neuronal cell line derived from Rikn *Prnp*$^{-/-}$ mice is more susceptive to SDP-induced apoptosis than that derived from the WT cell line, and *Prnp*$^{-/-}$ cell lines could be prevented from apoptosis by Bcl-2 or PrP transfection (13). Further studies have shown that PrP plays a role in the apoptosis prevention by inducing molecules such as SOD-1, Bcl-2 or Bcl-x_L with expression vector (6, 26). It has been shown that PrP potently inhibits Bax-induced cell death in human primary neurons, and octapeptide-repeats of PrP partially abolish the neuroprotective effect of PrP (3). SDP in neuronal cultures and Bax expression are both known to induce intracellular oxidative stress (1, 11). These results suggest that PrP played a role in inducing a certain protein(s) against oxidative stress, or PrP itself is an anti-oxidant

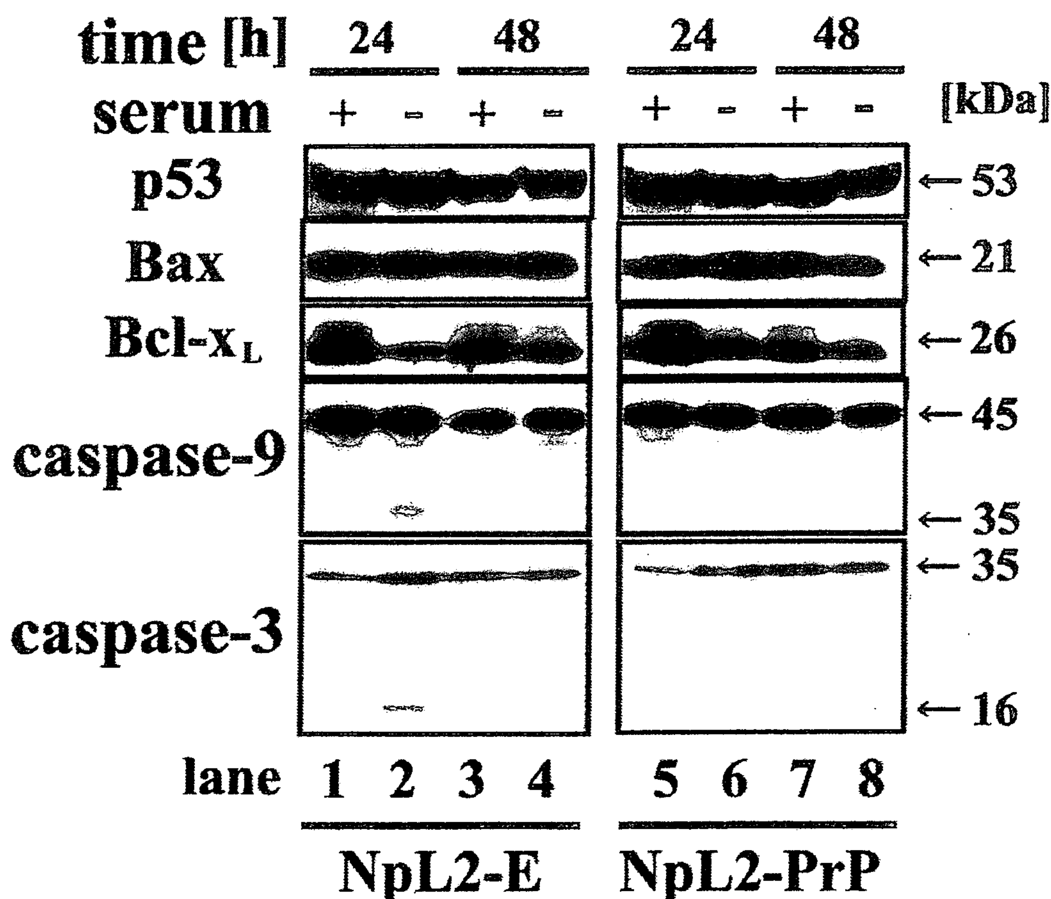

Fig. 3. Changes in signaling proteins during apoptosis. Western blot analysis of apoptotic proteins in neurons was performed to elucidate the underlying mechanism(s) of apoptosis. NpL2-E and NpL2-PrP cells were cultured in media with or without serum for 24 and 48 hr. Protein extracts were loaded from cultured neuronal cells and prepared to be 50 μg/lane. Samples were electrophoresed on an SDS-PAGE gel before being blotted onto a PVDF membrane. Specific antibodies were used to probe the presence of p53, Bax, Bcl-x_L, caspase-9 and caspase-3 on the blots. Cells in media with serum (+) (lanes 1, 3, 5 and 7) and without serum (−) (lanes 2, 4, 6, and 8) were portrayed by the blots. The positions relative to the molecular weight standards are represented on the right, while those p35 and p16 (respective activated fragments of caspase-9 and -3) are accordingly demarcated on the right by arrows.

protein. In this study, PrP prevented apoptosis in *Prnp*$^{-/-}$ cell lines after SDP without the influence of Dpl in Rikn *Prnp*$^{-/-}$ cell lines. When we examined if Dpl gene transfection enhanced apoptosis after SDP, no apparent differences were noted in the severity of SDP-induced apoptosis between NpL2-E and NpL2-Dpl. Transfection of the Dpl gene did not promote apoptotic cell death in NpL2 cells. In these cell lines, we only observed short-term cultures of 4 days. Abnormalities might be caused by Dpl if the study was conducted for a duration longer than 4 days. At any rate, this result demonstrated that apoptotic cell death was caused by PrP deficiency, and not by Dpl production.

PrP^C deficiency causes neurons to become more sensitive to oxidative stress induced by free radicals such as superoxides and hydrogen peroxide. *Prnp*$^{-/-}$ cerebellar neurons are more susceptible to copper toxicity and oxidative stress such as xanthine oxidase (4, 5). When the mitochondrial electron transport system may increase reactive oxygen species (ROS) production, SOD delays cell death induced by the increase of intracellular ROS. To explain this phenomenon, we focus on the relationship between PrP and SOD. Brown et al. have shown that PrP elicits SOD activity (6), which *in vitro* is increased by copper (II) ions (7). In a cell culture study, PrP overexpression induces upregulation of SOD activities without affecting the expression level of SOD in Rikn *Prnp*$^{-/-}$ cell lines (25, 28). In another report, stress-inducible protein (STI1) has been indicated to be responsible for the interaction between PrP and SOD molecules (29). STI1 is a candidate for the neuroprotective signals which interact with amino acids 113–129 of PrP (39). Truncated PrP enhances SOD and caspase-3/caspase-9 to levels much higher than the original Rikn *Prnp*$^{-/-}$ cell lines (25). The same STI1 signals might be responsible for the neuroprotective signals in ZrchI *Prnp*$^{-/-}$ cell lines as well. Our results using Dpl-nonexpressing ZrchI *Prnp*$^{-/-}$ cells and endogenous PrP substantiate these findings. Furthermore, Dpl expression did not enhance SOD activity in ZrchI *Prnp*$^{-/-}$ neuronal cell lines. It has been documented that Dpl has a copper-binding capacity similar to PrP (21). We therefore deduced that the introduction of Dpl would result in an increase of SOD activity in our cell lines, because the effect of PrP on SOD activity would be enhanced by the amount of copper (II) ions (7). However, Dpl expression failed to alter SOD activity in ZrchI *Prnp*$^{-/-}$ neuronal cell lines. Copper-binding to Dpl alone may not increase the SOD activity in neuronal cell lines, and the underlying mechanism(s) of the reaction remains unknown as yet. It must be clarified whether PrP has a neuroprotective role on the cell surface inducing antioxidant activity, or in an internal signaling pathway activating phosphatidylinositol 3-kinase (34).

Little is known about the events of apoptotic process that PrP^C intervened to protect neurons. Bounhar et al. have shown that PrP^C protect neurons against Bax-mediated cell death to a level equivalent to the neuroprotective action of Bcl-2 (3). In addition, PrP^C displays potent neuroprotective effect and prevents cell death of Bax-microinjected neurons. Bax is a major neuronal pro-apoptotic protein. In healthy cells, Bax usually resides in the cytoplasm and mitochondrial membrane. Bax is translocated from the cytoplasm to the mitochondrial membrane in apoptotic death (20, 35), and it promotes the release of cytochrome *c* from mitochondria (35). In addition, Bax is known to be a target for transcriptional activation by p53 in response to DNA damage (15). Significant changes of p53 and Bax expression were not observed in NpL2-E and NpL2-PrP cells treated with or without serum. Activations of caspase-9 and -3 were inhibited in serum-nontreated NpL2-PrP cells. Caspase-9 is the apical caspase of the mitochondrial pathway, while caspase-3 activates downstream in the mitochondrial pathway. These data suggest that PrP^C interacts with the factors effective in the upstream of apoptosis cascade.

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References

1) Atabay, C., Cagnoli, C.M., Kharlamov, E., Ikonomovic, M.D., and Manev, H. 1996. Removal of serum from primary cultures of cerebellar granule neurons induces oxidative stress and DNA fragmentation: protection with antioxidants and glutamate receptor antagonists. J. Neurosci. Res. **43**: 465–475.
2) Borchelt, D.R., Rogers, M., Stahl, N., Telling, G., and Prusiner, S.B. 1993. Release of the cellular prion protein from cultured cells after loss of its glycoinositol phospholipid anchor. Glycobiology **3**: 319–329.
3) Bounhar, Y., Zhang, Y., Goodyer, C.G., and LeBlanc, A. 2001. Prion protein protects human neurons against Bax-mediated apoptosis. J. Biol. Chem. **276**: 39145–39149.
4) Brown, D.R., Schulz-Schaeffer, W.J., Schmidt, B., and Kretzschmar, H.A. 1997. Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity. Exp. Neurol. **146**: 104–112.
5) Brown, D.R., Schmidt, B., and Kretzschmar, H.A. 1998. Effects of copper on survival of prion protein knockout neurons and glia. J. Neurochem. **70**: 1686–1693.
6) Brown, D.R., Wong, B.S., Hafiz, F., Clive, C., Haswell, S.J., and Jones, I.M. 1999. Normal prion protein has an

activity like that of superoxide dismutase. Biochem. J. **344**: 1–5.

7) Brown, D.R., Clive, C., and Haswell, S.J. 2001. Antioxidant activity related to copper binding of native prion protein. J. Neurochem. **76**: 69–76.
8) Büeler, H., Fischer, M., Lang, Y., Bluethmann, H., Lipp, H.P., DeArmond, S.J., Prusiner, S.B., Aguet, M., and Weissmann, C. 1992. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature **356**: 577–582.
9) Cepko, C.L. 1989. Immortalization of neural cells via retrovirus-mediated oncogene transduction. Annu. Rev. Neurosci. **12**: 47–65.
10) Gage, F.H., Ray, J., and Fisher, L.J. 1995. Isolation, characterization, and use of stem cells from the CNS. Annu. Rev. Neurosci. **18**: 159–192.
11) Kirkland, R.A., Windelborn, J.A., Kasprzak, J.M., and Franklin, J.L. 2002. A Bax-induced pro-oxidant state is critical for cytochrome *c* release during programmed neuronal death. J. Neurosci. **22**: 6480–6490.
12) Kretzschmar, H.A., Prusiner, S.B., Stowring, L.E., and DeArmond, S.J. 1986. Scrapie prion proteins are synthesized in neurons. Am. J. Pathol. **122**: 1–5.
13) Kuwahara, C., Takeuchi, A.M., Nishimura, T., Haraguchi, K., Kubosaki, A., Matsumoto, Y., Saeki, K., Matsumoto, Y., Yokoyama, T., Itohara, S., and Onodera, T. 1999. Prions prevent neuronal cell-line death. Nature **400**: 225–226.
14) Manson, J.C., Clarke, A.R., Hooper, M.L., Aitchison, L., McConnell, I., and Hope, J. 1994. 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. Mol. Neurobiol. **8**: 121–127.
15) Miyashita, T., and Reed, J.C. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell **80**: 293–299.
16) Moore, R.C., Lee, I.Y., Silverman, G.L., Harrison, P.M., Strome, R., Heinrich, C., Karunaratne, A., Pasternak, S.H., Chishti, M.A., Liang, Y., Mastrangelo, P., Wang, K., Smit, A.F., Katamine, S., Carlson, G.A., Cohen, F.E., Prusiner, S.B., Melton, D.W., Tremblay, P., Hood, L.E., and Westaway, D. 1999. Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. J. Mol. Biol. **292**: 797–817.
17) Moser, M., Colello, R.J., Pott, U., and Oesch, B. 1995. Developmental expression of the prion protein gene in glial cells. Neuron **14**: 509–517.
18) Nishida, N., Tremblay, P., Sugimoto, T., Shigematsu, K., Shirabe, S., Petromilli, C., Erpel, S.P., Nakaoke, R., Atarashi, R., Houtani, T., Torchia, M., Sakaguchi, S., DeArmond, S.J., Prusiner, S.B., and Katamine, S. 1999. A mouse prion protein transgene rescues mice deficient for the prion protein gene from purkinje cell degeneration and demyelination. Lab. Invest. **79**: 689–697.
19) Onodera, T., Sakudo, A., Wu, G., and Saeki, K. 2006. Bovine spongiform encephalopathy in Japan: history and recent studies on oxidative stress in prion diseases. Microbiol. Immunol. **50**: 565–578.
20) Putcha, G.V., Deshmukh, M., and Johnson, E.M. 1999. BAX translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, BCL-2, and caspases. J. Neurosci. **19**: 7476–7485.
21) Qin, K., Coomaraswamy, J., Mastrangelo, P., Yang, Y., Lugowski, S., Petromilli, C., Prusiner, S.B., Fraser, P.E., Goldberg, J.M., Chakrabartty, A., and Westaway, D. 2003. The PrP-like protein Doppel binds copper. J. Biol. Chem. **278**: 8888–8896.
22) Rossi, D., Cozzio, A., Flechsig, E., Klein, M.A., Rulicke, T., Aguzzi, A., and Weissmann, C. 2001. Onset of ataxia and Purkinje cell loss in PrP null mice inversely correlated with Dpl level in brain. EMBO J. **20**: 694–702.
23) Sakaguchi, S., Katamine, S., Nishida, N., Moriuchi, R., Shigematsu, K., Sugimoto, T., Nakatani, A., Kataoka, Y., Houtani, T., Shirabe, S., Okada, H., Hasegawa, S., Miyamoto, T., and Noda, T. 1996. Loss of cerebellar Purkinje cells in aged mice homozygous for a disrupted PrP gene. Nature **380**: 528–531.
24) Sakudo, A., Hamaishi, M., Hosokawa-Kanai, T., Tuchiya, K., Nishimura, T., Saeki, K., Matsumoto, Y., Ueda, S., and Onodera, T. 2003. Absence of superoxide dismutase activity in a soluble cellular isoform of prion protein produced by baculovirus expression system. Biochem. Biophys. Res. Commun. **307**: 678–683.
25) Sakudo, A., Lee, D.C., Saeki, K., Nakamura, Y., Inoue, K., Matsumoto, Y., Itohara, S., and Onodera, T. 2003. Impairment of superoxide dismutase activation by N-terminally truncated prion protein (PrP) in PrP-deficient neuronal cell line. Biochem. Biophys. Res. Commun. **308**: 660–667.
26) Sakudo, A., Lee, D.C., Saeki, K., Matsumoto, Y., Itohara, S., and Onodera, T. 2003. Tumor necrosis factor attenuates prion protein-deficient neuronal cell death by increases in anti-apoptotic Bcl-2 family proteins. Biochem. Biophys. Res. Commun. **310**: 725–729.
27) Sakudo, A., Lee, D.C., Yoshimura, E., Nagasaka, S., Nitta, K., Saeki, K., Matsumoto, Y., Lehmann, S., Itohara, S., Sakaguchi, S., and Onodera, T. 2004. Prion protein suppresses perturbation of cellular copper homeostasis under oxidative conditions. Biochem. Biophys. Res. Commun. **313**: 850–855.
28) Sakudo, A., Lee, D.C., Nishimura, T., Li, S., Tsuji, S., Nakamura, T., Matsumoto, Y., Saeki, K., Itohara, S., Ikuta, K., and Onodera, T. 2005. Octapeptide repeat region and N-terminal half of hydrophobic region of prion protein (PrP) mediate PrP-dependent activation of superoxide dismutase. Biochem. Biophys. Res. Commun. **326**: 600–606.
29) Sakudo, A., Lee, D.C., Li, S., Nakamura, T., Matsumoto, Y., Saeki, K., Itohara, S., Ikuta, K., and Onodera, T. 2005. PrP cooperates with STI1 to regulate SOD activity in PrP-deficient neuronal cell line. Biochem. Biophys. Res. Commun. **328**: 14–19.
30) Sakudo, A., Lee, D.C., Nakamura, I., Taniuchi, Y., Saeki, K., Matsumoto, Y., Itohara, S., Ikuta, K., and Onodera, T. 2005. Cell-autonomous PrP-Doppel interaction regulates apoptosis in PrP gene-deficient neuronal cells. Biochem. Biophys. Res. Commun. **333**: 448–454.
31) Sakudo, A., Onodera, T., Suganuma, Y., Kobayashi, T., Saeki, K., and Ikuta, K. 2006. Recent advances in clarifying prion protein functions using knockout mice and derived cell lines. Mini-Rev. Med. Chem. **6**: 589–601.
32) Sakudo, A., Onodera, T., and Ikuta, K. 2007. Prion protein

gene-deficient cell lines: powerful tools for prion biology. Microbiol. Immunol. **51**: 1–13.

33) Sakudo, A., Nakamura, I., Ikuta, K., and Onodera, T. 2007. Recent developments in prion disease research: diagnositic tools and *in vitro* cell-culture models. J. Vet. Med. Sci., in press.

34) Vassallo, N., Herms, J., Behrens, C., Krebs, B., Saeki, K., Onodera, T., Windl, O., and Kretzschmar, H.A. 2005. Activation of phosphatidylinositol 3-kinase by cellular prion protein and its role in cell survival. Biochem. Biophys. Res. Commun. **332**: 75–82.

35) Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G., and Youle, R.J. 1997. Movement of Bax from the cytosol to mitochondria during apoptosis. J. Cell Biol. **139**: 1281–1292.

36) Wong, B.S., Pan, T., Liu, T., Li, R., Petersen, R.B., Jones, I.M., Gambetti, P., Brown, D.R., and Sy, M.S. 2000. Prion disease: a loss of antioxidant function? Biochem. Biophys. Res. Commun. **275**: 249–252.

37) Yao, M., Bain, G., and Gottlieb, D.I. 1995. Neuronal differentiation of P19 embryonal carcinoma cells in defined media. J. Neurosci. Res. **41**: 792–804.

38) Yokoyama, T., Kimura, K.M., Ushiki, Y., Yamada, S., Morooka, A., Nakashiba, T., Sassa, T., and Itohara, S. 2001. *In vivo* conversion of cellular prion protein to pathogenic isoforms, as monitored by conformation-specific antibodies. J. Biol. Chem. **276**: 11265–11271.

39) Zanata, S.M., Lopes, M.H., Mercadante, A.F., Hajj, G.N., Chiarini, L.B., Nomizo, R., Freitas, A.R., Cabral, A.L., Lee, K.S., Juliano, M.A., de Oliveira, E., Jachieri, S.G., Burlingame, A., Huang, L., Linden, R., Brentani, R.R., and Martins, V.R. 2002. Stress-inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection. EMBO J. **21**: 3307–3316.

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Reduced response of splenocytes after mitogen-stimulation in the prion protein (PrP) gene-deficient mouse: PrPLP/Doppel production and cerebral degeneration

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Abstract

Splenocytes of wild-type (*Prnp*$^{+/+}$) and prion protein gene-deficient (*Prnp*$^{-/-}$) mice were treated with various activation stimuli such as T cell mitogen concanavalin A (ConA), phorbol 12-myristate 13-acetate (PMA) + ionomycin (Io), or B cell mitogen lipopolysaccharide (LPS). Cellular prion protein (PrP^{C}) expression was enhanced following ConA stimulation, but not PMA + Io or LPS in *Prnp*$^{+/+}$ splenocytes. Rikn *Prnp*$^{-/-}$ splenocytes elicited lower cell proliferations than *Prnp*$^{+/+}$ or Zrch I *Prnp*$^{-/-}$ splenocytes after LPS stimulation and showed sporadic nerve cells in the cerebral cortex and deeper structure. Around the degenerated nerve cells, mild vacuolation in the neuropil was observed. This neural alteration correlated well to the suppressed response of B cells in the spleen. The finding that discrete lesions within the central nervous systems induced marked modulation of immune function probably indicates the existence of a delicately balanced neural-endocrine network by PrP^{C} and PrPLP/Doppel.
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The nature of prion protein (PrP) has long been the subject of considerable controversies [1]. Little is known about the molecular state of the protein that constitutes the self-propagating, infectious particle per se [1]. PrP gene (*Prnp*)-deficient (*Prnp*$^{-/-}$) mice exhibit complete protection against scrapie infection, indicating that normal cellular prion protein (PrP^{C}) is an essential factor for inducing prion diseases [2]. However, studies on phenotype of *Prnp*$^{-/-}$ mice have documented inconsistent results. Zrch I *Prnp*$^{-/-}$ mice (type-1 *Prnp*$^{-/-}$ mice) display normal development and behavior [3], while Zrch II, Ngsk, Rcm0 and Rikn *Prnp*$^{-/-}$ mice (type-2 *Prnp*$^{-/-}$ mice) show late-onset ataxia [4–7]. This discrepancy may be due to genetic combinations without PrP and ectopic expression of PrP-like protein (PrPLP/Doppel) [4]. Several *in vitro* studies have suggested that PrP^{C} plays important roles in inhibiting apoptosis in neurons, and inflammatory responses (e.g., glial proliferation) [8–10]. While PrP^{C} is expressed in several peripheral tissues [11,12], most of the studies on normal functions of PrP^{C} have been restricted to the central nervous system (CNS) [13,14]. In this study, murine splenocytes were investigated to elucidate the function of PrP^{C},

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especially its responses to mitogen-stimulation and apoptosis in splenocytes.

Materials and methods

Cell culture. The entire *Prnp* gene open-reading frame (ORF) and the 3′-end of intron 2 were replaced with a pgk-neo gene cassette in Rikn $Prnp^{+/-}$ mice [7]. Genotyping of tested animals was performed by polymerase chain reaction (PCR) analysis and/or southern blotting of DNA prepared from the tails of mice. After sacrificing male $Prnp^{+/+}$ and Rikn $Prnp^{-/-}$ mice (50-week-old), the spleens were aseptically isolated. Single-cell suspensions were prepared as previously described [15]. Splenocytes, which were suspended in RPMI 1640 complete medium supplemented with 10% heat inactivated-fetal calf serum (HI-FCS), were cultured with 100 U/ml penicillin and 100 μg/ml streptomycin at a density of 1×10^6 cells/ml. The cultures contained 10 μg/ml T-cell mitogen concanavalin A (ConA; Sigma. St. Louis, MO), 25 ng/ml phorbol 12-myristate 13-acetate (PMA) + 1 μg/ml ionomycin (Io), 5 μg/ml, or B-cell mitogen lipopolysaccharide (LPS) were incubated for 48 h at 37 °C in a humidified 5% CO_2 incubator.

Cell proliferation assay. The tetra-color one-cell proliferation assay was performed according to manufacture's instructions (Seikagaku Corporation, Tokyo Japan).

Staining of CD4–CD8 cell-surface antigen. Cells were collected from cultures, and further incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 (Pharmingen, San Diego, CA) and phycoerythrin (PE)-conjugated anti-mouse CD8 (Pharmingen) antibodies in the dark at 4 °C for 1 h. The cells were washed twice after harvest and resuspended in phosphate-buffered saline (PBS) supplemented with 2% HI-FCS before analysis with the FACScan (Becton–Dickinson).

Intracellular cytokine staining. Intracellular staining of interleukin-2 (IL-2) was performed with the Cytofix/Cytoperm Plus™ (with GolgiPlug™) kit (Pharmingen), using PE-conjugated anti-mouse IL-2 (Pharmingen) and FITC-conjugated anti-mouse CD4 (Pharmingen) antibodies following the manufacturer's instruction.

DNA fragmentation assay. Flow cytometry analysis based on DNA fragmentation with the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method [16] was performed to quantify apoptotic cells. The TUNEL method was conducted with the *in situ* cell-death detection kit (Fluorescein; Roche Applied Science, Penzberg, Germany) according to manufacture's instructions with slight modification. Briefly, cells cultured in the presence and absence of mitogen at a density of 1×10^7 cells/well in a 24-well plate for 24 h were fixed with 2% para-formaldehyde overnight before further incubation in 0.1% Triton X-100 with 0.1% sodium citrate at 4 °C for 2 min. The TUNEL reaction was performed according to manufacture's instructions (Roche Applied Science). Fluorescence of FITC-dUTP was then analyzed with flow cytometry (FACScan).

Histological studies. Mice were anaesthetized with diethyl ether and perfused transcordially with PBS containing 4% para-formaldehyde fixative (pH 7.4) before the brain of each animal was isolated and immediately immersed in said fixative overnight at 4 °C. Coronal sections of the olfactory bulb and sagittal sections of the cerebrum and cerebellum were prepared. Tissue specimens dehydrated with a graded ethanol series were embedded in paraffin for serial sectioning. Serial sections (6-μm thickness) deparaffinized in xylene and re-hydrated in a series of graded ethanol were subjected to staining with the hematoxylin–eosin (H&E) reagent before observations under light microscopy.

Western blotting of PrPLP/Doppel. Doppel (Dpl) was detected as previously described [17]. To fractionate tissues samples, 10% (w/v) homogenate was prepared in sterile PBS followed by sonication. After initial centrifugation at 600*g* for 15 min at 4 °C, the supernatant was further ultracentrifuged at 100,000*g* for 2 h at 4 °C. The pellet was suspended in ice-cold radio-immunoprecipitation assay (RIPA) buffer containing 10 mM Tris–HCl (pH 7.4), 1% deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 2 μg/ml aprotinin, and 2 mM phenylsulfonyl fluoride (PMSF). The protein concentration was determined by the Bradford assay according to the manufacturer's instruction (DC protein assay kit; Bio-Rad Laboratories Inc., Tokyo). Lysates were boiled in 2-fold volume of sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer [1 M Tris–HCl (pH 6.8), 200 mM dithiothreitol, 10% SDS, 2% bromophenol blue, 20% glycerol] for 5 min. The samples were then subjected to electrophoresis in 12% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P, Amersham, Piscataway, NJ, USA) for 60 min (15 V). The membrane was blocked with Block Ace (Dainippon Pharmaceutical, Japan) overnight at room temperature. Primary anti-Dpl rabbit antiserum (a kind gift from Dr. Chen, Case Western University, Cleveland, OH, USA) diluted at 1:2500 in PBS/0.1% Tween 20 containing 10% Block Ace was incubated for 1 h at room temperature. After washing thrice with PBS/0.1% Tween 20, the membrane was further incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin antibodies (dilute at 1:5000 with PBS/0.1% Tween 20 containing 10% Block Ace) for 1 h at room temperature. Mouse hybridoma (T2) [18] was used for PrP detection in mouse brain. Hybridoma was purified with the MabTrapTM Kit (Amersham,) according to manufacturer's instructions. After treatments with primary antibodies, the blot was incubated with a horseradish peroxidase-conjugated anti-mouse Ig sheep F $(ab')_2$ fragment (dilution: 1:5000), and subsequently washed thrice in PBS/0.1% Tween 20 for 10 min. Blots were developed by exposing the enhanced chemiluminescence (ECL) reagent (Amersham) to Hyperfilm™ MP (Amersham) before being automatically processed in an X-ray film processor (Konica, Tokyo).

Results

Certain schools have previously characterized constitutive expression of PrP^C in murine immunocyte populations, using the Rikn $Prnp^{-/-}$ mouse as a negative control by flow cytometry. This suggests that PrP^C is involved in development of immunocytes [15]. In this study, splenocytes from $Prnp^{+/+}$ and Rikn $Prnp^{-/-}$ mice were stimulated with T-cell mitogen ConA, PMA + Io, or B-cell mitogen LPS to evaluate if PrP^C was involved in splenocytes activation.

First, flow cytometry analysis was performed to examine the effect of mitogen on population of $Prnp^{+/+}$ and Rikn or Zrch I $Prnp^{-/-}$ splenocytes. CD4–CD8 cell-surface antigens (CD4 and CD8) were used as markers to characterize the cell population. Splenocytes were treated with and without ConA, PMA + Io or LPS for 48 h before analyses of the respective cell populations for CD4 or CD8 expression were conducted. No differences in the cell population between $Prnp^{+/+}$ and $Prnp^{-/-}$ splenocytes were observed as previously reported (data not shown) [15]. Although, the percentages of CD4-positive and CD8-positive T cells were altered by mitogen, no differences between $Prnp^{+/+}$ and $Prnp^{-/-}$ splenocytes were encountered (data not shown). Moreover, the incidence of IL-2-positive cells appeared to have increased by mitogen as previously reported [19], albeit differences between $Prnp^{+/+}$ and $Prnp^{-/-}$ splenocytes were not observed (data not shown). We next investigated if PrP^C affected the responsiveness to mitogen, mitogen effects on cell proliferation in $Prnp^{+/+}$ and Rikn or Zrch I $Prnp^{-/-}$ splenocytes according to a previously described approach [20]. Cell counts of $Prnp^{+/+}$ splenocytes were elevated by ConA, PMA + Io, and LPS stimulation after 48-h incubation when compared with

non-stimulated $Prnp^{+/+}$ splenocytes (Fig. 1). Increases of the cell count in $Prnp^{-/-}$ splenocytes surpassed 2- and 3-fold with ConA and PMA + Io or LPS stimuli, respectively, when compared with non-stimulated $Prnp^{-/-}$ splenocytes (Fig. 1). However, the cell counts in $Prnp^{+/+}$ splenocytes registered a mere 1.3-fold increase with PMA + Io sitmulation as compared to similarly stimulated $Prnp^{-/-}$ splenocytes. Interestingly, Zrch I splenocytes proliferated more efficiently than Rikn $Prnp^{-/-}$ cells while Rikn $Prnp^{-/-}$ splenocytes proliferated less efficiently than $Prnp^{+/+}$ splenocytes after LPS stimulation (Fig. 1). These results suggest that PrPLP/Doppel-producing splenocytes were more sensitive to the toxic effects of LPS.

Since PrPLP/Doppel produces cytotoxicity in neurons [5], a similar effect would be expected to affect the proliferation rate of splenocytes. As apoptotic rates of $Prnp^{+/+}$ splenocytes, Rikn $Prnp^{-/-}$ splenocytes and Zrch I $Prnp^{-/-}$ splenocytes approximated 37% without any stimulation (controls), Rikn and Zrch I $Prnp^{-/-}$ splenocytes were thus equally susceptible to apoptosis compared with $Prnp^{+/+}$ splenocytes (Fig. 2). These susceptibility tendencies were also observed in ConA-, PMA + Io-, and LPS-stimulated splenocytes. Note that ConA, PMA + Io, and LPS suppressed apoptoses in $Prnp^{+/+}$, Rikn $Prnp^{-/-}$, and Zrch I $Prnp^{-/-}$ splenocytes, (Fig. 2), and the treatment with PMA + Io was especially marked in attenuating the apoptosis rate.

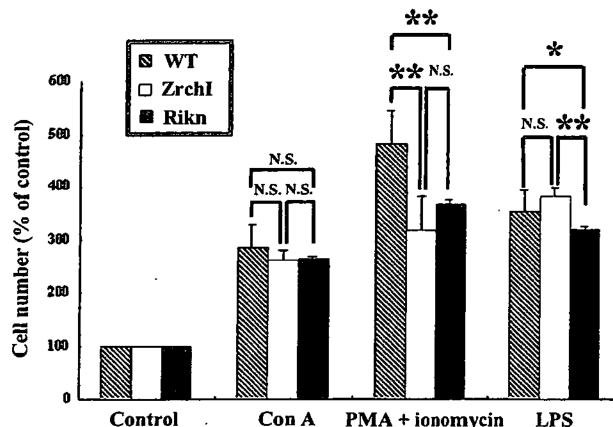

Fig. 1. Effects of mitogen on cell proliferations in wild-type prion proten (PrP) ($Prnp^{+/+}$) and PrP gene-deficient ($Prnp^{-/-}$) mouse splenocytes. $Prnp^{+/+}$ and Zrch I or Rikn $Prnp^{-/-}$ mouse splenocytes were cultured for 48 h in the absence or presence of T-cell mitogen concanavalin A (ConA), phorbol 12-myristate 13-acetate (PMA) + ionomycin (Io), and B-cell mitogen lipopolysaccharide (LPS). Cell counts were determined by the tetra-color one-cell proliferation assay. Cell counts of $Prnp^{-/-}$ splenocytes were compared with those of $Prnp^{+/+}$ splenocytes with non-treated cultures as controls (controls: 100%). Zrch I and Rikn $Prnp^{-/-}$ splenocytes revealed significantly lower cell proliferation rates than $Prnp^{+/+}$ splenocytes on exposure to PMA + Io. Furthermore, Rikn $Prnp^{-/-}$ splenocytes showed significantly lower cell proliferation rates than LPS-stimulated $Prnp^{+/+}$ splenocytes and Zrch I $Prnp^{-/-}$ splenocytes. Differences where $p < 0.05$ (*) or <0.01 (**) were statistically significant, or not significant (NS) compared with controls (unpaired t-test).

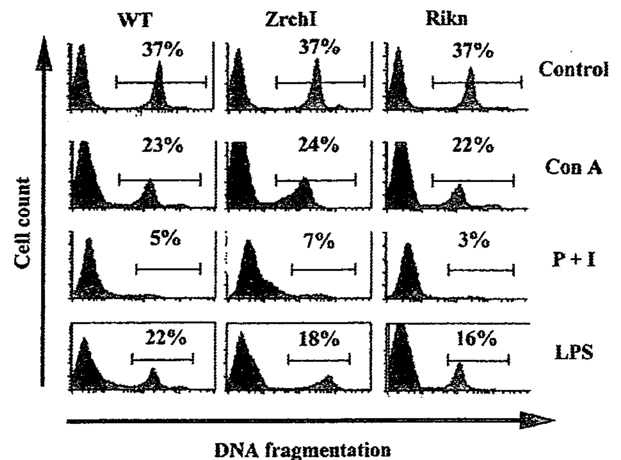

Fig. 2. Ratio of cell counts with fragmented DNA in $Prnp^{-/-}$ splenocytes after mitogenic stimulation. Splenocytes from Zrch I and Rikn $Prnp^{-/-}$ mice or $Prnp^{+/+}$ (WT) mice were untreated (Control) or treated with ConA, PMA + ionomycin (P + I) or LPS for 24 h. Quantitative analysis of apoptotic cells was performed with the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method. The numeral in each panel represents the ratio of the number of apoptotic cells (FITC-dUTP-positive cells) to total cell population. Refer to Fig. 1 for abbreviations.

Groups of 5 $Prnp^{-/-}$ and $Prnp^{+/+}$ mice each were histopathologically examined. Results indicated neuronal damage in either Zrch I $Prnp^{-/-}$ or $Prnp^{+/+}$ mice. In 5 Rikn $Prnp^{-/-}$ mice that displayed reduced responses to LPS stimulation, sporadic nerve cells were observed in the cerebral cortex and deeper brain structures (Fig. 3A and B). Around the degenerated nerve cells, mild vacuolation in neuropils was observed (Fig. 3B). In the cerebellum, most of the Purkinje cells were degenerated or beyond identification (Fig. 3C).

On detecting the brain of WT, ZrchI, and Rikn $Prnp^{-/-}$ mice with Western blot analysis using a polyclonal antibody against recombinant Dpl, a strong Dpl band was observed in samples of Rikn $Prnp^{-/-}$ mice, but not in those of ZrchI $Prnp^{-/-}$ and WT mice (Fig. 4A). Furthermore, PrP and Dpl were detected only in the brain of $Prnp^{+/-}$ mice. Age-related distributions of Dpl in the mouse brain, cerebrum, cerebellum, and olfactory bulb were traced in mice ranging from 5–100 weeks. Based on the expression-sites and age difference, the regional Dpl production levels were compared; viz., between the cerebrum, cerebellum and olfactory bulb. The molecular weight of Dpl in the respective sites approximated 25–30 kD, indicating Dpl molecules with a similar size prevailed in the different sites examined (Fig. 4B). Despite prevalence of such a commonality, histopathological changes were observed only in Rikn $Prnp^{-/-}$ mice.

Discussion

Certain schools have previously established the $Prnp^{-/-}$ neuronal cell lines and implicated an important role played

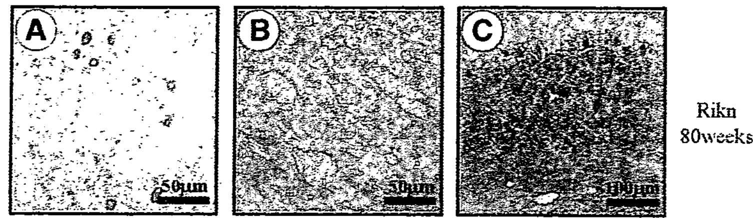

Fig. 3. Hematoxylin–eosin (H&E) staining of the cerebral cortex in a $Prnp^{-/-}$ mouse. Histopathological analysis by H&E staining of the brain obtained from an 80-week-old Rikn $Prnp^{-/-}$ mouse (A–C) under light microscopy (magnification 132×). Sagittal brain sections (6-μm thick) after fixation with 4% paraformaldehyde showed vacuolar degeneration and necrosis in the brainstem (A), lemniscus trigeminalis (B; higher magnification); and cerebellum (C).

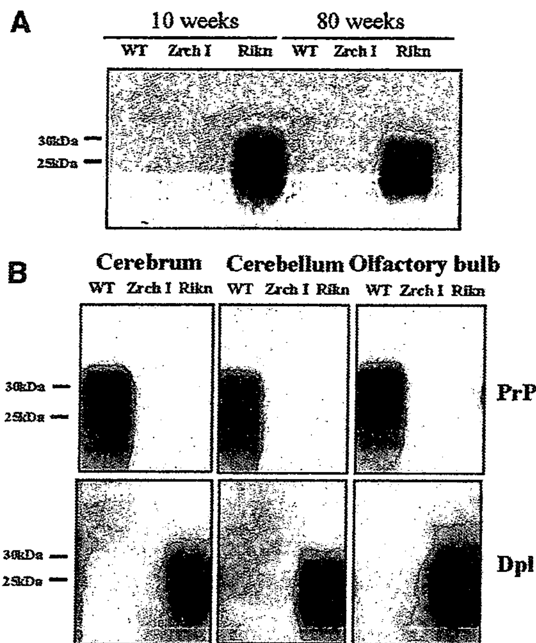

Fig. 4. Detection of Chimeric *Prnp/Prnd* mRNAs, prion pretein (PrP) and Doppel (Dpl) in brains of wild-type ($Prnp^{+/+}$) and PrP gene-deficient ($Prnp^{-/-}$) mice. (A) Western blot analysis of Dpl in brains of 10- or 80-week-old WT, ZrchI and Rikn $Prnp^{-/-}$ mice. Dpl was detected with a size of 25–30 kDa. (B) Western blot analyses of PrP and Dpl in the cerebrum, cerebellum and olfactory bulb of WT, $Prnp^{-/-}$ and $Prnp^{+/+}$ mice at 50 weeks of age. T2 antibodies were used to detect PrP. Western blotting was performed with anti-Dpl rabbit antiserum at a dilution rate of 1:2500. Each lane was loaded with 60 μg of protein.

by PrP^C in regulating apoptosis [10]. Although, PrPLP/Doppel reportedly displays certain neurotoxicities in the absence of PrP [5], it is not known whether PrPLP/Doppel induces cell death in other cells. Based on observations of said phenomenonal actions restricted to the CNS, apoptoses of $Prnp^{+/+}$, Rikn $Prnp^{-/-}$ (ectopically PrPLP/Doppel-producing strain) and Zrch I $Prnp^{-/-}$ (non-ectopically PrPLP/Doppel-producing strain) splenocytes were analyzed before and after mitogen-stimulation.

In this study, cell proliferations/populations of $Prnp^{+/+}$ and $Prnp^{-/-}$ splenocytes after mitogen stimulation were investigated. ConA-stimulated $Prnp^{+/+}$ splenocytes yielded higher PrP^C levels, confirming previous observations using human and murine lymphocytes [12,20]. However, the proliferation rates were not different between $Prnp^{+/+}$ and $Prnp^{-/-}$ splenocytes after ConA stimulations. However, PMA + Io stimulations enhanced the proliferation rate of $Prnp^{+/+}$ splenocytes compared with $Prnp^{-/-}$ splenocytes. Furthermore, Rikn $Prnp^{-/-}$ splenocytes showed decreased proliferation rates compared with $Prnp^{+/+}$ or Zrch I splenocytes after LPS stimulation. These findings suggest that PrP^C expression is regulated in the T cell, while inhibitory effects of PrPLP/Doppel on cell proliferation were probably induced in the B cell.

Garfin et al. have reported that the LPS-stimulated mitogen-response in splenocytes cultures (derived from experimental scrapie-infected mice) is reduced to 30–60% compared with controls [21]. In the brain of $Rikn^{-/-}$ mice, characteristic necrotic nerve cells were located throughout the cerebral cortex and deeper brain structures. Therefore, the reduced response of B cells may implicate that primary damage has been inflicted on the central nervous system by PrPLP/Doppel. In other words, PrP^C and PrPLP/Doppel might be critically involved in mitogen-responsiveness in T and B cells.

The CD4/CD8 ratios and IL-2 productions before and after mitogen stimulation were also investigated. In the absence and presence of mitogen stimulation, the CD4/CD8 ratios of $Prnp^{+/+}$ and $Prnp^{-/-}$ splenocytes were the same. Although mitogenic stimulations enhanced IL-2 productions, there was no difference between $Prnp^{+/+}$ and $Prnp^{-/-}$ splenocytes. Moreover, PrP^C plays a role in regulating apoptotic cell death in neurons and neuronal cell lines [10,22]. In the present study, we did not investigate differences in the ratio of apoptosis between $Prnp^{+/+}$ and $Prnp^{-/-}$ splenocytes, with and without mitogen-stimulation. PrP^C and PrPLP/Doppel expressions may not play an important role in apoptosis of splenocytes. Treatments with PMA + Io suppress apoptosis both in $Prnp^{+/+}$ and $Prnp^{-/-}$ splenocytes. Mitogen-stimulated T cells may secrete factors suppressing apoptosis. However, further studies on the relationships between PrP^C and cytokines or oxidative stress using lymphocytes are warranted to fully elucidate the role of PrP^C in lymphocytes. In conclusion, our findings suggest that PrP^C expression may have contributed to the regulation and proliferation in lymphocytes.

Through various investigations and clinical correlations, it has become evident that the neuronal and immunological systems are intricately linked via neuro-immunomodula-

tion [23–29]. It is well established that an ablation of specific brain areas is associated with dramatic alterations in immune functions [23,24,30,31], and mechanisms involved in brain-immunoreactions remain to be explored. The findings of receptors specific for hormones and neurotransmitters on the surface membrane of lymphocytes suggest that these soluble mediators may serve as the communicative link. Accumulating evidence has, in fact, advocated that these effects are the results of changes in the specific chemical neuroanatomy of the brain; viz., altered functions of neurotransmitter-containing neurons.

When significant neurodegenerations are induced in the cerebral cortex and deeper brain structures by PrPLP/Doppel, neural alterations would correlate well with the suppressed response of B cells in the spleen. Although this hypothesis remains to be elucidated, the finding of marked modulations of the immune function (derived from discrete lesions) within the central nervous systems indicates that the possible existence of a delicately balanced neural-endocrine network. Availability of a reliable animal model offers an excellent approach to dissecting the intricacies of this 'network' to facilitate further understanding of the regulatory mechanism of immune responses.

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References

[1] S.B. Prusiner, Prions, Proc. Natl. Acad. Sci. USA 95 (1998) 13363–13383.

[2] H. Büeler, A. Aguzzi, A. Sailer, R.A. Greiner, P. Autenried, M. Aguet, C. Weissmann, Mice devoid of PrP are resistant to scrapie, Cell 73 (1993) 1339–1347.

[3] H. Büeler, M. Fischer, Y. Lang, H. Bluethmann, H.P. Lipp, S.J. DeArmond, S.B. Prusiner, M. Aguet, C. Weissmann, Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein, Nature 356 (1992) 577–582.

[4] R.C. Moore, I.Y. Lee, G.L. Silverman, P.M. Harrison, R. Strome, C. Heinrich, A. Karunaratne, S.H. Pasternak, M.A. Chishti, Y. Liang, P. Mastrangelo, K. Wang, A.F. Smit, S. Katamine, G.A. Carlson, F.E. Cohen, S.B. Prusiner, D.W. Melton, P. Tremblay, L.E. Hood, D. Westaway, Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel, J. Mol. Biol. 292 (1999) 797–817.

[5] D. Rossi, A. Cozzio, E. Flechsig, M.A. Klein, T. Rülicke, A. Aguzzi, C. Weissmann, Onset of ataxia and Purkinje cell loss in PrP null mice inversely correlated with Dpl level in brain, EMBO J. 20 (2001) 694–702.

[6] S. Sakaguchi, S. Katamine, N. Nishida, R. Moriuchi, K. Shigematsu, T. Sugimoto, A. Nakatani, Y. Kataoka, T. Houtani, S. Shirabe, H. Okada, S. Hasegawa, T. Miyamoto, T. Noda, Loss of cerebellar Purkinje cells in aged mice homozygous for a disrupted PrP gene, Nature 380 (1996) 528–531.

[7] T. Yokoyama, K.M. Kimura, Y. Ushiki, S. Yamada, A. Morooka, T. Nakashiba, T. Sassa, S. Itohara, In vivo conversion of cellular prion protein to pathogenic isoforms, as monitored by conformation-specific antibodies, J. Biol. Chem. 276 (2001) 11265–11271.

[8] D.R. Brown, B. Schmidt, H.A. Kretzschmar, A neurotoxic prion protein fragment enhances proliferation of microglia but not astrocytes in culture, Glia 18 (1996) 59–67.

[9] G. Forloni, N. Angeretti, R. Chiesa, E. Monzani, M. Salmona, O. Bugiani, F. Tagliavini, Neurotoxicity of a prion protein fragment, Nature 362 (1993) 543–546.

[10] C. Kuwahara, A.M. Takeuchi, T. Nishimura, K. Haraguchi, A. Kubosaki, Y. Matsumoto, K. Saeki, Y. Matsumoto, T. Yokoyama, S. Itohara, T. Onodera, Prions prevent neuronal cell-line death, Nature 400 (1999) 225–226.

[11] P.E. Bendheim, H.R. Brown, R.D. Rudelli, L.J. Scala, N.L. Goller, G.Y. Wen, R.J. Kascsak, N.R. Cashman, D.C. Bolton, Nearly ubiquitous tissue distribution of the scrapie agent precursor protein, Neurology 42 (1992) 149–156.

[12] N.R. Cashman, R. Loertscher, J. Nalbantoglu, I. Shaw, R.J. Kascsak, D.C. Bolton, P.E. Bendheim, Cellular isoform of the scrapie agent protein participates in lymphocyte activation, Cell 61 (1990) 185–192.

[13] J. Collinge, M.A. Whittington, K.C. Sidle, C.J. Smith, M.S. Palmer, A.R. Clarke, J.G. Jefferys, Prion protein is necessary for normal synaptic function, Nature 370 (1994) 295–297.

[14] I. Tobler, S.E. Gaus, T. Deboer, P. Achermann, M. Fischer, T. Rülicke, M. Moser, B. Oesch, P.A. McBride, J.C. Manson, Altered circadian activity rhythms and sleep in mice devoid of prion protein, Nature 380 (1996) 639–642.

[15] A. Kubosaki, S. Yusa, Y. Nasu, T. Nishimura, Y. Nakamura, K. Saeki, Y. Matsumoto, S. Itohara, T. Onodera, Distribution of cellular isoform of prion protein in T lymphocytes and bone marrow, analyzed by wild-type and prion protein gene-deficient mice, Biochem. Biophys. Res. Commun. 282 (2001) 103–107.

[16] Y. Gavrieli, Y. Sherman, S.A. Ben-Sasson, Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation, J. Cell Biol. 119 (1992) 493–501.

[17] A. Sakudo, D.C. Lee, I. Nakamura, Y. Taniuchi, K. Saeki, Y. Matsumoto, S. Itohara, K. Ikuta, T. Onodera, Cell-autonomous PrP-Doppel interaction regulates apoptosis in PrP gene-deficient neuronal cells, Biochem. Biophys. Res. Commun. 333 (2005) 448–454.

[18] H. Hayashi, M. Tanaka, Y. Iwamaru, Y. Ushiki, K.M. Kimura, Y. Tagawa, M. Shinagawa, T. Yokoyama, Effect of tissue deterioration on postmortem BSE diagnosis by immunochemical detection of abnormal isoform of prion protein, J. Vet. Med. Sci. 66 (2004) 515–520.

[19] S.S. Yea, H.M. Kim, Y.J. Jeon, H.M. Oh, H.G. Jeong, K.H. Yang, Suppression of IL-2 and IL-4 gene expression by nodularin through the reduced NF-AT binding activity, Toxicol. Lett. 114 (2000) 215–224.

[20] N.A. Mabbott, K.L. Brown, J. Manson, M.E. Bruce, T-lymphocyte activation and the cellular form of the prion protein, Immunology 92 (1997) 161–165.

[21] D.E. Garfin, D.P. Stites, L.A. Zifnik, S.B. Prusiner, Suppression of polyclonal B cell activation in scrapie-infected C3H/HeJ mice, J. Immunol. 120 (1978) 1986–1990.

[22] Y. Bounhar, Y. Zhang, C.G. Goodyer, A. LeBlanc, Prion protein protects human neurons against Bax-mediated apoptosis, J. Biol. Chem. 276 (2001) 39145–39149.

[23] R.J. Cross, W.R. Markesbery, W.H. Brooks, T.L. Roszman, Hypothalamic immune interactions. I. The acute effect of anterior