ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research ((B)(2) 14370789) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan.

REFERENCES

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72:**248–254.

Chan, B. S., Satriano, J. A., Pucci, M., and Schuster, V. L. (1998). Mechanism of prostaglandin E_2 transport across the plasma membrane of HeLa cells and Xenopus oocytes expressing the prostaglandin transporter "PGT". *J. Biol. Chem.* **273:**6689–6697.

Dehouck, M.-P., Jolliet-Riant, P., Brée, F., Fruchart, J.-C., Cecchelli, R., and Tillement, J.-P. (1992). Drug transfer across the blood–brain barrier: Correlation between in vitro and in vivo models. *J. Neurochem.* **58:**1790–1797.

Ennis, S. R., Ren, X., and Betz, A. L. (1996). Mechanisms of sodium transport at the blood-brain barrier studied with in situ perfusion of rat brain *J. Neruochem.* **66:**756–763.

Follette, P. (2003). Prion disease treatment's early promise unravels. *Science* **299:**191–192.

Friedrich, A., George, R. L., Bridges, C. C., Prasad, P. D., and Ganapathy, V. (2001). Transport of cholin and its relationship to the expression of organic cation transporters in a rat brain microvessel cell line (RBE4). *Biochim. Biophys. Acta* **1512:**299–307.

Friedrich, A., Prasad, P. D., Freyer, D., Ganapathy, V., and Brust, P. (2003). Molecular cloning and functional characterization of the OCTN2 transporter at the RBE4 cells, an in vitro model of the blood-brain barrier. *Brain Res.* **968:**69–79.

Gorboulev, V., Ulzheimer, J. C., Akhoundova, A., Ulzheimer-Teuber, I., Karbach, U., Quester, S., Baumann, C., Lang, F., Busch, A. E., and Koepsell, H. (1997). Cloning and characterization of two human polyspecific organic cation transporters. *DNA Cell Biol.* **16:**871–881.

Grundemann, D., Gorboulev, V., Gambaryan, M., Vehyhl, M., and Koepsell, H. (1994). Drug excretion mediated by a new prototype of polyspecific transporter. *Nature* **372:**549–552.

Kekuda, R., Prasad, P. D., Wu, X., Wang, H., Fei, Y. J., Leibach, F. H., and Ganapathy, V. (1998). Cloning and functional characterization of a potential-sensitive, polyspecific organic cation transporter (OCT3) most abundantly expressed in placenta. *J. Biol. Chem.* **273:**15971–15979.

Koepsell, H. (1998). Organic cation transporters in intestine, kidney, liver, and brain. *Annu. Rev. Physiol.* **60:**243–266.

Korth, C., May, B. C. H., Cohen, F. E., and Prusiner, S. B. (2001). Acridine and phenothiazine derivatives as pharmacotherapeutics for prion disease. *Proc. Natl. Acad. Sci. U.S.A.* **98:**9836–9841.

Maegawa, H., Kato, M., Inui, K., and Hori, R. (1988). pH sensitivity of H^+/organic cation antiport system in rat renal brush-border membranes. *J. Biol. Chem.* **263:**11150–11154.

Miller, D. S., Villalobos, A. R., and Pritchard, J. B. (1999). Organic cation transport in rat choroid plexus cells studied by fluorescence microscopy. *Am. J. Physiol.* **276:**C955–C968.

Okuda, N., Saito, H., Urakami, Y., Takano, M., and Inui, K. I. (1996). cDNA cloning and functional expression of a novel rat kidney organic cation transporter. *Biochem. Biophys. Res. Commun.* **224:**500–507.

Sawada, N., Takanaga, H., Matsuo, H., Naito, M., Tsuruo, T., and Sawada, Y. (1999). Choline uptake by mouse brain capillary endothelial cells in culture. *J. Pharm. Pharmacol.* **51:**847–852.

Sweet, D. H., and Pritchard, J. (1999). rOCT2 is a basolateral potential-driven carrier, not an organic cation/proton exchanger. *Am. J. Physiol.* **277:**F890–F898.

Tamai, I., Ohashi, R., Nezu, J., Sai, Y., Kobayashi, D., Oku, A., Shimane, M., and Tsuji, A. (2000). Molecular and functional characterization of organic cation/carnitine transporter family in mice. *J. Biol. Biochem.* **275:**40064–40072.

Tamai, I., Yabuuchi, H., Nezu, J., Sai, Y., Oku, A., Shimane, M., and Tsuji, A. (1997). Cloning and characterization of a novel human pH-dependent organic cation transporter, OCTN1. *FEBS Lett.* **419:**107–111.

Tatsuta, T., Naito, M., Mikami, K., and Tsuruo, T. (1994). Enhanced expression by the brain matrix of P-glycoprotein in brain capillary endothelial cells. *Cell Growth Differ.* **5:**1145–1152.

Tatsuta, T., Naito, M., Oh-hara, T., Sugawara, I., and Tsuruo, T. (1992). Functional involvement of P-glycoprotein in blood–brain barrier. *J. Biol. Chem.* **267:**20383–20391.

Wu, X., George, R. L., Huang, W., Wang, H., Conway, S. J., Leibach, F. H., and Ganapathy, V. (2000). Structural and functional characteristics and tissue distribution pattern of rat OCTN1, an organic cation transporter, cloned from placenta. *Biochim. Biophys. Acta* **1466**:315–327.

Wu, X., Huang, W., Prasad, P. D., Seth, P., Rajan, D. P., Leibach, F. H., Chen, J., Conway, S. J., and Ganapathy, V. (1999). Functional characteristics and tissue distribution pattern of organic cation transporter 2 (OCTN2), an organic cation/carnitine transporter. *J. Pharmacol. Exp. Ther.* **290**:1482–1492.

Wu, X., Prasad, P. D., Leibach, F. H., and Ganapathy, V. (1998). cDNA sequence, transport function, and genomic organization of human OCTN2, a new member of the organic cation transporter family. *Biochem. Biophys. Res. Commun.* **246**:589–595.

Yabuuchi, H., Tamai, I., Nezu, J., Sakamoto, K., Oku, A., Shimane, M., Sai, Y., and Tsuji, A. (1999). Novel membrane transporter OCTN1 mediates multispecific, bidirectional, and pH-dependent transport of organic cations. *J. Pharmacol. Exp. Ther.* **289**:768–773.

Yamaoka, K., Tanigawara, Y., Nakagawa, T., and Uno, T. (1981). A pharmacokinetic analysis program (MULTI) for microcomputer. *J. Pharmacobiodyn.* **4**:879–885.

Cellular and Molecular Neurobiology, Vol. 24, No. 6, October 2004 (© 2004)

Toxicity of Quinacrine Can Be Reduced By Co-Administration of P-Glycoprotein Inhibitor in Sporadic Creutzfeldt-Jakob Disease

Katsuya Satoh,[1] Susumu Shirabe,[1,5] Katsumi Eguchi,[1] Atsushi Yamauchi,[2] Yasufumi Kataoka,[2] Masami Niwa,[3] Noriyuki Nishida,[4] and Shigeru Katamine[4]

Received March 22, 2004; accepted April 12, 2004

SUMMARY

1. Recent publication has suggested that quinacrine may be a candidate for treatment of Creutzfeldt-Jakob disease (CJD). But serious toxicity of quinacrine to liver and hematological system has been reported.

2. We disclosed the permeability of quinacrine can be enhanced by presence of p-glycoprotein inhibitor at blood–brain barrier in vitro. Therefore, we tried the protocol of combination of quinacrine and p-glycoprotein inhibitor, verapamil for patients with CJD.

3. When compared clinical effects by quinacrine and the combination therapy, improvement of clinical findings was observed at the same level without any adverse effects. Low-dose quinacrine with verapamil can be used as safe treatment of CJD.

KEY WORDS: quinacrine; sporadic Creutzfeldt-Jakob disease; p-glycoprotein inhibitor.

INTRODUCTION

Although there are number of promising agents to control prion protein in vitro or in vivo, no sufficiently safe agent has yet been discovered for patients with Creutzfeldt-Jakob disease (CJD) (Doh-ura *et al.*, 2000).

Quinacrine, originally used as an anti-malaria agent, was reported as a possible agent useful for treatment of CJD (Korth *et al.*, 2001). Recent report found that quinacine might present serious toxicity to the liver and hematological system (Scoazec *et al.*, 2003).

[1] The First Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Science, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.

[2] Department of Pharmaceutical Care and Health Sciences, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jo-nan ku, Fukuoka 812-8582, Japan.

[3] Department of Pharmacology, Nagasaki University Graduate School of Biomedical Science, 1-12-4 Sakamoto, Nagasaki 852-8501, Japan.

[4] Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Science, 1-12-4 Sakamoto, Nagasaki 852-8501, Japan.

[5] To whom correspondence should be addressed at Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Science, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan; e-mail: shirabe@net.nagasaki-u.ac.jp.

0272-4340/04/1200-0873/0

Quinacrine inhibited the accumulation of PrP^{Sc} in cultured infected cells, but did not have an apparent effect on PrP^{C} biosynthesis or turnover.

To develop some method of suppression of the adverse effects of quinacrine, we investigated the mechanism of quinacrine transport across the blood–brain barrier (BBB), and found that the permeability of quinacrine could be enhanced at the BBB by the presence of a p-glycoprotein inhibitor such as verapamil or cyclosporine (Dohgu *et al.*, 2003).

Therefore, we administrated a therapy regimen of combination of 200 mg/day of quinacrine and 120 mg/day of oral verapamil and compared it to one of 300–600 mg/day of quinacrine only.

We administrated quinacrine without verapamil for one patient, 64-year-old female who developed dementia and gait disturbance within two months. She was given 300 mg/day for the first two weeks, then the quantity was increased to 600 mg/day without p-glycoprotein inhibitor. Frequency of myoclonus, gaze, and smile were markedly improved. We stopped quinacrine administration due to liver dysfunction after four weeks. Two other sporadic CJD cases were treated by combination of quinacrine (200 mg/day) and verapamil (120 mg/day). The first case treated with combination therapy was a 71-year-old male, who had developed unstable gait, disorientation, and myoclonus. After two weeks administration of quinacrine and verapamil, frequency of myoclonus was dramatically decreased. Before starting medication, his eyes had rolled aimlessly. He began to gaze at his family and his doctor after the combination of quinacrine and verapamil. However, his symptoms returned to the non-medicated state after eight weeks, although he has been receiving medication.

The second case treated with combination therapy was a 65-year-old male. He was bedridden as a result of cerebellar ataxia and progressive dementia. Action myoclonus was observed. We started combination treatment of quinacrine and verapamil on him. After two weeks, his eye movement and myoclonus had improved markedly though the improvement was temporal. These three patients were diagnosed as possible CJD by based on clinical criteria of World Health Organization, diffusion-weighted MRI, and 14-3-3 protein in cerebrospinal fluid (CSF).

To determine whether quinacrine could be sufficiently transported to the brain, we measured the concentration of quinacrine in CSF at 4 weeks after administration of case in 2 and case 3 (Table I). Concentrations of quinacrine in CSF were measured by high-performance liquid chromatography method as described previously (Björkman and Elisson, 1987). The concentration of quinacrine in CSF, supposed to

Table I. Effects and Adverse Effects of Quinacrine in Patients with CJD

						Clinical effects	
Case	Co-adminisration	Conceratation of quinacrine in CSF	AST level[a]	Hematological dysfunction	Skin color change	Frequency of myoclonus	Improvement of gaze and smile
1	None	ND	158	—	+	Decreased	+
2	Verapamil	392 nM	24	—	+	Decreased	+
3	Verapamil	226 nM	53	—	+	Decreased	+

Note. Plus symbol shows that each patient has the indicated findings.
[a] ALT; peak data under quinacrine administration.

be approximately equal to the concentration of quinacrine in experimental treatment in vitro approx. 200–400 nM (Korth *et al.*, 2001).

When the clinical effects on the first patient were compared with the other two patients (combination of 200 mg/day of quinacrine and 120 mg/day of verapamil), improvement of the clinical findings in patients receiving a combination of low dose quinacrine and verapamil was observed to be approximately equal to the level improvement seen in the patient receiving quinacrine only. In two patients treated with the combination of low-dose quinacrine and verapamil, no liver dysfunction and hematological toxicity was observed. Although French National Surveillance Network of Prion Diseases recommended to use quinacrine 1000 mg the first day, then 300 mg each day, we conclude that low-dose quinacrine can be used as a safe and effective treatment of CJD when given in combination with a p-glycoprotein inhibitor such as verapamil.

REFERENCES

Doh-ura, K., Mekada, E., Ogomori, K., and Iwaki, T. (2000). Enhanced CD9 expression in the mouse and human brains infected with transmissible spongiform encephalopathies. *J. Neuropathol. Exp. Neurol.* **59**(9):774–785.

Korth, C., May, B. C., Cohen, F. E., and Prusiner, S. B. (2001). Acridine and phenothiazine derivatives as pharmacotherapeutics for prion disease. *Proc. Natl. Acad. Sci.* **98**(17):9836–9841.

Scoazec, J. Y., Krolak-Salmon, P., Casez, O., Besson, G., Thobois, S., Kopp, N., *et al.* (2003). Quinacrine-induced cytolytic hepatitis in sporadic Creutzfeldt-Jakob disease. *Ann. Neurol.* **53**(4):546–547.

Dohgu, S., Yamauchi, A., Takata, F., Sawada, Y., Higuchi, S., Naito, M., Tsuruo, T., Shirabe, S., Niwa, M., Katamine, S., and Kataoka, Y. (2003). Uptake and Efflux of Quinacrine, a Candidate for the Treatment of Prion Diseases at the Blood-Brain Barrier. *Cell Mol. Neurobiol.* (in press).

Björkman, S., and Elisson, L. O. (1987). Determination of quinacrine (mepacrine) in plasma by high-performance liquid chromatography with fluorimetric detection. *J Chromatograph* **420**:341–348.

EXCLI Journal 2004;3:82-90 – ISSN 1611-2156
received: 19. October 2004, accepted: 27. October 2004, published: 28. October 2004

Original article:

The Absence of Prion-Like Infectivity in Mice expressing Prion Protein-Like Protein

Ryuichiro Atarashi[1,4], Suehiro Sakaguchi[1,3,4,*], Kazuto Shigematsu[2], Shigeru Katamine[1]

[1]Department of Molecular Microbiology and Immunology, [2]Department of Pathology, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-12-4, Nagasaki 852-8523, JAPAN, Phone: +81-95-849-7059, Fax: +81-95-849-7060, [3]PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama, Japan, [4]These authors contributed equally. E-mail: suehiros-ngs@umin.ac.jp (*corresponding author)

ABSTRACT

Cellular prion protein, PrP^{C}, undergoes pathogenic structural conversion into the proteinase K (PK)-resistant isoform, PrP^{Sc}, to constitute a nucleic acid-free infectious agent, so called a prion. To determine whether a recently identified PrP-like protein, named PrPLP/Dpl, could also be transformed to a prion-like protein, we intracerebrally inoculated a mouse-adapted Fukuoka-1 prion into Ngsk and Zrch I mice either homozygously ($Prnp^{0/0}$) or heterozygously ($Prnp^{0/+}$) devoid of PrP^{C}. Only the former expressed PrPLP/Dpl ectopically in the brains, particularly in neurons. Ngsk $Prnp^{0/+}$ and Zrch I $Prnp^{0/+}$ mice similarly developed the disease. The diseased Ngsk $Prnp^{0/+}$ mice transmitted the disease to the mice expressing PrP^{C} but not to the mice expressing PrPLP/Dpl, showing abundant accumulation of PrP^{Sc} but not PK-resistant PrPLP/Dpl in the brains. Moreover, the inoculated Ngsk $Prnp^{0/0}$ mice neither developed the disease nor produced any infectivity transmissible to PrPLP/Dpl-expressing mice. These results indicate that PrPLP/Dpl have no potential to undergo pathogenic conversion to form a prion-like infectious particle.

Key words: Prion protein, prion protein-like protein, prion, knockout mice

INTRODUCTION

Transmissible spongiform encephalopathies, or prion diseases including Creutzfeldt-Jakob disease in humans and scrapie in animals, are infectious neurodegenerative disorders characterized by deposition of an abnormally folded, proteinase K (PK)-resistant isoform of prion protein, PrP^{Sc}, in the central nervous system (Prusiner, 1998). PrP^{Sc} is a highly fibrinogenic protein generated by the structural conversion of the normal PK-sensitive PrP (PrP^{C}), a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein abundantly expressed by neurons (Prusiner, 1998). Mice

devoid of PrP^C ($Prnp^{0/0}$) were resistant to the diseases without accumulation of PrP^{Sc} or propagation of infectious agents, or prions, in the brain (Bueler et al., 1993, Prusiner et al., 1993, Sakaguchi et al., 1995), strongly arguing for the "protein-only" hypothesis (Prusiner, 1998). This hypothesis postulates that a prion is mainly composed of PrP^{Sc} and propagates via the pathogenic conversion of PrP^C into PrP^{Sc} (Prusiner, 1998). Intermolecular interaction of these two molecules is thought to be essential in the conversion (Prusiner, 1998).

The gene designated *Prnd* encoding a PrP-like protein, PrPLP/Doppel (Dpl), was recently identified 16 kb downstream of the murine PrP gene, *Prnp* (Li et al., 2000, Moore et al., 1999). PrPLP/Dpl is also a GPI-anchored glycoprotein of the raft membrane and shares 23% identical amino acids with PrP (Li et al., 2000, Moore et al., 1999). Its protein structure is also extremely similar to that of the C-terminal two-third of PrP^C, comprising three α-helices and two short β-strands (Mo et al., 2001). These topological and structural similarities of the two proteins raised the question of whether PrPLP/Dpl could modify the pathogenesis of prion diseases by interfering with the interaction between PrP^C and PrP^{Sc}. Moore et al. and Tuzi et al. recently showed that PrPLP/Dpl had no potential to affect pathogenesis by demonstrating that mice expressing PrPLP/Dpl ectopically in the brains developed the disease with incubation periods and pathologies indistinguishable from those in control mice (Moore et al., 2001, Tuzi et al., 2002). However, another intriguing question remains to be addressed, that is, whether PrPLP/Dpl itself can undergo pathogenic conversion and become an infectious isoform that can propagate like a prion.

In the present study, we inoculated a mouse-adapted Fukuoka-1 prion into Ngsk $Prnp^{0/0}$ and Ngsk $Prnp^{0/+}$ mice, both ectopically expressing PrPLP/Dpl in the brain due to an unusual intergenic RNA splicing conducted on the Ngsk targeted allele (Li et al., 2000), and then examined whether PrPLP/Dpl-associated infectivity could be generated in these mice by inoculating the brain homogenates of these mice into Ngsk $Prnp^{0/0}$ mice expressing PrPLP/Dpl only.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Japan SLC. Ngsk $Prnp^{0/0}$ and Zrch I $Prnp^{0/0}$ mice were generated as previously described (Bueler et al., 1992, Sakaguchi et al., 1995). To produce Ngsk $Prnp^{0/+}$ and Zrch I $Prnp^{0/+}$ mice, Ngsk $Prnp^{0/0}$ and Zrch I $Prnp^{0/0}$ mice were intercrossed with C57BL/6 mice, respectively. The mice were handled in accordance with the Guidelines of Animal Experimentation of Nagasaki University.

Prion Inoculation

10% (w/v) brain homogenates of mice suffering from terminal disease after inoculation of a Fukuoka-1 prion (kindly provided by Dr. Tateishi) were prepared in phosphate-buffered saline (PBS), and an aliquot (20 μl) of the homogenates was intracerebrally inoculated into each mouse under anesthetization by the inhalation of diethyl ether (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Western Blotting

Total proteins extracted from mouse brains in a buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA) were treated with or without 20 μg/ml PK (Wako Pure Chemical Industries) at 37°C for 30 min, and then boiled in the presence of Laemmli's buffer for 10 min to halt the PK digestion. The proteins were separated on 12% SDS-polyacrylamide gel and electrically transferred onto a nitrocellulose membrane (Millipore Corporation, Bedford, MA). After 1 hr blocking at room temperature (RT) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20) containing 5% dry skim milk, the membrane was incubated with an indicated first antibody in TBST containing 1% dry skim milk for 2 hr at RT. Anti-PrP and anti-PrPLP/Dpl sera were raised against recombinant mouse PrP23-231 in Ngsk *Prnp*$^{0/0}$ mice and recombinant mouse PrPLP/Dpl24-154 in rabbit, respectively. The immunocomplexes were detected by using horseradish peroxidase-conjugated antibodies (Amersham Pharmacia Biotech, Buckinghamshire, UK) and an ECL system (Amersham Pharmacia Biotech).

Immunohistochemistry

Deparaffinized sections were digested with 1 mg/ml trypsin for 15 min at 37°C, and then placed in 3% H_2O_2 in methanol for 30 min at room temperature to eliminate endogenous peroxidase activity. After treatment with normal rabbit serum for 30 min, the tissue sections were incubated overnight at 4°C with anti-GFAP (1:50 [Dako, Kyoto, Japan]). To detect the glial fibrillary acidic protein (GFAP) immunoreactivity, we used the Polymer-Immuno Complex method in accordance with the manufacturer's recommendations (Dako). The antibody-bound peroxidase was revealed with 0.04 % diamino-benzidine (Sigma Chemical Co., St. Louis, MO).

RESULTS

A mouse-adapted Fukuoka-1 prion was inoculated intracerebrally into Ngsk *Prnp*$^{0/0}$, Ngsk *Prnp*$^{0/+}$, and Zrch I *Prnp*$^{0/+}$ mice. The former two mouse lines expressed PrPLP/Dpl in the brains, particularly in neurons, but the latter did not (Li et al., 2000). In keeping with our previous report (Sakaguchi et al., 1995), none of the Ngsk *Prnp*$^{0/0}$ mice exhibited the disease-specific neurological symptoms for up to 600 days post-inoculation (p.i.) (Table 1). By contrast, all of the Ngsk *Prnp*$^{0/+}$ and Zrch I *Prnp*$^{0/+}$ mice developed the disease with similar incubation times of 282.3±22.5 and 286.4±27.0 days p.i., respectively (Table 1), and with similar clinical symptoms such as body weight loss, greasy yellowish hair, kyphosis, and flaccid paralysis of legs. Comparable levels of PrP^{Sc} with the same patterns of glycosylation were detected in the brains of these diseased mice on Western blotting (Figure 1). Histological findings in the affected brain tissues were also indistinguishable between the two lines of mice: the brains were markedly atrophic (data not shown) and many vacuoles were detectable mainly in the cerebral cortex and hippocampus (Figure 2, upper panels). Moreover, hypertrophic astrocytes with strong immunoreactivities of GFAP were similarly infiltrated in the same brain regions of these mice on immunohistochemical examinations (Figure 2, lower panels).

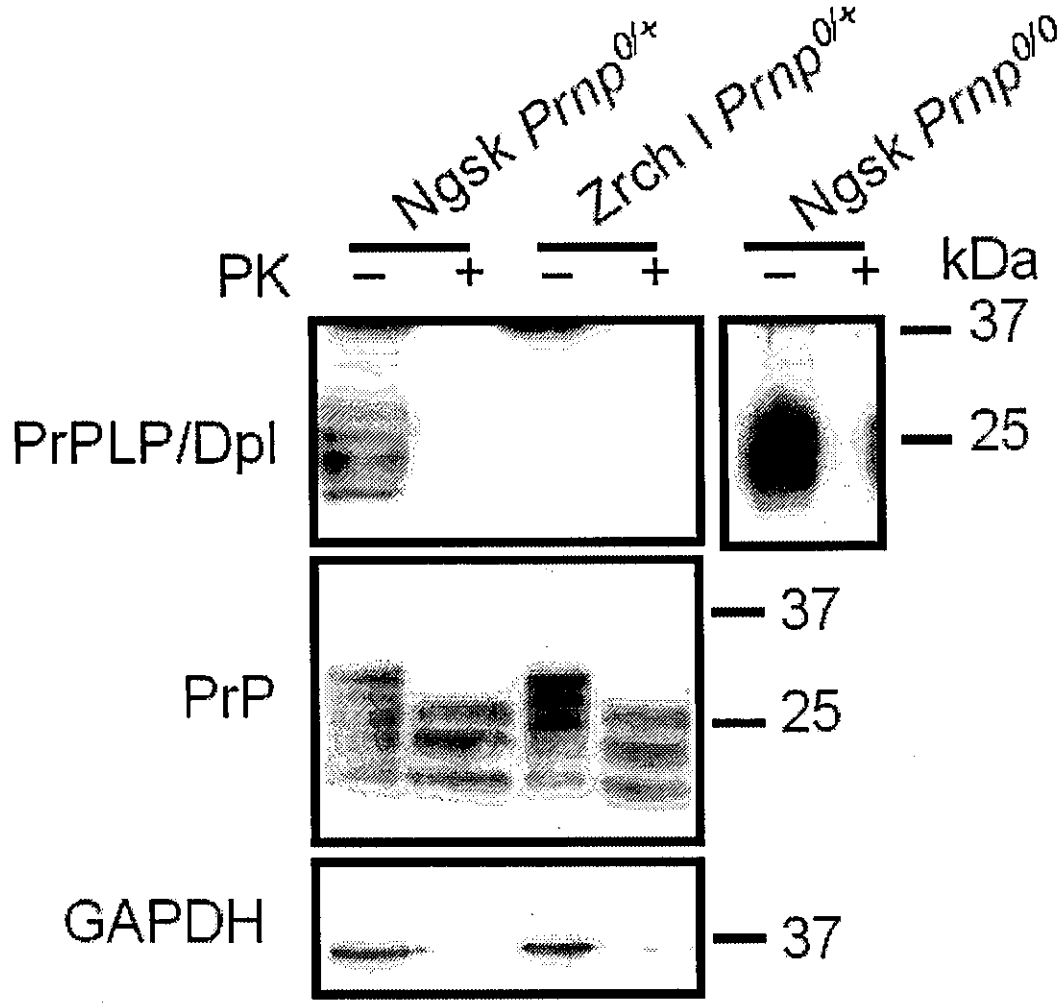

Figure 1: Western blotting of the brains of Ngsk *Prnp*$^{0/+}$ and Zrch I *Prnp*$^{0/+}$ mice with neurological symptoms and of Ngsk *Prnp*$^{0/0}$ mice 360 days after inoculation with the Fukuoka-1 prion. The brain homogenates treated with (+) or without (-) proteinase K (PK) were probed by rabbit antiserum against recombinant mouse PrPLP/Dpl, mouse antiserum against recombinant mouse PrP, and mouse monoclonal antibody against rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH; HyTest, Turku, Finland).

PrP^C undergoes conformational conversion into a PK-resistant PrP, PrP^{Sc}, and mediates the prion transmission. It has been suggested that PrP^C is also converted into a PK-sensitive but infectious intermediate form of PrP, termed PrP*, and that it supports the prion propagation (Aguzzi and Weissmann, 1997). To determine whether PrPLP/Dpl could be similarly transformed into a PK-resistant or a PK-sensitive but infectious intermediate isoform, we first subjected the brains of both the diseased Ngsk *Prnp*$^{0/+}$ and the Ngsk *Prnp*$^{0/0}$ mice sacrificed at 360 days p.i. to Western blotting. On Western blotting,

anti-PrP antibodies detected PK-resistant PrP^{Sc} abundantly accumulated in the brains of Ngsk *Prnp*$^{0/+}$ mice (Figure 1), but not in the Ngsk *Prnp*$^{0/0}$ mice (data not shown). By contrast, the PrPLP/Dpl detectable in the brains of these mice by anti-PrPLP/Dpl antibodies raised against recombinant mouse PrPLP/Dpl in rabbit was digested to an undetectable level by the PK treatment (Figure 1).

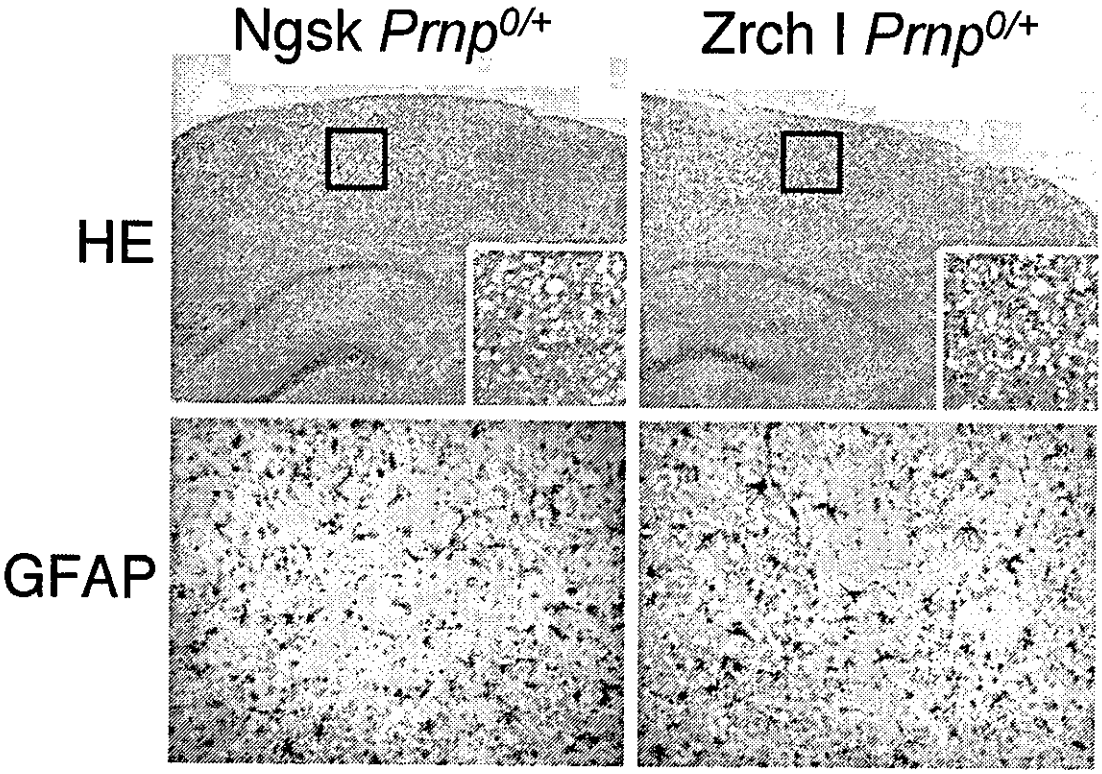

Figure 2: Hematoxylin-eosin staining (upper panel) and GFAP immunohistochemical staining (lower panel) of the brains of Ngsk *Prnp*$^{0/+}$ and Zrch I *Prnp*$^{0/+}$ mice with neurological symptoms after inoculation with the Fukuoka-1 prion. (Magnification, ×12.5; Inset magnification, ×50).

We next inoculated the brain homogenate of the diseased Ngsk *Prnp*$^{0/+}$ mice intracerebrally into wild-type, Ngsk *Prnp*$^{0/+}$ and Ngsk *Prnp*$^{0/0}$ mice to determine whether PrPLP/Dpl could be converted into a prion-like infectious protein. All of the wild-type and Ngsk *Prnp*$^{0/+}$ mice developed the disease at 161±11 and 269±27 days p.i., respectively (Table 1). The clinical symptoms in these mice were indistinguishable from those of the corresponding mice inoculated by the brain homogenates of infected wild-type mice (data not shown). By contrast, none of the Ngsk *Prnp*$^{0/0}$ mice exhibited disease-specific symptoms for up to 600 days p.i. (Table 1). Additionally, we inoculated the brain homogenate from the Ngsk *Prnp*$^{0/0}$ mice sacrificed at 112 weeks p.i. into indicator Ngsk *Prnp*$^{0/0}$ mice. However, no disease-specific neurological symptoms could be detected in the indicator mice for up to 600 days p.i. (Table 1).

Table 1. No PrPLP/Dpl-associated infectivity in Ngsk *Prnp*$^{0/+}$ and Ngsk *Prnp*$^{0/0}$ mice

donor mouse	recipient mouse	diseased mice /inoculated mice	incubation time (mean±SE; days)
wild-type (diseased)	Ngsk *Prnp*$^{0/0}$	0/5	>600
	Ngsk *Prnp*$^{0/+}$	5/5	282.3±22.5
	Zrch I *Prnp*$^{0/+}$	5/5	286.4±27.0
Ngsk *Prnp*$^{0/+}$ (diseased)	wild-type	7/7	161.4±10.9
	Ngsk *Prnp*$^{0/+}$	8/8	296.3±12.1
	Ngsk *Prnp*$^{0/0}$	0/5	>600
Ngsk *Prnp*$^{0/0}$ (112-wk p.i.)	Ngsk *Prnp*$^{0/0}$	0/5	>600

DISCUSSION

In the present study, we first showed that Ngsk *Prnp*$^{0/+}$ mice expressing PrPLP/Dpl ectopically in neurons succumbed to the disease after the infection of a Fukuoka-1 prion with incubation periods identical to that of Zrch I *Prnp*$^{0/+}$ mice expressing no PrPLP/Dpl. PrP^{Sc} was similarly accumulated in the brains of both lines of *Prnp*$^{0/+}$ mice, and pathological changes such as vacuolation and astrocyte infiltration patterns were indistinguishable between them. These findings are consistent with the results reported previously by Moore et al. (Moore et al., 2001) and Tuzi et al. (Tuzi et al., 2002), indicating that PrPLP/Dpl has no potential to affect the pathogenesis of prion disease. By contrast, Ngsk *Prnp*$^{0/0}$ mice were free of the disease for up to 600 days p.i. with no accumulation of PrP^{Sc} in the brains after inoculation of the prion, confirming that PrP^{C} is crucial for the pathogenesis of the diseases via its conversion into PrP^{Sc}.

The pathogenesis of prion diseases is yet to be elucidated. *Prnp*$^{0/0}$ mice were shown to spontaneously develop neurological abnormalities similar to those often observed in prion diseases, including impairment of memory and learning, alteration of circadian rhythms, and demyelination in the spinal cord and peripheral nerves (Collinge et al., 1994, Nishida et al., 1999, Tobler et al., 1996), strongly suggesting that the functional loss of PrP^{C} is involved in the pathogenesis of the diseases at least in part. Unlike the accumulation of PrP^{Sc}, the constitutive conversion of PrP^{C} into PrP^{Sc} is thought to cause the reduction of PrP^{C} in the affected brain, which in turn might lead to the functional impairment of PrP^{C}. It was recently shown that PrPLP/Dpl ectopically expressed in neurons impairs the function of PrP^{C}, causing neuronal cell death, particularly in Purkinje cells, granule cells, and hippocampal pyramidal cells (Anderson et al., 2004, Moore et al., 2001, Yamaguchi et al., 2004). In the present study, no exacerbation of prion pathogenesis was demonstrated in Ngsk *Prnp*$^{0/+}$ mice expressing PrPLP/Dpl in neurons as compared with Zrch I *Prnp*$^{0/+}$ mice

expressing no PrPLP/Dpl. This result seems to refute the idea that some aspects of the prion pathogenesis are attributable to the functional loss of PrP^C. We cannot, however, rule out the possibility that PrP^C is functionally inactivated in prion diseases in a way different from that of PrPLP/Dpl.

We next showed that, in contrast to PrP^{Sc} abundantly accumulated in the brains of the diseased Ngsk *Prnp*$^{0/+}$ mice, no PK-resistant PrPLP/Dpl could be detected in the brains of these mice nor in the Ngsk *Prnp*$^{0/0}$ mice sacrificed at 360 days p.i. These results were consistent with those of Tuzi et al. (Tuzi et al., 2002). We further showed that intracerebral inoculation of the brain homogenates of the diseased Ngsk *Prnp*$^{0/+}$ mice resulted in the disease only in mice expressing PrP^C, such as wild-type and Ngsk *Prnp*$^{0/+}$ mice, but not in Ngsk *Prnp*$^{0/0}$ mice which express PrPLP/Dpl but are devoid of PrP^C. Similarly, no disease-specific symptoms were detected in indicator Ngsk *Prnp*$^{0/0}$ mice, which were intracerebrally inoculated with the brain homogenates of the Ngsk *Prnp*$^{0/0}$ mice sacrificed at 112 weeks p.i.. The homologous combination of PrP^C and PrP^{Sc} is important for the efficient transmission of prions. Thus, taken together with the finding that PK-resistant PrPLP/Dpl were undetected in the diseased Ngsk *Prnp*$^{0/+}$ mice and the Ngsk *Prnp*$^{0/0}$ mice sacrificed at 360 days p.i., this unsuccessful transmission of the disease from these mice into Ngsk *Prnp*$^{0/0}$ mice strongly indicates that PrPLP/Dpl does not undergo pathogenic conversion to a prion-like infectious protein. On the contrary, the disease transmission is dependent on the expression of PrP^C in the recipient mice and the presence of PrP^{Sc} in the inocula, reaffirming the central role of PrP in prion diseases.

Behrens et al. previously showed that PrPLP/Dpl is not indispensable to either the pathogenesis or the PrP^{Sc} generation by demonstrating that scrapie prions induced typical features of prion pathology including PrP^{Sc} accumulation in neuronal grafts derived from embryonic stem cells homozygous for a disrupted *Prnd* (Behrens et al., 2001). PrPLP/Dpl is the first identified host protein exhibiting high similarities in amino acid composition and protein conformation to PrP. No other PrP-like proteins have been reported to date. Together with these results, our findings strongly indicate that prion diseases are disorders specifically associated with the pathogenic conformation of PrP.

Acknowledgement: This study was supported in part by a Research for Comprehensive Promotion of Study of Brain and a Grand-in-Aid for Scientific Research on Priority Areas – Advanced Brain Science Project – from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by a Research on Specific Diseases from the Ministry of Health, Labour and Welfare, Japan.

REFERENCES

Aguzzi A ,Weissmann C. Prion research: the next frontiers. *Nature* 1997;389:795-8

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 U S A* 2004;101:3644-9

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;2:347-52

Bueler H, Fischer M, Lang Y, Bluethmann H, Lipp HP, DeArmond SJ, Prusiner SB, Aguet M ,Weissmann C. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature* 1992;356:577-82

Bueler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, Aguet M ,Weissmann C. Mice devoid of PrP are resistant to scrapie. *Cell* 1993;73:1339-47

Collinge J, Whittington MA, Sidle KC, Smith CJ, Palmer MS, Clarke AR ,Jefferys JG. Prion protein is necessary for normal synaptic function. *Nature* 1994;370:295-7

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;20:553-67

Mo H, Moore RC, Cohen FE, Westaway D, Prusiner SB, Wright PE ,Dyson HJ. Two different neurodegenerative diseases caused by proteins with similar structures. *Proc Natl Acad Sci U S A* 2001;98:2352-7

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

Moore RC, Mastrangelo P, Bouzamondo E, Heinrich C, Legname G, Prusiner SB, Hood L, Westaway D, DeArmond SJ, Tremblay P. Doppel-induced cerebellar degeneration in transgenic mice. *Proc Natl Acad Sci U S A* 2001;98:15288-93

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;79:689-97

Prusiner SB, Groth D, Serban A, Koehler R, Foster D, Torchia M, Burton D, Yang SL ,DeArmond SJ. 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;90:10608-12

Prusiner SB. Prions. *Proc Natl Acad Sci U S A* 1998;95:13363-83

Sakaguchi S, Katamine S, Shigematsu K, Nakatani A, Moriuchi R, Nishida N, Kurokawa K, Nakaoke R, Sato H, Jishage K ,et al. Accumulation of proteinase K-resistant prion protein (PrP) is restricted by the expression level of normal PrP in mice inoculated with a mouse-adapted strain of the Creutzfeldt-Jakob disease agent. *J Virol* 1995;69:7586-92

Tobler I, Gaus SE, Deboer T, Achermann P, Fischer M, Rulicke T, Moser M, Oesch B, McBride PA ,Manson JC. Altered circadian activity rhythms and sleep in mice devoid of

prion protein. *Nature* 1996;380:639-42

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;83:705-11

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;319:1247-52

Neuropathology and Applied Neurobiology (2005), **31**, 80–87 doi: 10.1111/j.1365-2990.2004.00592.x

Fatal familial insomnia with an unusual prion protein deposition pattern: an autopsy report with an experimental transmission study

K. Sasaki*, K. Doh-ura*, Y. Wakisaka*, H. Tomodat and T. Iwaki*

**Department of Neuropathology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, and †Department of Neurology, Imazu Red Cross Hospital, Imazu, Fukuoka, Japan*

K. Sasaki, K. Doh-ura, Y. Wakisaka, H. Tomoda and T. Iwaki (2005) *Neuropathology and Applied Neurobiology* 31, 80–87

Fatal familial insomnia with an unusual prion protein deposition pattern: an autopsy report with an experimental transmission study

We recently performed a *post-mortem* examination on a Japanese patient who had a prion protein gene mutation responsible for fatal familial insomnia (FFI). The patient initially developed cerebellar ataxia, but finally demonstrated insomnia, hyperkinetic delirium, autonomic signs and myoclonus in the late stage of the illness. Histological examination revealed marked neuronal loss in the thalamus and inferior olivary nucleus; however, prion protein (PrP) deposition was not proved in these lesions by immunohistochemistry. Instead, PrP deposition and spongiform change were both conspicuous within the cerebral cortex, whereas particular PrP deposition was also observed within the cerebellar cortex. The abnormal protease-resistant PrP (PrP^{res}) molecules in the cerebral cortex of this case revealed PrP^{res} type 2 pattern and were compatible with those of FFI cases, but the transmission study demonstrated that a pathogen in this case was different from that in a case with classical FFI. By inoculation with homogenate made from the cerebral cortex, the disease was transmitted to mice, and neuropathological features that were distinguishable from those previously reported were noted. These findings indicate the possibility that a discrete pathogen was involved in the disease in this case. We suggest that not only the genotype of the PrP gene and some other as yet unknown genetic factors, but also the variation in pathogen strains might be responsible for the varying clinical and pathological features of this disease.

Keywords: Creutzfeldt-Jakob disease, NZW mouse, prion disease, thalamic form, transmissible spongiform encephalopathy

Introduction

Fatal familial insomnia (FFI) is one of the disease entities of prion disease or transmissible spongiform encephalopathy (TSE) and it is linked to a mutation at codon 178 of the prion protein gene (PRNP), aspartic acid to asparagine substitution (D178N), in conjunction with methionine at the polymorphic position 129 of the mutant allele [1]. The neuropathological hallmark of FFI is the predominance of lesions within the thalamus [2]. Clinically this disorder is characterized by progressive insomnia, dysautonomia and motor signs [3]. The D178N mutation is also associated with familial Creutzfeldt-Jakob disease (CJD). The disease phenotypes have been considered to depend on the polymorphism at codon 129 of the mutant allele, methionine (129Met) in FFI and valine (129Val) in CJD [4]. However, the FFI genotype reveals diverse clinical expression including cerebellar ataxia, dementia and autonomic abnormalities with or without insomnia [5,6]. In Japan, one FFI case [7] and some cases of the 'sporadic'

Correspondence: Kensuke Sasaki, Department of Neuropathology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan. Tel: +81-92-6425539; Fax: +81-92-6425540; E-mail: ksasaki@np.med.kyushu-u.ac.jp

thalamic form of CJD [8,9] have been reported, and these have indicated a discrepancy between PRNP genotype and the disease phenotype.

We recently performed a *post-mortem* examination on a Japanese patient with a 27-month history of familial prion disease with PRNP D178N-129Met mutation. The clinical data on this patient and his family have been published in part [10]. Here we report additional clinical data and *post-mortem* neuropathological findings, as well as findings in mice infected with the patient's material.

Case report

The pedigree is presented in Figure 1. In October 1997, a 50-year-old Japanese man (Patient II-5) developed an unsteady gait, followed within a month by difficulty in speech. Although these symptoms worsened rapidly, he did not immediately develop either dementia or insomnia. He was admitted to a hospital for neurological evaluation in February 1998, and PRNP D178N-129Met mutation (heterozygous for 129Met/Val) was revealed as previously reported [10]. From October 1998, either insomnia or delirium was clearly apparent. Hyperthermia without any signs indicative of infection or inflammation, thus suggesting an autonomic sign, was also observed. He often showed reality disturbance and restlessness. He became progressively demented, developed trismus, myoclonus and horizontal nystagmus, and demonstrated increased muscle tone. Finally he became bedridden with flexion contracture. Brain computed tomography revealed mild atrophy of the cerebellum and brainstem. Electroencephalograms showed a background of 9 Hz diffuse α activities, but periodic synchronous discharges were not detected during the clinical course. Sleep activities with rapid eye movements were not recorded in sleep electroencephalograms. In January 2000, he died of pneumonia at the age of 52 years, about 27 months after the onset of disease.

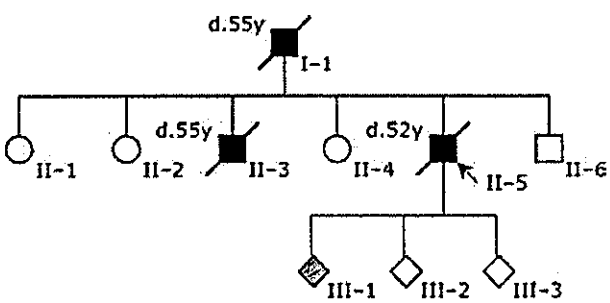

Figure 1. Family tree of the present pedigree. Patients who developed rapidly progressive cerebellar ataxia are depicted by closed symbols, along with their age at death (years old). One of the children of the present case has fatal familial insomnia genotype D178N-129Met/Met (grey symbol).

Patient II-3, one of the brothers of Patient II-5, also showed rapidly progressive cerebellar ataxia. He developed an ataxic gait, forgetfulness and dysarthria at the age of 55 years. Brain computed tomography demonstrated moderate cerebellar atrophy, and electroencephalograms showed diffuse intermittent slow activities without periodic synchronous discharges. He developed myoclonic jerks, akinetic mutism with a decorticate posture, and died 7 months after the onset. Patient I-1, the father of Patients II-5 and II-3, had also developed an ataxic gait and dementia at the age of 55 years. He died of unknown causes after a clinical course of 12 months. Neither autopsy nor PRNP analysis was carried out in either Patient I-1 or Patient II-3. One of the children of Patient II-5 was revealed to have PRNP D178N-129Met mutation (homozygous for 129Met).

Materials and methods

Autopsy was performed 6 h *post-mortem*. A frontal tip of the right cerebral hemisphere and a cerebellar tip were sampled and frozen for Western blot analysis. The remaining brain was immersion-fixed in 10% formalin for 2 weeks. Tissue blocks were immersed in 98% formic acid for 1 h and paraffin-embedded. Hematoxylin and eosin (HE) stain, Klüver-Barrera stain and Bodian's method were performed on 7-μm-thick sections. Immunohistochemical analyses were performed by a standard indirect method for glial fibrillary acidic protein (GFAP) (polyclonal, Dako, Denmark, or monoclonal, clone G-A-5, Roche, Switzerland), ferritin (polyclonal, Dako), β-amyloid precursor protein (APP) (monoclonal, clone LN27, Zymed, USA), SNAP-25 (monoclonal, clone MAB331, Chemicon, USA) and prion protein (PrP) (monoclonal, clone 3F4, Senetek, USA). For anti-PrP immunohistochemistry, sections were pretreated with hydrolytic autoclaving as previously reported [11]. Western blot analysis for protease-resistant PrP (PrP^{res}) was performed using frontal cortical and cerebellar tissue tips from this case, applying phosphotungstic acid precipitation of PrP^{res} as described previously [12] with 50 μg/ml proteinase K (PK) digestion, along with a control case with sporadic

CJD (77-year-old man, duration of illness was 9 months, 129Met/Met). Transmission study was performed as described previously [13]. Briefly, frontal cortical tissue tips were aseptically homogenized with nine volumes of saline, and after removal of debris by low-speed centrifugation the supernatant was used as 10% homogenate. Twenty microliters of 10% homogenate were injected intracerebrally into female NZW mice or female Tg7 mice expressing hamster PrP but not endogenous murine PrP. Sections of infected mice were analysed by HE stain and also by immunohistochemistry for PrP (polyclonal, PrP-C, IBL, Japan) and GFAP (clone G-A-5, Roche). Permission for the animal experiments was obtained from the Animal Experiment Committee of Kyushu University.

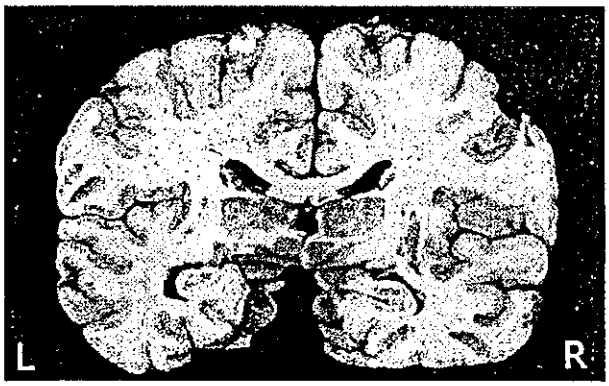

Figure 2. Coronal section at the thalamic level. The medial part of the thalamus is atrophic and the third ventricle is dilated.

Results

The brain weighed 1350 g before fixation. The cerebellum showed slight atrophy, whereas the volume of the forebrain was preserved. Coronal sections showed atrophy of the medial part of the thalamus and symmetrical dilatation of the third ventricle (Figure 2).

The summary of histological examination is shown in Figure 3. Marked neuronal loss and moderate astrogliosis in the thalamus were observed, most prominently in its centromedial nucleus and dorsomedial nucleus. However, spongiform change was imperceptible (Figure 4A,B). Neuronal loss and gliosis in the medial portion of the inferior olivary nucleus were also apparent (Figure 4C,D). In the cerebellum there was mild loss of granular cells, and the molecular layer was slightly atrophic. There were localized lesions of spongiosis in the cerebellar molecular layer. Purkinje's cells appeared not to be decreased in number, but they often demonstrated shrunken features. The cerebellar white matter showed diffuse myelin pallor. The cerebral cortex showed uneven distribution of spongiform change and neuronal loss (Figure 4E). There was no apparent difference in the intensities of the cortical lesions among the lobes of the cerebrum except that the lesions are more prominent in the entorhinal cortex and less in the occipital lobe. Moderate astrogliosis was associated with the spongiform lesions (Figure 4F).

Immunohistochemistry for PrP revealed that there was no punctate or plaque-type immunoreactivity in the thal-

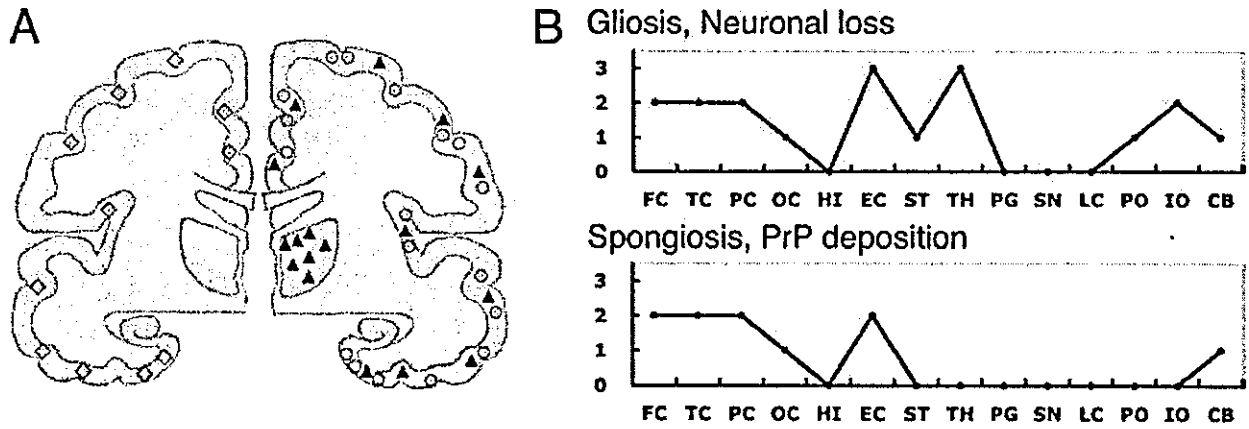

Figure 3. Lesion profiles of the present case. A: schematic drawing of the distribution of prion protein (PrP) deposition (diamonds), spongiosis (circles) and neuronal loss (triangles) in this case, which can be compared to that of fatal familial insomnia (FFI) and 178CJD shown in ref. [19]. B: lesion profiles in respect to gliosis/neuronal loss and spongiosis/PrP deposition. Brain regions studied were: frontal cortex (FC), temporal cortex (TC), parietal cortex (PC), occipital lobe (OC), hippocampus (HI), entorhinal cortex (EC), striatum (ST), thalamus (TH), substantia nigra (SN), periaqueductal grey (PG), pons (PO), locus ceruleus (LC), medulla oblongata (ME), cerebellum (CB). The vertical axis is the degree of lesion graded as follows. 0: not detectable; 1: mild; 2: moderate; 3: severe.

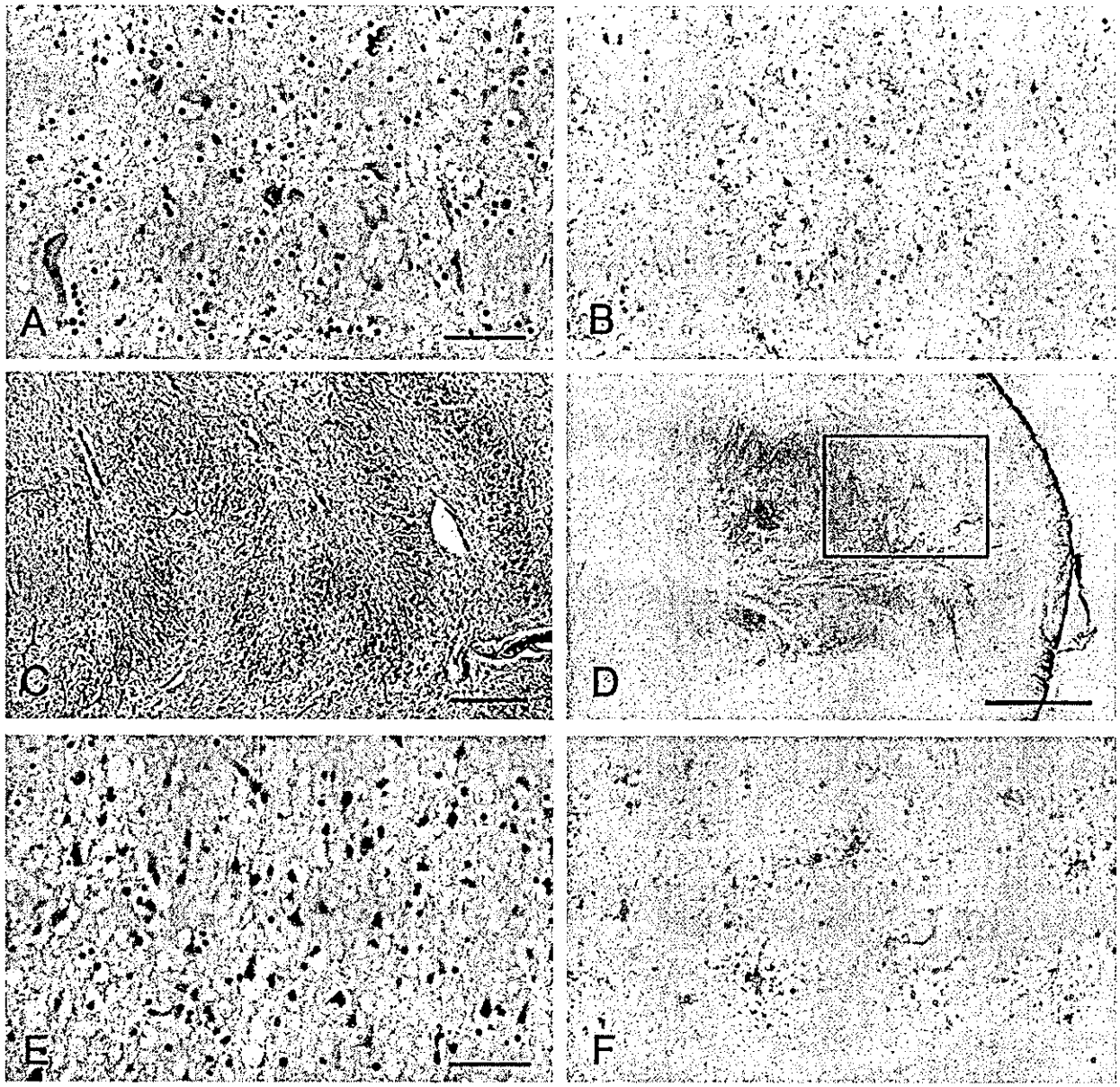

Figure 4. Neuronal loss and gliosis revealed by haematoxylin and eosin stain (A, E), Bodian's stain (C) or immunohistochemistry for glial fibrillary acidic protein (GFAP) (B, D, F). A, B: centromedial nucleus of the thalamus. C, D: inferior olivary nucleus. Neuronal loss is more evident in the medial part (left side of panel C, which represents the rectangular area depicted in panel D). E, F: cerebral cortex (frontal lobe). Both spongiform change and gliosis are remarkable. Bars: 50 μm (A, B, E, F), 200 μm (C), 1 mm (D).

amus or inferior olivary nucleus (Figure 5C,D). In the cerebellar molecular layer, punctate deposits of PrP were focally observed (Figure 5A), and the regions with these deposits were coincident with the extent of spongiform change. Likewise, fine granular deposition of PrP was also detected together with spongiform degeneration in the cerebral cortex (Figure 5B). The distribution of PrP deposits appeared to be more broad and noticeable in the cerebral cortex than in the cerebellum. As a unique finding, the anti-PrP antibody revealed swollen and/or frizzled axons in the deeper parts of the cerebral white matter, in the corpus callosum, or at the borders of the thalamus and caudate nucleus (Figure 5E). Axonal transported substances, APP (Figure 5E, inset) and SNAP-25 (data not shown) were also detected immunohistochemically in those axons.

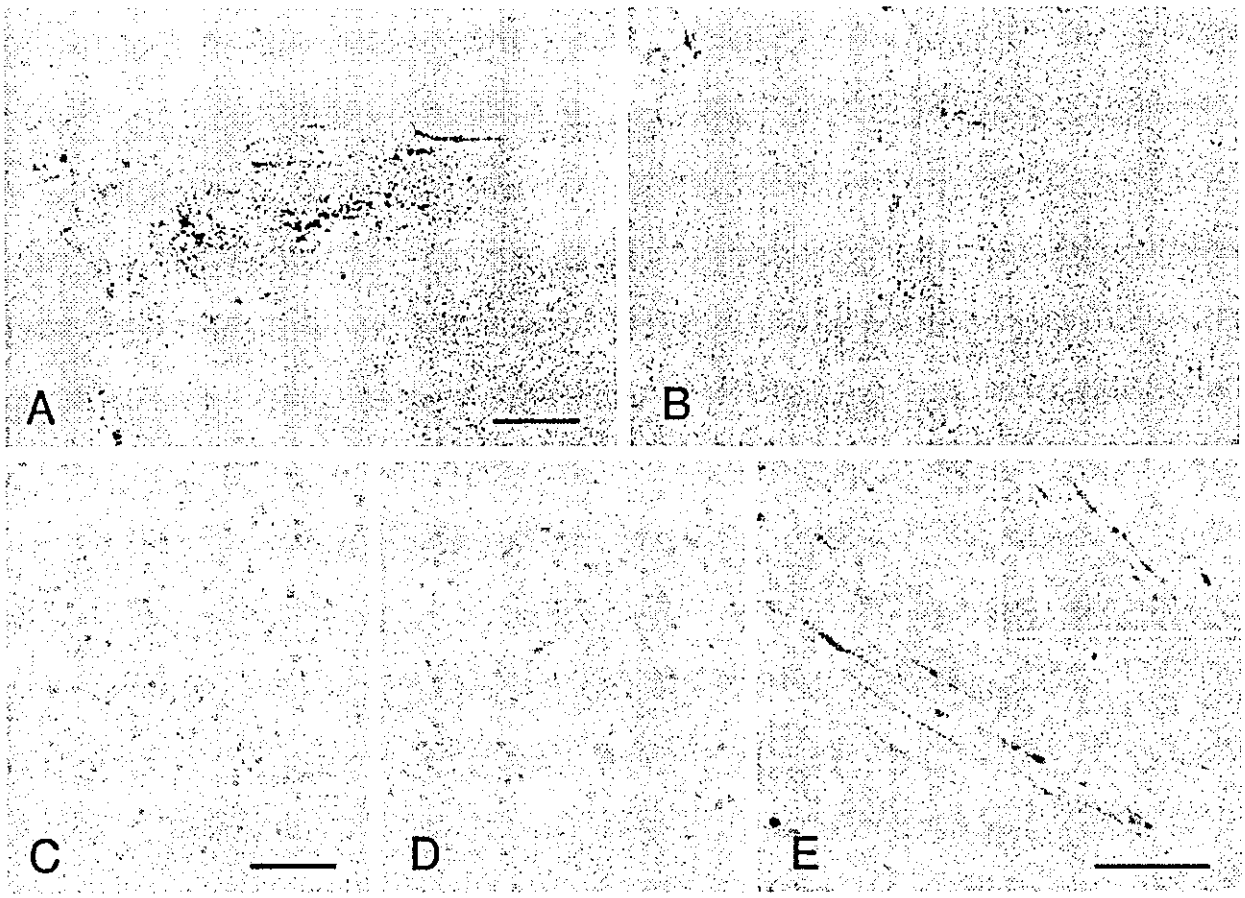

Figure 5. Immunohistochemistry for prion protein (PrP) deposition. A: cerebellum. B: frontal cortex. C: centromedial nucleus of the thalamus. D: inferior olivary nucleus. PrP deposition can not be detected in the thalamus or the inferior olivary nucleus, but coarse or fine granular PrP deposition is visible within the cerebral cortex and the cerebellar molecular layer. E: axons with swollen and/or frizzled features can be detected in the white matter at the border of the thalamus. These axons are also immunostained with anti-APP (amyloid precursor protein) antibody (inset). Bars: 50 μm (A, B, E), 100 μm (C, D).

Although the conventional method of Western blot analysis for PrP^{res} failed to detect any particular signal (data not shown), by application of phosphotungstic acid precipitation that preferably concentrates PrP^{res} but not cellular PrP [12], Western blot analysis of the extract from the frontal cortex of this case revealed a detectable amount of PrP^{res} (Figure 6). The molecular weight of non-glycosylated form of PrP was about 19 kDa (PrP^{res} type 2 pattern) and also the PrP^{res} glycoform ratio was compatible with that of FFI, which has been previously reported [14]. The extract from the cerebellum showed no significant signal in Western blot analysis even with phosphotungstic acid precipitation (data not shown).

The disease of this case was successfully transmitted to some of the mice inoculated with tissue homogenate from the frontal cortex. The incubation time was 571.6 ± 61.1 days (5/7 of the inoculated mice developed TSE) in the NZW mice and 736 ± 64.4 (5/8) in the Tg7 mice, respectively. Although not all the mice developed TSE, diseased mice demonstrated lethargy in the terminal stage rather than excitability. In the TSE-developed mice pathological examination of the brain showed that spongiform change and gliosis were prominent in the cerebral cortex in addition to the thalamus (Figure 7). Immunohistochemistry for PrP revealed that diffuse granular PrP deposition was present within the deep layer of the cerebral cortex as well as in the lateral portion of the thalamus (Figure 7B,E).

Discussion

It is established that there is an overlapping spectrum between classical FFI and CJD in association with PRNP

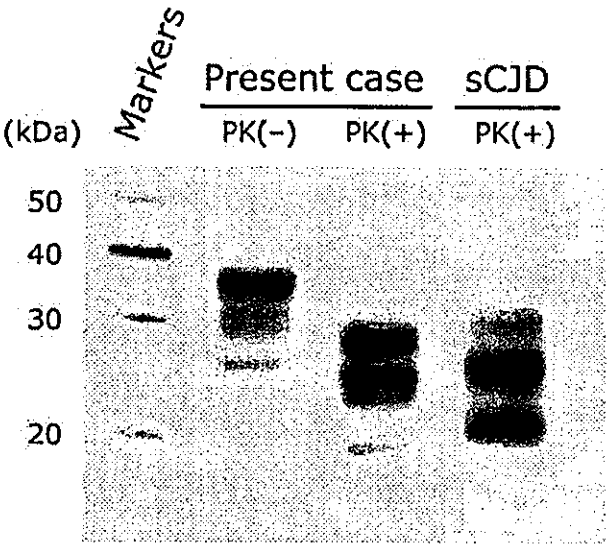

Figure 6. Western blot analysis for protease-resistant prion protein (PrP^{res}). Brain homogenate of the frontal cortex of this case is treated with or without proteinase K (PK), and then PK-digested sample is followed by the 40-times concentration with phosphotungstic acid precipitation for PrP^{res}. The abnormal PrP molecules in the frontal cortex of this case migrate as PrP^{res} type 2. PrP molecules in the lane sporadic Creutzfeldt-Jakob disease (sCJD) are also shown as a standard type 1 PrP (MM1). Molecular sizes (kDa) are indicated on the left.

D178N [6]; however, this case adds to our knowledge about this disease. Although the present case had FFI genotype, the clinical features were initially characterized by prominent cerebellar ataxia, and the neuropathological findings were also atypical in the following respects. First, PrP deposition and spongiform change in the cerebral cortex were more conspicuous than in the thalamus or inferior olivary nucleus, both of which are extremely vulnerable sites for FFI. It has been previously reported that heterozygotes Met/Val at codon 129 result in a longer clinical course than homozygotes [4], and it is therefore possible that the lesions seen in the cerebral cortex were more prominent simply because of the longer course of illness in this patient. However, a further noteworthy point about this case is rather that there was no PrP deposition either in the thalamus or in the inferior olivary nucleus.

Second, immunohistochemical examination detected a peculiar deposition of PrP within the molecular layer of the cerebellum. The localized lesions of granular deposits of PrP and spongiform change in the cerebellar molecular layer seemed to be similar to those reported in a patient from an Austrian FFI family [15]. The cerebellar ataxia of this case could have attributed to the loss of granular neurones and degeneration of Purkinje's cells, in addition to the lesions of the inferior olivary nucleus, although the pathology related to PrP deposition could have also been responsible.

A third atypical feature is that the neuronal loss in the thalamus was most noticeable in the centromedial nucleus. A previous study revealed that severe atrophy of the anterior ventral and dorsomedial thalamic nuclei was consistently observed, whereas that of other thalamic nuclei was less severe and they were inconsistently affected [2]. In this case, the medial portion of the thalamus was indeed damaged crucially, but the principal lesion was different from the typical pathology of FFI.

In addition, an interruption of axonal transport was suggested. Some of the axons were swollen and associated with PrP accumulation, and both APP and SNAP-25 were also accumulated in those axons. APP and SNAP-25 are presynaptic protein and APP is considered as the most effective marker for axonal injury [16]. Aberration in recruitment of PrP might be involved in the pathogenesis of TSE, as described previously [17,18].

This case showed a small amount of specific PrP^{res} in the cerebral cortex but not in the cerebellum as detected by Western blotting. The ratio of PrP^{res} quantity in those regions was visually correlated with that of immunohistochemical reactivity for PrP. Although fresh frozen samples from the thalamus or the inferior olivary nucleus were not obtained for Western blot analyses, we suspect that PrP^{res} in such regions would be too sparse to be detected by Western blotting even in combination with phosphotungstic acid precipitation. The type 2 migration pattern and the glycoform ratio of PrP^{res} in this case were compatible with those in the typical FFI [14]; however, it remains to be elucidated whether these abnormal proteins that can be classified in the same PrP^{res} type may have different influences on the neurodegeneration processes.

Finally, the transmission study revealed that a pathogen in the frontal cortex of this case might be different from that of an FFI case previously reported by Dr Tateishi and his colleagues [13]. NZW mice infected with a thalamic tissue sample of a typical FFI case exhibited excitability as the principal clinical sign and demonstrated PrP deposition predominantly localized within the thalamus. On the other hand, NZW mice infected with a frontal cortical tissue sample from the present case showed lethargy as a clinical sign, and demonstrated diffuse PrP deposition within the deep layer of the cerebral cortex, as well as in the lateral portion of the thalamus. The PrP deposition