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書籍

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大久保憲.	手術室での感染防止.	中田精三編著.	手術室看護の知識と実際	メディカ出版	東京	2009	66-87

雑誌

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Hizume M, Kobayashi A, Teruya K, Ohashi H, Ironside JW, Mohri S, Kitamoto T.	Human Prion Protein (PrP) 219K is converted to PrPSc but shows heterozygous inhibition in variant Creutzfeldt-Jakob disease infection.	J Biol Chem.	284	3603-3609	2009
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Human Prion Protein (PrP) 219K Is Converted to PrP^{Sc} but Shows Heterozygous Inhibition in Variant Creutzfeldt-Jakob Disease Infection*[S]

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Prion protein gene (*PRNP*) E219K is a human polymorphism commonly occurring in Asian populations but is rarely found in patients with sporadic Creutzfeldt-Jakob disease (CJD). Thus the polymorphism E219K has been considered protective against sporadic CJD. The corresponding mouse prion protein (PrP) polymorphism variant (mouse PrP 218K) is not converted to the abnormal isoform (PrP^{Sc}) and shows a dominant negative effect on wild-type PrP conversion. To define the conversion activity of this human molecule, we herein established knock-in mice with human PrP 219K and performed a series of transmission experiments with human prions. Surprisingly, the human PrP 219K molecule was converted to PrP^{Sc} in variant CJD infection, and the conversion occurred more efficiently than PrP 219E molecule. Notably the knock-in mice with *PRNP* codon 219E/K showed the least efficient conversion compared with their hemizygotes with *PRNP* codon 219E/0 or codon 219K/0, or homozygotes with *PRNP* codon 219E/E or codon 219K/K. This phenomenon indicated heterozygous inhibition. This heterozygous inhibition was observed also in knock-in mice with *PRNP* codon 129M/V genotype. In addition to variant CJD infection, the human PrP 219K molecule is conversion-competent in transmission experiments with sporadic CJD prions. Therefore, the protective effect of *PRNP* E219K against sporadic CJD might be due to heterozygous inhibition.

Human prion diseases have been classified into infectious, inherited, and sporadic forms. Infectious human prion disease was demonstrated first in Kuru (1) and recently in Creutzfeldt-Jakob disease (CJD)[3] with dura mater-grafted CJD, pituitary hormone-associated CJD, and variant CJD (vCJD) (2, 3). Familial CJD, Gerstmann-Straeussler syndrome, and fatal familial insomnia are human inherited prion diseases (4). Sporadic CJD (sCJD) is of unknown etiology. These prion diseases are caused by the accumulation of an abnormal isoform (PrP^{Sc}) of prion protein (PrP), which is converted from the normal cellular isoform (PrP^{C}) (5). The human PrP contains 253 amino acids encoded by prion protein gene (*PRNP*), which is located on chromosome 20. Numerous point mutations or insertional mutations in the open reading frame of *PRNP* have been reported in inherited prion diseases. In addition, normal polymorphisms of *PRNP* appear to influence the susceptibility to sporadic or infectious prion diseases. Homozygosity at the polymorphic *PRNP* codon 129 (methionine or valine) may cause a predisposition to sporadic or iatrogenic CJD in Europeans (6, 7). All cases of vCJD are homozygous for methionine at *PRNP* codon 129 (129M/M) (8).

In 1994, we reported that glutamate to lysine substitution at codon 219 is a polymorphism occurring in the Japanese population (9). This is a common polymorphism (allele frequency; 6%), which was later found also in other populations in the East Asia, the South Asian subcontinent, and the Pacific region, but has not been reported in Europeans (10–12). It has been reported that the *PRNP* genotype at codon 219 influences the clinicopathological features of Gerstmann-Straeussler syndrome with a codon 102 mutation (13), and that the codon 219K genotype appears to have a protective effect for sCJD (14). In addition, the codon 218K variant (corresponding to the human 219K) in the murine prion protein gene (*prnp*) was not converted to PrP^{Sc} and also showed a dominant negative effect on wild-type PrP conversion both in scrapie-infected neuroblastoma cells (15) and in transgenic mice (16). This dominant negative effect of the mouse PrP 218K variant was proposed at first to be mediated by protein X (15) but was also observed in *in vitro* fibril formation without protein X (17). In contrast to the murine *prnp* 218K variant, the human PrP 219K molecule did

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[3] The abbreviations used are: CJD, Creutzfeldt-Jakob disease; vCJD, variant CJD; sCJD, sporadic CJD; PrP, prion protein; PrP^{C}, normal cellular isoform of PrP; PrP^{Sc}, abnormal isoform of PrP; FDC, follicular dendritic cell; *PRNP*, human prion protein gene; *prnp*, murine prion protein gene.

not prevent the onset of dura mater-grafted CJD (14) or familial CJD (18). Therefore, it remains unclear whether the human PrP 219K molecule is conversion-competent or not.

In the present study, we newly established knock-in mice expressing the human PrP 219K molecule (Ki-Hu219K/K) and compared the conversion activity with other human PrP polymorphic molecules (19). We found that human PrP 219K is readily converted to PrP^{Sc} in vCJD infection, and also report the inhibition of PrP conversion in the heterozygous knock-in models.

EXPERIMENTAL PROCEDURES

Production of Humanized Knock-in Mouse with Homozygous, Heterozygous, or Hemizygous Genetic Background—Knock-in mice and transgenic mice were generated as reported previously (20). The open reading frame was replaced with human PrP gene with lysine at codon 219 (Fig. 1*A*). The 5′-primer was designed to incorporate a SmaI site. The PCR fragment was ligated to the mouse sequence using the SmaI site. Consequently, after processing of the N-terminal signal peptide during post-translational modification, the resulting molecule was identical with human PrP. The knock-in mice with human PrP 129M or 129V were already established (19, 21). We produced knock-in mouse crossed with the PrP knock-out mouse (20) to provide the hemizygous genetic background in the present study.

Sources of Prion Inocula and Transmission Experiment—Human brain tissues were obtained at autopsy from CJD patients after receiving informed consent for research use. Brain homogenate was prepared from four patients with vCJD (96/02, 96/07, 96/45, or 05/02), or two cases with sCJD (MM1 and MV1). The open reading frame of *PRNP* was analyzed by PCR direct sequencing (22). Human brain homogenates (10%) were prepared as described previously (23). Transmission studies were performed using 20 μl of the homogenates for intracerebral inoculation or 50 μl for intraperitoneal inoculation. Mice were sacrificed at 75 days post-inoculation for follicular dendritic cell (FDC) bioassay. Our previous study showed that the level of PrP^{Sc} accumulated in the spleen of knock-in mouse expressing chimeric human/mouse PrP with 129 M/M reached a plateau at 45 days post-inoculation (20). Thus, we decided to perform FDC assay at 75 days post-inoculation (19). Half of the spleen was immediately frozen for Western blotting, and the remaining half was fixed in 10% buffered formalin for the immunohistochemistry. Intracerebrally inoculated mice were sacrificed after the onset of the disease or examined when postmortem. One hemisphere of the brain was immediately frozen for Western blotting, and the other hemisphere was fixed in formalin for the immunohistochemistry.

Immunohistochemistry—Mouse tissues were fixed with 10% buffered formalin, and treated with 60% formic acid before embedding in paraffin. Tissue sections were processed for PrP immunohistochemistry using hydrolytic autoclaving pretreatment (24). The PrP-N antiserum (25) or ChW antiserum (19) were used as the primary antibody. A goat anti-rabbit immunoglobulin polyclonal antibody labeled with a peroxidase-conjugated dextran polymer, EnVision (DakoCytomation, Denmark), was used as the secondary antibody.

Western Blotting—PrP^{Sc} was extracted from either spleen or brain with collagenase treatment as previously described (26) with modifications. PrP^{C} was measured in the membrane fractions of the brain isolated from the knock-in mice. For the quantitative analysis, samples (corresponding to 7.5 mg wet weight of spleen tissue for PrP^{Sc}, or 500 μg wet weight of brain tissue for PrP^{C}) were subjected to 13.5% SDS-PAGE and transferred to polyvinylidene difluoride membrane. ChW antiserum or 3F4 antibody was used as the primary antibody. Anti-rabbit or anti-mouse EnVision was used as the secondary antibody. Enhanced chemiluminescence detection (GE Healthcare) was used to visualize Western blots. The signal intensities of the Western blots were quantified with Quantity One software using an imaging device, Vasa Doc 5000 (Bio-Rad). Western blot analysis was repeated at least three times, and signal intensities were expressed as mean ± S.E.

To check the protease resistance in each PrP polymorphism, we did the following experiment with the infected or uninfected knock-in mouse brains. Brain tissues of the Ki-Hu219K/K or Ki-Hu219E/E infected with MM1 prions or the uninfected control brain tissues were homogenized with 10 times the amount of the buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and protease inhibitors (Complete, Roche Applied Science). The homogenates were centrifuged at 1000 × *g* for 10 min to discard the nuclear fraction. The supernatants were homogenized again with a glass homogenizer adding the Sarkosyl solution at a final concentration of 2%. The homogenate was centrifuged again at 2000 × *g* for 10 min. The final supernatant was digested at 37°C for 60 min with Proteinase K at concentrations of 0, 1, 3, 10, 30, 100, and 300 μg/ml. The digested samples were added with the equal volume of Laemmli sample buffer and boiled for 15 min for the Western blot analysis.

Statistical Analysis—Incubation times and the signal intensities of Western blots are expressed as mean ± S.E.

RESULTS

Knock-in Mouse as a Model to Provide the Physiological Expression Level of Recombinant PrP—In comparison with transgenic technology, knock-in mice produced by the homologous recombination technique have the advantage of providing a constant expression level (20, 27). Therefore, as reported previously (19), the expression level of PrP^{C} in the spleens of the heterozygous knock-in mice (Ki-Hu129M/V) was the same as that of the homozygous knock-in mice (Ki-Hu129M/M or Ki-Hu129V/V). It is impossible to establish a heterozygous animal model without the homologous recombination technique. However, a transgenic model with the *PRNP* 129M/V genotype was reported previously, but this model (Tg45/152) showed uneven expression (M:V = 1:1.5) and overexpression of the gene (4–6 fold) (28). In addition to the advantage described above, here we present that the expression levels of PrP^{C} in the brains of the hemizygous knock-in mice (Ki-Hu129M/0 or Ki-Hu129V/0) showed almost half the intensities of those seen in the homozygous mice (Ki-Hu129M/M or Ki-Hu129V/V) (Fig. 1, *B* and *C*). Thus, in this study, we can analyze two different expression levels of recombinant PrP: 1 copy of gene expression in the hemizygous knock-in mice and 2 copies of

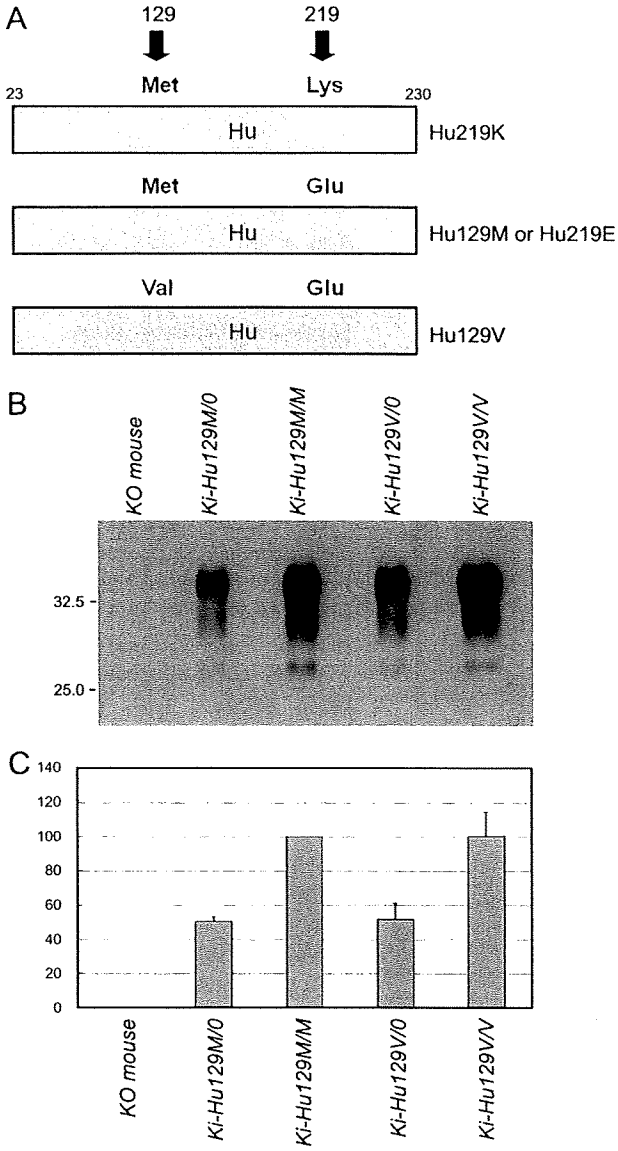


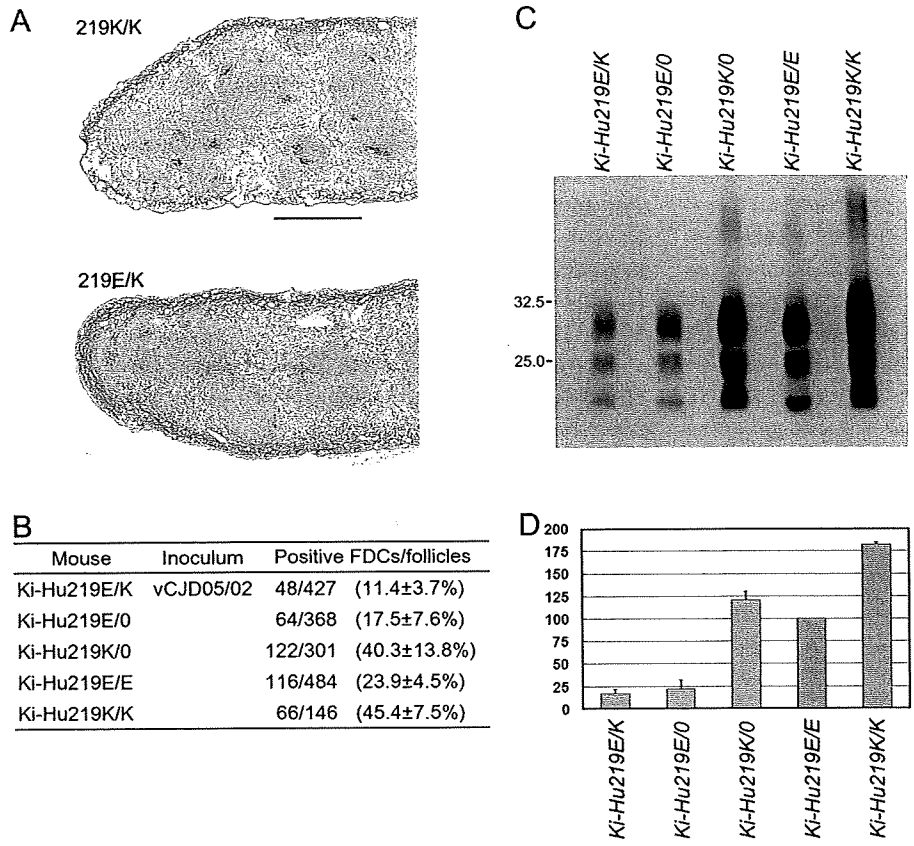
FIGURE 1. **Characterization of the knock-in mice.** *A*, the open reading frames of the knock-in vectors. The Hu219K vector encodes methionine at codon 129 and lysine at codon 219. The Hu129M (a synonym for Hu219E) and the Hu129V vectors were reported previously (19). *B*, Western blot analysis of the membrane fraction of the brains from the knock-out mouse, the hemizygous knock-in mice, and the homozygous knock-in mice. The position of molecular size standards is shown on the *left* (kilodaltons). *C*, the comparatively corrected signal intensities (numbers/mm^2) are presented. We assigned a signal intensity of 100/mm^2 for Ki-Hu129M/M. The corrected signal intensities are as follows. Ki-Hu129M/0, 50.7 ± 2.7; Ki-Hu129V/0, 51.8 ± 9.3; and Ki-Hu129V/V, 100.5 ± 13.9/mm^2 (mean ± S.E.).

gene expression in the homozygous or heterozygous knock-in mice.

Human PrP 219K Molecule Is Conversion-competent in vCJD Infection—At first, we examined the transmission experiment with vCJD prions, since a case of vCJD patient was reported in Japan (29). When considering the possibility of secondary infection from human to human transmission in Japan, it is important to know whether the Japanese populations with the codon 219E/E, E/K or K/K genotype are susceptible to vCJD prions. In secondary vCJD infection, a direct intracerebral route of exposure is only likely to occur during neurosurgical procedures, whereas a peripheral route of infection via blood transfusion, tissue transplantation or general surgery is far more likely. Thus, we analyzed the susceptibility to vCJD prions using the FDC assay after the peripheral route of infection in a murine model.

It was surprising that positive PrP immunolabeling was observed in the FDC of the spleens from Ki-Hu219K/K intraperitoneally inoculated with vCJD prions (Fig. 2*A*). To compare the conversion efficiency between PrP 219E and PrP 219K, we prepared knock-in mice with the following genotypes: Ki-Hu219E/K, Ki-Hu219E/0, Ki-Hu219K/0, Ki-Hu219E/E, and Ki-Hu219K/K. These knock-in mice were inoculated with vCJD prions from 2 different patients and were sacrificed at 75 days post-inoculation to analyze PrP^{Sc} in the FDC by immunohistochemistry or Western blotting. The immunohistochemical analysis showed positive FDC staining in almost all knock-in mice inoculated with the vCJD prions (supplemental Fig. S1*A*). Although the vCJD infection was established in almost all mice irrespective of the codon 219 genotype, the numbers of positively stained FDCs differed in each knock-in mouse (Fig. 2*A*). Therefore, we counted the total number of lymphoid follicles and the number of positively stained FDCs in all mice. The positive rate in the FDC assay was lowest in Ki-Hu219E/K, followed by Ki-Hu219E/0, Ki-Hu219E/E and Ki-Hu219K/0, and was highest in Ki-Hu219K/K (Fig. 2*B*). Western blot analysis showed that the quantity of PrP^{Sc} was highest in the spleens of Ki-Hu219K/K, followed by Ki-Hu219K/0, Ki-Hu219E/E and Ki-Hu219E/0, and was lowest in Ki-Hu219E/K (Fig. 2, C and D). Despite the different genotype in the knock-in mice (219K/K) and vCJD prions (219E/E), the most effective conversion was observed in Ki-Hu219K/K mice. Even the hemizygous Ki-Hu219K/0 showed more PrP^{Sc} accumulation than did Ki-Hu219E/E. This observation in Western blot was reproducible in a transmission experiment using another vCJD inoculum (vCJD96/07) (supplemental Fig. S1*B*). In both transmission experiments, the heterozygous model showed the lowest efficiency of conversion.

Heterozygous Inhibition Is Also Observed in the PRNP Codon 129M/V Genotype—As reported previously (19), Ki-Hu129M/M and Ki-Hu129M/V mice were susceptible to vCJD prions as revealed by FDC assay, but Ki-Hu129V/V mice were not. Because the human PrP 129V molecule is conversion-incompetent in vCJD infection, it was the best model to compare the amount of PrP^{Sc} between Ki-Hu129M/V and Ki-Hu129M/0 to examine the influence of heterozygosity. We prepared knock-in mice with the following genotypes: Ki-Hu129M/V, Ki-Hu129M/0, Ki-Hu129M/M (a synonym for Ki-Hu219E/E), and Ki-Hu129V/V. All of these knock-in mice had Glu at codon 219 of *PRNP* (Fig. 1*A*). These mice were inoculated intraperitoneally with vCJD prions (vCJD05/02). In Western blot analysis, Ki-Hu129M/M and Ki-Hu129M/V had PrP^{Sc} in the spleen, but Ki-Hu129V/V did not (Fig. 3, *A* and *B*). However, the amount of PrP^{Sc} in the spleens of Ki-Hu129M/V was consistently less than that in Ki-Hu129M/0. This observation in Western blot was reproducible in a transmission experiment using another vCJD inoculum (vCJD96/07) (supplemental Fig. S2). Therefore, the conversion-incompetent human PrP 129V molecule also appears to show an inhibitory effect on the accumulation of human PrP 129M PrP^{Sc}.



B

Mouse	Inoculum	Positive FDCs/follicles	
Ki-Hu219E/K	vCJD05/02	48/427	(11.4±3.7%)
Ki-Hu219E/0		64/368	(17.5±7.6%)
Ki-Hu219K/0		122/301	(40.3±13.8%)
Ki-Hu219E/E		116/484	(23.9±4.5%)
Ki-Hu219K/K		66/146	(45.4±7.5%)

FIGURE 2. **Transmission experiment using the knock-in mice with codon 219 polymorphism.** *A*, immunohistochemistry analysis of the spleen from Ki-Hu219K/K or from Ki-Hu219E/K. The follicular dendritic cells were positively immunolabeled with PrP antibody. *Scale bar*, 500 μm. *B*, summary of the immunohistochemical analysis. We used the hemizygous models (Ki-Hu219E/0 and Ki-Hu219K/0), the homozygous models (Ki-Hu219E/E and Ki-Hu219K/K), and the heterozygous model (Ki-Hu219E/K) intraperitoneally inoculated with vCJD05/02. We counted the total number of positive FDCs and the total number of lymphoid follicles in the spleens from all mice. The positive rate of FDCs for each animal is expressed as mean % value ± S.D. *C*, Western blot analysis of PrP^{Sc} from the knock-in mice inoculated with vCJD05/02. The position of molecular size standards is shown on the *left* (kilodaltons). *D*, the comparatively corrected signal intensities (numbers/mm^2) are presented. We assigned a signal intensity of 100/mm^2 for Ki-Hu219E/E. The corrected signal intensities are as follows. Ki-Hu219E/K, 17.6 ± 3.9; Ki-Hu219E/0, 21.9 ± 10.1; Ki-Hu219K/0, 121.0 ± 8.4; and Ki-Hu219K/K, 182.1 ± 2.1/mm^2 (mean ± S.E.).

Transmission Studies via the Intracerebral Administration—It was established that the human PrP 219K molecule is converted in vCJD infection, but it remains uncertain as to whether this molecule could be converted by infection with other prions. To examine the transmissibility of other prions, we performed intracerebral inoculation of 10% brain homogenates from a patient with sCJD. In the transmission experiment using sCJD prions, all Ki-Hu219K/K showed PrP^{Sc} accumulation in the brain. Compared with Ki-Hu219E/E (467 ± 24 days), Ki-Hu219K/K showed a longer incubation period (573 ± 103 days) after inoculation with sCJD-MM1 prions (129M/M, 219E/E, and type 1 PrP^{Sc}). It is significant that human PrP 219K could be converted by sCJD-MM1 or MV1 prions (129M/V, 219E/E, and type 1 PrP^{Sc}) (Fig. 4*A*). Western blot analysis of the brains from Ki-Hu219K/K showed a similar PrP^{Sc} isoform on blot to that seen in Ki-Hu219E/E (Fig. 4*B*). Thus the human PrP 219K is conversion-competent also in sCJD prion infection.

We also summarize the transmission data of vCJD prions with the intracerebral inoculation (Fig. 4*A*). In vCJD (vCJD05/02) infection, Ki-Hu219K/K mice showed PrP^{Sc} accumulation in the brain after the incubation period of 412 ± 6 days. Ki-Hu129M/M (the same mouse as Ki-Hu219E/E) showed a longer incubation period compared with Ki-Hu219K/K. In addition to these homozygous models, the Ki-Hu129M/V heterozygous mice show no clinical signs and still are alive after >740 days of incubation (Fig. 4*A*). Therefore, heterozygous inhibition was similarly observed as in intracerebral infections.

DISCUSSION

It was not expected that the Hu219K PrP molecule was converted to the abnormal isoform. Therefore, it should be important to check the protease sensitivity in the normal isoforms and abnormal isoforms of Hu219K and Hu219E. At first, we checked the protease sensitivity of the uninfected or infected brain samples with MM1 prion (supplemental Fig. S3). The pattern in the protease sensitivity of PrP^{C} and the protease resistance of PrP^{Sc} was almost the same in Ki-Hu219E/E and Ki-Hu219K/K. In addition to this result, the positive rate in the immunohistochemical analysis of the FDC assay infected with vCJD prions correlated to the quantitative data in the Western blot (Fig. 2 and supplemental Fig. S4). In addition, Ki-Hu219K/K mice showed a shorter incubation period compared with Ki-Hu129M/M (Ki-Hu219E/E) in the central nervous system infection. Therefore, it was concluded that the Hu219K molecule is readily converted in vCJD prions compared with Hu219E. From our data, the Hu219K molecule might be more suitable as a substrate to amplify vCJD prions by the PMCA technique (30) compared with Hu219E (31). Although many transmission studies have shown the importance of homology at the polymorphic codons between PrP^{C} and PrP^{Sc} for efficient conversion (32), vCJD prions represent an exception as far as *PRNP* codon 219 is concerned. This exception can be explained by the fact that bovine PrP has a amino acid substitution of glutamine corresponding to codon 219. The structure of PrP^{Sc} with Hu219K might have a similar structure of BSE PrP^{Sc} with 219Q compared with that of Hu219E. Based on the codon 219 substitution, we can design a better PrP^{C} substrate to amplify vCJD prions.

Despite the conversion competence of the Hu219K and Hu219E PrP molecules, heterozygous inhibition was observed in Ki-Hu219E/K as well as Ki-Hu129M/V mice with conversion-competent 129M and conversion-incompetent 129V molecules. In addition to the peripheral route infection in the FDC assay, Ki-Hu129M/V mice showed heterozygous inhibition of

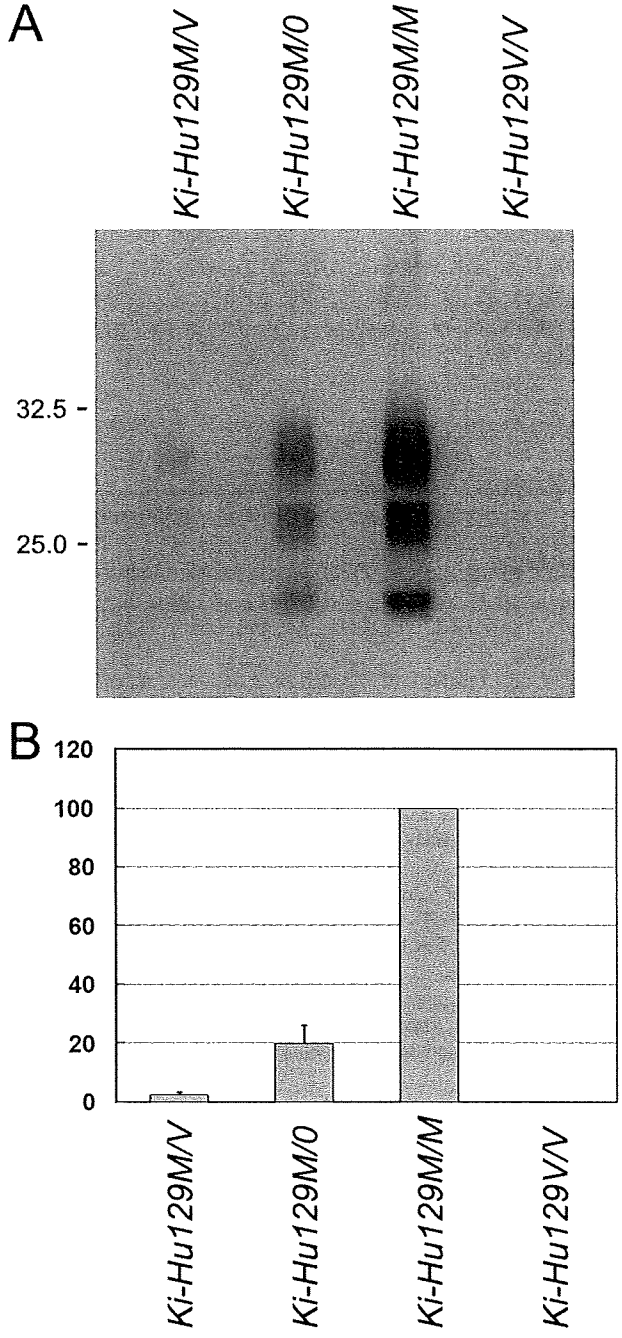


FIGURE 3. **Transmission experiment using the knock-in mice with the codon 129 polymorphism.** *A*, Western blot analysis of PrP^{Sc} from the knock-in mice with the 129 polymorphism. The hemizygous model (Ki-Hu129M/0), the homozygous models (Ki-Hu129M/M or Ki-Hu129V/V), or the heterozygous model (Ki-Hu129M/V) were intraperitoneally inoculated with vCJD05/02. The position of molecular size standards is shown on the *left* (kilodaltons). *B*, the comparatively corrected signal intensities (numbers/mm^2) are presented. We assigned a signal intensity of 100/mm^2 for Ki-Hu129M/M. The corrected signal intensities are as follows. Ki-Hu129M/V, 2.6 ± 0.7; and Ki-Hu129M/0, 19.7 ± 6.3/mm^2 (mean ± S.E.).

A

Mouse	Inoculum	Incubation period
Ki-Hu129M/M	sCJD MM1(H3)	467±24 (8/8)
Ki-Hu219K/K	sCJD MM1(H3)	573±103 (5/5)
Ki-Hu219K/K	sCJD MV1(Su)	643±146 (5/5)
Ki-Hu219K/K	vCJD05/02	412± 6 (3/4)
Ki-Hu129M/M	vCJD05/02	607, 622, >700 (2/5)
Ki-Hu129M/V	vCJD05/02	>742 (0/5)

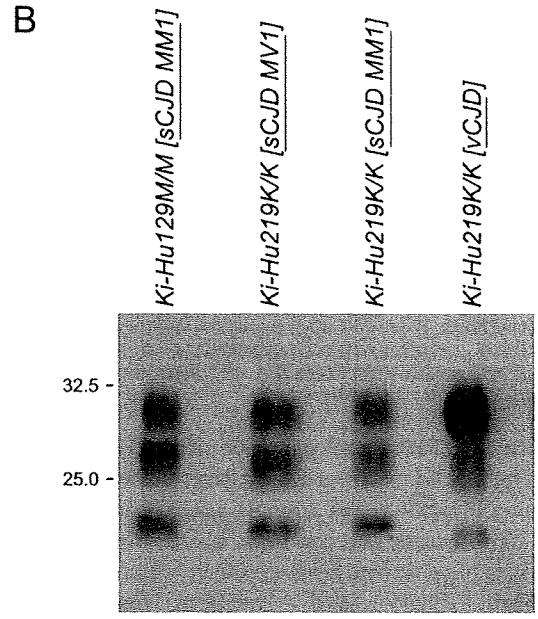


FIGURE 4. **Transmission experiment in Ki-Hu219K/K inoculated intracerebrally with sCJD prions.** *A*, summary of the transmission experiments. The MM1 inoculum is the same in both Ki-Hu219K/K and Ki-Hu129M/M. The vCJD inoculum is also the same in Ki-Hu219K/K, Ki-Hu129M/M, and Ki-Hu129M/V. The incubation period was expressed by days (number of positive transmissions/number of total animals). *B*, Western blot analysis of PrP^{Sc} in the brains. The knock-in mouse [*the inoculated sample*] is designated *above each lane*. The position of molecular size standards is shown on the *left* (kilodaltons).

vCJD prion infection in the central nervous system. Therefore, the heterozygous inhibition is a universal feature of prion infections both in peripheral infection and central nervous system infection.

The effect of *PRNP* polymorphisms have been studied recently in an *in vitro* model (17, 33), in which fibril formation revealed the β-oligomer state (34). It was suggested that the β-oligomer was not on the pathway to amyloid formation and that the refolding and dissociation of the β-oligomer into the α-monomer most likely preceded the fibril formation. The kinetics of dissociation of the β-oligomer was 100-fold slower in the 129M/V heterogenous β-oligomer than those in either the 129M or 129V homogenous β-oligomer (33). The inhibition of amyloid formation was also reported in a fibrillization model mixed with murine wild-type PrP with 218Q and murine PrP 218K molecule (17). In this system, the murine PrP 218K molecule was converted into a fibril, but the conversion efficiency was lowered. It was interesting that the murine PrP 218K molecules were incorporated into fibrils as often as the wild-type molecules. In another model (35), transgenic mice expressing conversion-incompetent PrP-Fc_2 showed a reduced conversion of wild-type PrP by dimeric PrP-Fc_2. Interestingly, the dimeric PrP-Fc_2 was also incorporated in protease-resistant fibrils.

Based on these previous findings, we propose a possible mechanism to explain the heterozygous inhibition. This mechanism is entirely based on the distinct structure of each PrP^{Sc} molecule (36, 37) (Fig. 5). At first, PrP^{C} is converted to PrP^{Sc},

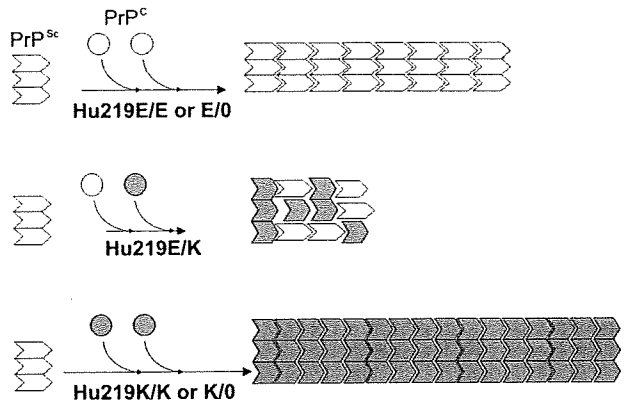


FIGURE 5. **A possible mechanism underlying the heterozygous inhibition in vCJD infection.** *Upper*, PrP^{Sc} proliferation model in Ki-Hu219E/E or Ki-Hu219E/0. There is only one PrP^{Sc}. The Hu219E PrP^{Sc} is piled up into the protease-resistant amyloid fibril. *Middle*, the heterozygous inhibition model. There are two distinct structural PrP^{Sc} in the same mouse. It takes time to pile up amyloid fibrils like a stone fence, because there are two distinct types of PrP^{Sc} blocks. Thus, PrP^{Sc} formation can be inhibited in the heterozygous animals. *Lower*, PrP^{Sc} proliferation model in Ki-Hu219K/K or Ki-Hu219K/0. The initial seed is Hu219E PrP^{Sc}. However, the resulting Hu219K PrP^{Sc} acts as a new seed, and the increasing Hu219K PrP^{Sc} is piled up rapidly into the protease-resistant amyloid fibrils. There was no or negligible Hu219E PrP^{Sc} as a decelerator. In this figure, each *circle* or *block* designates the following: *open circles*, Hu219E PrP^{C}; *open hexagonal blocks*, Hu219E PrP^{Sc}; *filled circles*, Hu219K PrP^{C}; and *filled hexagonal blocks*, Hu219K PrP^{Sc}.

then the converted PrP^{Sc} is piled up into amyloid fibrils according to the nucleated polymerization hypothesis (38). In the homozygous and hemizygous animals, there is only one structural PrP^{Sc}. This means that the same blocks (the same structural PrP^{Sc}) are piled up into amyloid fibrils with no other influence on fibril formation and elongation. However, in the heterozygous animals, there are at least two distinct structural PrP^{Sc} composed of the Hu219E or Hu219K molecule. To form and elongate amyloid fibrils in the heterozygous animal, it takes time to pile up amyloid fibrils, because there are two types of blocks (PrP^{Sc}) with a distinct structure (Fig. 5). The two different-shaped PrP^{Sc} may act as decelerators of each other. In the FDC assay of Ki-Hu129M/V, the amyloid fibril formation of Hu129M was inhibited by the Hu129V molecule, which was conversion-incompetent. This phenomenon corresponds to the dominant negative effect as reported previously. We can explain the dominant negative phenomenon by this decelerator hypothesis if the conversion-incompetent Hu129V molecules significantly reduce the rate of Hu129M amyloid formation and elongation.

The decelerator hypothesis may explain some unusual phenomena in prion infections. We propose that PrP^{Sc} molecules with different amino acid sequences act as decelerators in the process of amyloid formation. Because PrP molecules with different amino acid sequences are likely to have different conformations, we can infer that PrP^{Sc} molecules with different conformations act as decelerators in the process of amyloid formation. This decelerator hypothesis can explain the phenomenon described as interference (39), which is observed in an animal inoculated with two different prion strains. A distinct strain should have a distinct conformation of PrP^{Sc}. This PrP^{Sc} with a distinct conformation may inhibit another type of PrP^{Sc} amyloid formation. Recently, it has been reported that the prion interference is due to a reduction of the strain-specific PrP^{Sc} level (40). Our decelerator hypothesis can account for such prion interference.

In this report, we clearly show heterozygous inhibition of PrP^{Sc} formation using a knock-in mouse model of prion infection. *PRNP* heterozygosity may be important in determining resistance to human prion diseases (12). Although the incubation period after the intracerebral transmission of sCJD prions in Ki-Hu219E/K remains to be determined, the present study suggests that the absence of patients with the 219E/K *PRNP* genotype in sCJD might be due to heterozygous inhibition, because Hu219K is conversion-competent also in sCJD prions infection.

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Review

Heterozygous inhibition in prion infection

The stone fence model

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Abbreviations: PrP, prion protein; PrP^{C}, normal cellular isoform of PrP; PrP^{Sc}, abnormal isoform of PrP; CJD, Creutzfeldt-Jakob disease; vCJD, variant CJD; sCJD, sporadic CJD; PRNP, human PrP gene

Key words: prion protein, Creutzfeldt-Jakob disease, polymorphism, knock-in mouse, conversion, heterozygous inhibition, stone fence model

The human PrP gene (*PRNP*) has two major polymorphic codons: 129 for methionine (M) or valine (V) and 219 for glutamate (E) or lysine (K). The *PRNP* heterozygotes appear to be protected from sporadic CJD compared to the *PRNP* homozygotes. The molecular mechanism responsible for these protective effects of *PRNP* heterozygosity has remained elusive. In this review, we describe the inhibition of PrP conversion observed in a series of transmission studies using *PRNP* heterozygous animal models. In vCJD infection, the conversion incompetent human PrP 129V molecules showed an inhibitory effect on the conversion of human PrP 129M molecules in the 129M/V heterozygous mice. Furthermore, though the human PrP 219E and PrP 219K were both conversion competent in vCJD infection, these conversion competent PrP molecules showed an inhibitory effect in the 219E/K heterozygous animals. To explain this heterozygous inhibition, we propose a possible mechanism designated as the stone fence model.

Introduction

Creutzfeldt-Jakob disease (CJD), scrapie, and bovine spongiform encephalopathy are lethal transmissible neurodegenerative diseases caused by an abnormal isoform of prion protein (PrP^{Sc}) that is converted from the normal cellular isoform (PrP^{C}).[1] The human PrP gene (*PRNP*) has two major polymorphic codons: 129 for methionine (M) or valine (V), and 219 for glutamate (E) or lysine (K).[2,3] These *PRNP* polymorphisms affect the susceptibility to sporadic (sCJD), variant (vCJD), or iatrogenic CJD.[4-8] In particular, the *PRNP* heterozygotes appear to be protected from sCJD compared to the *PRNP* homozygotes. The frequency of the PRNP 129M/V genotype in sCJD is significantly lower than that in the normal population.[5] Moreover, the *PRNP* 219E/K genotype is absent in sCJD patients.[9] The molecular mechanism responsible for these protective effects of *PRNP* heterozygosity has remained elusive.

In this review, we describe the inhibition of PrP conversion observed in a series of transmission studies using *PRNP* heterozygous animal models.[10,11] To explain this heterozygous inhibition, we propose a possible mechanism designated as the stone fence model.

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Two Modes of Heterozygous Inhibition

Inhibition by the conversion incompetent PrP molecules. vCJD prions (genotype: 129M/M and 219E/E) can be transmitted to knock-in mice expressing human PrP with 129M/M (Ki-Hu129M/M) or with 129M/V (Ki-Hu129M/V), but not to those with 129V/V (Ki-Hu129V/V).[10] In transmission experiments using vCJD prions, we found an inhibitory effect of the conversion incompetent PrP 129V molecules in the 129M/V heterozygous animals. The amount of PrP^{Sc} in the spleens of Ki-Hu129M/V mice intraperitoneally inoculated with vCJD prions was much lower than that in the spleens of Ki-Hu129M/M mice (Fig. 1).[11] Moreover, the amount of PrP^{Sc} in Ki-Hu129M/V mice was even lower than that in hemizygous knock-in mice expressing human PrP 129M from one allele (Ki-Hu129M/0), which express half the level of PrP 129M compared with Ki-Hu129M/M mice. Thus, we confirmed that the decreased PrP^{Sc} accumulation in Ki-Hu129M/V mice was not due only to the expression level of PrP 129M. These findings clearly showed that the conversion incompetent PrP 129V molecules exerted an inhibitory effect on the conversion of PrP 129M molecules in the 129M/V heterozygous animals.

Previous studies have demonstrated that the conversion incompetent PrP molecules exhibit inhibitory effects on the

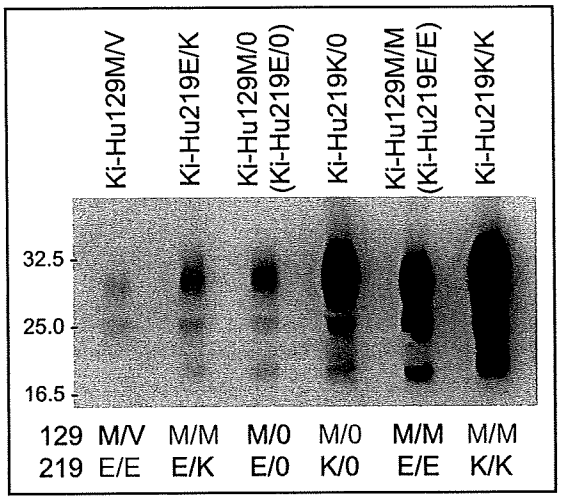

Figure 1. Heterozygous inhibition in vCJD infection. Western blot analysis of PrP^{Sc} in the spleens of knock-in mice intraperitoneally inoculated with vCJD prions. The amount of PrP^{Sc} in the 129M/V heterozygous mice was even lower than that in the 129M/0 hemizygous mice. Furthermore, the amount of PrP^{Sc} was the highest in the 219K/K mice, whereas the PrP^{Sc} accumulation in the 219E/K heterozygous mice was even lower than that in the 219E/0 hemizygous mice or 219K/0 hemizygous mice. Therefore, we found that both the conversion incompetent PrP and the conversion competent PrP showed inhibitory effects in the heterozygous animals.

conversion of the co-existing conversion competent PrP. This type of inhibition has been referred to as a dominant negative effect. When the endogenous mouse PrP gene was ablated, transgenic mice expressing exogenous human PrP or hamster PrP became more susceptible to human prions or hamster prions, respectively.[12,13] In a cell-free conversion system using mouse and hamster PrP, the conversion but not the binding to PrP^{Sc} was inhibited by the conversion incompetent PrP in a dose-dependent manner.[14] Furthermore, dominant negative mutations in mouse PrP have been studied intensively due to their potential for therapeutic applications.[15-22] In accord with these reports, the conversion incompetent human PrP 129V molecules in our study showed an inhibitory effect on the conversion of the human PrP 129M molecules.

Inhibition by the conversion competent PrP molecules. When we performed intraperitoneal inoculation of vCJD prions into knock-in mice expressing human PrP with 129M/M and 219E/E (Ki-Hu219E/E, a synonym of Ki-Hu129M/M), 129M/M and 219K/K (Ki-Hu219K/K), or 129M/M and 219E/K (Ki-Hu219E/K), we made two important findings. (1) Ki-Hu219K/K mice showed high susceptibility to vCJD prions. (2) Nevertheless, the heterozygous Ki-Hu219E/K mice showed the lowest susceptibility among the knock-in mice with polymorphism at codon 219.[11] The amount of PrP^{Sc} and the number of PrP-positive follicular dendritic cells in the spleens of Ki-Hu219K/K mice were higher than those of Ki-Hu219E/E mice (Fig. 1). By contrast, the amount of PrP^{Sc} accumulation in Ki-Hu219E/K mice was even lower than that in the hemizygous Ki-Hu219E/0 mice or Ki-Hu219K/0 mice. Thus, though the human PrP 219E and PrP 219K were both conversion competent in vCJD infection, these conversion competent PrP molecules showed an inhibitory effect in the 219E/K heterozygous animals.

The Stone Fence Model of Heterozygous Inhibition

The molecular mechanisms responsible for the inhibitory effect of the conversion incompetent PrP have been studied previously.[14,23,24] Briefly, though the conversion incompetent PrP molecules are not converted into PrP^{Sc}, they bind to PrP^{Sc} and are incorporated into amyloid fibrils with the conversion competent PrP molecules. Based on these previous findings, we propose a possible mechanism to explain the inhibitory effect of the conversion competent PrP as well as the conversion incompetent PrP. We designated the mechanism as the stone fence model (Fig. 2).

PrP^{C} is converted to PrP^{Sc} and then piled up into amyloid fibrils according to the nucleated polymerization hypothesis.[25] In homozygous (219E/E or 219K/K) or hemizygous (219E/0 or K/0) animals, the conversion results in a single PrP^{Sc} population (Fig. 2). This means that the same blocks (the same PrP^{Sc}) would be piled up into the amyloid fibrils without any delay. By contrast, in the heterozygous 219E/K animals, the conversion results in at least two distinct PrP^{Sc} populations (219E PrP^{Sc} or 219K PrP^{Sc}) with different structures. These two PrP^{Sc} blocks would be piled up into the same fibril just like a stone fence composed of heterologous blocks. However, the fibril elongation would be delayed because the two types of PrP^{Sc} blocks interfere with each other due to their incompatible structures. Thus, the two PrP^{Sc} populations with different structures can act as decelerators of each other in the process of stacking.

The decelerator hypothesis based on the stone fence model is compatible with the possible mechanism for the inhibitory effect

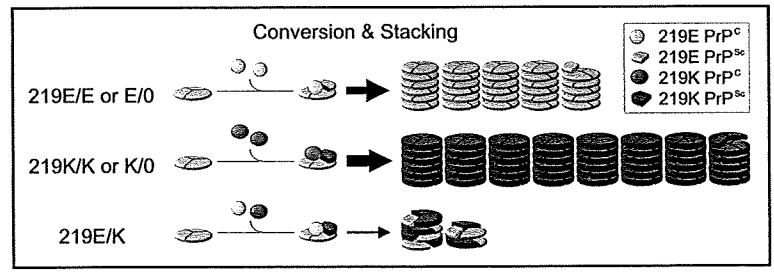

Figure 2. The stone fence model: a possible mechanism of the heterozygous inhibition in vCJD infection. PrP^{C} is converted to PrP^{Sc} and then piled up into amyloid fibrils according to the nucleated polymerization hypothesis and the trimeric models.[25-28] In the homozygous (219E/E or K/K) or the hemizygous (219E/0 or K/0) animals, the PrP^{Sc} blocks are piled up into the amyloid fibrils without delay because only a uniform PrP^{Sc} population exists. Though the initial seed is 219E PrP^{Sc} also in the 219K/K or 219K/0 animals, the resulting 219K PrP^{Sc} acts as a new seed in the subsequent steps and are efficiently piled up. Therefore, the inhibitory effect of the initial 219E PrP^{Sc} seed is negligible in these animals. By contrast, in the heterozygous (219E/K) animals, at least two PrP^{Sc} populations are generated. These two PrP^{Sc} blocks are piled up into the same fibril just like a stone fence composed of heterologous blocks. The two types of PrP^{Sc} blocks interfere with each other due to their incompatible structures and delay the fibril elongation. Thus, the distinct PrP^{Sc} populations act as decelerators of each other in the heterozygous animals.

of the conversion incompetent PrP molecules.[14,23] The conversion incompetent PrP is incorporated into amyloid fibrils with the conversion competent PrP, but acts as a decelerator at the conversion step. Therefore, the heterozygous inhibition can be caused both by the conversion incompetent PrP and the conversion competent PrP, and can occur both at the conversion step and the stacking step. Since it remains to be determined whether the human PrP 219E is efficiently converted by 219K PrP^{Sc}, we cannot rule out the possibility that the inhibition in the 219E/K heterozygous animals also occurs at the conversion step in addition to the stacking step.

Mysterious Phenomena in Prion Diseases Revisited With The Stone Fence Model

The stone fence model can explain the prion strain interference.[29,30] The preceding infection with a prion strain prior to the superinfection with another prion strain interferes with the replication of the superinfected strain.[31] According to the stone fence model (Fig. 2), the pre-existing PrP^{Sc} and the superinfected PrP^{Sc} might be piled up into the same fibril, but act as decelerators of each other due to their incompatible structures. To pile up the two distinct PrP^{Sc} populations into the same fibril, still unidentified interactions between the PrP^{Sc} molecules such as inter-oligomer interaction[32] or protofibril stacking[33] might underlie the stacking step. Thus, if the pre-existing PrP^{Sc} dominates enough, the elongation of the amyloid fibrils seeded by the superinfected PrP^{Sc} is decelerated by the pre-existing PrP^{Sc}. Meanwhile, the efficacy of interference depends on the combination of the prion strains co-infected. Scrapie 22L strain but not Chandler strain interfered with the replication of the superinfected Gerstmann-Sträussler-Scheinker disease Fukuoka-1 strain.[34] The compatibility between the two types of PrP^{Sc} blocks might determine the efficacy of interference.

The stone fence model can also account for the absence of the *PRNP* 219E/K genotype in sCJD patients.[9] In our experiments using vCJD prions, the human PrP 219E and PrP 219K were both conversion competent, whereas the susceptibility of the 219E/K heterozygous animals was even lower than that of the hemizygous animals. Furthermore, intracerebral transmission experiments using sCJD prions revealed that the human PrP 219E and PrP 219K were both conversion competent also in sCJD infection.[11,35] Though the susceptibility of the 219E/K heterozygous animals to sCJD prions remains to be determined, the absence of the PRNP 219E/K genotype in sCJD patients is probably due to the heterozygous inhibition. Even though the human PrP 219E or PrP 219K spontaneously converts into PrP^{Sc}, subsequent fibril formation and elongation would be decelerated because the heterologous PrP^{Sc} blocks act as decelerators of each other.

Lessons From the Heterozygous Inhibition Experiments

Mouse PrP 218K (corresponding to human PrP 219K) molecules are conversion incompetent in mouse scrapie infection and show dominant negative effects both in vitro and in vivo.[15-20] Therefore, the absence of the *PRNP* 219E/K genotype in sCJD patients was formerly explained by the dominant negative effect. However, our study revealed that the human PrP 219K molecules are conversion competent in sCJD infection as well as vCJD infection. This discrepancy suggested that the mutations in mouse PrP exhibit different effects from those of the corresponding mutations in human PrP as regards the conversion competence. This could have great significance for transgenic models expressing mouse PrP with mutations corresponding to the human pathogenic mutations. Otherwise, the distinct prion strains used in the experiments might underlie the discrepancy.[19]

To compare precisely the susceptibility of the experimental animals with different PrP genotypes, knock-in mice including heterozygous mice have an advantage over transgenic mice because they have identical genetic backgrounds, identical PrP expression levels, and equivalent expression from the heterozygous genes.[10] Furthermore, the hemizygous knock-in mice express exactly half the level of PrP compared to the homozygous mice.[11] Since the expression level of PrP affects the length of the incubation period regardless of the PrP genotype, the heterozygous and the hemizygous knock-in mice are both indispensable to analyze the heterozygous inhibition.

Conclusion

The present study, together with evidence from other groups, suggests that heterozygous inhibition is a universal phenomenon that can be caused by both conversion incompetent PrP and conversion competent PrP, or by both PrP^{C}-heterozygosity and PrP^{Sc}-heterozygosity. The decelerator hypothesis based on the stone fence model paves the way for the solution of this phenomenon. To determine whether the efficacy of heterozygous inhibition is affected by the infected prion strain or the host PrP genotype, other heterozygous models need to be examined.

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Symposium: Prion diseases — Updated

A traceback phenomenon can reveal the origin of prion infection

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The transmission of prions to animals with incongruent prion protein (PrP) gene (referred to as cross-sequence transmission) results in a relatively long incubation period and can generate a new prion strain with unique transmissibility designated as a traceback phenomenon. For example, cross-sequence transmission of bovine spongiform encephalopathy (BSE) prions to human generated variant Creutzfeldt-Jakob disease (vCJD) prions which retained the transmissibility to mice expressing bovine PrP. This finding suggests that traceback studies could enable us to identify the origin of prions. There are two distinct phenotypes in dura mater graft-associated Creutzfeldt-Jakob disease (dCJD), with the majority represented by a non-plaque-type of dCJD (np-dCJD) and the minority by a plaque-type of dCJD (p-dCJD). To identify the origin of p-dCJD, we performed a traceback study using mice expressing human PrP with methionine homozygosity (129M/M) or valine homozygosity (129V/V) at polymorphic codon 129. The characteristics of p-dCJD such as the accumulation of abnormal isoform of PrP (PrP^{Sc}) intermediate in size between type 1 and type 2, and plaque-type PrP deposition in the brain were maintained after transmission to the 129M/M mice. Furthermore, the 129V/V mice were more susceptible to p-dCJD prions than the 129M/M mice and produced type 2 PrP^{Sc} that were identical in size to those from the 129V/V mice inoculated with sporadic CJD prions from a patient with 129V/V and type 2 PrP^{Sc} (sCJD-VV2). In addition, we performed intracerebral transmission of sCJD-VV2 prions to the 129M/M mice as an experimental model for p-dCJD. These 129M/M mice showed the accumulation of the intermediate type PrP^{Sc} and plaque-type PrP deposition in the brain. These results suggest that p-dCJD could be caused by cross-sequence transmission of sCJD-VV2 prions to individuals with the 129M/M genotype.

Key words: Creutzfeldt-Jakob disease, polymorphism, prion protein, traceback, transmission.

INTRODUCTION

Creutzfeldt-Jakob disease (CJD), scrapie, and bovine spongiform encephalopathy (BSE) are lethal transmissible neurodegenerative diseases caused by an abnormal isoform of prion protein (PrP^{Sc}), which is converted from the normal cellular isoform (PrP^{C}).[1] The homology of the prion protein (PrP) gene between inoculated animals and the inoculum determines the susceptibility to prion infection.[2–4] The transmission of prions to animals with incongruent PrP gene (referred to as cross-sequence transmission) results in a relatively long incubation period.

The potential for cross-sequence transmission should be considered in the iatrogenic transmission of CJD prions, for example dura mater graft-associated CJD (dCJD), because codon 129 of the human PrP gene shows methionine (M) / valine (V) polymorphism. The genotype (M/M, M/V, or V/V) at codon 129 and type (type 1 or type 2) of PrP^{Sc} in the brain are major determinants of the clinicopathological phenotypes of sporadic CJD (sCJD).[5–7] Type 1 and type 2 PrP^{Sc} are distinguishable according to the size of the proteinase K-resistant core of PrP^{Sc} (PrP^{res}) (21 and 19 kDa, respectively), reflecting differences in the proteinase K-cleavage site (at residues 82 and 97, respectively).[5,8] According to this molecular typing system, sCJD can be classified into six subgroups (MM1, MM2, MV1, MV2, VV1, or VV2). In Europe, 28.4% of sCJD patients are valine homozygotes (129V/V) or methionine/valine heterozygotes at codon 129 (129M/V).[6] Meanwhile, the

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A

Inoculum			Susceptibility[†](n/n°)[‡]
vCJD		96/02	5/5
		96/07	4/5
		Jpn	4/4
sCJD	MM1	H3	0/5
	MM2 (Cortical)	KZ	0/5
	MM2 (Thalamic)	NG	0/4
	MV1	SM	0/5
	MV2	PH	0/5
	VV2	AK	0/6
dCJD	np-d	GF	0/3
		TC	0/4
	p-d	KR	0/5
		TV	0/5

[†]Positives were confirmed by immunohistochemical analysis of the spleens.
[‡]n, number of animals with positive labeling of abnormal PrP in the spleens; n°, number of inoculated animals.

B

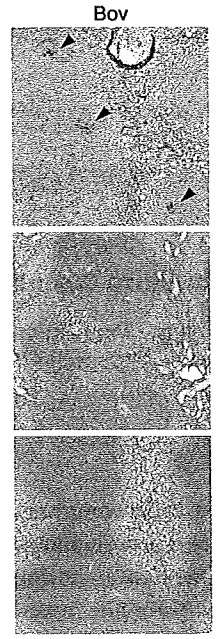


Fig. 1 The traceback phenomenon in variant Creutzfeldt-Jakob disease (vCJD) prion transmission. (a) Summary of intraperitoneal transmission of vCJD prions, sporadic CJD (sCJD) prions, or dura mater graft-associated CJD (dCJD) prions to prion protein (PrP)-bovinized mice. The transmission study was performed with 50 μL of 10% brain homogenates from vCJD patients, sCJD patients, or dCJD patients. Knock-in mice expressing bovine PrP (Ki-Bov/Bov) were highly susceptible to vCJD prions but not to other CJD prions. (b) Immunohistochemical analysis of the spleens after hydrolytic autoclaving pretreatment using anti-PrP antiserum PrP-N.[23,24] The PrP-bovinized mice inoculated with vCJD prions showed PrP deposition in follicular dendritic cells (arrowheads).

population data show a high prevalence (91.6%) of methionine homozygosity (129M/M) in Japanese people.[9] Since the dural grafts used in Japan were manufactured by German companies,[10–12] these data raise the possibility that part of the Japanese dCJD cases might have been caused by cross-sequence transmission of sCJD prions. Indeed, there are two distinct phenotypes in dCJD, with the majority represented by a non-plaque-type of dCJD (np-dCJD) and the minority by a plaque-type of dCJD (p-dCJD).[13–17] The clinicopathological features of np-dCJD are similar to those of sCJD-MM1.[11] By contrast, despite the fact that the 129M/M genotype is identical to np-dCJD patients, p-dCJD shows unique features characterized by: (i) a clinical course of long duration; (ii) the absence or late occurrence of myoclonus and periodic synchronous discharges on electroencephalogram; and (iii) plaque-type PrP deposition in the brain.[13–21] The reason for the existence of the two distinct phenotypes in dCJD has remained elusive.

In this review, we describe a new strategy to identify the origin of prions. We found that prion strains emerged through cross-sequence transmission had unique transmissibility designated as the traceback phenomenon. The traceback study revealed that p-dCJD could be caused by cross-sequence transmission of sCJD-VV2 prions to individuals with the 129M/M genotype.

DISCOVERY OF TRACEBACK PHENOMENON

To investigate the transmissibility of variant CJD (vCJD) prions, we previously performed intraperitoneal inoculation of brain homogenates from vCJD patients into PrP-humanized mice or bovinized mice.[22] Immunohistochemical analysis of the spleens at 75 days post-inoculation revealed that knock-in mice expressing bovine PrP (Ki-Bov/Bov) were highly susceptible to vCJD prions despite cross-sequence transmission (number of diseased animals/number of inoculated animals = 13/14) (Fig. 1). By contrast, other CJD prions including sCJD (MM1, MM2, MV1, MV2, or VV2) or dCJD (np-d or p-dCJD) showed negative transmissibility to Ki-Bov/Bov mice. These phenomena can be explained as follows: cross-sequence transmission of BSE prions to human generated vCJD prions, which retained the memory of the parental BSE prions within its conformational properties and/or its PrP^{Sc} subpopulation. Therefore, PrP-bovinized mice showed high susceptibility to vCJD prions. We designated these phenomena as traceback and considered that a traceback study could be a powerful tool to identify the origin of prions.

TRACEBACK OF PLAQUE-TYPE OF DURA-CJD PRIONS

To identify the origin of p-dCJD prions, we performed a traceback study using PrP-humanized mice with the 129M/M or 129V/V genotype. We performed intracerebral inoculation of a brain homogenate from a p-dCJD patient into knock-in mice expressing human PrP with 129M/M (Ki-Hu129M/M) or 129V/V (Ki-Hu129V/V).[25] The mean incubation time of Ki-Hu129M/M mice was 500 ± 59 days (4/4), which was longer than that of Ki-Hu129V/V mice (304 ± 13 days [6/6]) (Fig. 2a). Thus, the 129V/V mice

A

Inoculum		Incubation period in days±SEM (n/n°)[1] 129M/M Tg+Ki-Hu129M$_{4R}$/M$_{4R}$ (9.8×)[2]	129M/M Ki-Hu129M/M (1×)	129V/V Ki-Hu129V/V (1×)
np-dCJD	GF	161±5 (5/5)	N.D.[3]	N.D.
	TC	208±2 (5/5)	N.D.	N.D.
p-dCJD	KR	420±10 (5/5)	N.D.	259±6 (6/6)
	KD	398±10 (5/5)	500±59 (4/4)	304±13 (6/6)

[1] n, number of diseased animals; n°, number of inoculated animals.
[2] The expression levels of human PrP in the brains.
[3] N.D., not done.

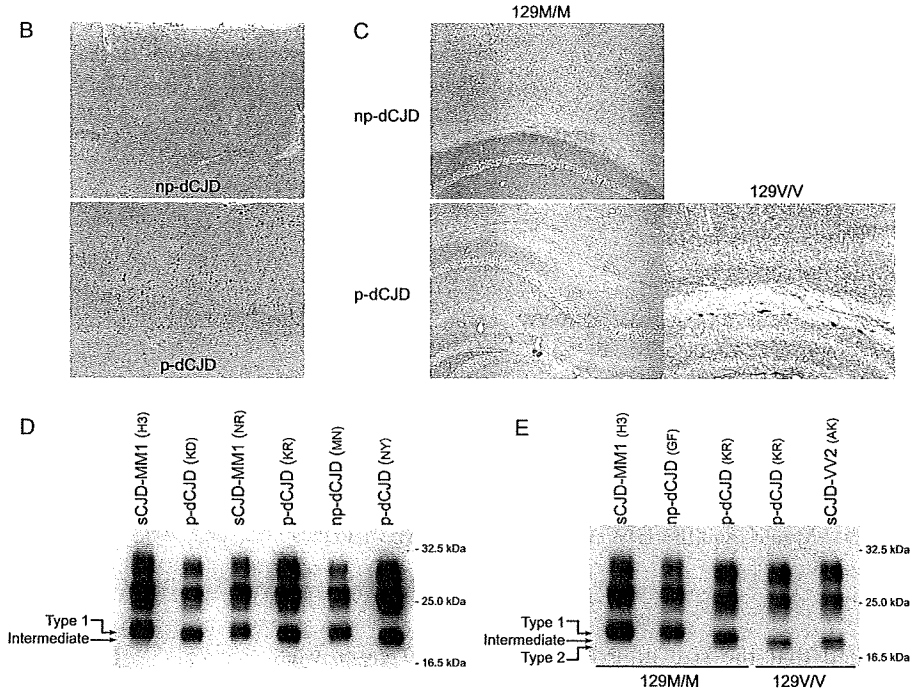


Fig. 2 The traceback phenomenon in p-dCJD prions transmission. (a) Summary of intracerebral transmission of dura mater graft-associated CJD (dCJD) prions to prion protein (PrP)-humanized mice with the methionine homozygosity (129M/M) or valine homozygosity (129V/V) genotype. The transmission study was performed with 20 μL of 10% brain homogenates from non-plaque-type of dCJD (np-dCJD) patients or plaque-type dCJD (p-dCJD) patients. The 129V/V mice were more susceptible to p-dCJD prions than the 129M/M mice. By contrast, the 129M/M mice were highly susceptible to np-dCJD prions. (b) Plaque-type PrP deposition in the brain is a characteristic feature of p-dCJD cases. np-dCJD cases show diffuse synaptic-type PrP deposition in the grey matter. (c) Immunohistochemical analysis of the brains after hydrolytic autoclaving pretreatment using anti-PrP antiserum PrP-N. The 129M/M mice and the 129V/V mice showed a few plaque-type PrP deposits besides the diffuse synaptic-type deposition in the grey matter when inoculated with p-dCJD prions. The 129M/M mice inoculated with np-dCJD prions showed synaptic-type PrP deposition. (d) Western blot analysis of the brains from dCJD patients or those from sporadic CJD (sCJD)-MM1 patients after proteinase K-digestion using anti-PrP antibody 3F4. The intermediate type proteinase K-resistant core of the abnormal isoform of prion protein (PrP^{res}) was a common form in p-dCJD cases. (e) Western blot analysis of the brains from PrP-humanized mice after proteinase K-digestion using anti-PrP antibody 3F4. The 129M/M mice inoculated with p-dCJD prions produced the intermediate type PrP^{res} that were smaller than type 1 PrP^{res} from the 129M/M mice inoculated with np-dCJD prions. Moreover, the 129V/V mice inoculated with p-dCJD prions produced type 2 PrP^{res} that were identical in size and glycoform ratio to type 2 PrP^{res} from the 129V/V mice inoculated with sCJD-VV2 prions.

were highly susceptible to p-dCJD prions despite cross-sequence transmission. Furthermore, we also verified that the 129V/V mice were more susceptible to p-dCJD prions than PrP-humanized mice overexpressing (9.8×) human PrP with 129M/M. For p-dCJD prions, the mean incubation times of knock-in mice crossed with transgenic mice expressing human PrP with 129M/M and four octapeptide repeats (Tg+Ki-Hu129M$_{4R}$/M$_{4R}$) were 420 ± 10 (5/5) and 398 ± 10 (5/5) despite the high expression level of human PrP. By contrast, the mean incubation times of Tg+Ki-Hu129M$_{4R}$/M$_{4R}$ mice inoculated with np-dCJD prions were 161 ± 5 (5/5) and 208 ± 2 days (5/5).

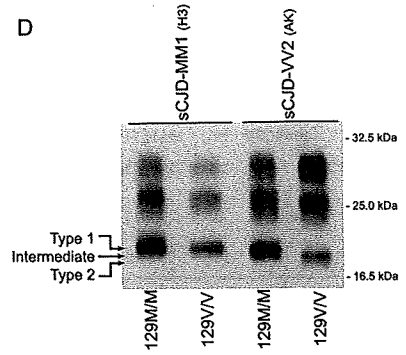
p-dCJD are very different from np-dCJD in the patterns of PrP deposition (Fig. 2b). In p-dCJD cases, plaque-type deposition and perineuronal deposition spread throughout the cerebral grey matter in addition to the diffuse synaptic-type deposition. Meanwhile, np-dCJD cases show only diffuse synaptic-type PrP deposition. Similarly, immunohistochemical analysis of the brains from the PrP-humanized mice inoculated with p-dCJD prions showed characteristic patterns of PrP deposition. The 129M/M mice inoculated with np-dCJD prions showed diffuse synaptic-type PrP deposition in the cerebral grey matter (Fig. 2c). By contrast, for p-dCJD prions, the 129M/M mice and the 129V/V mice showed a few plaque-type PrP deposits besides the diffuse synaptic-type deposition.

To date, we have classified p-dCJD cases into MM1. However, there has been an exceptional case of p-dCJD that showed the accumulation of intermediate-sized PrP^{res} located between type 1 and type 2.[15] Therefore, we re-examined carefully the size of PrP^{res} in the brains from three p-dCJD patients (Fig. 2d). Western blot analysis

A

Inoculum		Incubation period in days±SEM (n/n°)[1] 129M/M Tg+Ki-Hu129M$_{4R}$/M$_{4R}$ (9.8×)[2]	129V/V Ki-Hu129V/V (1×)
sCJD-MM1	H3	175±4 (9/9)	542, 648 (2/5)[3]
sCJD-VV2	AK	505±14 (5/5)	312±7 (4/4)

[1]n, number of diseased animals; n°, number of inoculated animals.
[2]The expression levels of human PrP in the brains.
[3]Three animals died of causes other than prion disease.



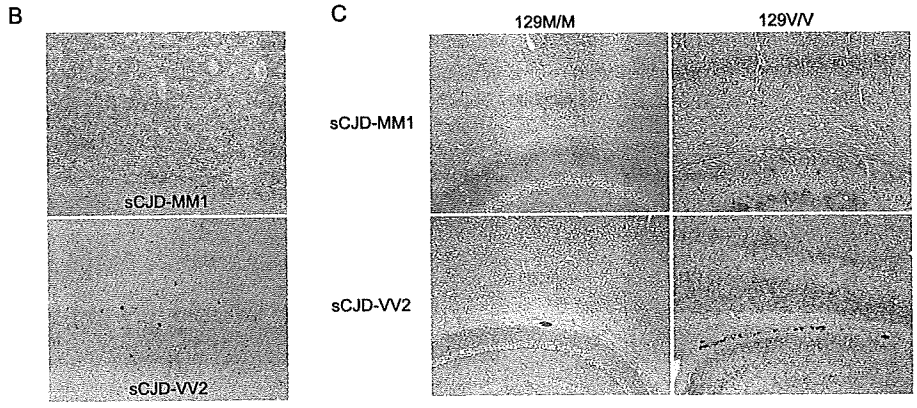


Fig. 3 Cross-sequence transmission of sporadic Creutzfeldt-Jakob disease (sCJD)-MM1 prions or sCJD-VV2 prions. (a) Summary of intracerebral transmission of sCJD prions to prion protein (PrP)-humanized mice with the methionine homozygosity (129M/M) or valine homozygosity (129V/V) genotype. The transmission study was performed with 20 μL of 10% brain homogenate from a sCJD-MM1 patient or a sCJD-VV2 patient. Cross-sequence transmission resulted in relatively long incubation times. (b) The characteristic patterns of PrP deposition in sCJD-MM1 or sCJD-VV2 cases. sCJD-MM1 cases show diffuse synaptic-type PrP deposition in the grey matter. sCJD-VV2 cases show plaque-type PrP deposition in addition to the synaptic-type deposition. (c) Immunohistochemical analysis of the brains after hydrolytic autoclaving pretreatment using anti-PrP antiserum PrP-N. The 129M/M mice inoculated with sCJD-VV2 prions showed a few plaque-type PrP deposits similar to those in the 129M/M mice inoculated with plaque-type dura mater graft-associated CJD (p-dCJD) prions. For sCJD-MM1 prions, the 129M/M mice and the 129V/V mice showed diffuse synaptic-type PrP deposition in the cerebral grey matter. (d) Western blot analysis of the brains after proteinase K-digestion using anti-PrP antibody 3F4. The 129M/M mice inoculated with sCJD-VV2 prions produced the intermediate type proteinase K-resistant core of the abnormal isoform of prion protein (PrP^{res}) with an upward size shift from the inoculated type 2 template. For sCJD-MM1 prions, the 129M/M mice and the 129V/V mice produced type 1 PrP^{res}.

revealed that the size of PrP^{res} from p-dCJD cases was smaller than that of MM1 PrP^{res} from sCJD-MM1 or np-dCJD cases. Thus, it appeared that the intermediate type PrP^{res} is not rare but rather a common form in p-dCJD cases. Furthermore, the 129M/M mice inoculated with p-dCJD prions produced the intermediate type PrP^{res}, whereas the 129M/M mice inoculated with np-dCJD prions produced type 1 PrP^{res} (Fig. 2e). Thus, the characteristics of p-dCJD such as the intermediate type PrP^{res} and plaque-type PrP deposits in the brain were maintained after transmission to the 129M/M mice. Meanwhile, the 129M/M mice inoculated with np-dCJD prions showed the accumulation of type 1 PrP^{res} and synaptic-type PrP deposits in the brain. These results clearly demonstrate that p-dCJD and np-dCJD are distinct subtypes of d-CJD caused by distinct prion strains. Moreover, the 129V/V mice inoculated with p-dCJD prions produced type 2 PrP^{res} with a downward size shift from the intermediate type template (Fig. 2e). Thus, the traceback study revealed that the 129V/V mice inoculated with p-dCJD prions showed high susceptibility and accumulation of type 2 PrP^{res}. These findings raised the possibility that p-dCJD might be caused by cross-sequence transmission of sCJD-VV2 prions.

EXPERIMENTAL MODEL FOR PLAQUE-TYPE OF DURA-CJD

To address this possibility, we performed intracerebral inoculation of a brain homogenate from a sCJD-MM1 patient or that from a sCJD-VV2 patient into the PrP-humanized mice as experimental models for dCJD. For sCJD-MM1 prions, the mean incubation time of Tg+Ki-Hu129M$_{4R}$/M$_{4R}$ mice was 175 ± 4 days (9/9), which was shorter than that of Ki-Hu129V/V mice (542 and 648 days, 2/5) (Fig. 3a). For sCJD-VV2 prions, the mean incubation time of Tg+Ki-Hu129M$_{4R}$/M$_{4R}$ mice was 505 ± 14 days (5/5), which was longer than that of Ki-Hu129V/V mice (312 ± 7 days, 4/4).

sCJD-MM1 cases and sCJD-VV2 cases show quite different patterns of PrP deposition in the brain (Fig. 3b). sCJD-MM1 cases show diffuse synaptic-type deposition, whereas sCJD-VV2 cases show plaque-type deposition and perineuronal deposition besides the diffuse synaptic pattern. Similarly, immunohistochemical analysis of the brains revealed that the 129M/M mice and the 129V/V mice showed diffuse synaptic-type PrP deposition in the cerebral grey matter when inoculated with sCJD-MM1