Human Prion Protein (PrP) 219K Is Converted to PrPSc but Shows Heterozygous Inhibition in Variant Creutzfeldt-Jakob Disease Infection*§

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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 (PrPSc) 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 PrPSc 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-Straussler 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 (PrPSc) of prion protein (PrP), which is converted from the normal cellular isoform (PrPc) (5). The human PrP contains 253 amino acids encoded by prion protein gene (PRNP), which is located on chromosome 20. Numerous point mutations or insertion 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-Straussler 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 PrPSc 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 vitro fibril formation without protein X (17). In contrast to the murine prnp 218K variant, the human PrP 219K molecule did not show a dominant negative effect either in scrapie-infected neuroblastoma cells (15) or in transgenic mice (16). This dominant negative effect of the human 219K variant in our experiments may be due to heterozygous inhibition.

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** The abbreviations used are: CJD, Creutzfeldt-Jakob disease; vCJD, variant CJD; sCJD, sporadic CJD; PrP, prion protein; PrPc, normal cellular isoform of PrP; PrPSc, abnormal isoform of PrP; FDC, follicular dendritic cell; PRNP, human prion protein gene; prnp, murine prion protein gene.

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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 PrPSc 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. 1A). 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 MMV1). 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 PrPSc 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 post-mortem. 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.

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 PrPSc in 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 PrPSc 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
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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. 2A). 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 PrPSc 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. S1A). 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. 2A). 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. 2B). Western blot analysis showed that the quantity of PrPSc 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 PrPSc 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. S1B). 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 PrPSc 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 PrPSc. Western blot analysis, Ki-Hu129M/M and Ki-Hu129M/V had PrPSc in the spleen, but Ki-Hu129V/V did not (Fig. 3A and B). However, the amount of PrPSc 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 PrPSc.
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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 PrPSc 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-M1 prions (129M/M, 219E/E, and type 1 PrPSc). It is significant that human PrP has a amino acid substitution of glutamine corresponding to codon 219. The structure of PrPSc with Hu219K might have a similar structure of BSE PrPSc with 219Q compared with that of Hu219E. Based on the codon 219 substitution, we can design transmissible prion models. 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-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 showed no clinical signs and still are alive after >740 days of incubation (Fig. 4A). 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 PrPSc and the protease resistance of PrPSc 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-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 showed no clinical signs and still are alive after >740 days of incubation (Fig. 4A). Therefore, heterozygous inhibition was similarly observed as in intracerebral infections.

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/E and Ki-Hu219K/K), 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 PrPSc 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/mm²) are presented. We assigned a signal intensity of 100/mm² for Ki-Hu219E/E. The corrected signal intensities are as follows: Ki-Hu219E/E, 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/mm² (mean ± S.E.).
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-Fc2 showed a reduced conversion of wild-type PrP by dimeric PrP-Fc2. Interestingly, the dimeric PrP-Fc2 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 PrPβSc molecule (36, 37) (Fig. 5). At first, PrPβC is converted to PrPβSc,
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![Diagram showing the conversion and inhibitory effect of PrP 219K.]

FIGURE 5. A possible mechanism underlying the heterozygous inhibition in vCJD infection. Upper, PrPSc proliferation model in Ki-Hu219E/K or Ki-Hu219E/K. The initial seed is Hu219E PrPSc. However, the resulting Hu219K PrPSc acts as a decelerator. In this figure, each circle or block designates the following: open circles, Hu219E PrPSc; open hexagonal blocks, Hu219E PrPSc; filled circles, Hu219K PrPSc; and filled hexagonal blocks, Hu219K PrPSc.

then the converted PrPSc is piled up into amyloid fibrils according to the nucleated polymerization hypothesis (38). In the homozygous and hemizygous animals, there is only one structural PrPSc. This means that the same blocks (the same structural PrPSc) 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 PrPSc 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 (PrPSc) with a distinct structure (Fig. 5). The two different-shaped PrPSc 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 PrPSc 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 PrPSc 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 PrPSc. This PrPSc with a distinct conformation may inhibit another type of PrPSc amyloid formation. Recently, it has been reported that the prion interference is due to a reduction of the strain-specific PrPSc level (40). Our decelerator hypothesis can account for such prion interference.

In this report, we clearly show heterozygous inhibition of PrPSc 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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