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Abstract

The clinicopathological phenotypes of sporadic Creutzfeldt-Jakob disease (sCJD) correlate with the allelotypes (M or V) of the polymorphic codon 129 of the human prion protein (PrP) gene and the electrophoretic mobility patterns of abnormal prion protein (PrP(Sc)). Transmission of sCJD prions to mice expressing human PrP with a heterologous genotype (referred to as cross-sequence transmission) results in prolonged incubation periods. We previously reported that cross-sequence transmission can generate a new prion strain with unique transmissibility designated as a traceback phenomenon. To verify experimentally the traceback of sCJD-VV2 prions, we inoculated sCJD-VV2 prions into mice expressing human PrP with the 129M/M genotype. These 129M/M mice showed altered neuropathology and a novel PrP(Sc) type after a long incubation period. We then passaged the brain homogenate from the 129M/M mouse inoculated with sCJD-VV2 prions into further 129M/M or 129V/V mice. Despite cross-sequence transmission, 129V/V mice were highly susceptible to these prions compared to the 129M/M mice. The neuropathology and PrP(Sc) type of the 129V/V mice inoculated with the 129M/M mouse-passaged sCJD-VV2 prions were identical to those of the 129V/V mice inoculated with sCJD-VV2 prions. Moreover, we generated for the first time type 2 PrP(Sc)-specific antibody in addition to type 1 PrP(Sc)-specific antibody and discovered that drastic changes in the PrP(Sc) subpopulation underlie the traceback phenomenon. Here we report the first direct evidence of the traceback in prion infection.
Experimental Verification of a Traceback Phenomenon in Prion Infection

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ABSTRACT

The clinicopathological phenotypes of sporadic Creutzfeldt-Jakob disease (sCJD) correlate with the allelotype (M or V) of the polymorphic codon 129 of the human prion protein (PrP) gene and the electrophoretic mobility patterns of abnormal prion protein (PrPSc). Transmission of sCJD prions to mice expressing human PrP with a heterologous genotype (referred to as cross-sequence transmission) results in prolonged incubation periods. We previously reported that cross-sequence transmission can generate a new prion strain with unique transmissibility designated as a traceback phenomenon. To verify experimentally the traceback of sCJD-VV2 prions, we inoculated sCJD-VV2 prions into mice expressing human PrP with the 129M/M genotype. These 129M/M mice showed altered neuropathology and a novel PrPSc type after a long incubation period. We then passaged the brain homogenate from the 129M/M mouse inoculated with sCJD-VV2 prions into further 129M/M or 129V/V mice. Despite cross-sequence transmission, 129V/V mice were highly susceptible to these prions compared to the 129M/M mice. The neuropathology and PrPSc type of the 129V/V mice inoculated with the 129M/M mouse-passaged sCJD-VV2 prions were identical to those of the 129V/V mice inoculated with sCJD-VV2 prions. Moreover, we generated for the first time type 2 PrPSc-specific antibody in addition to type 1 PrPSc-specific antibody and discovered that drastic changes in the PrPSc subpopulation underlie the traceback phenomenon. Here we report the first direct evidence of the traceback in prion infection.
INTRODUCTION

Creutzfeldt-Jakob disease (CJD) is a lethal transmissible neurodegenerative disease caused by an abnormal isoform of prion protein (PrP<sup>Sc</sup>), which is converted from the normal cellular isoform (PrP<sup>C</sup>) (1, 23). The genotype (M/M, M/V, or V/V) at polymorphic codon 129 of the human prion protein (PrP) gene and the type (type 1 or type 2) of PrP<sup>Sc</sup> in the brain are major determinants of the clinicopathological phenotypes of sporadic CJD (sCJD) (15-18). Type 1 and type 2 PrP<sup>Sc</sup> are distinguishable according to the size of the proteinase K-resistant core of PrP<sup>Sc</sup> (PrP<sup>res</sup>) (21 and 19 kDa, respectively), reflecting differences in the proteinase K-cleavage site (at residues 82 and 97, respectively) (15, 18). According to this molecular typing system, sCJD can be classified into six subgroups (MM1, MM2, MV1, MV2, VV1, or VV2).

The homology of the PrP gene between inoculated animals and the inoculum determines the susceptibility to prion infection. Transmission of sCJD prions to mice expressing human PrP with a non-homologous genotype (referred to as cross-sequence transmission) results in a relatively long incubation period (10, 12). Meanwhile, the cross-sequence transmission can generate a new prion strain.

Transmission of sCJD-VV2 prions to mice expressing human PrP with the 129M/M genotype generates unusual PrP<sup>res</sup> intermediate in size between type 1 and type 2 (10). We have designated this unusual PrP<sup>res</sup> with an upward size shift (Sh+) from the inoculated type 2 template as MM[V2]<sup>2<sup>Sh+</sup></sup>PrP<sup>res</sup>: host genotype [inoculated prions] type of generated PrP<sup>res</sup>.

Similar to the MM[V2]<sup>2<sup>Sh+</sup></sup>PrP<sup>res</sup>, the intermediate-sized PrP<sup>res</sup> has been observed in the plaque-type of dura mater graft-associated CJD (p-dCJD) (10, 13). Furthermore, a transmission study using p-dCJD prions revealed that PrP-humanized mice with the 129V/V genotype were highly susceptible to p-dCJD
prions despite cross-sequence transmission (10). In addition, these 129V/V mice inoculated with p-dCJD prions produced type 2 PrP<sub>res</sub> (10). These findings suggest that p-dCJD could be caused by cross-sequence transmission of sCJD-VV2 prions to individuals with the 129M/M genotype. We have designated these phenomena as “traceback”. The traceback phenomenon was discovered for the first time by a transmission study using variant CJD (vCJD) prions (2). Mice expressing bovine PrP were highly susceptible to vCJD prions because vCJD was caused by cross-sequence transmission of bovine spongiform encephalopathy prions to human. These findings suggest that a traceback study can be a powerful tool to identify the origin of prions (2, 10, 11). However, the traceback phenomenon has not been verified experimentally despite the abundant circumstantial evidence described above.

To verify the traceback of sCJD-VV2 prions, we inoculated sCJD-VV2 prions into PrP-humanized mice with the 129M/M genotype as an experimental model of p-dCJD. Thereafter, we inoculated these MM[VV2]<sub>2</sub>Sh<sup>+</sup> prions into PrP-humanized mice with the 129M/M or 129V/V genotype, and compared the incubation period, neuropathology, and the type of PrP<sub>res</sub> in the brain. Here we report the first direct evidence of the traceback in prion infection.

**MATERIALS AND METHODS**

**Production of PrP<sub>res</sub> type-specific polyclonal antibodies**

A synthetic peptide corresponding to human PrP 82-98 was used as the immunogen for type 1 PrP<sub>res</sub>-specific antibody Tohoku 1 because residues 82-96 were retained in type 1 PrP<sub>res</sub> but not in type 2 PrP<sub>res</sub> after proteinase K-digestion (18). For type 2 PrP<sub>res</sub>-specific antibody Tohoku 2, a short synthetic peptide corresponding human PrP 97-103 was used as the immunogen because the length of the
immunogen peptide is critical for the production of proteolytic cleavage site-specific polyclonal antibodies (25, 26). Cysteine residues were added to the C-terminus of each peptide and was utilized for conjugation to bovine thyroglobulin via EMCS (N-(6-Maleimidocaproyloxy)succinimide) (Dojin). For the initial injection, 100 μg of conjugates were emulsified in complete Freund’s adjuvant and subcutaneously injected into rabbits. For the boosting injections, 100 μg of conjugates were emulsified in incomplete Freund’s adjuvant and were subcutaneously injected on days 7, 21, 35, 49, 63, 84, and day 91. At day 98, the rabbits were sacrificed and sera were collected. Antibodies were purified by affinity chromatography using the immunogen peptides. Another type 1 PrP<sup>res</sup>-specific monoclonal antibody, POM2, reacts with repeated octapeptide epitopes 59-65, 67-73, 75-81, and 83-89 of human and murine PrP (21, 22).

Production of knock-in mice and transgenic mice

The production of knock-in mice expressing human PrP with 129M/M (Ki-Hu129M/M) and Ki-Hu129V/V mice has been reported previously (2). Ki-Hu129M/M mice and knock-in mice expressing human PrP with 129M/M and 4 octapeptide repeats (Ki-Hu129M<sub>4R</sub>/M<sub>4R</sub>) were crossed with transgenic mice expressing human PrP with 129M (Tg-Hu129M) and Tg-Hu129M<sub>4R</sub>, respectively (10). The expression levels of human PrP in the brains from Tg+Ki-Hu129M/M and Tg+Ki-Hu129M<sub>4R</sub>/M<sub>4R</sub> were 1.2x and 9.8x, respectively, the levels observed in Ki-Hu129M/M mice.

Human brain inocula

Brain tissues were obtained at autopsy from CJD patients after receiving informed consent for research.
use. The diagnosis of CJD and the type of PrP\textsuperscript{Sc} were confirmed by neuropathological examination, PrP\textsuperscript{Sc} immunohistochemistry and western blotting as described (7, 27). The genotype and the absence of mutations in the open reading frame of the PrP gene were determined by sequence analysis (8). The CJD cases selected for the transmission studies were typical of the sCJD-MM1 and sCJD-VV2 subgroups. In the sCJD-VV2 case (AK), the plaque-type PrP deposition in the brain and the absence of periodic synchronous discharges on electroencephalogram were confirmed. More detailed information of the patient was reported previously (4). sCJD-MM1 (H3) and sCJD-VV2 (AK) showed the same transmissibilities to PrP-humanized mice as those of the other sCJD-MM1 and sCJD-VV2 isolates, respectively (27).

Transmission experiments

Human brain homogenates (10%) and mouse brain homogenates (10%) were prepared as described (9). Intracerebral transmission was performed using 20 µl of the homogenates (27). The inoculated mice were sacrificed after the onset of disease, and their brains were immediately frozen or fixed in 10% buffered formalin.

Immunohistochemistry

Formalin-fixed mouse brains were treated with 60% formic acid for 1 hour to inactivate the infectivity and embedded in paraffin. Tissue sections were pretreated by hydrolytic autoclaving before PrP immunohistochemistry (7). The PrP-N antiserum was used as the primary antibody (6). Goat-anti-rabbit immunoglobulins polyclonal antibody labeled with the peroxidase-conjugated dextran polymer,
Expression of Glutathione S-transferase (GST)-fusion recombinant PrP

The open reading frame of the human PrP gene was amplified by PCR with human DNA. The amplified fragment was cloned into pBluescript plasmid (Stratagene). With the plasmid construct, N-terminally or C-terminally truncated human PrP gene fragments were amplified by PCR. The primers used for the amplification are shown in Table 1. These primers introduced BamH I sites at the 5’ end of the fragments and Xho I sites at the 3’ end of the fragments. The amplified fragments were cloned into pGEM-T Easy plasmid (Promega). After digestion with BamH I and Xho I, the fragments were inserted into the BamH I/Xho I sites of the expression vector pGEX-4T-1 (GE Healthcare). Escherichia coli BL21 (DE3) were transformed with the pGEX-4T-1 plasmid constructs, and GST- fusion recombinant PrP fragments were purified using glutathione sepharose 4B beads (GE Healthcare) according to the manufacturer’s instructions. GST-fusion recombinant PrP fragments were subjected to 13% SDS-PAGE and western blotting.

Enzyme-linked immunosorbent assay (ELISA)

Synthetic peptides corresponding to human PrP97-103 and 93-103 were used as the antigens. Plates were individually coated with 50 ng/50 µl/well antigen or with 0.1% bovine serum albumin (BSA). The polyclonal antibody Tohoku 2 was serially diluted and added to each well as the primary antibody. Goat-anti-rabbit IgG Fab’ fraction labeled with horseradish peroxidase was used as the secondary antibody. The color was developed with o-phenylenediamine.
Western blotting

PrP<sup>Sc</sup> was extracted from mouse brains with collagenase treatment as described (5) with some modifications. Samples were subjected to 13% SDS-PAGE and western blotting as described (2). The 3F4 monoclonal antibody (Signet Laboratories), PrP-N (Immuno-Biological Laboratories), PrP-C (Immuno-Biological Laboratories), POM2, Tohoku 1, and Tohoku 2 were used as the primary antibodies. Anti-mouse EnVision+ and anti-rabbit EnVision+ were used as the secondary antibodies. The signal intensities of the western blots were quantified with Quantity One software using an imaging device VersaDoc 5000 (Bio-Rad Laboratories).

Statistical analysis

Incubation times and signal intensities of PrP<sup>res</sup> bands are expressed as mean ± SEM.

RESULTS

Transmission of MM[VV2]<sup>2</sup><sup>Sh+</sup> prions to PrP-humanized mice with the 129M/M or 129V/V genotype

To verify the traceback of sCJD-VV2 prions, we performed intracerebral inoculation of a brain homogenate from a sCJD-VV2 patient into Tg+Ki-Hu129M/M mice. Thereafter, we performed the second passage of the brain homogenate from Tg+Ki-Hu129M/M mouse inoculated with sCJD-VV2 prions (MM[VV2]<sup>2</sup><sup>Sh+</sup> prions: host genotype [inoculated prions] type of generated PrP<sup>res</sup>). Since Tg+Ki-Hu129M/M mice were established before the Ki-Hu129M/M mice were produced, we used
them in the primary transmission of sCJD-VV2 prions. The data of the primary transmission were already reported previously (10). The mean incubation time of Tg+Ki-Hu129M/M mice inoculated with sCJD-VV2 prions was 723 ± 79 days (number of diseased animals / number of inoculated animals = 4/4) (Table 2). In the second passage, we inoculated intracerebrally the MM[VV2]2Sh+ prions into Ki-Hu129M/M mice or Ki-Hu129V/V mice. Ki-Hu129M/M mice inoculated with MM[VV2]2Sh+ prions showed long incubation times of 685 ± 17 days (6/6). By contrast, the mean incubation time of Ki-Hu129V/V mice inoculated with MM[VV2]2Sh+ prions was shortened to 309 ± 3 days (7/7). In spite of cross-sequence transmission, the mean incubation time of Ki-Hu129V/V mice was much shorter than that of Ki-Hu129M/M mice. Immunohistochemical analysis of the brains from Tg+Ki-Hu129M/M mice inoculated with sCJD-VV2 prions showed large plaque-type PrP deposits spread throughout the cerebral grey matter and thalamus (Fig. 1). Ki-Hu129M/M mice inoculated with MM[VV2]2Sh+ prions showed similar patterns of PrP deposition to those of Tg+Ki-Hu129M/M mice inoculated with sCJD-VV2 prions. By contrast, Ki-Hu129V/V mice inoculated with MM[VV2]2Sh+ prions showed diffuse synaptic-type PrP deposits in the grey matter and small plaque-type deposits restricted to within the cerebral white matter. These patterns of PrP deposition were identical to those of Ki-Hu129V/V mice inoculated with sCJD-VV2 prions. Thus, we confirmed that Ki-Hu129V/V mice were highly susceptible to MM[VV2]2Sh+ prions that originated from sCJD-VV2 prions, and that the neuropathology of Ki-Hu129V/V mice inoculated with MM[VV2]2Sh+ prions was identical to that of the Ki-Hu129V/V mice inoculated with the parental sCJD-VV2 prions.

Characterization of PrP<sup>res</sup> in the mouse brains using PrP<sup>res</sup> type-specific antibodies
Tg+Ki-Hu129M/M mice inoculated with sCJD-VV2 prions produced unusual PrP\textsuperscript{res} with an upward size shift from the inoculated type 2 template (10). To characterize these type 2\textsuperscript{sh} PrP\textsuperscript{res}, we produced PrP\textsuperscript{res}-type-specific antibodies. Type 1 PrP\textsuperscript{res}-specific polyclonal antibody Tohoku 1 reacted with epitopes located between residues 82 and 96 of human PrP (Fig. 2). Type 2 PrP\textsuperscript{res}-specific polyclonal antibody Tohoku 2 reacted with a synthetic peptide corresponding to human PrP\textsuperscript{97-103} (the immunogen peptide) but not with peptide 93-103 (Fig. 3). The amino group at the N-terminus of the immunogen peptide might constitute an essential part of the epitopes for Tohoku 2 as reported in other proteolytic cleavage site-specific antibodies (25, 26). Therefore, Tohoku 1 should specifically detect type 1 PrP\textsuperscript{res}, and Tohoku 2 should specifically detect the N-terminal cleavage site of type 2 PrP\textsuperscript{res} after proteinase K digestion (Fig. 4A).

Firstly, with these PrP\textsuperscript{res} type-specific antibodies, we performed western blot analysis of the PrP\textsuperscript{res} in the human brain inocula used in this transmission study (Fig. 4B). In addition to the newly generated type-specific antibodies, we used the monoclonal antibody POM2, which also specifically detects type 1 PrP\textsuperscript{res} (Fig. 2) (21), as a reference antibody. Conventional typing of PrP\textsuperscript{res} using monoclonal antibody 3F4, which detects all PrP\textsuperscript{res} types, showed only a single PrP\textsuperscript{res} type in the brain of a sCJD-MM1 patient or in that of a sCJD-VV2 patient. With the PrP\textsuperscript{res} type-specific antibodies, however, small amounts of POM2/Tohoku 1-reactive subpopulations were observed in the sCJD-VV2 brain (Fig. 4B, bar graphs).

The mean signal intensities of PrP\textsuperscript{res} in the sCJD-MM1 brain were assigned as 100 / mm\textsuperscript{2} in each experiment using 3F4, POM2, or Tohoku 1 (n = 3). In the western blot analysis using 3F4, the mean signal intensities of PrP\textsuperscript{res} in the sCJD-VV2 brain were 224 / mm\textsuperscript{2} (white bars). By contrast, the signal intensities of POM2/Tohoku 1-reactive PrP\textsuperscript{res} bands in the sCJD-VV2 brain were 12 / mm\textsuperscript{2} (hatched bar).
and 48 / mm² (grey bar), respectively. Thus, using type 1 PrPres-specific antibody, the sCJD-VV2 brain contained minority subpopulations that could be detected by type 1 PrPres-specific antibodies as reported previously (21, 28). The sizes of POM2/Tohoku 1-reactive bands were smaller than those of type 1 PrPres in the sCJD-MM1 brain. Thus, POM2 and Tohoku 1 could detect the intermediate-sized PrPres in addition to type 1 PrPres. Furthermore, trace amounts of the Tohoku 2-reactive subpopulation were observed in the sCJD-MM1 brain (Fig. 4B, black bar). The mean signal intensities of PrPres in the sCJD-VV2 brain were assigned as 100 / mm² in each experiment using Tohoku 2 (n = 3). The mean signal intensities of Tohoku 2-reactive PrPres bands in the sCJD-MM1 brain were 2 / mm². Thus, using type 2 PrPres-specific antibody, the minority type 2 PrPres subpopulation could be detected even in the sCJD-MM1 brain.

Secondly, we performed western blot analysis of PrPres in the mouse brains using the PrPres type-specific antibodies. Western blot analysis using 3F4 showed that Tg+Ki-Hu129M/M mice inoculated with sCJD-VV2 prions produced type 2PrPres that was located between type 1 PrPres from Ki-Hu129M/M mice inoculated with sCJD-MM1 prions and type 2 PrPres from Ki-Hu129V/V mice inoculated with sCJD-VV2 prions (Fig. 4C) (10). These PrPres were probed with type-specific antibodies Tohoku 1, Tohoku 2, or POM2. The brains from Tg+Ki-Hu129M/M mice inoculated with sCJD-VV2 prions contained POM2/Tohoku 1-reactive PrPres subpopulations of which the sizes were smaller than those of type 1 PrPres from Ki-Hu129M/M inoculated with sCJD-MM1 prions. However, the signal intensities of these POM2/Tohoku 1-reactive bands were apparently decreased compared to those detected by 3F4 (Fig. 4C, bar graphs). The mean signal intensities of PrPres from Ki-Hu129M/M mice inoculated with sCJD-MM1 prions were assigned as 100 / mm² in each experiment using 3F4, POM2.
In the brains from Tg+Ki-Hu129M/M mice inoculated with sCJD-VV2 prions, the mean signal intensities of 3F4-reactive PrP\textsuperscript{res} bands were 154 / mm\textsuperscript{2} (white bars). However, the mean signal intensities of POM2/Tohoku 1-reactive PrP\textsuperscript{res} bands were 34 / mm\textsuperscript{2} (hatched bar) and 113 / mm\textsuperscript{2} (grey bar), respectively. Since the epitopes for Tohoku 1 were located more at the C-terminal compared with that of POM2 (Fig. 2), the signal intensities of Tohoku 1-reactive bands might be higher than those of POM2-reactive bands. Thus, the brains from Tg+Ki-Hu129M/M mice inoculated with sCJD-VV2 prions contained the intermediate-sized PrP\textsuperscript{res}, but certain subpopulations that could not be detected by POM2 or Tohoku 1 must also have been present. The Tohoku 2-reactive subpopulation was not observed in Tg+Ki-Hu129M/M mice inoculated with sCJD-VV2 prions or Ki-Hu129M/M mice inoculated with sCJD-MM1 prions (Fig. 4C, black bars). The mean signal intensities of PrP\textsuperscript{res} from Ki-Hu129V/V mice inoculated with sCJD-VV2 prions were assigned as 100 / mm\textsuperscript{2} in each experiment using Tohoku 2 (n = 4). The brains from Ki-Hu129V/V mice inoculated with sCJD-VV2 prions contained small amounts of POM2/Tohoku 1-reactive subpopulations in addition to the Tohoku 2-reactive majority subpopulation. Thus, in the cross-sequence transmission of sCJD-VV2 prions to Tg+Ki-Hu129M/M mice, POM2/Tohoku 1-reactive subpopulations were increased, whereas the Tohoku 2 reactive-subpopulation was decreased. Therefore, the upward size shift from type 2 to type 2\textsuperscript{Sh+} in the western blot analysis using 3F4 reflected the shift of the majority PrP\textsuperscript{res} subpopulation from the Tohoku 2-reactive subpopulation to the POM2/Tohoku 1-reactive subpopulation.

In the second passage of the brain homogenate from Tg+Ki-Hu129M/M mouse inoculated with sCJD-VV2 prions (MM[V2]\textsuperscript{2Sh+} prions: host genotype [inoculated prions] type of generated PrP\textsuperscript{res}), Ki-Hu129M/M mice inoculated with MM[V2]\textsuperscript{2Sh+} prions produced the intermediate-sized PrP\textsuperscript{res} that...
were identical in size to parental MM[V2]2Sh+ prions when probed with 3F4 (Fig. 4C). Similar to the brains from Tg+Ki-Hu129M/M mice inoculated with sCJD-VV2 prions, the brains from Ki-Hu129M/M mice inoculated with MM[V2]2Sh+ prions contained POM2/Tohoku 1-reactive subpopulations but not the Tohoku 2-reactive subpopulation. In the brains from Ki-Hu129M/M mice inoculated with MM[V2]2Sh+ prions, the mean signal intensities of 3F4, POM2, and Tohoku 1-reactive PrP\(^{res}\) bands were 176 / mm\(^2\) (white bars), 85 / mm\(^2\) (hatched bar), and 139 / mm\(^2\) (grey bar), respectively. Meanwhile, western blot analysis using 3F4 showed that Ki-Hu129V/V mice inoculated with MM[V2]2Sh+ prions produced type 2 PrP\(^{res}\), i.e., the intermediate-sized PrP\(^{res}\) reverted to type 2 when MM[V2]2Sh+ prions were transmitted to Ki-Hu129V/V mice. Moreover, in the traceback transmission of MM[V2]2Sh+ prions to Ki-Hu129V/V mice, POM2/Tohoku 1-reactive subpopulations were decreased, whereas the Tohoku 2 reactive-subpopulation predominated. In the brains from Ki-Hu129V/V mice inoculated with MM[V2]2Sh+ prions, the mean signal intensities of 3F4, POM2, and Tohoku 1-reactive PrP\(^{res}\) bands were 179 / mm\(^2\) (white bars), 13 / mm\(^2\) (hatched bar), and 43 / mm\(^2\) (grey bar), respectively, whereas the mean signal intensities of Tohoku 2-reactive PrP\(^{res}\) bands were 159 / mm\(^2\) (black bar). Thus, PrP\(^{res}\) type-specific antibodies revealed that cross-sequence transmission of sCJD-VV2 prions generated a new prion strain (MM[V2]2Sh+ prions) with an altered proportion of PrP\(^{res}\) subpopulations, and that the altered proportion reverted to the original proportion through the traceback transmission to Ki-Hu129V/V mice.

Traceback study of p-dCJD prions reevaluated with the PrP\(^{res}\) type-specific antibodies

We reported previously that p-dCJD prions showed the intermediate-sized PrP\(^{res}\), and that Ki-Hu129V/V
mice inoculated with p-dCJD prions showed accumulation of type 2 PrPres (10). To characterize these PrPres in the brains from PrP-humanized mice inoculated with p-dCJD prions, we performed western blot analysis using PrPres type-specific antibodies (Fig. 5). Since Tg+Ki-Hu129M<sub>4R</sub>/M<sub>4R</sub> mice were already established before the Ki-Hu129M/M mice were produced, we used them in the traceback study of p-dCJD or np-dCJD prions. Subsequently, we confirmed that Tg+Ki-Hu129M<sub>4R</sub>/M<sub>4R</sub>, Ki-Hu129M/M, and Tg+Ki-Hu129M/M mice produced PrPres identical in size in the transmission studies using various CJD prions (10). POM2/Tohoku 1-reactive subpopulations existed in the brains from Tg+Ki-Hu129M<sub>4R</sub>/M<sub>4R</sub> mice inoculated with p-dCJD prions, but the signal intensities were apparently decreased compared to those detected by 3F4 (Fig. 5, bar graphs). The mean signal intensities of PrPres from Tg+Ki-Hu129M<sub>4R</sub>/M<sub>4R</sub> mice inoculated with sCJD-MM1 prions were assigned as 100 / mm² in each experiment using 3F4, POM2, or Tohoku 1 (n = 3). In the brains from Tg+Ki-Hu129M<sub>4R</sub>/M<sub>4R</sub> mice inoculated with p-dCJD prions, the mean signal intensities of 3F4-reactive PrPres bands were 105 / mm² (white bars). However, the mean signal intensities of POM2/Tohoku 1-reactive PrPres bands were 23 / mm² (hatched bar) and 45 / mm² (grey bar), respectively. The sizes of POM2/Tohoku 1-reactive bands were smaller than those of type 1 PrPres from Tg+Ki-Hu129M<sub>4R</sub>/M<sub>4R</sub> mice inoculated with non-plaque-type of dCJD (np-dCJD) prions or sCJD-MM1 prions. In addition, trace amounts of the Tohoku 2-reactive subpopulation were observed in the brains from Tg+Ki-Hu129M<sub>4R</sub>/M<sub>4R</sub> mice inoculated with p-dCJD prions, np-dCJD prions, or sCJD-MM1 prions (Fig. 5, black bars). The mean signal intensities of PrPres from Ki-Hu129V/V mice inoculated with sCJD-VV2 prions were assigned as 100 / mm² in each experiment using Tohoku 2 (n = 3). Since the sizes of these Tohoku 2-reactive bands were identical to those of type 2 PrPres from Ki-Hu129V/V mice inoculated with sCJD-VV2 prions, we used them in the traceback study of p-dCJD or np-dCJD prions.
inoculated with sCJD-VV2 prions, Tohoku 2 could specifically detect type 2 PrP\textsuperscript{res}. Meanwhile, in the transmission of p-dCJD prions to Ki-Hu129V/V mice, POM2/Tohoku 1-reactive subpopulations were decreased, whereas the Tohoku 2 reactive-subpopulation predominated. In the brains from Ki-Hu129V/V mice inoculated with p-dCJD prions, the mean signal intensities of 3F4, POM2, and Tohoku 1-reactive PrP\textsuperscript{res} bands were 65 / mm\textsuperscript{2} (white bars), 12 / mm\textsuperscript{2} (hatched bar), and 13 / mm\textsuperscript{2} (grey bar), respectively, whereas the mean signal intensities of Tohoku 2-reactive PrP\textsuperscript{res} bands were 45 / mm\textsuperscript{2} (black bar). Thus, the changes in PrP\textsuperscript{res} subpopulation observed in this traceback study of p-dCJD prions were identical to those observed in the traceback study of MM[VV2]\textsuperscript{Sh+} prions.

**DISCUSSION**

In order to protect humans and animals from infectious diseases, it is often crucial to determine the origin of those isolates that may lie at the origin of epidemics. In the case of conventional pathogens, this is relatively simple and primarily involves the sequencing of pathogen-associated nucleic acids. Because prions lack informational nucleic acids, however, the unambiguous assignment of a given infection to a specific source is very often impossible. Therefore, methods aimed at characterizing stable prion properties after passaging through hosts would be extremely valuable.

Here we demonstrate the first direct evidence of traceback in prion infection. Ki-Hu129V/V mice were highly susceptible to the Tg+Ki-Hu129M/M mouse-passaged sCJD-VV2 prions (MM[VV2]\textsuperscript{Sh+} prions) despite cross-sequence transmission (Fig. 6). In addition, MM[VV2]\textsuperscript{Sh+} prions and sCJD-VV2 prions exhibited similar neuropathology and the identical PrP\textsuperscript{res} type when inoculated into Ki-Hu129V/V mice, i.e., the altered disease phenotypes and unusual PrP\textsuperscript{res} type of MM[VV2]\textsuperscript{Sh+} prions reverted to
those of the parental sCJD-VV2 prions. Furthermore, we generated for the first time type 2 PrP\textsuperscript{res}-specific polyclonal antibody Tohoku 2 in addition to type 1 PrP\textsuperscript{res}-specific polyclonal antibody Tohoku 1. These PrP\textsuperscript{res} type-specific antibodies revealed that drastic changes in the PrP\textsuperscript{res} subpopulations underlie the traceback phenomenon.

The present study clearly shows that traceback studies can be a reliable tool to identify the origin of prions if atypical prion strains emerge through cross-sequence transmission. Although the numbers of animals and human brain inocula used for the transmission were limited in the present study, we demonstrated experimentally the traceback phenomenon: Ki-Hu129V/V mice were highly susceptible to MM[VV2]2\textsuperscript{Sh} prions that originated from sCJD-VV2 prions. In the cross-sequence transmission of sCJD-VV2 prions to Tg+Ki-Hu129M/M mice, POM2/Tohoku 1-reactive subpopulations were increased, whereas the Tohoku 2 reactive-subpopulation was decreased. By contrast, the altered proportion of PrP\textsuperscript{res} subpopulations reverted to the original proportion through the traceback transmission to Ki-Hu129V/V mice. Similar changes in the PrP\textsuperscript{res} subpopulations were observed in the traceback transmission of p-dCJD prions to Ki-Hu129V/V mice. Therefore, the present study shows again that p-dCJD could be caused by cross-sequence transmission of sCJD-VV2 prions to individuals with the 129M/M genotype.

The drastic changes in the PrP\textsuperscript{res} subpopulations can be the molecular basis of the traceback phenomena. We suppose that the subpopulation change observed in the cross-sequence transmission is due to adaptation and/or a selection process (3, 20), which requires a relatively long incubation period.

By contrast, the subpopulation change observed in the traceback transmission might be due to reemergence of the parental prions. Since the emerging prion strain generated by the cross-sequence
transmission retains the memory of the parental prions within its conformational properties or repertoire of PrP<sup>Sc</sup> subpopulations, the parental PrP<sup>Sc</sup> subpopulation reemerges and becomes predominant if the emerging prion strain is transmitted to the original host. Therefore, the incubation period can be shortened, and the altered disease phenotypes revert to the original ones in traceback transmission.

Unexpectedly, type 2 PrP<sup>res</sup>-specific antibody Tohoku 2 revealed that trace amounts of type 2 PrP<sup>res</sup> coexisted with type 1 PrP<sup>res</sup> in the brain of a sCJD-MM1 patient. In addition, PrP-humanized mice with the 129M/M genotype inoculated with sCJD-MM1 prions could produce trace amounts of type 2 PrP<sup>res</sup> in addition to type 1 PrP<sup>res</sup>. The additional type 2 PrP<sup>res</sup> was detected in Tg+Ki-Hu129M<sub>4R</sub>M<sub>4R</sub> but not in Ki-Hu129M/M mice. Since Tg+Ki-Hu129M<sub>4R</sub>M<sub>4R</sub> mice express human PrP with 4 octapeptide repeats at 9.8x the level observed in Ki-Hu129M/M mice, these differences might account for the subtle change.

Further large-scale studies are needed to determine whether an additional type 2 PrP<sup>res</sup> can be detected by Tohoku 2 in other human CJD cases formerly classified as type 1.

The present study raises the possibility that co-occurrence of multiple PrP<sup>res</sup> subpopulations in the same brain might be a general phenomenon. Both type 1 and type 2 PrP<sup>res</sup> can be detected in the same brain in 35% of sCJD patients examined (19, 24). By type 1 PrP<sup>res</sup>-specific antibodies, the minority type 1 subpopulation can be detected with type 2 in all sCJD patients or variant CJD patients formerly classified as type 2 (21, 28). In accord with these reports, small amounts of type 1 (and the intermediate-sized) PrP<sup>res</sup> were detected by type 1 PrP<sup>res</sup>-specific antibodies in Ki-Hu129V/V mice inoculated with sCJD-VV2 prions in the present study. In addition, trace amounts of type 2 PrP<sup>res</sup> were detected by type 2 PrP<sup>res</sup>-specific antibody in the sCJD-MM1 case or Tg+Ki-Hu129M<sub>4R</sub>M<sub>4R</sub> mice inoculated with sCJD-MM1 prions. These findings are in line with a report that diverse PrP<sup>res</sup> fragments
can be detected by N-terminal amino acid sequencing in the same brain even though only a single PrP<sup>res</sup> type is detected by conventional western blot analysis (18). Since the conventional western blot analysis using antibodies that react with all PrP<sup>res</sup> types failed to detect type 1 PrP<sup>res</sup> unless type 1 PrP<sup>res</sup> represented more than 30-40% of total PrP<sup>res</sup> in experimentally mixed type 1 + 2 brain samples, the co-occurrence of multiple PrP<sup>res</sup> subpopulations might be underestimated (21, 28). Therefore, the “type” of PrP<sup>Sc</sup> determined by the conventional typing system might merely represent the predominant PrP<sup>res</sup> subpopulation among multiple subpopulations.

However, the biological importance of the minority PrP<sup>res</sup> subpopulation detected by the PrP<sup>res</sup> type-specific antibodies or by N-terminal amino acid sequencing remains to be determined. Insufficient proteinase K-digestion can generate type 1 specific antibody-reactive PrP bands in brain samples from sCJD or vCJD patients classified as type 2 (14). Otherwise, the size of the PrP<sup>res</sup> fragment might not always reflect the conformation of PrP<sup>Sc</sup>, e.g., the minority MM1 PrP<sup>res</sup> subpopulation detected in sCJD-MM2 patients might differ from the genuine MM1 PrP<sup>res</sup> of sCJD-MM1 patients. It remains unknown whether the minority PrP<sup>res</sup> subpopulation has infectivity and pathogenicity to cause prion disease. A concise and attractive explanation would be that the proportion of PrP<sup>Sc</sup> subpopulations in the brain determines the disease phenotype, transmissibility, and the type of PrP<sup>Sc</sup> determined by the conventional typing system, but further studies are needed to elucidate why multiple PrP<sup>res</sup> subpopulations can be detected in the same brain. Therefore, the significance of the conventional molecular typing system using antibodies that react with all PrP<sup>res</sup> types is likely to continue to be used in the classification of sCJD.

In conclusion, we verified experimentally that traceback studies can be a reliable tool to identify the
origin of prions. The present study shows that the changes in PrPres subpopulations correlate with the changes in prion strain-specific properties, e.g., transmissibility and disease phenotypes, in the traceback transmission. Hereafter, the proportion of PrPres subpopulations in human CJD cases should be analyzed quantitatively using PrPres type-specific antibodies, Tohoku 1 and Tohoku 2.

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REFERENCES


FIGURE LEGENDS

1 Fig. 1  The changes in neuropathology through cross-sequence transmission and traceback transmission. Immunohistochemical analysis of PrP\textsuperscript{sc} in the brains from PrP-humanized mice inoculated with sCJD-VV2 prions or Tg+Ki-Hu129M/M mouse-passaged sCJD-VV2 prions (MM[VV2]\textsuperscript{Sh+} prions: host genotype [inoculated prions] type of generated PrP\textsuperscript{res}). Tg+Ki-Hu129M/M mice inoculated with sCJD-VV2 prions or Ki-Hu129M/M mice inoculated with MM[VV2]\textsuperscript{Sh+} prions showed prominent plaque-type PrP deposits throughout the cerebral grey matter. By contrast, plaque-type PrP deposits were restricted to within the white matter in the brains from Ki-Hu129V/V mice inoculated with sCJD-VV2 prions or MM[VV2]\textsuperscript{Sh+} prions. G: grey matter. W: white matter.

Fig. 2  Epitope mapping of polyclonal antibody Tohoku 1. (A) GST-fusion C-terminally truncated human PrP fragments were probed with PrP-N, POM2, or Tohoku 1. POM2 (21) reacted with all PrP fragments. By contrast, Tohoku 1 reacted with PrP fragments 23-94, 23-95, and 23-96. In addition, weak reactivity to PrP fragments 23-89, 23-90, 23-91, 23-92, and 23-93 was also observed. (B) GST-fusion N-terminally truncated human PrP fragments were probed with 3F4, PrP-C, POM2, or Tohoku 1. POM2 reacted only with PrP fragment 82-230. Tohoku 1 reacted with PrP fragments 82-230, 84-230, 86-230, 88-230, and 90-230. PrP fragment 97-230 corresponding to proteinase K-digested type 2 PrP\textsuperscript{res} fragment was not detected by Tohoku 1 or POM2. Low molecular weight bands lacking the PrP-C epitope should be degradation products.
Fig. 3 Characterization of polyclonal antibody Tohoku 2 by peptide ELISA. Tohoku 2 specifically reacted with a synthetic peptide corresponding to human PrP 97-103 (■), but not with peptide 93-103 (●). Control wells were coated with 0.1% BSA (▲).

Fig. 4 Characterization of PrP\(^{\text{res}}\) using PrP\(^{\text{res}}\) type-specific antibodies. (A) The epitopes for type 1 PrP\(^{\text{res}}\)-specific polyclonal antibody Tohoku 1 or type 2 PrP\(^{\text{res}}\)-specific polyclonal antibody Tohoku 2. POM2 also specifically detects type 1 PrP\(^{\text{res}}\) (21). 3F4 detects all types of PrP\(^{\text{res}}\). (B) Characterization of the human brain inocula used for the transmission studies. Western blot analysis using POM2 and Tohoku 1 revealed that the sCJD-VV2 brain contained minority subpopulations that could be detected by type 1 PrP\(^{\text{res}}\)-specific antibodies, as reported previously (21, 28). Meanwhile, using type 2 PrP\(^{\text{res}}\)-specific antibody Tohoku 2, the minority type 2 PrP\(^{\text{res}}\) subpopulation could be detected even in the sCJD-MM1 brain. The mean signal intensities of PrP\(^{\text{res}}\) in the sCJD-MM1 brain were assigned as 100 / mm\(^2\) in each experiment using 3F4 (white bars), POM2 (hatched bars), or Tohoku 1 (grey bars). The mean signal intensities of PrP\(^{\text{res}}\) in the sCJD-VV2 brain were assigned as 100 / mm\(^2\) in each experiment using Tohoku 2. The signal intensities of PrP\(^{\text{res}}\) are expressed as mean ± SEM (n = 3). (C) Western blot analysis using PrP\(^{\text{res}}\) type-specific antibodies revealed that drastic changes in the PrP\(^{\text{res}}\) subpopulations underlie the traceback phenomenon. In the cross-sequence transmission of sCJD-VV2 prions to Tg+Ki-Hu129M/M mice, POM2/Tohoku 1-reactive subpopulations were increased, whereas the Tohoku 2 reactive-subpopulation was decreased. Conversely, in the traceback transmission of MM[VV2]\(^{2\text{Shv}}\) prions to Ki-Hu129V/V mice, POM2/Tohoku 1-reactive subpopulations were decreased, whereas the Tohoku 2 reactive-subpopulation predominated. The signal intensities of PrP\(^{\text{res}}\) from
Ki-Hu129M/M mice inoculated with sCJD-MM1 were assigned as 100 / mm² in each experiment using 3F4 (white bars), POM2 (hatched bars), or Tohoku 1 (grey bars). The signal intensities of PrP⁰ from Ki-Hu129V/V mice inoculated with sCJD-VV2 were assigned as 100 / mm² in each experiment using Tohoku 2 (black bars). The signal intensities of PrP⁰ are expressed as mean ± SEM (n = 4).

**Fig. 5** Traceback study of p-dCJD prions reevaluated with PrP⁰ type-specific antibodies. In the traceback transmission of p-dCJD prions to Ki-Hu129V/V mice, POM2/Tohoku 1-reactive subpopulations were decreased, whereas the Tohoku 2 reactive-subpopulation predominated. In addition, trace amounts of the Tohoku 2-reactive subpopulation were observed in the brains from Tg+Ki-Hu129M₄₆/M₄₆ mice inoculated with p-dCJD prions, np-dCJD prions, or sCJD-MM1 prions. The signal intensities of PrP⁰ from Tg+Ki-Hu129M₄₆/M₄₆ mice inoculated with sCJD-MM1 were assigned as 100 / mm² in each experiment using 3F4 (white bars), POM2 (hatched bars), or Tohoku 1 (grey bars). The signal intensities of PrP⁰ from Ki-Hu129V/V mice inoculated with sCJD-VV2 were assigned as 100 / mm² in each experiment using Tohoku 2 (black bars). The signal intensities of PrP⁰ are expressed as mean ± SEM (n = 3).

**Fig. 6** Diagram of the traceback studies. The cross-sequence transmission of sCJD-VV2 prions to Tg+Ki-Hu129M/M mice generated a new prion strain (MM[V/V]²Sh+ prions) with altered conformational properties and disease phenotypes after a long incubation period. In the secondary transmission, Ki-Hu129V/V mice were highly susceptible to these MM[V/V]²Sh+ prions despite cross-sequence transmission. Furthermore, the altered conformational properties and disease phenotypes
reverted to the original ones. If atypical prion strains emerge through cross-sequence transmission, traceback studies can be a reliable tool to identify the origin of prions.
Table 1  Primers used for the amplification of truncated human PrP gene fragments

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<td>for C-terminally truncated fragments</td>
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<tr>
<td><em>BamH I</em>-23</td>
<td>5'-GGATCCAAGAAGCGCCCGAAGCCTGGAGGA-3'</td>
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<tr>
<td>and</td>
<td></td>
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<tr>
<td>89-<em>Xho I</em></td>
<td>5'-CTCGAGTCACCAGCCACCACCACATGAGGCTG-3'</td>
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<td>5'-CTCGAGTCACCAGCCACCACCACATGAGGCTG-3'</td>
</tr>
<tr>
<td>91-<em>Xho I</em></td>
<td>5'-CTCGAGTCACCAGCCACCACCACATGAGGCTG-3'</td>
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<td>92-<em>Xho I</em></td>
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<td>95-<em>Xho I</em></td>
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<td>96-<em>Xho I</em></td>
<td>5'-CTCGAGTCACCAGCCACCACCACATGAGGCTG-3'</td>
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| for N-terminally truncated fragments |  |
| *BamH I*-82 | 5'-CGTGGATCCCGGACAGCCTCATGAGTCGGGTGCTG-3' |
| *BamH I*-84 | 5'-CGTGGATCCCGGACAGCCTCATGAGTCGGGTGCTG-3' |
| *BamH I*-86 | 5'-CGTGGATCCCGGACAGCCTCATGAGTCGGGTGCTG-3' |
| *BamH I*-88 | 5'-CGTGGATCCCGGACAGCCTCATGAGTCGGGTGCTG-3' |
| *BamH I*-90 | 5'-CGTGGATCCCGGACAGCCTCATGAGTCGGGTGCTG-3' |
| *BamH I*-92 | 5'-CGTGGATCCCGGACAGCCTCATGAGTCGGGTGCTG-3' |
| *BamH I*-94 | 5'-CGTGGATCCCGGACAGCCTCATGAGTCGGGTGCTG-3' |
| *BamH I*-97 | 5'-CGTGGATCCCGGACAGCCTCATGAGTCGGGTGCTG-3' |
| and |  |
| 230-*Xho I* | 5'-CCGCTCGAGTCACGATCCTCCTCTCTCTCTTGAATAGGCCGTG-3' |
Table 2  Transmission of sCJD prions to humanized mice with 129M/M or 129V/V

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Incubation period in days ± SEM (n/n&lt;sub&gt;0&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>sCJD-MM1 (H3)</td>
<td>Tg+Ki-Hu129M/M (1.2X)&lt;sup&gt;b&lt;/sup&gt; 429 ± 6 (6/6)</td>
</tr>
<tr>
<td>sCJD-VV2 (AK)</td>
<td>723 ± 79 (4/4)</td>
</tr>
<tr>
<td>MM[VV2]2&lt;sup&gt;sh+&lt;/sup&gt;</td>
<td>N.D.&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup> n, number of diseased animals; n<sub>0</sub>, number of inoculated animals.

<sup>b</sup> The expression levels of human PrP in the brains.

<sup>c</sup> N.D., not done.
Figure 3
Figure 4

A

Tohoku 1
POM2
3F4
sCJD-MM1
sCJD-VV2

Kl-Hu129M/M
Tg+Kl-Hu129M/M
Kl-Hu129V/V
Kl-Hu129M/M
Kl-Hu129V/V

MM[V]212

B

Tohoku 2
Tohoku 1
POM2
3F4
sCJD-MM1
sCJD-VV2

Kl-Hu129M/M
Tg+Kl-Hu129M/M
Kl-Hu129V/V
Kl-Hu129M/M
Kl-Hu129V/V

MM[V]212

C

Tohoku 2
Tohoku 1
POM2
3F4
sCJD-MM1
sCJD-VV2

Kl-Hu129M/M
Tg+Kl-Hu129M/M
Kl-Hu129V/V
Kl-Hu129M/M
Kl-Hu129V/V

MM[V]212
Figure 6