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Biochemical and Biophysical Research Communications 342 (2006) 293–299
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vCJD prion acquires altered virulence through trans-species infection

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Received 25 January 2006
Available online 7 February 2006

Abstract

Variant Creutzfeldt–Jakob disease (vCJD) appears to be caused by infection with the bovine spongiform encephalopathy (BSE) agent. To date, all patients with vCJD are homozygous for methionine at codon 129 of the PrP gene. To investigate the relationship between polymorphism at codon 129 and susceptibility to BSE or vCJD prions, we performed splenic follicular dendritic cell assay with humanized knock-in mice through peripheral infection. All humanized knock-in mice showed little or no susceptibility to BSE prions. Only the subset of humanized knock-in mice with codon 129 Met/Met genotype showed weak susceptibility by Western blotting. Surprisingly, we succeeded in the transmission of vCJD prions to humanized knock-in mice not only with codon 129 Met/Met but also with codon 129 Met/Val. Humanized knock-in mice with codon 129 Val/Val were not susceptible. The results suggest that human heterozygotes at codon 129 are also at risk for secondary infection with vCJD.

Keywords: BSE; vCJD; PrP; Polymorphism; Prion; Bovinized mouse; Humanized mouse; Follicular dendritic cell; Virulence; Traceback

Variant Creutzfeldt–Jakob disease (vCJD) appears to be caused by infection with the bovine spongiform encephalopathy (BSE) agent [1]. The evidence for a causal relationship between vCJD and BSE has been considerably strengthened by epidemiological and clinicopathological studies [2,3], the results of experimental strain typing [4], and biochemical studies of the disease-associated isoform of the prion protein (PrP^{Sc}) in inbred and transgenic mice [5,6] and primates [7]. Studies of the prion protein gene (*PRNP*) in patients with CJD have revealed that a naturally occurring polymorphism at codon 129 is a genetic risk factor for this disease [8–10]. To date, all patients with vCJD are homozygous for methionine at codon 129 in the *PRNP* (129MM) [11]. As the 129MM genotype is found in 37% of the normal population of the United Kingdom [12], it appears that the possession of 129MM genotype represents a higher risk for vCJD infection than either methionine/valine heterozygosity (129MV) or valine homozygosity (129VV).

The infectious agent of CJD or scrapie can replicate in the lymphoreticular system prior to involvement of the central nervous system [13]. Follicular dendritic cells (FDCs) are the sites of PrP^{Sc} accumulation within the lymphoreticular system [14]. The preclinical diagnostic value of FDC has been confirmed by the examination of tonsilar tissues taken from scrapie-infected sheep [15,16] and appendix from a patient prior to the onset of vCJD [17]. For the purpose of assessing the PrP^{Sc} accumulation in the splenic FDC by intraperitoneal administration, we should use knock-in mice, because transgenic mice do not express recombinant PrP^{C} (denoted cellular isoform of the prion

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.01.149

protein) in splenic FDC [18]. We have generated knock-in mice (Ki-ChM mice) expressing a human-mouse chimeric prion protein [18]. In splenic FDCs of Ki-ChM mice, PrP^{Sc} accumulation was detected within 14 days following intraperitoneal administration of human prions. Therefore, the splenic FDC assay using knock-in mice appears to be a sensitive and rapid system to estimate the susceptibility for BSE and vCJD, just as the prevalence of vCJD in the United Kingdom can be estimated on PrP^{Sc} accumulation in the FDC of tonsil or appendix samples [19].

To clarify the relationship between the polymorphism at codon 129 in *PRNP* and the susceptibility to BSE or vCJD prions, we attempted to transmit by intraperitoneal administration to knock-in mice expressing the complete human PrP sequence. We reveal herein that vCJD prions are transmissible to codon 129 heterozygous humanized knock-in mice, while BSE prions are not.

Materials and methods

Production of knock-in and transgenic mice. Knock-in mice and transgenic mice were generated as reported previously [18]. The open-reading frame (ORF) was replaced with either the human or bovine PrP gene sequences. ORFs of the human PrP gene with either methionine or valine at codon 129 were isolated by PCR amplification from human genomic DNA [18]. The 5′ primer was designed to incorporate a *Sma*I site into position 115 [20]. Following amplification by PCR, this fragment was joined to the mouse sequence using the *Sma*I site (Fig. 1A). The ORF of the bovine PrP gene was amplified by PCR from bovine genomic DNA. The PCR product contained six octarepeats and a polymorphism at codon 218 [AAG (K) to GAG (E)]. As for the human PrP gene primer, the 5′ primer for the bovine PrP ORF was designed to incorporate a *Sma*I site, and the amplified fragment was joined to the mouse sequence using the *Sma*I site (Fig. 1A). Consequently, after processing of the N-terminal signal peptide (residues 1–22) during post-translational modification, the resulting molecule of prion proteins resembles either human or bovine-specific PrP residues.

We also created knock-in (Ki) mouse crossed with transgenic (Tg) mouse bearing the same PrP construct (Ki + Tg mouse) in the present study. However, transgenic mouse did not show PrP^{C} expression in the FDC as previously reported [18]. Therefore, we used Ki + Tg mouse as the laboratory model in FDC assay, which had the same sensitivity as knock-in mouse.

Sources of prion inocula and transmission experiments. Human brain tissues were isolated at autopsy, after obtaining informed consent for research use, from neuropathologically confirmed cases of sporadic CJD (sCJD), dura graft-associated CJD (dCJD), and vCJD. The sCJD case was classified as MM1 (codon 129 Met/Met and type 1 PrP^{Sc}) [10]. The dCJD case was accompanied by florid-type plaques [21]. The samples from vCJD patients (96/02, 96/07, and 96/45) were obtained from the UK National CJD Surveillance Unit. BSE1 and BSE2 samples were acquired from BSE cattle in Japan. BSE3 and BSE4 samples were obtained from the

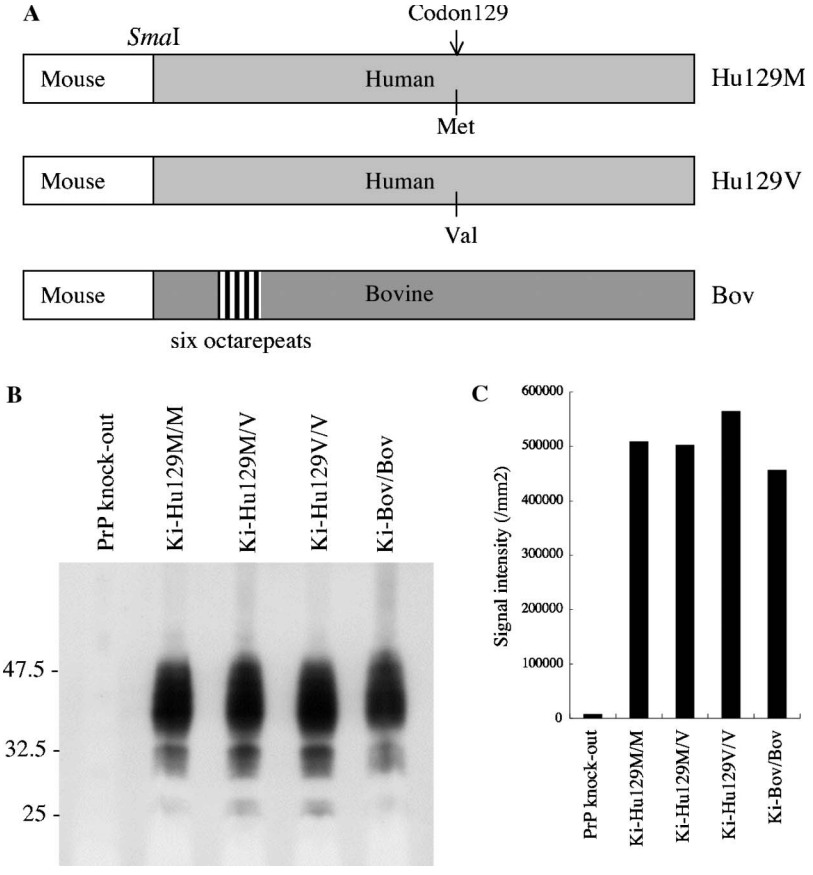

Fig. 1. Characterization of knock-in mice. (A) The ORFs of the knock-in and transgenic vectors. The Hu129M vector encodes a methionine at codon 129. The Hu129V vector encodes a valine at codon 129. The Bov vector has six octarepeats. All of these ORFs encode the mouse PrP sequence at N-terminus before the *Sma*I site. (B) Western blot analysis of spleen membrane fractions from knock-in mice for determinating the quantity of PrP^{C}. Western blots were analyzed using the ChW polyclonal antibody. The identities of the spleen samples are designated above each lane. Numbers show the molecular size standards (kDa). (C) Signal intensity (mm^{-2}) of Western blots (B).

Veterinary Laboratories Agency, Weybrige, UK. Each knock-in mouse was intraperitoneally inoculated with 50 μl of a 10% (wt/vol) brain homogenate from either CJD patients or BSE cattle. Mice were sacrificed at 75 days post-inoculation for FDC bioassay.

Immunohistochemistry. Mouse splenic tissues were immersion-fixed in 10% buffered formalin. After treatment with 99% formic acid for 1 h to inactivate infectivity, samples were embedded in paraffin and then cut into 5 μm sections. Tissue sections were processed for PrP immunohistochemistry by hydrolytic autoclaving pretreatment [22]. The PrP-N antiserum [23] was used as the primary antibody. Goat anti-rabbit immunoglobulin labeled with a peroxidase-conjugated dextran polymer, EnVision® (DakoCytomation, Denmark), was used as the secondary antibody.

Western immunoblots. PrP^{Sc} was extracted from spleen with collagenase treatment as previously described [24] with modifications. The amount of PrP^{C} was measured in membrane fractions of splenic tissue, as previously described [22], isolated from knock-in mice. *N*-Glycosidase F (PNGaseF®, New England BioRabs Inc., USA) was used for deglycosylation of PrP^{Sc}. Samples (corresponding to 7.5 mg wet weight for PrP^{Sc} or 2.5 mg for PrP^{C} of spleen tissue) were subjected to 15% SDS–PAGE and transferred to Immun-Blot® PVDF membrane (Bio-Rad Laboratories, USA). Anti-ChW antiserum, rabbit-derived polyclonal antibodies against recombinant protein corresponding to the residues 122 –231 of PrP in Ki-ChM mouse, was used as the primary antibody. Anti-rabbit EnVision® was used as the secondary antibody. Enhanced chemiluminescent detection (Amersham Biosciences, UK) was used to visualize Western blots. The signal intensities (mm^{-2}) of Western blotting were quantified with Quantity One® software using an imaging device Vasa Doc 5000® (Bio-Rad Laboratories, USA).

Results and discussion

Expression of splenic PrP^{C} in knock-in mice or Ki + Tg mice

We constructed two human PrP genes with either methionine or valine at codon 129 and one bovine PrP gene, designated as Hu129M, Hu129V, and Bov, respectively (Fig. 1A). We generated the following four knock-in mice, expressing human PrP with homozygosity for methionine at codon 129 (Ki-Hu129M/M), homozygosity for valine (Ki-Hu129V/V), heterozygosity for methionine/valine (Ki-Hu129M/V), or expressing bovine PrP (Ki-Bov/Bov).

Expression levels of the knock-in PrP genes were determined by Western blot analysis of membrane fractions derived from splenic tissue, using the polyclonal antibody ChW (Fig. 1B) and quantifying the intensity of signals seen in Western blots (Fig. 1C). The humanized knock-in mice, Ki-Hu129M/M, Ki-Hu129M/V, and Ki-Hu129V/V, expressed similar levels of PrP^{C}. A PrP knock-out mouse was used as a negative control. The molecular weight of PrP^{C} was relatively high in Ki-Bov/Bov mouse, because of the six repeat sequences within the bovine *PRNP*. The similarity of PrP^{C} expression levels in humanized knock-in mice indicated that PrP gene homologous replacement had proceeded as planned and enabled us to compare the susceptibility of each knock-in mouse to inoculated prions under identical conditions.

We also generated transgenic mice (Tg-V or Tg-Bov) expressing either Hu129V or Bov and crossed with knock-in mouse bearing the same PrP construct. The transgenic mice lines utilized in this study are designated Tg-V#139, Tg-V#144, and Tg-Bov#32, with copy numbers of recombinant PrP gene of 4, 5, and 2 copies, respectively (data not shown). The relative expression levels of the recombinant PrP^{C} in brain of transgenic mice were 1×, 1.3×, and 0.8× that of knock-in mice, respectively (data not shown). In splenic FDCs of transgenic mice, however, the recombinant PrP^{C} were negligible as reported previously [18], and the expression levels of the recombinant PrP^{C} in spleen of Ki + Tg mice were almost equal to those of knock-in mice (data not shown). Therefore, we equated the effect of PrP^{C} expression on susceptibility of Ki + Tg mice with that of knock-in mice in this FDC assay.

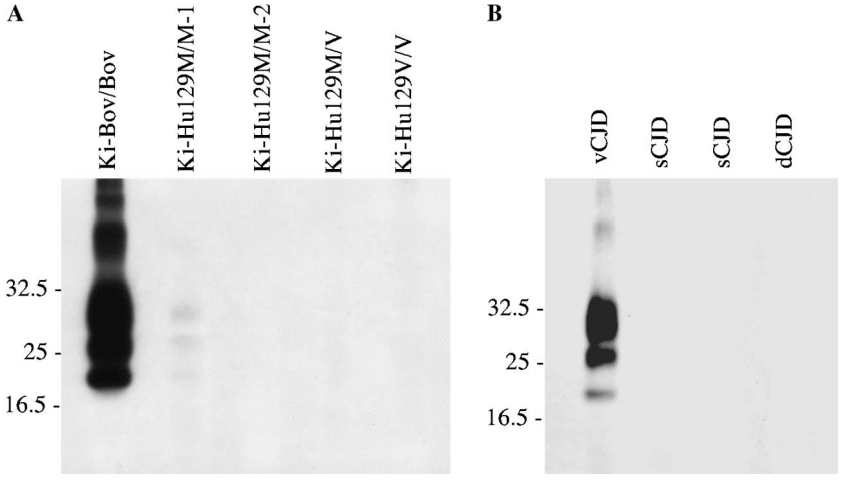

Fig. 2. Western blot analysis for PrP^{Sc} of proteinase K (PK)-treated spleen homogenates from prion-inoculated knock-in mice. (A) Each knock-in mouse was injected with BSE prion (BSE3). The identities of the spleen samples are designated above each lane (Ki-Hu129M/M-1, A3822-3827; Ki-Hu129M/M-2, A3461-3466). (B) Ki-Bov/Bov mice or Ki-Bov/Bov + Tg-Bov mice were injected with human prions. The identities of the spleen sample are, from the left side, vCJD prion-inoculated Ki-Bov/Bov + Tg-Bov mouse, sCJD prion-inoculated Ki-Bov/Bov mouse, sCJD prion-inoculated Ki-Bov/Bov + Tg-Bov mouse, and dCJD prion-inoculated Ki-Bov/Bov mouse. Western blots were analyzed using anti-ChW antiserum as the primary antibody. Numbers show the molecular size standards (kDa).

Table 1
Summary of intraperitoneal transmission of prions to Ki-mice or Ki + Tg-mice

	Mouse type	Mouse code	Inoculum	IHC	WB
A	Ki-Bov/Bov	A3074–3078	BSE1	5/5	+
	Ki-Bov/Bov	A3048–3052	BSE2	4/5	+
	Ki-Bov/Bov	A3402–3410	BSE3	9/9	+
	Ki-Bov/Bov + Tg-Bov#32	A3429–3433	BSE3	5/5	+
B	Ki-Hu129M/M	A3131–3134	BSE1	0/4	−
	Ki-Hu129M/M	A3107–3111	BSE2	0/5	−
	Ki-Hu129M/M	A3461–3466	BSE3	0/6	−
	Ki-Hu129M/M	A3822–3827	BSE3	0/6	+[a]
	Ki-Hu129M/V	A3967–3970	BSE3	0/4	−
	Ki-Hu129M/V	A3971–3976	BSE4	0/6	−
	Ki-Hu129V/V	A3089–3092	BSE1	0/4	−
	Ki-Hu129V/V	A2922–2924	BSE2	0/3	−
	Ki-Hu129V/V	A3113–3116	BSE2	0/4	−
	Ki-Hu129V/V	A3440–3446	BSE3	0/7	−
	Ki-Hu129V/V + Tg-V#139	A3447–3452	BSE3	0/6	−
	Ki-Hu129V/V + Tg-V#144	A3456–3460	BSE3	0/5	−
C	Ki-Hu129M/M	A3384–3388	vCJD 96/02	2/5	+
	Ki-Hu129M/M	A3276–3280	vCJD 96/07	5/5	+
	Ki-Hu129M/V	A3801–3806	vCJD 96/02	6/6	+[a]
	Ki-Hu129M/V	A3807–3812	vCJD 96/07	4/6	+[a]
	Ki-Hu129M/V	A3936–3940	vCJD 96/45	3/5	+[a]
	Ki-Hu129V/V	A3286–3288	vCJD 96/07	0/3	−
	Ki-Hu129V/V + Tg-V#139	A3377–3382	vCJD 96/02	0/6	−
	Ki-Hu129V/V + Tg-V#139	A3102–3106	vCJD 96/07	0/5	−
	Ki-Hu129V/V + Tg-V#139	A3503–3508	vCJD 96/45	0/6	−
D	Ki-Bov/Bov	A4208–4213	vCJD 96/02	n.d.	+
	Ki-Bov/Bov	A4214–4218	vCJD 96/07	n.d.	+
	Ki-Bov/Bov + Tg-Bov#32	A3281–3285	vCJD 96/07	5/5	+
	Ki-Bov/Bov	A3472–3476	sCJD	0/5	−
	Ki-Bov/Bov + Tg-Bov#32	A3477–3480	sCJD	0/4	−
	Ki-Bov/Bov	A3396–3401	dCJD	0/6	−

IHC, immunohistochemistry of spleen tissue. (The number of mice with positive labeling of abnormal PrP in follicular dendritic cells)/(the number of examined mice.) WB, Western blot analysis of spleen tissue for PrP^{Sc}. n.d., not determined.
[a] Western blots showed weak immunoreactivity.

Susceptibility of humanized knock-in mice to BSE prions

Almost all the Ki-Bov/Bov mice showed positive immunoreactivity for PrP^{Sc} in spleen tissues by Western blot analysis (Fig. 2A, lane Ki-Bov/Bov) and immunohistochemical analysis (Table 1A). Briefly, both Ki-Bov/Bov mice and Ki-Bov/Bov + Tg-Bov mice were highly susceptible to BSE prions. In contrast, none of the humanized knock-in mice showed a positive reaction for PrP^{Sc} by immunohistochemistry (Table 1B). Although one group (A3822-3827) of Ki-Hu129M/M mice showed weak immunoreactivity for PrP^{Sc} by Western blot analysis (Fig. 2A, Lane Ki-Hu129M/M-1), the remaining Ki-Hu129M/M mice and all the Ki-Hu129M/V and Ki-Hu129V/V mice showed negative immunoreactivity (Fig. 2A, Table 1B).

As judged by immunoreactivity for PrP^{Sc}, humanized knock-in mice with either the 129MV or 129VV genotypes were highly resistant to BSE prions. The inefficient transmission of BSE prions to humanized knock-in mice, even those with the 129MM genotype, appeared attributable to the species barrier between cattle and human. These results support that vCJD remains at a low prevalence considering the number of exposures to BSE prions in the United Kingdom. If there had not been a species barrier for BSE prions, the number of vCJD patients would not be limited to the 187 cases worldwide to date.

Susceptibility of humanized knock-in mice to vCJD prions

The results of Western blot analysis are summarized in Table 1C. Each humanized knock-in mouse showed different immunoreactivity for PrP^{Sc} (Fig. 3A). Ki-Hu129M/M mice showed positive, and Ki-Hu129M/V mice showed weakly positive immunoreactivity. In contrast, none of the Ki-Hu129V/V or Ki-Hu129V/V + Tg-V mice showed positive immunoreactivity. Although Western blot analysis of PrP^{Sc} showed atypical signals (26 kDa) located close to position of monoglycosylated PrP^{Sc} (Fig. 3A, indicated by filled arrow), these 26 kDa signals were also detectable only with anti-rabbit IgG antibody alone (data not shown) and

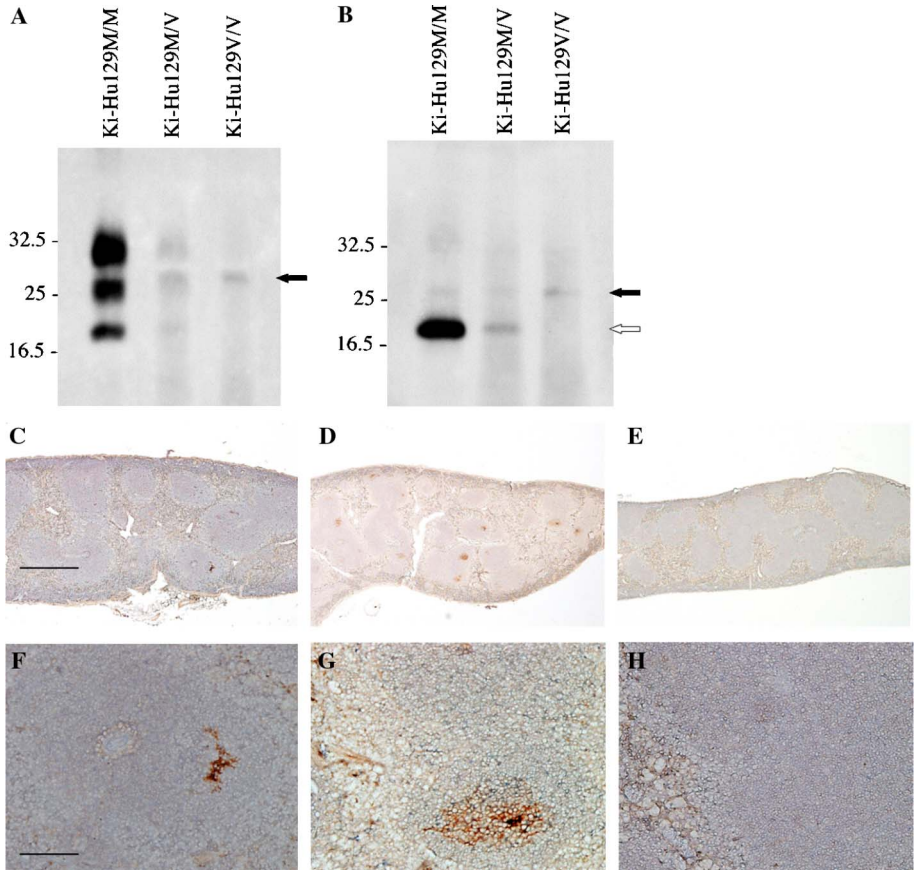

Fig. 3. Susceptibility of humanized knock-in mice to vCJD prions. Each knock-in mouse was injected intraperitoneally with vCJD prion (vCJD 96/07). (A,B) Western blot analysis for PrP^{Sc} of PK-treated (A) or PK and PNGaseF-treated (B) spleen homogenates from vCJD prion-inoculated knock-in mice. Western blots were analyzed using anti-ChW antiserum as the primary antibody. Numbers show the molecular size standards (kDa). Filled arrows show 26 kDa atypical signals. Open arrow shows deglycosylated PrP. (C–H) Immunohistochemical analysis for PrP^{Sc} in the spleens of vCJD prion-inoculated knock-in mice. Anti-PrP-N antiserum was used as the primary antibody. Small granular staining of abnormal PrP^{Sc} is observed in follicular dendritic cells in the spleens of Ki-Hu129M/M mouse (A3276) (C) [enlarged in (F)] and Ki-Hu129M/V mouse (A3807) (D) [enlarged in (G)]. (E,H) Abnormal PrP immunoreactivity is absent in Ki-Hu129V/V mouse (A3286). Scale bars: 500 μm (C–E), 100 μm (F–H).

did not shift after deglycosylation (Fig. 3B, deglycosylated PrP is indicated by open arrow). Therefore, we considered that these 26 kDa signals were derived from immunoglobulin molecules, but not from PrP molecules.

Although Ki-Hu129M/V mice showed a weak immunoreactivity for PrP^{Sc} by Western blot analysis, the immunohistochemical analysis revealed definitely abnormal PrP^{Sc} staining in splenic FDCs (Figs. 3D and G), similar to the immunohistochemical results of Ki-Hu129M/M mice (Figs. 3C and F). None of the Ki-Hu129V/V mice showed positive staining for PrP^{Sc} in splenic FDCs (Figs. 3E and H).

The susceptibility of Ki-Hu129M/M and Ki-Hu129M/V mice to vCJD prions indicated that vCJD prions became more virulent than BSE prions to humans following trans-species transmission. In other words, not only human with 129MM genotype but also with 129MV are at risk of secondary infection with vCJD prions. A case of vCJD who received a blood transfusion from a donor who later died of vCJD [25], and autopsy evidence of possible transfusion transmission of vCJD in an individual with the 129MV genotype [26] were reported previously. The transmissibility of vCJD prions to Ki-Hu129M/M and Ki-Hu129M/V mice provides support for these clinical reports. The normal population of the United Kingdom has genotype frequencies of 37% for Met/Met, 51% for Met/Val, and 12% for Val/Val at codon 129 [12]. Thus, 88% of the population of the United Kingdom may be at risk for secondary infection with vCJD prions. The present evidence of susceptibility to vCJD in humanized knock-in mice raises concerns about further infection with vCJD prions through transfusion or surgical instruments.

Transgenic mice studies have reported the influences of 129M or 129V on the transmission of BSE or vCJD prions to humans [27–29]. The present results confirm the previous transgenic data in respect of both inefficient transmission rate of BSE prions to the humanized mice, and much higher transmission rate of BSE or vCJD prions to the humanized mice with 129M than with 129V. The present results, however, differ from the transgenic data in that Ki-129V/V mice were completely resistant to vCJD prions, whereas vCJD prions could transmit to Tg-129V mice [28].

In those transgenic studies, the overexpression of PrP^C in brain and/or the intracerebral administration might enhance susceptibility to vCJD prions. However, secondary infection with vCJD prions is most likely to be the result of exposure by peripheral route through transfusion or surgical instruments. On this point, the intraperitoneal administration with knock-in mice is more practical to assess the risk of secondary infection with vCJD prions than the intracerebral administration with transgenic mice. Furthermore, in the transgenic mice studies, it is impossible to estimate the susceptibility of 129MV genotype. The present results provide the first experimental evidence of the susceptibility of 129MV genotype to vCJD prions.

Susceptibility of Ki-Bov/Bov mice or Ki-Bov/Bov + Tg-Bov mice to vCJD prion and the other human prions

Ki-Bov/Bov mice or Ki-Bov/Bov + Tg-Bov mice were challenged by transmission with human prions derived from cases of vCJD, sCJD, and dCJD. vCJD prion-inoculated Ki-Bov/Bov mice and vCJD prion-inoculated Ki-Bov/Bov + Tg-Bov mice showed positive immunoreactivity for PrP^{Sc} by Western blot analysis (Fig. 2B, vCJD). In contrast, sCJD or dCJD prion-inoculated Ki-Bov/Bov mice and sCJD prion-inoculated Ki-Bov/Bov + Tg-Bov mice showed negative immunoreactivity for PrP^{Sc} (Fig. 2B, sCJD and dCJD). The results of Western blot analysis were confirmed by immunohistochemistry (Table 1D).

These present data suggest that vCJD prions can trace their infectivity back to bovinized mice, as vCJD prions retained their virulence for Ki-Bov/Bov mice even after trans-species transmission. This traceback phenomenon has also been reported in bovine PrP transgenic mice that were highly susceptible to vCJD prions as to BSE prions [6,30]. This phenomenon was also supported by the fact that FTIR spectral patterns of PrP amyloid fibrils were maintained through trans-species infection between the hamster and the mouse [31]. We can use this phenomenon to help clarify the origin of infectious prion diseases, such as Kuru, human growth hormone-associated CJD, and dura-associated CJD.

Acknowledgments

This study was supported by a grant from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (S.M., N.T., and T.K.). In addition, we thank H. Kudo for technical assistance.

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