[9] M. Nakajima, T. Yamada, T. Kusuhara, Results of quinacrine administration to patients with Creutzfeldt–Jakob disease, Dement. Geriatr. Cogn. Disord. 17 (2004) 158–163.
[10] J.F. Martinez-Lage, A. Rabano, J. Bermejo, M.M. Perez, M.C. Guerrero, M.A. Contreras, A. Lunar, Creutzfeldt–Jakob disease acquired via a dural graft: failure of therapy with quinacrine and chlorpromazine, Surg. Neurol. 64 (2005) 542–545.
[11] J. Collinge, M. Gorham, F. Hudson, A. Kennedy, G. Keogh, S. Pal, M. Rossor, P. Rudge, D. Siddique, M. Spyer, D. Thomas, S. Walker, T. Webb, S. Wroe, J. Darbyshire, A partially randomised patient preference trial to assess the safety and activity of quinacrine in human prion disease: the PRION-1 trial, Lancet Neurol. 8 (2009) 334–344.
[12] T.W.J. Furlow, R.J. Whitley, F.J. Wilmes, Repeated suppression of Creutzfeldt–Jakob disease with vidarabine, Lancet 2 (1982) 564–565.
[13] C. Masullo, G. Macchi, Y.G. Xi, M. Pocchiari, Failure to ameliorate Creutzfeldt–Jakob disease with amphotericin B therapy, J. Infect. Dis. 165 (1992) 784–785.
[14] M.G. Terzano, E. Montanari, S. Calzetti, D. Mancia, A. Lechi, The effect of amantadine on arousal and EEG patterns in Creutzfeldt–Jakob disease, Arch. Neurol. 40 (1983) 555–559.
[15] Y. Huang, H. Okochi, B.C.H. May, G. Legname, S.B. Prusiner, Quinacrine is mainly metabolized to mono-desethyl quinacrine by CYP3A4/5 and its brain accumulation is limited by P-glycoprotein, Drug Metab. Dispos. 34 (2006) 1136–1144.
[16] S. Ghaemmaghami, M. Ahn, P. Lessard, K. Giles, G. Legname, S.J. DeArmond, S.B. Prusiner, Continuous quinacrine treatment results in the formation of drug-resistant prions, PLoS Pathog. 5 (2009) e1000673.
[17] B.C.H. May, A.T. Fafarman, S.B. Hong, M. Rogers, L.W. Deady, S.B. Prusiner, F.E. Cohen, Potent inhibition of scrapie prion replication in cultured cells by bis-acridines, Proc. Natl. Acad. Sci. USA 100 (2003) 3416–3421.
[18] R. Klingenstein, S. Lober, P. Kujala, S. Godsave, S.R. Leliveld, P. Gmeiner, P.J. Peters, C.J. Korth, Tricyclic antidepressants, quinacrine and a novel, synthetic chimera thereof clear prions by destabilizing detergent-resistant membrane compartments, J. Neurochem. 98 (2006) 748–759.
[19] H.T. Nguyen Thi, C.Y. Lee, K. Teruya, W.Y. Ong, K. Doh-ura, M.L. Go, Antiprion activity of functionalized 9-aminoacridines related to quinacrine, Bioorg. Med. Chem. 16 (2008) 6737–6746.
[20] P. Bazzini, C.G. Wermuth, Substituent groups. in: C.G. Wermuth (Ed.), The Practice of Medicinal Chemistry. Academic Press, London, 2008, pp. 445–447.
[21] H.B. Jeon, Y. Lee, C. Qiao, H. Huang, L.M. Sayre, Inhibition of bovine plasma amine oxidase by 1,4-diamino-2-butenes and -2-butynes, Bioorg. Med. Chem. 11 (2003) 4631–4641.
[22] M.K. Hu, L.J. Wu, G. Hsiao, M.H. Yen, Homodimeric tacrine congeners as acetylcholinesterase inhibitors, J. Med. Chem. 45 (2002) 2277–2282.
[23] P. Kutschy, M. Dzurilla, M. Takasugi, M. Torok, I. Achbergerova, R. Homzova, M. Racova, New syntheses of indole phytoalexins and related compounds, Tetrahedron 54 (1998) 3549–3566.
[24] K. Doh-Ura, T. Iwaki, B. Caughey, Lysosomotropic agents and cysteine protease inhibitors inhibit scrapie-associated prion protein accumulation, J. Virol. 74 (2000) 4894–4897.
[25] S. Dollinger, S. Lober, R. Klingenstein, C. Korth, P.A. Gmeiner, A chimeric ligand approach leading to potent antiprion active acridine derivatives: design, synthesis, and biological investigations, J. Med. Chem. 49 (2006) 6591–6595.
[26] A. Kimata, H. Nakagawa, R. Ohyama, T. Fukuuchi, S. Ohta, K. Doh-ura, T. Suzuki, N. Miyata, New series of antiprion compounds: Pyrazolone derivatives have the potent activity of inhibiting protease-resistant prion protein accumulation, J. Med. Chem. 50 (2007) 5053–5056.
[27] S. Kawatake, Y. Nishimura, S. Sakaguchi, T. Iwaki, K. Doh-ura, Surface plasmon resonance analysis for the screening of anti-prion compounds, Biol. Pharm. Bull. 29 (2006) 927–932.
[28] F. Touil, S. Pratt, R. Mutter, B. Chen, Screening a library of potential prion therapeutics against cellular prion proteins and insights into their mode of biological activities by surface plasmon resonance, J. Pharm. Biomed. Anal. 40 (2006) 822–832.
[29] L. Di, E.H. Kerns, K. Fan, O.J. McConnell, G.T. Carter, High throughput artificial membrane permeability assay for blood-brain barrier, Eur. J. Med. Chem. 38 (2003) 223–232.
[30] E.H. Kerns, L. Di, Blood–brain barrier. in: E.H. Kerns, L. Di (Eds.), Drug-like Properties: Concepts, Structure Design and Methods: From ADME to Toxicity Optimization. Academic Press, Amsterdam, 2008, pp. 122–136.
[31] S. Dohgu, A. Yamauchi, F. Takata, Y. Sawada, S. Higuchi, Uptake and efflux of quinacrine, a candidate for the treatment of prion diseases, at the blood–brain barrier, Cell Mol. Biol. 24 (2004) 205–217.
[32] D.S. Miller, A.R. Villalobos, J.B. Pritchard, Organic cation transport in rat choroid plexus cells studied by fluorescence microscopy, Am. J. Phys. 276 (1999) C955–C968.
[33] J. Hosokawa-Muto, Y.O. Kamatari, H.K. Nakamura, K. Kuwata, Variety of antiprion compounds discovered through an in silico screen based on cellular-form prion protein structure: correlation between antiprion activity and binding affinity, Antimicrob. Agents Chemother. 53 (2009) 765–771.
[34] M.J. Thompson, J.C. Louth, G.K. Greenwood, F.J. Sorrell, S.G. Knight, N.B.P. Adams, B. Chen, Improved 2,4-diarylthiazole-based antiprion agents: switching the sense of the amide group at C5 leads to an increase in potency, ChemMedChem 5 (2010) 1476–1488.
[35] W. Heal, M.J. Thompson, R. Mutter, H. Cope, J.C. Louth, B. Chen, Library Synthesis and screening: 2,4-diphenylthiazoles and 2,4-diphenyloxazoles as potential novel prion disease therapeutics, J. Med. Chem. 50 (2007) 1347–1353.
[36] C. Korth, B.C.H. May, F.E. Cohen, S.B. Prusiner, Acridine and phenothiazine derivatives as pharmacotherapeutics for prion disease, Proc. Natl. Acad. Sci. USA 98 (2004) 9836–9841.
[37] H.M. Sim, C.Y. Lee, P.L.R. Ee, M.L. Go, Dimethoxyaurones: potent inhibitors of ABCG2 (breast cancer resistance protein), Eur. J. Pharm. Sci. 35 (2008) 293–306.

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ORIGINAL RESEARCH

Effects of a Brain-Engraftable Microglial Cell Line Expressing Anti-Prion scFv Antibodies on Survival Times of Mice Infected with Scrapie Prions

Koji Fujita · Yoshitaka Yamaguchi · Tsuyoshi Mori · Naomi Muramatsu · Takahito Miyamoto · Masashi Yano · Hironori Miyata · Akira Ootsuyama · Makoto Sawada · Haruo Matsuda · Ryuji Kaji · Suehiro Sakaguchi

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Abstract We first verified that a single chain Fv fragment against prion protein (anti-PrP scFv) was secreted by HEK293T cells and prevented prion replication in infected cells. We then stably expressed anti-PrP scFv in brain-engraftable murine microglial cells and intracerebrally injected these cells into mice before or after infection with prions. Interestingly, the injection before or at an early time point after infection attenuated the infection marginally but significantly prolonged survival times of the mice. These suggest that the ex vivo gene transfer of anti-PrP scFvs using brain-engraftable cells could be a possible immunotherapeutic approach against prion diseases.

Keywords Prion · Immunotherapy · ScFv · Ex vivo gene transfer · Brain-engraftable cells

K. Fujita · Y. Yamaguchi · T. Mori · N. Muramatsu · T. Miyamoto · M. Yano · S. Sakaguchi (✉)
Division of Molecular Neurobiology, The Institute for Enzyme Research, The University of Tokushima, Kuramoto-cho 3-18-15, Tokushima 770-8503, Japan
e-mail: sakaguch@ier.tokushima-u.ac.jp

K. Fujita · R. Kaji
Department of Clinical Neuroscience, The University of Tokushima Graduate School, Tokushima 770-8503, Japan

H. Miyata
Animal Research Center, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi, Kitakyushu 807-8555, Japan

A. Ootsuyama
Department of Radiation Biology and Health, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi, Kitakyushu 807-8555, Japan

M. Sawada
Department of Brain Functions, Division of Stress Adaptation and Protection, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Aichi 464-8601, Japan

H. Matsuda
Department of Molecular and Applied Biosciences, Graduate School of Biosphere Science, Laboratory of Immunology, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8528, Japan

Introduction

Prion diseases including Creutzfeldt–Jakob disease in humans are a group of fatal neurodegenerative disorders caused by the so-called prion (Prusiner 1998). Prions consist of the partially proteinase K (PK)-resistant, amyloidogenic isoform of PrP (designated PrP^{Sc}), and propagate by catalyzing conformational conversion of the host-encoded PK-sensitive, normal isoform of PrP (PrP^{C}) into PrP^{Sc} (Prusiner 1998). The constitutive conversion of PrP^{C} to PrP^{Sc} results in marked accumulation of PrP^{Sc} in the brain, suggesting that the accumulated PrP^{Sc} may be toxic to neurons, thereby causing degenerative neuronal cell death in prion diseases. Indeed, mice devoid of PrP^{C} ($Prnp^{0/0}$) are resistant to prions, neither producing PrP^{Sc} or prions in the brain nor developing the disease (Bueler et al. 1993; Manson et al. 1994; Prusiner et al. 1993; Sakaguchi et al. 1995). However, the molecular pathogenesis underlying the neurodegeneration remains largely unknown.

It has been shown that certain anti-PrP antibodies (Abs) exhibit an anti-prion activity, reducing PrP^{Sc} levels in prion-infected mouse neuroblastoma N2a cells and eventually curing those cells when added to the culture media (Enari et al. 2001; Peretz et al. 2001). These results give rise to the possibility of immunotherapy against prion diseases. Indeed, Song et al. directly infused 31C6 anti-PrP

monoclonal Ab (mAb) via an intraventricular route into the brains of mice that had been infected by Chandler or Obihiro scrapie prions, showing that the mAb could attenuate their resulting experimental prion diseases (Song et al. 2008). However, the effectiveness was very marginal, with the survival times extended only by ~10 days. This very limited anti-prion effect of the intraventricularly infused 31C6 mAbs is probably because, due to its large molecular size, the mAb could not efficiently infiltrate into the brain regions that are relevant to the therapy against prion diseases. Therefore, reduction of the molecular size of anti-PrP Abs may be beneficial to augmentation of their anti-prion effects in vivo. Importantly, it was already shown that anti-PrP single chain Fv (scFv) antibodies were effective against prions in infected cells (Campana et al. 2008; Donofrio et al. 2005).

Microglia are glial cells that infiltrate and accumulate at the pathological lesions affected by prions (Kopacek et al. 2000). One of us showed that an immortalized rat microglial cell line expressing the ex vivo transfected-lacZ gene could be engrafted into the rat brain, surviving for at least the 3 weeks it was monitored (Sawada et al. 1998), and also successfully established the immortalized murine microglial Ra2 cell line (Kanzawa et al. 2000). Another colleague produced 3S9 anti-PrP mAb that exhibited strong anti-prion activity (0.6 nM IC_{50}) in infected N2a cells (Miyamoto et al. 2005). We are therefore interested in investigating the possible effects of Ra2 cell-mediated ex vivo gene transfer of 3S9 anti-PrP scFv (3S9scFv) on prion diseases. In this study, we established a Ra2 microglial cell line expressing 3S9scFv tagged with a myc-epitope and showed that intracerebral injection of these cells before or at early time points after experimental prion infection was marginally but still significantly effective, prolonging survival times in mice.

Materials and Methods

Construction of Lentivirus Expression Vectors

To construct lentivirus expression vectors encoding 3S9scFv, each cDNA for the heavy (H) and light (L) chains of 3S9 mAb were first cloned using the SMART™ RACE cDNA Amplification Kit (Clontech, California, USA) according to the user manual (GenBank accession No. HM627495 and HM627496). In brief, total RNA extracted from 3S9 hybridoma was subjected to first strand cDNA synthesis followed by nested polymerase chain reaction (PCR). The first PCR was performed using the Universal Primer (Clontech) as a sense primer and an H chain-specific antisense primer 1 (Table 1) or a kappa L chain-specific antisense primer 1 (Table 1). The second PCR was subsequently done using the Nested Universal Primer (Clontech) as a sense primer and an H chain-specific antisense primer 2 (Table 1) or a kappa L chain-specific antisense primer 2 (Table 1). Each PCR product was cloned into a pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), resulting in pTOPO-3S9H and pTOPO-3S9L.

To construct CS-CA-3S9scFv, the DNA fragment for the H chain variable region of 3S9 mAb was amplified by PCR using pTOPO-3S9H as a template with a scFv sense primer containing an *Eco*RI site (Table 1) and a sh-HV-a3 antisense primer including a *Sal*I site as well as a linker-coding sequence (Table 1). The DNA fragment for the L chain variable region of 3S9 mAb was also amplified by PCR using pTOPO-3S9L as a template with a sh-LV-S4 primer containing a *Sal*I site (Table 1) and a scFv-myc-*Xho*I antisense primer including a myc tag-coding sequence, two copies of a stop codon and an *Xho*I site (Table 1). The *Eco*RI–*Sal*I DNA fragment of the H chain variable region and the *Sal*I–*Xho*I DNA fragment of the L chain variable region with a myc tag were ligated in tandem into the *Eco*RI–*Xho*I digested CS-CA-MCA vector (provided from RIKEN BioResource Center, Tsukuba, Japan), resulting in CS-CA-3S9scFv.

For construction of CS-CA-3S9scFv/IRES-hrGFP, the *Xho*I–*Bsp*1407 I DNA fragment including an internal ribosome entry site (IRES) sequence followed by the 5′ region of humanized recombinant green fluorescent protein (hrGFP) sequence was isolated from pIRES-hrGFP-2a (Clontech), and the 3′ region of hrGFP was amplified by PCR using pIRES-hrGFP-2a as a template with an hrGFP-*Bsp*1407 I sense primer (Table 1) and an hrGFP-*Xho*I antisense primer (Table 1). The isolated *Xho*I–*Bsp*1407 I DNA fragment and the amplified *Bsp*1407 I–*Xho* I fragment of the 3′ region of hrGFP were ligated in tandem into the *Xho*I-digested CS-CA-3S9scFv vector, resulting in CS-CA-3S9scFv/IRES-hrGFP. CS-CA-GFP was from RIKEN BioResource Center.

Preparation of Lentiviral Vectors

Lentiviral vectors were prepared as described elsewhere (Miyoshi et al. 1999). In brief, human embryonic kidney HEK293T cells were transfected with CS-CA-3S9scFv/IRES-hrGFP or CS-CA-GFP, together with pCAG-HIVgp (RIKEN BioResource Center) and pCMV-VSV-G-RSV-Rev (RIKEN BioResource Center) using Lipofectamine 2000 reagent (Invitrogen). The culture media containing lentivirus vectors were collected 48 h after transfection and filtered through a 0.45-μm filter. An aliquot of the media was stored at −30°C until use. The others were ultracentrifuged using an SCP85H ultracentrifuge (Hitachi, Tokyo, Japan) and the resulting pellet was suspended in Hank's Balanced Salt Solution (Invitrogen) and stored at −80°C until use.

Table 1 Primers used in the present study

Products	Primers	Sequence	Recital
3S9 mAb H chain ORF	IgG1 H chain-specific antisense primer 1	5′-tcctgtaggaccagagggctccaaggacac-3′	
	IgG1 H chain-specific antisense primer 2	5′-cctctaga**tcatca**tttaccaggagagtgg-3′	Underlined, *Xba*I site; bold, two copies of a stop codon
3S9 mAb L chain ORF	Kappa L chain-specific antisense primer 1	5′-gcctccaagaccttagaagggaagatagga-3′	
	Kappa L chain-specific antisense primer 2	5′-cctctaga**ctacta**acactcattcctgttg-3′	Underlined, *Xba* I site; bold, two copies of a stop codon
3S9scFv	*Eco*RI-scFv sense primer	5′-ccgaattcctaact**atg**ggatggaac-3′	Underlined, *Eco*RI site; bold, a start codon
	sh-HV-a3 antisense primer	5′-acgcgtcgac*cgcgctgccgccgctgccgccgccgcc gctgccgccgccgccgctgctt*gaggagactgtgag-3′	Underlined *Sal*I site; italic, a linker-coding sequence
	sh-LV-S4 primer	5′-acgcgtcgacgtgatattgtactaactcagt-3′	Underlined, Sal I site
	scFv-myc-*Xho*I antisense primer	5′-gcctcgag**tcatca***cagatcctcttctgagatg agtttttgtt*cttgtatttccaactt-3′	Underlined, *Xho*I site; bold, two copies of stop codon; italic, a myc-epitope tag-coding sequence
Mouse PrP	PrP *Bam*HI sense primer	5′-tcggatcccgtcatc**atg**gcg-3′	Underlined, *Bam*HI site; bold, a start codon
	PrP *Xba*I antisense primer	5′-cctctagagc**tca**tccaca-3′	Underlined, *Xba*I site; bold, a stop codon
3S9scFv/IRES-hrGFP	hrGFP-Bsp1407I sense primer	5′-cgaggtggtgtacatgaacg-3′	Underlined, Bsp1407 I site
	hrGFP-Xho I antisense primer	5′-ctgcacgagtgggtg**taataa**ctcgagcg-3′	Underlined, *Xho*I site; bold, a stop codon
3S9scFv (RT-PCR)	3S9 sense primer	5′-cctacaaccagaaattcgagg-3′	
	3S9 antisense primer	5′-tgttgataccagtgtaggtgg-3′	
Mouse GAPDH (RT-PCR)	moGAPDH sense primer	5′-atggccttccgtgttcctac-3′	
	moGAPDH antisense primer	5′-cctctcttgctcagtgtcct-3′	

Enzyme-Linked Immunosorbent Assay (ELISA)

Mouse recombinant PrP (mo-recPrP) with a 6 × His tag was purified elsewhere (Yamanaka et al. 2006). Each well of a 96-well plate was coated with mo-recPrP by overnight incubation in 50 mM carbonate buffer at 4°C and then blocked with Blocking One reagent (Nacalai tesque, Kyoto, Japan) for 1 h at room temperature (RT). The wells were incubated for 1 h at RT with the culture medium (100 μl/well) from HEK293T cells transfected with either CS-CA-3S9scFv or CS-CA-MCS, and washed with 0.05% Tween 20-containing phosphate-buffered saline (PBS). Immune complexes were detected using mouse anti-c-myc mAb (Biomol, Farmingdale, NY), horseradish peroxidase (HRP)-conjugated sheep anti-mouse Ab (GE Healthcare, Buckinghamshire, England), and 1-Step™ Ultra TMB-ELISA (Pierce, Rockford, IL). Colorimetric values were measured at 450 nm using MULTISKAN JX (Thermo Electron Corporation, Waltham, MA).

Fluorescence-Activated Cell Sorter (FACS) Analysis

PrP^C-deficient hippocampal neurons, designated HpL3-4 (Kuwahara et al. 1999), and HpL3-4 cells overexpressing exogenous PrP^C, designated HpL3-4TR (Kuwahara et al. 1999) (kindly provided by Prof. Onodera, The University of Tokyo, Japan), were harvested in PBS containing 20 mM EDTA. The cells were then incubated for 1 h on ice with the culture medium from HEK293T cells transfected with either CS-CA-3S9scFv or CS-CA-MCS, and washed with BSS buffer (140 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 1 mM $CaCl_2$, pH 7.0). Immune complexes were detected using mouse anti-c-myc Ab (Biomol) and Alexa Fluor® 546 goat anti-mouse IgG (Invitrogen) and analyzed using a flow cytometer (EPICS® XL-MCL, Beckman Coulter, Brea, CA). Microglial cells were harvested in PBS using rubber scrapers and analyzed for expression of hrGFP using a flow cytometer (BD FACSCanto™ II Flow Cytometer, BD Biosciences, San Jose, CA).

In vitro Anti-Prion Activity Assay

Mouse PrP^C-overexpressing N2a cells, designated N2aC24, were established by transfection with pEF1/Myc-His A (Invitrogen) encoding mouse PrP^C, designated pEF1-moPrP, and by subsequent cloning of G418 (Nacalai tesque)-resistant colonies using cloning cylinders. The pEF1-moPrP was constructed by insertion of the PCR-amplified mouse PrP ORF (GenBank accession No. M13685). N2aC24 cells were

then exposed to 22L and Chandler scrapie prions by incubation with a lysate of 22L or Chandler prion-infected N2a58 cells (kindly provided by Prof. Nishida, Nagasaki University, Japan) (Atarashi et al. 2006), and 22L and Chandler prion-infected N2aC24 clones, designated N2aC24L1-3 and N2aC24Chm, respectively, were obtained by limiting dilution. To assess the in vitro anti-prion activity of 3S9scFv, the infected cells were incubated with the 3S9scFv-containing culture medium for 72 h, lysed in a buffer (150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris–HCl, pH 7.5), and subjected to Western blotting for PrP^{Sc}.

Cloning of 3S9scFv/GFP-Ra2 and GFP-Ra2

The microglial cell line Ra2 was cultured in Eagle's MEM (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum, 0.1% of glucose, 2 μg/l of recombinant mouse GM-CSF (R&D Systems, Minneapolis, MN) and 5 mg/l of bovine insulin (Sigma, St. Louis, MO). Ra2 was infected by the concentrated lentivirus encoding Sh3.9scFv/IRES-hrGFP or GFP alone with 1 multiplicity of infection, and 3S9scFv/GFP-Ra2 and GFP-Ra2 cells were cloned by limiting dilution.

Reverse Transcription-PCR (RT-PCR) Analysis

RT-PCR was performed with SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* High Fidelity (Invitrogen). In brief, total RNA extracted from cells was treated with DNase I for 15 min at RT and heated at 65°C for 10 min to destroy the DNase I activity. The treated RNA was then subjected to 1st strand cDNA synthesis using SuperScript™ III reverse transcriptase with random hexamer. After treatment with RNase H at 37°C for 20 min, the first strand cDNAs for 3S9scFv and mouse glyceraldehyde-3-phosphate dehydrogenase (moGAPDH) were amplified by PCR using primer pairs of 3S9scFv-myc sense and antisense primers (Table 1) or moGAPDH sense and antisense primers, respectively (Table 1).

Western Blot Analysis

Proteins were denatured in Laemli's sample buffer, separated on a 12% SDS-polyacrylamide gel, and onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Thereafter, the membrane was blocked with 5% fat-free dry milk in TBST buffer (10 mM Tris–HCl, pH 7.8, 100 mM NaCl, 0.1% Tween 20) for 1 h at RT and incubated with mouse anti-c-myc mAb (Biomol), rabbit anti-c-myc polyclonal Ab (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan), or goat anti-PrP M-20 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight in TBST buffer containing 1% fat-free dry milk. Immune complexes were detected using 1 h-incubation with HRP-conjugated anti-mouse IgG (GE Healthcare), anti-rabbit IgG (GE Healthcare), or anti-goat IgG (Millipore), and then visualized by LAS-4000 (Fujifilm, Tokyo, Japan) using ECL Plus Western Blotting System (GE Healthcare) or Immobilon™ Western Chemiluminesent HRP substrate (Millipore).

Prion Inoculation

Brains were removed from ddY mice showing terminal symptoms due to infection with Chandler or 22L scrapie prions and a 10% (w/v) brain homogenate was prepared in MEM (Invitrogen). Five-week-old C57BL/6 mice (CLEA

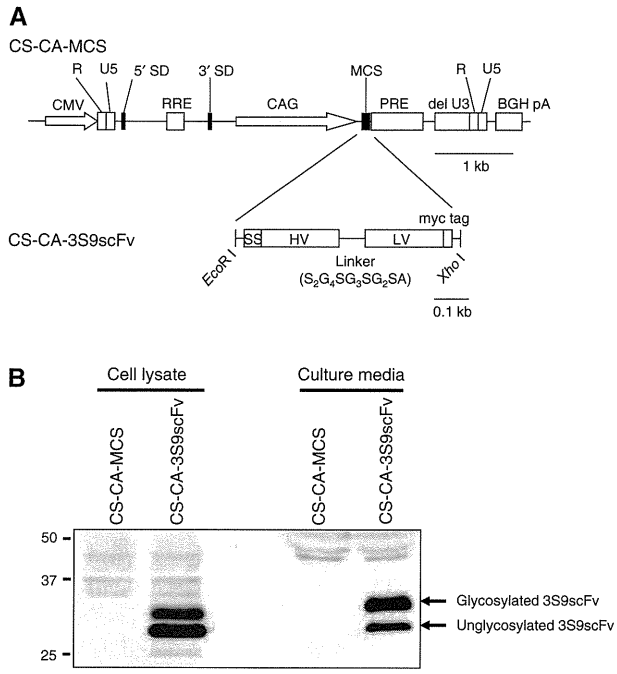

Fig. 1 3S9scFv is produced by HEK293T cells. **a** Schematic representations of CS-CA-MCS and CS-CA-3S9scFv lentivirus expression vectors. CMV represents human cytomegalovirus immediate early promoter; R, the R region of the 5′ or 3′ long term repeat (LTR) from human immunodeficiency virus type 1 (HIV-1); U5, the U5 region of the 5′ or 3′ LTR from HIV-1; del U3, deletion of enhancer and promoter sequences in the U3 region of LTR from HIV-1; 5′ SD, 5′ splicing donor site; 3′ SD, 3′ splicing acceptor site; RRE, Rev responsive element; PRE, Woodchuck hepatitis virus posttranscriptional regulatory element; CAG, CMV immediate early enhancer and chicken β-actin gene promoter; BGH pA, bovine growth hormone polyadenylation signal; MCS, multiple cloning site; SS, signal sequence; HV, the variable region of 3S9 heavy chain; LV, the variable region of 3S9 light chain. **b** Western blotting of 3S9scFv produced by HEK293T cells. Cell lysates and culture media from HEK293T cells transfected with CS-CA-MCS or CS-CA-3S9scFv were subjected to Western blotting with mouse anti-c-myc mAb. Two discrete bands correspond to glycosylated and unglycosylated 3S9scFvs

Japan, Tokyo, Japan) were intracerebrally inoculated with a 20 μl-aliquot of the homogenate, containing 1.1×10^4 ID_{50} RML prions or 0.94×10^3 ID_{50} 22L prions. Mice were cared for in accordance with the Guidelines for Animal Experimentation of The University of Tokushima.

Microglia Inoculation

1×10^6 microglial cells suspended in 20 μl PBS were intracerebrally injected into each mouse at the indicated times before or after inoculation with prions. Mice were cared for in accordance with the Guidelines for Animal Experimentation of The University of Tokushima.

Determination of the Terminal Stage of the Disease

Mice were observed daily and diagnosed as terminal when they developed more than five of the following features: emaciation, decreased locomotion, ruffled body hair, ataxic gait, kyphosis, priapism, upright tail, and paralysis of the hind legs.

Results

Characterization of Recombinant 3S9scFv Produced by HEK293T Cells

We transiently transfected CS-CA-3S9scFv and control CS-CA-MCS expression vectors (Fig. 1a) into HEK293T cells and then carried out western blotting of the cell lysates prepared 2 days after transfection using anti-myc antibody. No signals could be detected in the control CS-CA-MCS-transfected cells (Fig. 1b). In contrast, two strong discrete signals were observed in the CS-CA-3S9scFv-transfected cells (Fig. 1b). They are glycosylated and unglycosylated recombinant 3S9scFvs because the glycosylation signal is found in the variable region of the L

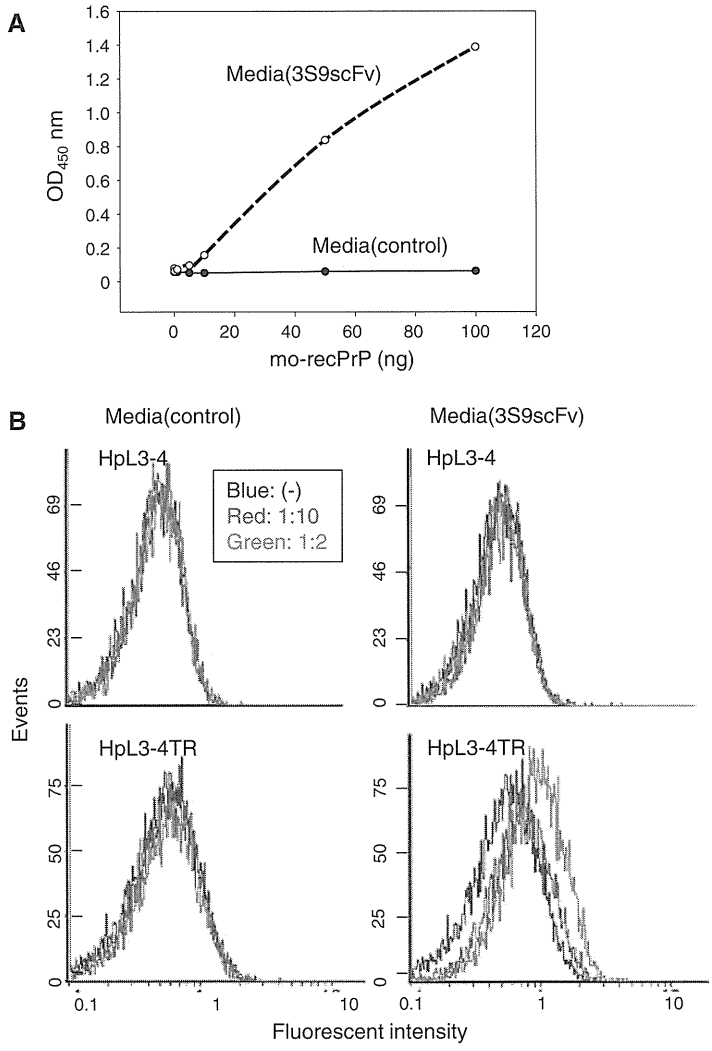

Fig. 2 Binding of 3S9scFv produced by HEK293T cells to PrP. **a** ELISA analysis of 3S9scFv showing its binding activity to mo-recPrP. 3S9scFv-containing culture media showed higher colorimetric values at OD_{450} in a dose-dependent manner of mo-recPrP. In contrast, no binding was detected with the culture media from control cells. **b** FACS analysis of 3S9scFv showing its binding activity to PrP^C expressed on the cell surface. PrP^C-negative HpL3-4 cells showed no signal shift with both the control and the 3S9scFv-containing culture media (*upper panels*). On the other hand, PrP^C-expressing HpL3-4TR cells exhibited positive signals with 3S9scFv-containing culture media, but not with the control media (*lower panels*)

chain of 3S9 mAb and the upper band was shifted to the lower band after PNGase F treatment (data not shown). The same signals were detected in the media of CS-CA-3S9scFv-transfected cells (hereafter referred to as the 3S9scFv media), but not in the media of control CS-CA-MCS-transfected cells (hereafter referred to as the control media) (Fig. 1b). These results indicate that recombinant 3S9scFv is produced by HEK293T cells and secreted into the culture media.

We then investigated the recombinant 3S9scFv for its binding ability to PrP by an ELISA assay using mo-recPrP. No increase in colorimetric value at 450 nm was detected with the control media (Fig. 2a). In contrast, the colorimetric value of mo-recPrP in 3S9scFv media increased in a dose-dependent manner (Fig. 2a). These results clearly indicate that recombinant 3S9scFv binds to mo-recPrP. We also investigated whether the recombinant 3S9scFv could bind to native PrP or PrP^C expressed on the cell surface using HpL3-4 and HpL3-4TR cells by FACS analysis. HpL3-4 cells are devoid of PrP^C while HpL3-4TR cells express abundant PrP^C on their surface. Both media induced no signal shift of HpL3-4 cells (Fig. 2b). In contrast, the signal peak of HpL3-4TR cells was shifted to the right by the 3S9scFv media in a dose-dependent manner whereas no signal shift caused by the control media could be detected (Fig. 2b). These results indicate that 3S9scFv produced by HEK293T cells recognizes PrP^C expressed on the cell surface.

Finally, we investigated the recombinant 3S9scFv for its anti-prion activity. 22L prion-infected N2aC24L1-3 and Chandler prion-infected N2aC24Chm cells were incubated with either 3S9scFv media or the control media for 3 days and subjected to western blotting for detection of PrP^{Sc}. No decrease in PrP^{Sc} levels could be detected in both infected cells when incubated with the control media (Fig. 3). In contrast, the 3S9scFv media markedly reduced PrP^{Sc} levels in both N2aC24L1-3 and N2aC24Chm cells in a dose-dependent manner (Fig. 3). No cell death was observed in these cells treated with the 3S9scFv media (data not shown). These results clearly indicate that recombinant 3S9scFv produced by HEK293T cells is effective against both prions.

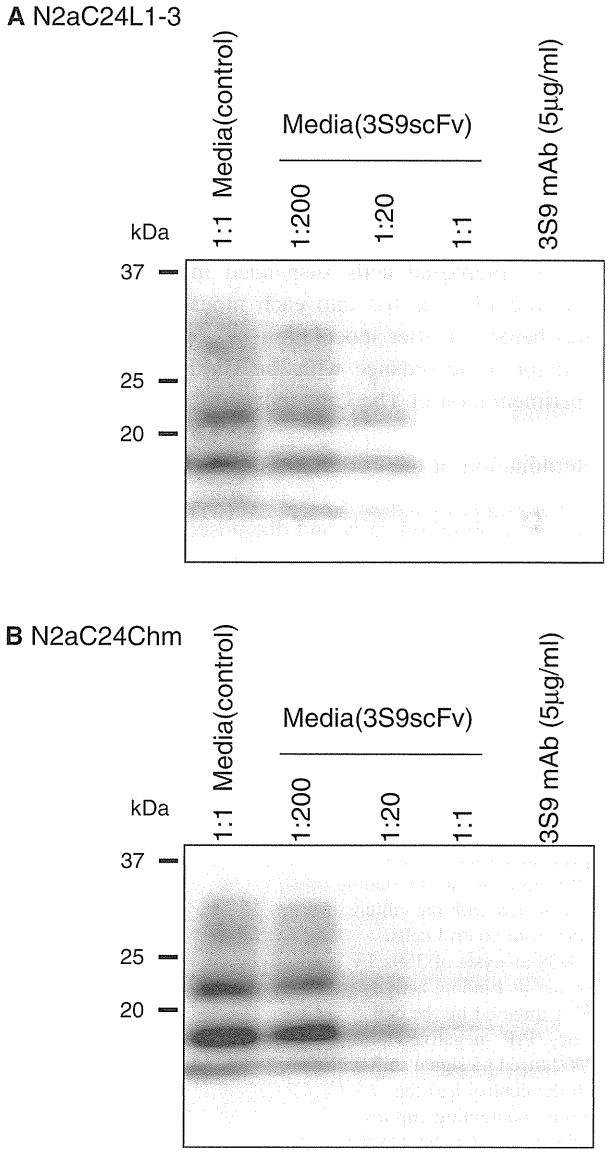

Fig. 3 In vitro anti-prion activity of 3S9scFv produced by HEK293T cells. The control and 3S9scFv-containing culture media were added at the indicated ratio into the culture medium of 22L prion-infected N2aC24L1-3 cells (**a**) or Chandler prion-infected N2aC24Chm cells (**b**). PrP^{Sc} levels in these infected cells were markedly reduced in a dose-dependent manner of 3S9scFv-containing culture media added

Establishment of a Ra2 Microglial Cell Clone Stably Expressing 3S9scFv

We constructed CS-CA-3S9scFv/IRES-hrGFP expression vector by introducing IRES-hrGFP sequence downstream of the 3S9scFv sequence in CS-CA-3S9scFv expression vector (Fig. 4a) and produced lentivirus vector encoding 3S9scFv and hrGFP. We then infected Ra2 microglial cells with the lentivirus vector and successfully cloned a 3S9scFv/GFP-Ra2 cell line (Fig. 4b) by limiting dilution. Subsequent FACS analysis revealed that about 90% of the cells were positive for hrGFP expression (Fig. 4b). We also cloned control GFP-Ra2 cells, which express GFP alone. The expression of 3S9scFv was confirmed using RT-PCR and western blotting. No 3S9scFv mRNA expression was detected in control GFP-Ra2 cells (Fig. 4c). In contrast, 3S9scFv mRNA was expressed in 3S9scFv/GFP-Ra2 cells (Fig. 4c). Consistently, western blotting showed that 3S9scFv was expressed in 3S9scFv/GFP-Ra2 cells, but not in GFP-Ra2 cells (Fig. 4d).

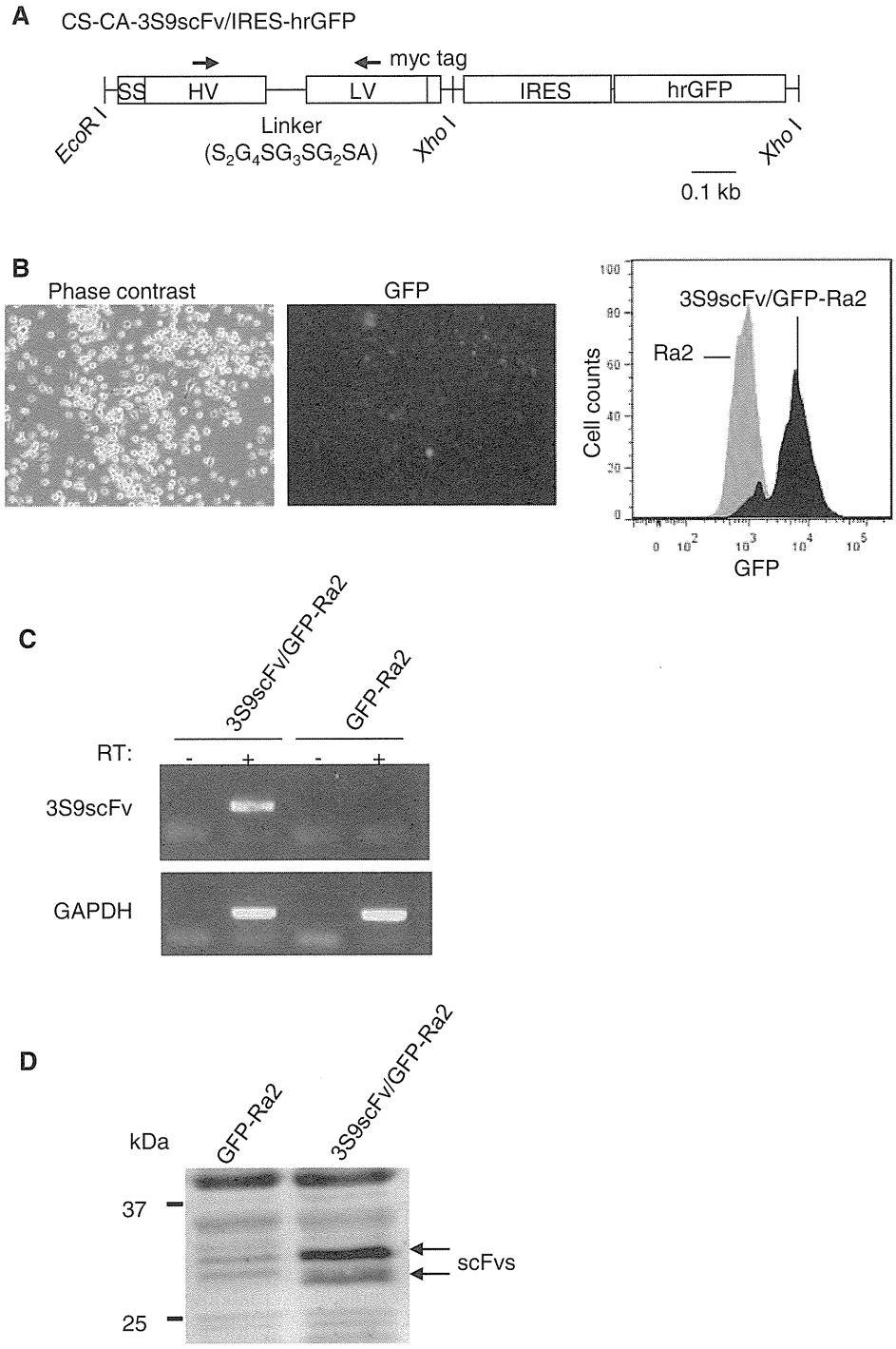

Fig. 4 3S9scFv expressed by Ra2 cells. **a** A schematic representation of part of the CS-CA-3S9scFv/IRES-hrGFP expression vector. Primer pairs used for RT-PCR are indicated by arrows. **b** Phase contrast picture (*left panel*), fluorescent microscopic picture (*middle panel*), and FACS analysis (*right panel*) of 3S9scFv/GFP-Ra2 cells. **c** RT-PCR for 3S9scFv. 3S9scFv mRNA expression was detected in 3S9scFv/GFP-Ra2 cells but not in GFP-Ra2 cells. GAPDH mRNA was similarly expressed in both cells. **d** Western blotting for 3S9scFv. Lysates from GFP-Ra2 and 3S9scFv/GFP-Ra2 cells were probed with rabbit polyclonal anti-c-myc antibodies. Glycosylated and unglycosylated 3S9scFvs were expressed in 3S9scFv/GFP-Ra2 cells but not in GFP-Ra2 cells

Effects of 3S9scFv/GFP-Ra2 Cells on Scrapie Prions in Mice

To investigate the possible effects of the brain-engraftable cells-mediated ex vivo gene transfer of 3S9scFv on prions in mice, we directly injected 3S9scFv/GFP-Ra2 microglial cells into the right cerebrum at 1 week and into the left cerebrum at 3 weeks before inoculation of mouse-adapted Chandler prions into the right cerebrum. As controls, we similarly injected GFP-Ra2 microglial cells. Survival times of the mice injected with 3S9scFv/GFP-Ra2 cells were marginally but still significantly elongated, compared to those of the control mice ($P = 0.014$, Logrank test, Fig. 5a). No differences in PrP^{Sc} levels were detected between the brains of mice treated with GFP-Ra2 and 3S9scFv/GFP-Ra2 microglial cells (Fig. 5b). We also

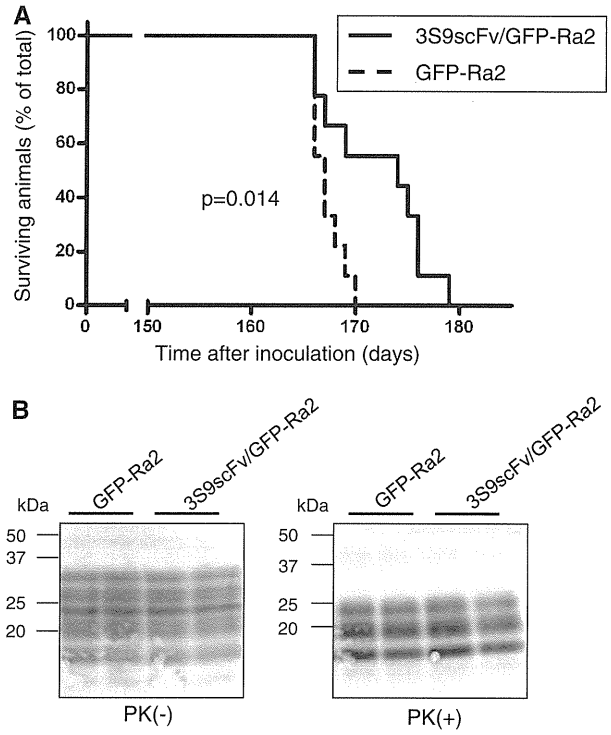

Fig. 5 **a** Survival curves of mice prophylactically injected with 3S9scFv/GFP-Ra2 cells. Mice ($n = 10$) intracerebrally injected with 3S9scFv/GFP-Ra2 cells before infection with Chandler prions survived significantly longer than those ($n = 10$) injected with control GFP-Ra2 cells ($P = 0.014$, Logrank test). **b** Western blotting of the brains of two mice from each mouse group. No decrease in PrP^{Sc} levels was observed in the brains of 3S9scFv/GFP-Ra2 cells-injected mice, compared to the GFP-Ra2 cells-injected control mice

injected 3S9scFv/GFP-Ra2 microglial cells into the right cerebrum of mice 7 or 13 weeks after infection with Chandler or 22L scrapie prions. No significant elongation of the survival times could be observed in the mice infected with Chandler prions (Fig. 6). However, 3S9scFv/GFP-Ra2 cells were partially but still significantly effective against 22L prions when injected 7 weeks after infection ($P = 0.035$, Logrank test, Fig. 6), but not 13 weeks after infection (Fig. 6). Western blotting showed no decrease of PrP^{Sc} in the brains of terminally ill mice injected with 3S9scFv/GFP-Ra2 cells (data not shown). We also failed to detect the injected 3S9scFv/GFP-Ra2 cells in the brains of terminally ill mice, even at the injection sites by immunohistochemistry using anti-hrGFP and anti-c-myc Abs (data not shown).

Discussion

Since it was shown that anti-PrP mAbs or Fab fragments prevented PrP^{Sc} formation and prion propagation in prion-infected N2a cells, eventually rescuing the cells from prion infection (Enari et al. 2001; Peretz et al. 2001), the prospect of immunotherapy against prion diseases has attracted considerable attention. On the other hand, neurotoxic adverse effects were reported for some anti-PrP mAbs and $F(ab')_2$ fragments when they were injected into the brains of normal and prion-infected mice (Lefebvre-Roque et al. 2007; Solforosi et al. 2004), giving rise to great caution for development of the immunotherapy against prion diseases. However, no adverse effects such as neurotoxicity and autoimmune reactions were observed in mice intraventricularly administered with 31C6 and 3S9 mAbs (Sakaguchi et al. 2009; Song et al. 2008). This therefore indicates that anti-PrP mAb-mediated neurotoxicity appears to be associated with specific epitopes on PrP. Thus, an appropriate selection of non-toxic anti-PrP mAbs, such as 31C6 and 3S9 mAbs, may be required for use in immunotherapy of prion diseases.

No effective regimens for the immunotherapy of prion diseases have been developed. Direct intraventricular infusion of anti-PrP mAbs had very limited or no effect on survival times in mice that had been experimentally infected with mouse-adapted prions (Sakaguchi et al. 2009; Song et al. 2008). Wuertzer et al. (2008) reported the possibility of an alternative regimen using recombinant adeno-associated vector 2 (rAAV2)-mediated gene transfer of anti-PrP scFvs to the brain. They showed that prophylactic injection of the rAAV2 vector encoding anti-PrP scFvs into the thalamus and striatum 1 month before intraperitoneal infection with Chandler prions extended survival times by 50 days in mice. However, no data were available for the therapeutic injection of anti-PrP scFv-encoding rAAV2 vectors. In this study, we presented another possibility for the ex vivo gene transfer of anti-PrP scFvs using brain-engraftable cells as an immunotherapeutic approach against prion diseases.

We showed that recombinant 3S9scFv produced by HEK293T cells could bind to both recombinant PrP and PrP^{C}, native PrP, and prevent PrP^{Sc} formation in both 22L and Chandler prion-infected N2a cells, N2aC24L1-3, and N2aC24Chm cells. Similar results were reported by other investigators, showing that 6H4 anti-PrP mAb-derived scFv, which was produced by stably transfected human rhabdomyosarcoma RD-4 cells, reduced PrP^{Sc} levels in prion-infected N2a/Bos2 cells (Donofrio et al. 2005). These results clearly indicate that recombinant anti-PrP scFvs secreted from cultured cells are active against prions. We then established a 3S9scFv/GFP-Ra2 cell line, a brain-engraftable Ra2 microgial cell line stably expressing 3S9scFv, and intracerebrally injected these cells into mice before or after infection with Chandler or 22L prions. No autoimmune responses such as lymphocyte infiltration were observed in the brain (data not shown), suggesting that, similarly to parent 3S9 anti-PrP mAb, 3S9scFv

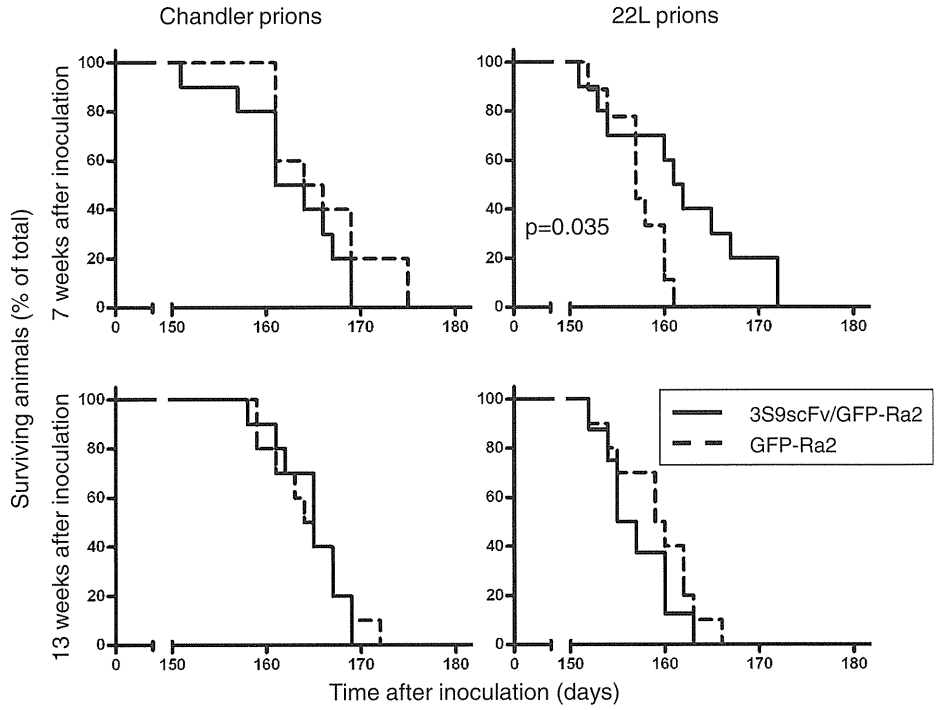

Fig. 6 Survival curves of mice intracerebrally injected with 3S9scFv/GFP-Ra2 cells 7 and 12 weeks after infection with prions. No anti-prion effects of 3S9scFv/GFP-Ra2 cells were detected in mice infected with Chandler prions. In contrast, injection of 3S9scFv/GFP-Ra2 cells 7 weeks after infection with 22L prions significantly extended survival times in mice, compared to injection of GFP-Ra2 cells ($P = 0.035$, Logrank test). No extension in survival times could be detected in 22L prion-infected mice when 3S9scFv/GFP-Ra2 cells were injected 13 weeks after infection commenced. Each group comprises 9–10 mice

might not induce autoimmune reactions. At present, we do not know whether or not 3S9scFv/GFP-Ra2 cells themselves could be infected with prions in the brain. However, transient transfection of the CS-CA-3S9scFv expression vector prevented PrP^{Sc} formation in N2aC24L1-3 cells (data not shown), suggesting the unlikelihood of 3S9scFv/GFP-Ra2 cells becoming infected. Nonetheless, the injection of 3S9scFv/GFP-Ra2 cells either before or at an early time point after the infection, significantly prolonged survival times of the mice. Altogether, these results suggest that the ex vivo gene transfer of 3S9scFv using brain-engraftable Ra2 microglial cells might be effective against prions.

The anti-prion effects were different against 22L and Chandler prions. Different anti-prion effects were also reported with 31C6 anti-PrP mAb. The mAb was more effective against Chandler prions than Obihiro prions when intraventricularly infused into mice (Song et al. 2008). This is possibly because different strains of prions affect different brain regions and therefore the brain regions relevant to therapy might be somewhat different from one strain to another. It is also possible that, since 22L prions were inoculated into mice with titers more than tenfold less than the Chandler prions, the different effects of 3S9scFv/GFP-Ra2 cells against 22L and Chandler prions in the mice might result from difference in the inoculation titers. Moreover, the anti-prion effects were very marginal. This might be due to the short lifetime of Ra2 cells in vivo. The injected 3S9scFv/GFP-Ra2 cells were not found in the brains of terminally ill mice by immunohistochemistry using anti-hrGFP and anti-c-myc Abs (data not shown). Alternatively, low expression of 3S9scFv in 3S9scFv/GFP-Ra2 cells might be attributable to the marginal anti-prion effects. Or, both might be mutually associated with the marginal anti-prion effects.

The ex vivo gene transfer of anti-PrP Abs into the brain may be advantageous as an immunotherapeutic approach against prion diseases over the direct intraventricular infusion or the virus vector-mediated gene transfer methods. The intraventricularly infused Abs and the scFvs delivered by virus vectors could spread to only restricted regions that are close to sites where the Abs or virus vectors are injected, resulting in a limited effect on PrP^{Sc} formation and prion propagation in the brain. Greater therapeutic effect may be expected by the ex vivo gene transfer of anti-PrP Abs using brain-engraftable cells, because these cells have a potential to migrate to broad regions after engraftment into the brain. Indeed, it was recently reported that bone marrow-derived mesenchymal stem cells could spread widely as differentiated cells including astrocytes, oligodenrocytes and neurons, throughout the brains of mice that had been infected with prions, when engrafted into the hippocampus or even intravenously injected (Song et al. 2009). Taken together with our present results, these indicate that the brain-engraftable cells-mediated ex vivo gene transfer of anti-PrP Abs might be a possible immunotherapeutic approach against prion diseases. However, further studies are necessary for this kind of immunotherapeutic approach against prion diseases to be practically effective.

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References

Atarashi R, Sim VL, Nishida N, Caughey B, Katamine S (2006) Prion strain-dependent differences in conversion of mutant prion proteins in cell culture. J Virol 80:7854–7862

Bueler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, Aguet M, Weissmann C (1993) Mice devoid of PrP are resistant to scrapie. Cell 73:1339–1347

Campana V, Zentilin L, Mirabile I, Kranjc A, Casanova P, Giacca M, Prusiner S, Legname G, Zurzolo C (2008) Development of antibody fragments for immunotherapy of prion diseases. Biochem J 418:507–515

Donofrio G, Heppner FL, Polymenidou M, Musahl C, Aguzzi A (2005) Paracrine inhibition of prion propagation by anti-PrP single-chain Fv miniantibodies. J Virol 79:8330–8338

Enari M, Flechsig E, Weissmann C (2001) Scrapie prion protein accumulation by scrapie-infected neuroblastoma cells abrogated by exposure to a prion protein antibody. Proc Natl Acad Sci USA 98:9295–9299

Kanzawa T, Sawada M, Kato K, Yamamoto K, Mori H, Tanaka R (2000) Differentiated regulation of allo-antigen presentation by different types of murine microglial cell lines. J Neurosci Res 62:383–388

Kopacek J, Sakaguchi S, Shigematsu K, Nishida N, Atarashi R, Nakaoke R, Moriuchi R, Niwa M, Katamine S (2000) Upregulation of the genes encoding lysosomal hydrolases, a perforin-like protein, and peroxidases in the brains of mice affected with an experimental prion disease. J Virol 74:411–417

Kuwahara C, Takeuchi AM, Nishimura T, Haraguchi K, Kubosaki A, Matsumoto Y, Saeki K, Matsumoto Y, Yokoyama T, Itohara S, Onodera T (1999) Prions prevent neuronal cell-line death. Nature 400:225–226

Lefebvre-Roque M, Kremmer E, Gilch S, Zou WQ, Feraudet C, Gilles CM, Sales N, Grassi J, Gambetti P, Baron T, Schatzl H, Lasmezas CI (2007) Toxic effects of intracerebral PrP antibody administration during the course of BSE infection in mice. Prion 1:198–206

Manson JC, Clarke AR, McBride PA, McConnell I, Hope J (1994) PrP gene dosage determines the timing but not the final intensity or distribution of lesions in scrapie pathology. Neurodegeneration 3:331–340

Miyamoto K, Nakamura N, Aosasa M, Nishida N, Yokoyama T, Horiuchi H, Furusawa S, Matsuda H (2005) Inhibition of prion propagation in scrapie-infected mouse neuroblastoma cell lines using mouse monoclonal antibodies against prion protein. Biochem Biophys Res Commun 335:197–204

Miyoshi H, Smith KA, Mosier DE, Verma IM, Torbett BE (1999) Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. Science 283:682–686

Peretz D, Williamson RA, Kaneko K, Vergara J, Leclerc E, Schmitt-Ulms G, Mehlhorn IR, Legname G, Wormald MR, Rudd PM, Dwek RA, Burton DR, Prusiner SB (2001) Antibodies inhibit prion propagation and clear cell cultures of prion infectivity. Nature 412:739–743

Prusiner SB (1998) Prions. Proc Natl Acad Sci USA 95:13363–13383

Prusiner SB, Groth D, Serban A, Koehler R, Foster D, Torchia M, Burton D, Yang SL, DeArmond SJ (1993) Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies. Proc Natl Acad Sci USA 90:10608–10612

Sakaguchi S, Katamine S, Shigematsu K, Nakatani A, Moriuchi R, Nishida N, Kurokawa K, Nakaoke R, Sato H, Jishage K, Kuno J, Noda T, Miyamoto T (1995) Accumulation of proteinase K-resistant prion protein (PrP) is restricted by the expression level of normal PrP in mice inoculated with a mouse-adapted strain of the Creutzfeldt-Jakob disease agent. J Virol 69: 7586–7592

Sakaguchi S, Ishibashi D, Matsuda H (2009) Antibody-based immunotherapeutic attempts in experimental animal models of prion diseases. Expert Opin Ther Pat 19:907–917

Sawada M, Imai F, Suzuki H, Hayakawa M, Kanno T, Nagatsu T (1998) Brain-specific gene expression by immortalized microglial cell-mediated gene transfer in the mammalian brain. FEBS Lett 433:37–40

Solforosi L, Criado JR, McGavern DB, Wirz S, Sanchez-Alavez M, Sugama S, DeGiorgio LA, Volpe BT, Wiseman E, Abalos G, Masliah E, Gilden D, Oldstone MB, Conti B, Williamson RA (2004) Cross-linking cellular prion protein triggers neuronal apoptosis in vivo. Science 303:1514–1516

Song CH, Furuoka H, Kim CL, Ogino M, Suzuki A, Hasebe R, Horiuchi M (2008) Effect of intraventricular infusion of anti-prion protein monoclonal antibodies on disease progression in prion-infected mice. J Gen Virol 89:1533–1544

Song CH, Honmou O, Ohsawa N, Nakamura K, Hamada H, Furuoka H, Hasebe R, Horiuchi M (2009) Effect of transplantation of bone marrow-derived mesenchymal stem cells on mice infected with prions. J Virol 83:5918–5927

Wuertzer CA, Sullivan MA, Qiu X, Federoff HJ (2008) CNS delivery of vectored prion-specific single-chain antibodies delays disease onset. Mol Ther 16:481–486

Yamanaka H, Ishibashi D, Yamaguchi N, Yoshikawa D, Nakamura R, Okimura N, Arakawa T, Tsuji T, Katamine S, Sakaguchi S (2006) Enhanced mucosal immunogenicity of prion protein following fusion with B subunit of *Escherichia coli* heat-labile enterotoxin. Vaccine 24:2815–2823

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Short Communication

Atypical L-Type Bovine Spongiform Encephalopathy (L-BSE) Transmission to Cynomolgus Macaques, a Non-Human Primate

Fumiko Ono, Naomi Tase[1], Asuka Kurosawa[3], Akio Hiyaoka, Atsushi Ohyama, Yukio Tezuka, Naomi Wada[2], Yuko Sato[3], Minoru Tobiume[3], Ken'ichi Hagiwara[4], Yoshio Yamakawa[4]*, Keiji Terao[1], and Tetsutaro Sata[3]

The Corporation for Production and Research of Laboratory Primates, Tsukuba 305-0843;
[1]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba 305-0843;
[2]Department of Veterinary Medicine, Yamaguchi University, Yamaguchi 753-8515; and
[3]Department of Pathology and [4]Department of Biochemistry and Cell Biology,
National Institute of Infectious Diseases, Tokyo 162-8640, Japan

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SUMMARY: A low molecular weight type of atypical bovine spongiform encephalopathy (L-BSE) was transmitted to two cynomolgus macaques by intracerebral inoculation of a brain homogenate of cattle with atypical BSE detected in Japan. They developed neurological signs and symptoms at 19 or 20 months post-inoculation and were euthanized 6 months after the onset of total paralysis. Both the incubation period and duration of the disease were shorter than those for experimental transmission of classical BSE (C-BSE) into macaques. Although the clinical manifestations, such as tremor, myoclonic jerking, and paralysis, were similar to those induced upon C-BSE transmission, no premonitory symptoms, such as hyperekplexia and depression, were evident. Most of the abnormal prion protein (PrP^{Sc}) was confined to the tissues of the central nervous system, as determined by immunohistochemistry and Western blotting. The PrP^{Sc} glycoform that accumulated in the monkey brain showed a similar profile to that of L-BSE and consistent with that in the cattle brain used as the inoculant. PrP^{Sc} staining in the cerebral cortex showed a diffuse synaptic pattern by immunohistochemistry, whereas it accumulated as fine and coarse granules and/or small plaques in the cerebellar cortex and brain stem. Severe spongiosis spread widely in the cerebral cortex, whereas florid plaques, a hallmark of variant Creutzfeldt-Jakob disease in humans, were observed in macaques inoculated with C-BSE but not in those inoculated with L-BSE.

Bovine spongiform encephalopathy (BSE) is a fatal transmissible neurodegenerative disorder of cattle caused by the BSE prion. This disease first emerged among cattle in the United Kingdom in 1987 (1) and subsequently spread throughout Europe, Japan, and North America within the next decade (2,3). Based on similarities in disease phenotype, brain pathology, brain lesion profile, and the glycoform profile of the proteinase-resistant core of prion protein (PrP^{Sc}), it was initially believed that the disease was caused by transmission of a single prion strain conferring classical BSE (C-BSE) (4–7). However, two types of BSE, with distinct biochemical and pathological characteristics from those of C-BSE, have been detected in the European Union (EU), Japan, and the United States (8–14). These atypical BSE types have been classified as H- and L-BSE (15) in the light of the high and low molecular mass fragments of the non-glycosylated PrP molecule in the proteinase K resistant core of PrP^{Sc}. In addition, the L-type BSE prion is distinguishable by its high content of monoglycosylated molecules in the core. To date, 27 cases of L-BSE and 24 cases of H-BSE have been reported worldwide (16), thus meaning that the prevalence of atypical BSE is considerably lower than that of C-BSE. However, recent studies showed that L-BSE is easily transmissible to transgenic mice expressing human (17,18) or bovine (19,20) prion protein, as well as to non-human primates (21), with shorter incubation periods than for the transmission of C-BSE to these animals. The virulent nature of L-BSE has stimulated new concern for human public health since the transmission of C-BSE to humans resulted in variant Creutzfeldt-Jakob disease (vCJD) (4–7), a new emergent prion disease.

Since September 2001, 36 BSE cattle have been found in Japan after blanket BSE testing of approximately 10 million cattle (22) by screening and active surveillance programs conducted by the Ministry of Health, Labour and Welfare and the Ministry of Agriculture, Forestry and Fisheries (23). Two cattle, slaughtered at abattoirs, were identified as having atypical BSE by confirmatory examinations involving Western blotting (WB) and/or immunohistochemistry (IHC). Both these cases were classified as L-BSE on the basis of their glycoform profiles. The first L-BSE case was reported in a young Holstein steer (23 months old; BSE/JP8) (12), whereas the second case was reported in an old Japanese meat cow (196 months old; BSE/JP24) (13). Transmission of the first L-BSE case to bovinized mouse (transgenic

*Corresponding author: Mailing address: Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111 ext. 2127, Fax: +81-3-5285-1157, E-mail: yamakawa@nih.go.jp

mice over-expressing bovine PrP) was unsuccessful, probably due to the extremely low amount of PrP^{Sc} and the limited sample size (24). In contrast, the second L-BSE case was readily transmitted to both bovinized mice (25) and bovine (20,26), with shorter incubation periods than those required for the transmission of C-BSE to these animals. The glycoform profile of PrP^{Sc} propagated in the recipient animals was similar to that accumulated in the brain of the donor cattle (BSE/JP24) (20,25,26). To date, the biochemical and histopathological characteristics of the JP24 case analyzed with bovinized mice and bovine have been reported to be similar to those reported for the bovine amyloidotic spongiform encephalopathy (BASE) case, a representative L-BSE originally identified and characterized in Italy in 2003 (8,18,19). In this study, we inoculated a brain homogenate of BSE/JP24 into cynomolgus macaques to investigate disease manifestation and the characteristics of L-BSE in primates in comparison with C-BSE.

Two macaques simultaneously developed neurological signs and symptoms 19–20 months post-inoculation (mpi) with the brain homogenate of BSE/JP24. The monkeys entered the terminal stage of the disease (total paralysis) at 24–25 mpi. Both the onset and duration of the disease were shorter than those reported for the transmission of C-BSE to macaques by us and other groups (27,28). The clinical manifestations such as tremor, myoclonic jerking, and paralysis were similar to those observed during the transmission of C-BSE to macaques, whereas the premonitory abnormal behaviors, such as hyperekplexia and depression, seen upon transmission of C-BSE to macaques were not evident (27).

Histopathological analysis and IHC, performed as described previously (29), showed that severe spongiform changes and the accumulation of PrP^{Sc} with various patterns were detectable in the brains of both monkeys (Fig. 1). Vacuolization was profound throughout the cerebral cortex, from the frontal to the occipital lobes (Fig. 1a). Likewise, synaptic-type PrP^{Sc} precipitation (30) was observed in the whole cerebral cortex and basal ganglia by IHC (Figs. 1b and c). Dense precipitates and plaques of PrP^{Sc}, which had been observed in cattle (JP24) brain (13), were not detected in the cerebrum of the monkeys. PrP^{Sc}, in the form of small plaques or coarse granules, was, however, detected in the molecular layer of the cerebellum (Fig. 1e). Despite the severe spongiosis in the cerebral cortex, florid plaques, which are large PrP^{Sc} plaques surrounded by vacuoles, a hallmark of vCJD (4–7,30) and C-BSE transmission to macaques (27,28), were not observed. The histopathology of the brain was therefore similar to that reported for the brain of L-BSE (BASE)-transmitted macaques (21).

Figure 2 shows the results of WB analysis of PrP^{Sc} in the brain and peripheral nerves (dorsal root ganglia). Despite the region-specific morphology of PrP^{Sc} deposition, the proportions of the di/mono/non-glycosylated forms of PrP^{Sc} propagated in various regions of the

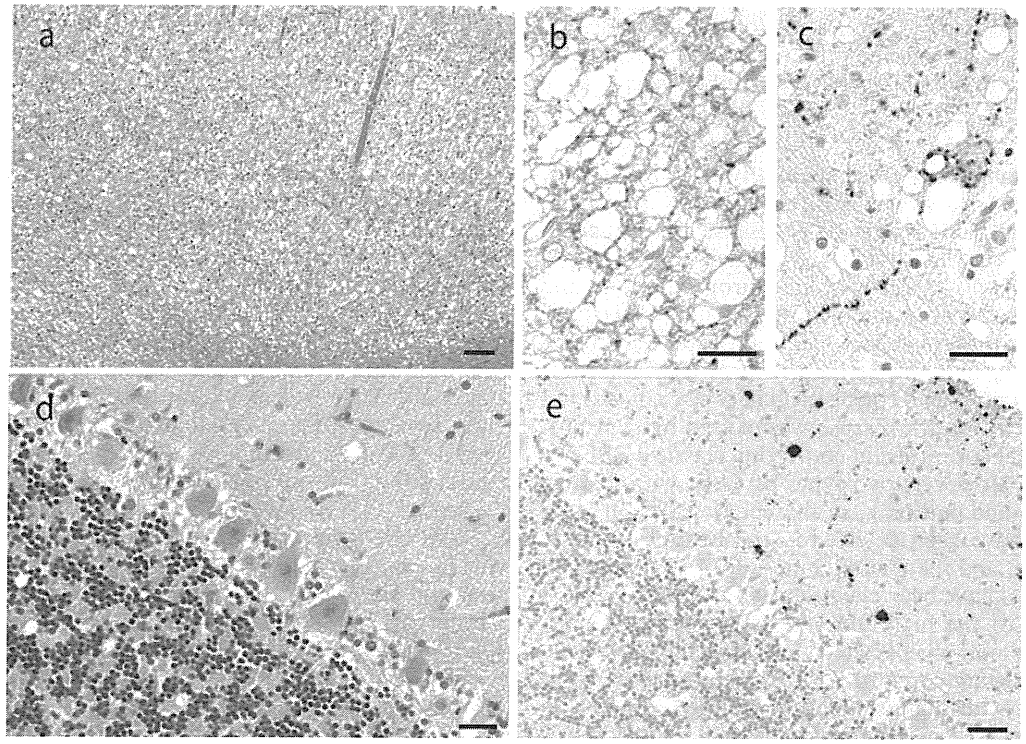

Fig. 1. Histopathology (HE) and PrP^{Sc} immunostaining of monkey brain. HE-staining of brain sections corresponding to the cortex of the cerebrum (a) and the cerebellum (d); PrP^{Sc} immunostaining of the cerebral cortex (b) and basal ganglia (c) and the cerebellum (e); a consecutive section of (d). Bar = 100 μm (a); 20 mm (b, c, d, and e). HE- and PrP^{Sc} immunostaining were performed as described previously (29). Anti-prion protein antibody T4, a rabbit polyclonal antibody raised against the synthetic peptide correspond to codons 211–239 of bovine prion protein (38), was used as the primary antibody. The CSA II amplification system (Biotin-free Catalyzed Amplification System; Dako, Kyoto, Japan) was used to enhance signal intensity instead of the Envision+ immuno-enhancing system.

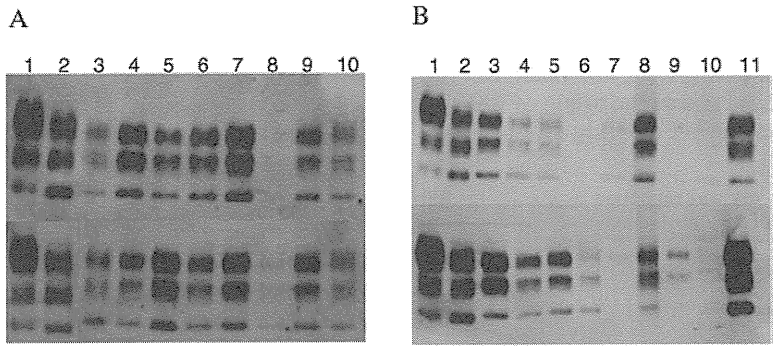

Fig. 2. PrP^{Sc} accumulated in the brain (A) and peripheral nerves (B) of euthanized monkeys. Upper and lower panels represent the brains of different monkeys, #14 and #15, respectively. (A) Lane 1, PrP^{Sc} of C-BSE (25 μg tissue equivalent); lane 2, PrP^{Sc} of L-BSE (50 μg equivalent); lanes 3–10, PrP^{Sc} accumulated in the brain of a monkey (10 μg brain tissue equivalent): lane 3, frontal lobe; lane 4, temporal lobe; lane 5, parietal lobe; lane 6, occipital lobe; lane 7, thalamus; lane 8, mid brain/medula oblongata; lane 9, cerebellum; lane 10, olfactory bulb. (B) Lanes 1–2, PrP^{Sc} of C-, L-BSE (25 and 50 μg brain equivalent); lanes 3–6, spinal cord of cervical thoracic and lumber regions (200 μg tissue equivalent): lane 6, dorsal root ganglia (1 mg tissue equivalent); lane 7, optic nerve (1 mg tissue equivalent); lane 8, retina (1 mg tissue equivalent); lane 9, trigeminal nerve (1 mg tissue equivalent); lane 10, sciatic nerve (5 mg tissue equivalent); lane 11, olfactory bulb (20 μg tissue equivalent). All techniques used in this analysis, including preparation of tissue homogenates, proteinase K treatment, poly-acrylamide gel electrophoresis on 12% gels (NuPAGE; Invitrogen, Carlsbad, Calif., USA) and transfer of protein onto PVDF membrane, were performed as described previously (27,29). Anti-prion protein antibody 6H4 (mouse monoclonal; Roche Applied Science, Basel, Switzerland) and peroxidase-labeled anti-mouse IgG antibody $(Fab)_2$, were used as the primary and secondary antibodies, respectively. Immunoreactive protein on the blot was reacted with ECLplus chemiluminescent reagent and signals were recorded and processed on a Lumino-image analyzer LAS-3000 mini (Fuji film, Tokyo, Japan). PrP^{Sc} of C-BSE and L-BSE were prepared from cattle brain by the same method and electrophoresed as in-house references to evaluate the whole process.

brain were similar to each other and to those observed in the original JP24 cattle (Fig. 2A). Although it appeared that the non-glycosylated PrP^{Sc} accumulated in the monkey tissue showed a slight upper-shift of its electrophoretic mobility towards that of C-BSE in the present WB analysis (for example, Fig. 2A lower panel), it is not known whether this minor shift was significant as the non-glycosylated PrP^{Sc} in the BSE/JP24 cattle originally had a mobility similar to, or only slightly different from, that of the C-BSE case (13,25). Further transmission experiments and a more accurate WB analysis are therefore needed to confirm such subtle differences in the non-glycosylated molecule of BSE prions after transmission to macaques. Both IHC and WB successfully detected PrP^{Sc} in retina, trigeminal ganglia, and dorsal root ganglia (Fig. 2B). PrP^{Sc} was barely detected in the sciatic nerve of monkey #15 by WB (Fig. 2B lowere panel lane 10). However, the amount of PrP^{Sc} in the sciatic nerve was estimated to be less than 1/1,000 of the cerebral PrP^{Sc}. Deposition of PrP^{Sc} in lymphoid tissues (spleen and tonsils) or lymph nodes (inguinal, axillary, submandibular, deep cervical, mesenteric, subiliac, and hilar lymph nodes) was not detected by IHC or WB. Considering the sensitivity of WB analysis and the amount of tissue (5 mg/lane) used in this experiment, the amount of PrP^{Sc} in lymphoid tissues must therefore be lower than 1/5,000 of that in the brain.

The results of an enzyme-linked immunosorbent assay for bovine PrP^{Sc} (BSE TeSeE; BioRad, Mornes-la-Coquette, France) suggested that the amount of PrP^{Sc} in the inoculum used in this study was as low as 1/5 than in a brain homogenate of C-BSE (JP6; $10^{5.2}$ LD_{50}/g using bovinized mice; see ref. 24) previously used for the transmission of C-BSE to macaques (27). Despite the low concentration of PrP^{Sc}, both the incubation period and the duration of the disease were approximately 2/3 shorter than those required for the transmission of C-BSE to macaques, although they were similar to those reported for the transmission of BASE to macaques (21). These results may therefore indicate that the L-BSE agent is more virulent in non-human primates. However, further experiments involving oral administration would be required to assess the risk of L-BSE transmission from affected cattle to humans through the consumption of beef products.

We were unable to detect PrP^{Sc} in lymphatic tissues or lymph nodes by WB or IHC. However, this does not necessarily indicate the absence of infectivity of those organs. Determination of the infectivity using inbred mice was difficult since they are reportedly insensitive or have limited sensitivity, if any, to L-BSE (25,31). The BSE/JP24 isolate could not be transmitted to three different lines of inbred mice, even 700 days after inoculation (Hagiwara, unpublished). Further analysis using transgenic mice expressing bovine or human PrP, or in vitro amplification of PrP^{Sc} by protein misfolding cyclic amplification (32,33) or quaking-induced conversion (34), would be necessary to detect trace amounts of PrP^{Sc} or infectivity in the lymphatic tissues or lymph nodes of the macaques.

The origin of atypical BSE is generally unknown, except for one H-BSE case in the United States that is considered to result from a heritable pathogenic mutation in the PrP gene (35). Epidemiological evidence (8–10,13,15) suggests that most cases of atypical BSE are found in old cattle (>8 years of age), and the relatively even birth-year distributions lead to speculation that atypical BSE is a sporadic prion disorder of cattle similar to sporadic CJD in humans (36). In this context, the conversion of BASE prion and other L-BSE prions

into C-BSE-like phenotypes during interspecies transmission to inbred mice or transgenic mice expressing ovine PrP has been reported (31,37,38). These findings are therefore of interest as regards the origin of BSE and the possible divergent evolution of prion strains.

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Conflict of interest None to declare.

REFERENCES

1. Wells, G.A.H., Scott, A.C., Johnson, C.T., et. al. (1987): A novel progressive spongiform encephalopathy in cattle. Vet. Rec., 121, 419–420.
2. World Organization for Animal Health. Numbers of Cases of Bovine Spongiform Encephalopathy (BSE) Report in the United Kingdom. Online at 〈http://www.oie.int/eng/info/en_esbru_original.htm〉.
3. World Organization for Animal Health. Numbers of Cases of Bovine Spongiform Encephalopathy (BSE) in Farm Cattle Worldwide (excluding the United Kingdom). Online at 〈http://www.oie.int/eng/info/copie2_en_esbmonde.htm〉.
4. Will, R.G., Ironside, J.W., Zeidler, M., et al.(1996): A new variant of Creutzfeldt-Jakob disease in the UK. Lancet, 347, 921–925.
5. Collinge, J., Sidle, K.C.L., Meads, J., et al. (1996): Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature, 383, 685–690.
6. Hill, A.F., Desbruslais, M., Joiner, S., et al. (1997): The same prion strain causes vCJD and BSE. Nature, 389, 448–450.
7. Bruce, M.E., Will, R.G., Ironside, J.W., et al. (1997): Transmission to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature, 389, 498–501.
8. Casalone, C., Zanusso, G., Acutis, P., et al (2004): Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. Proc. Natl. Acad. Sci. USA, 101, 3065–3070.
9. Biacabe, A-G., Laplanche, J-L., Ryder, S., et al. (2004): Distinct molecular phenotypes in bovine prion diseases. EMBO Rep., 5, 110–115.
10. Bushmann, A., Gretzschel, A., Biacabe, A.-G., et al. (2006): Atypical BSE in Germany-proof of transmissibility and biochemical characterization. Vet. Microbiol., 117, 103–116.
11. Baron, T.G.M., Biacabe, A-G., Bencsik, A., et al. (2006): Transmission of new bovine prion to mice. Emg. Infect. Dis., 12, 1125–1128.
12. Yamakawa, Y., Hagiwara, K., Nohtomi, K., et al. (2003): Atypical proteinase K-resistant prion protein (PrP^{res}) observed in an apparently healthy 23-month-old Holstein steer. Jpn. J. Infect. Dis., 56, 221–222.
13. Hagiwara, K., Yamakawa, Y., Sato, Y., et al. (2007): Accumulation of mono-glycosylated form-rich, plaque-forming PrP^{Sc} in the second atypical bovine spongiform encephalopathy case in Japan. Jpn. J. Infect. Dis., 60, 305–308.
14. Richt, J.A., Kunkle, R.A., Nicholson, E.M., et al. (2007): Identification of two bovine spongiform encephalopathy cases diagnosed in the United States. J. Vet. Diagn. Invest., 19, 142–154.
15. Jacobs, J.G., Langeveld, J-P.M., Biacabe, A-G., et al. (2007): Molecular discrimination of atypical bovine spongiform encephalopathy strain from geographical region spanning a wide area in Europe. J. Clin. Microbiol., 45, 1821–1829.
16. Stack, M.J., Focosi-Snyman, R., Cawthraw, S., et al. (2009): Third atypical BSE case in Great Britain with an H-type molecular profile. Vet. Rec., 14, 605–606.
17. Béringue, V., Herzog, L., Reine, F., et al. (2008): Transmission of atypical bovine prion to mice transgenic for human prion protein. Emerg. Infect. Dis., 14, 1898–1901.
18. Kong, Q., Zheng, M., Casalone, C., et al. (2008): Evaluation of the human transmission risk of an atypical bovine spongiform encephalopathy prion strain. J. Virol., 82, 3697–3701.
19. Lombardi, G., Casalone, C., D'Angelo, A., et al. (2008): Interspecies transmission of BASE induces clinical dullness and amyotrophic change. PLoS Pathog., 4, e1000075.
20. Fukuda, S., Iwamura, Y., Imamura, M., et al. (2009): Interspecies transmission of L-type-like bovine spongiform encephalopathy detected in Japan. Microbiol. Immunol., 53, 704–707.
21. Comoy, E.E., Casalone, C., Lescoutra-Etchegaray, N., et al. (2008): Atypical BSE (BASE) transmitted from asymptomatic aging cattle to a primate. PLoS ONE, 3, e3017.
22. Ministry of Health, Labour and Welfare: Results on the BSE Screening. Online at 〈http://www.mhlw.go.jp/houdou/0110/h1018-6.html〉 (in Japanese).
23. Yamanouchi, K. and Yoshikawa, Y. (2007): Bovine spongiform encephalopathy (BSE) safety measure in Japan. J. Vet. Med. Sci., 69, 1–6.
24. Yokoyama, T., Masujin, K., Yamakawa, Y., et al. (2007): Experimental transmission of two young and one suspended bovine spongiform encephalopathy (BSE) cases to bovinized transgenic mice. Jpn. J. Infect. Dis., 60, 317–320.
25. Masujin, K., Shu, Y., Yamakawa, Y., et al. (2008): Biological and biochemical characterization of L-type-like bovine spongiform encephalopathy (BSE) in Japanese black cattle. Prion, 2, 123–128.
26. Iwamaru, Y., Imamura, M., Matsuura, Y., et al. (2009): Accumulation of L-type bovine prions in peripheral nerve tissues. Emeg. Infect. Dis., 16, 1151–1154.
27. Ono, F., Terao, K., Tase, N., et al. (2011): Experimental transmission of bovine spongiform encephalopathy (BSE) to cynomolgus macaques, a non-human primate. Jpn. J. Infect. Dis., 64, 50–54.
28. Lasmézas, C.I., Deslys, J-P., Demaimay, R., et al. (1996): BSE transmission to macaques. Nature, 381, 743–744.
29. Iwata, N., Sato, Y., Higuchi, Y., et al. (2006): Distribution of PrP^{Sc} in cattle with bovine spongiform encephalopathy slaughtered at abattoirs in Japan. Jpn. J. Infect. Dis. 59, 100–107.
30. Liberski, P.P and Ironside, J.W. (2005): Neuropathology of transmissible spongiform encephalopathies (prion diseases). p. 13–48. *In* Brown, D.R. (ed.), Neurodegeneration and Prion Diseases. Springer, New York.
31. Capobianco, R., Casalone, C., Suardi, S., et al. (2007): Conversion of the BASE prion strain into the BSE strain: the origin of BSE? PLoS Pathog., 3, e31.
32. Saborio, G.P., Permanne, P. and Soto, C. (2001): Sensitive detection of prion protein by cyclic amplification of protein misfolding. Nature, 411, 810–813.
33. Chen, B., Morales, R., Barria, M.A., et al. (2010): Estimating prion concentration in fluids and tissue by quantitative PMCA. Nat. Methods, 7, 519–520.
34. Atarashi, R., Wilham, J.M., Christensen, L., et al. (2010): Simplified ultrasensitive prion detection by recombinant PrP conversion with shaking. Nat. Methods, 5, 211–212.
35. Richt, J.A. and Hall, S.M. (2008): BSE case associated with prion protein gene mutation. PLoS Pathog., 4, e1000156.
36. Brown, P., McShane, L.M., Zanusso, G., et al. (2006): On the question of sporadic or atypical bovine spongiform encephalopathy and Creutzfeldt-Jakob diseases. Emerg. Infect. Dis., 12, 1816–1821.
37. Béringue, V., Andréoletti, O., Le Dur, A., et al. (2007): A bovine prion acquires an epidemic bovine spongiform encephalopathy strain-like phenotype on interspecies transmission. J. Neurosci., 27, 6965–6971.
38. Takahashi, H., Takahashi, R.H., Hasegawa, H., et al. (1999): Characterization of antibodies raised against bovine PrP-peptides. J. Neurovirol., 5, 300–307.

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Original Article

Experimental Transmission of Bovine Spongiform Encephalopathy (BSE) to Cynomolgus Macaques, a Non-Human Primate

Fumiko Ono, Keiji Terao[1], Naomi Tase[1], Akio Hiyaoka, Atsushi Ohyama, Yukio Tezuka, Naomi Wada[2], Asuka Kurosawa[3], Yuko Sato[3], Minoru Tobiume[3], Ken'ichi Hagiwara[4], Yoshio Yamakawa[4]*, and Tetsutaro Sata[3]

The Corporation for Production and Research of Laboratory Primate, Tsukuba 305-0843;
[1]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba 305-0843;
[2]Department of Veterinary Medicine, Yamaguchi University, Yamaguchi 357-8515; and
[3]Department of Pathology and [4]Department of Biochemistry and Cell Biology,
National Institute of Infectious Diseases, Tokyo 162-8640, Japan

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SUMMARY: Bovine spongiform encephalopathy (BSE) was transmitted to three macaques by intracerebral inoculation of a brain homogenate from affected cattle detected in Japan. All monkeys developed abnormal behavioral signs, such as intermittent anorexia and hyperekplexia, around 24 months after inoculation. Neuronal symptoms, such as tremor, myoclonic jerking, and paralysis, appeared 27–44 months after inoculation. These symptoms worsened and total paralysis ensued within a year after onset. The disease duration was approximately 8–12 months. Both the incubation period and the duration of disease were shortened in the secondary transmission experiment to macaques. Heavy accumulation of disease-causing conformer(s) of prion protein (PrP^{Sc}), with a similar glycoform profile to the PrP^{Sc} contained in the inoculum, and severe spongiform changes in the histology of the brain, confirmed the successful transmission of BSE to monkeys. Florid plaques, a characteristic histological hallmark of variant Creutzfeldt-Jakob disease, were prominent in the cerebral cortex, in which a prion antigen was detected by immunohistochemistry (IHC). PrP^{Sc} was mostly confined to the central nervous system, although small amounts of PrP^{Sc} accumulated in the peripheral nerves of monkeys, as detected by Western blotting (WB). Neither IHC nor WB detected PrP^{Sc} in the lymphatic organs/tissues, such as the tonsils, spleen, and appendix.

INTRODUCTION

Transmissible spongiform encephalopathies, or prion diseases, are fatal neurodegenerative disorders characterized by severe spongiform changes and the accumulation of abnormal form(s) of prion protein (PrP^{Sc}) in the central nervous system (CNS) (1-3). PrP^{Sc} is a disease-causing conformer(s) of PrP encoded on the host DNA (4,5) that exhibits, with a few exceptions, partial resistance to digestion by proteinase K (1). Human prion diseases are known to be either sporadic, genetic or infectious disorders, such as sporadic Creutzfeldt-Jakob disease (CJD), Gerstmann-Straüssler-Scheinker syndrome, fatal familial insomnia, or kuru, iatrogenic CJD, and variant CJD (vCJD), respectively (1,6). In ruminants, the diseases emerge as scrapie in sheep and goats (1), bovine spongiform encephalopathy (BSE) in cattle (1,7), and chronic wasting disease in elk and deer (1).

vCJD was first recognized in 1996 as a CJD-like fatal neurological disorder that emerged among teenagers in the UK (6). The brain pathology and biochemical characteristics associated with PrP^{Sc} strongly suggested that vCJD was induced by the causative agent of BSE upon the consumption of meat products contaminated by risk materials such as the CNS and/or spinal ganglia of BSE-affected cattle (8–10). As a result of the BSE pandemic, more than 223 CJD patients have been identified to date across the world, with 175 cases in the UK and 48 cases in 10 other countries (11). The annual incidence of vCJD has been reported to be in decline, although some reports predict that the number of vCJD patients may reach some 136,000 in the future, depending on the incubation period of BSE in humans (12–14).

The emergence of vCJD raised another public-health concern regarding the possible iatrogenic transmission of the disease from human to human through blood transfusion or the administration of biological products of human origin, because of the peripheral distribution of PrP^{Sc} in lymphoid follicles of the tonsils and appendix (15,16) even before the onset of clinical symptoms (17). To date, at least three definite cases of transfusion-associated vCJD infection have been reported (11). Appropriate animal models for vCJD are therefore greatly needed in order to study the pathogenesis of the disease and to develop therapeutic interventions.

In this study, we report the pathological and biochemical analyses of an animal model for vCJD using cynomolgus macaques inoculated with classical-type BSE prion isolated from Japanese cattle. We also report a second successful transmission experiment.

*Corresponding author: Mailing address: Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111 ext. 2127, Fax: +81-3-5285-1157, E-mail: yamakawa@nih.go.jp

MATERIALS AND METHODS

Monkeys: The cynomolgus macaques used in this experiment were bred at the Tsukuba Primate Research Center at the National Institutes of Biomedical Innovation (Ibaraki, Japan). All monkeys were male and were 2–2.4 years old when inoculated. The amino acids at codons 129 and 219 of the prion protein gene (Prnp) were methionine/methionine (M/M) and glutamic acid/glutamic acid (E/E), respectively. Monkeys were bred and dissected in biosafety level 3 facilities according to the Biosafety Guidelines of the National Institute of Biomedical Innovation and the National Institute of Infectious Diseases (Tokyo, Japan). The Animal Ethics Committee and the Animal Care and Use Committee at the National Institute of Biomedical Innovation approved this study.

Inoculum: The inoculum was prepared from the brain tissue of BSE cattle (classical-BSE [C-BSE], BSE/JP6) which had been detected in 2003 as a result of the BSE inspection program (18). A 10% homogenate (w/v) of the brain was prepared by shaking the brain tissue (thalamic region) with zirconia beads (d = 2 mm; Nikkato, Tokyo, Japan) in PBS for 5 min using a Multi-beads Shocker apparatus (Yasui Kikai, Tokyo, Japan). This homogenate was then stored at −80°C until use. Prior to inoculation, the homogenate was further dispersed by brief sonication. Two hundred microliters of the homogenate was introduced into the hypothalamus on the right side of the brain using 23G needles, which were passed through a hole in the skull made by a micro-drill. The position of the distal end of the needle was adjusted using brain map coordination under anesthesia with ketamine-HCl and xylazine. A brain homogenate of a diseased monkey (#7) was also prepared as an inoculum for the second transmission.

Histopathology and immunohistochemistry (IHC): For histological examination and IHC, tissue samples were fixed in buffered formalin, treated with formic acid, and processed as described previously (19). The sections were prepared and subjected to routine histological examination by hematoxylin and eosin (HE) staining and IHC. For the IHC of PrP^{Sc}, the sections were pretreated by hydrolytic autoclaving in 1 mM HCl at 121°C for 20 min or 100 mM NaOH, 2% NaCl and 0.1% *N*-lauroylsarcosine at 60°C for 10 min (20). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in 100% methanol for 30 min at room temperature. The sections were treated with an anti-prion protein antibody T4, a rabbit polyclonal antibody (21). The Envision$^+$ system (Dako, Kyoto, Japan) was used for signal detection. A negative control section derived from a healthy monkey was also subjected to IHC.

Preparation of PrP^{Sc} and Western blotting (WB): Tissue homogenates (5–20% (w/v) depending on the sample size) were prepared by shaking diced tissues with zirconia beads (d = 2 mm) in PBS as described above. PrP^{Sc} was prepared by successive hydrolysis of homogenates with DNase, collagenase, and proteinase K in 50 mM Tris-HCl buffer pH 7.5 containing 0.1 M NaCl and 2% twittergent 4-12, and precipitated by the addition of a 0.5 volume of 2-butanol/methanol mixture (5:1, v/v) containing 3 mM phenylmethylsulfonyl fluoride, as described previously (19). The precipitate was dissolved in a minimum volume (10–40 μl) of SDS-sample buffer (0.065 M Tris-HCl buffer pH 6.8 containing 5% glycerol, 3 mM ethylenediaminetetraacetic acid, 5% sodium dodecyl sulfate, 4% β-mercaptoethanol, and 0.04% bromophenol blue) and heated for 5 min at 100°C. The sample, with or without dilution, was electrophoresed in a 12% NuPAGE polyacrylamide gel with NuPAGE MOPS SDS running buffer (Invitrogen, Carlsbad, Calif., USA), then the proteins were electrophoretically transferred onto PVDF membrane in a NuPAGE transfer buffer (Invitrogen) following the manufacturer's instructions. The membrane was blocked by soaking in 50 mM Tris-HCl buffer, pH 7.5, containing 10% non-fat milk and 5% fetal calf serum. The blot was then incubated with anti-prion protein monoclonal antibody 6H4 (Prionics AG, Zurich, Switzerland) (0.125 μg/ml) followed by a horseradish peroxidase-conjugated sheep antibody $(Fab')_2$ against mouse IgG (NA 9310; Amersham Biosciences, Piscataway, N.J., USA). An ECLplus detection kit (Amersham Biosciences) was used to detect immunoreactive proteins and the signal was recorded and processed using an image analyzer (LAS-3000-mini; Fuji Film, Tokyo Japan). The detection threshold for PrP^{Sc} by WB was estimated to be equivalent to 1 μg of brain tissue (data not shown).

RESULTS

Clinical signs and symptoms: The clinical signs and symptoms observed during the course of the disease are listed in Table 1. Upon primary inoculation, two of the three monkeys (#7 and #10) simultaneously developed abnormal behavioral signs, such as intermittent anorexia and hyperekplexia, 26 and 27 months post-inoculation (mpi). Neurological disorders, such as tremor, myoclonic jerking, and paralysis, became apparent at 29 and 27 mpi. All monkeys displayed total paralysis at the terminal stage of the disease (35–37 mpi). The third monkey (#11) also developed similar symptoms,

Table 1. Signs and symptoms observed during the transmission of BSE to macaques

Transmission	Primary			Secondary	
Monkey #	#7	#10	#11	#16	#17
Inoculated age (mo)	24	28	29	25	24
Onset (mo)	29	27	44	15	13
Disease duration (mo)	8	8	15	5	6
Abnormal behaviors:					
Depression	−	+++	−	+	−
Self harm behavior	+	−	+	−	−
Anorexia	+	−	+	−	+
Scordinema	−	+	−	−	−
Hyperekplexia	+++	+++	++	++	++
Neuronal symptoms:					
Ataxia	+++	+++	+++	++	+++
Tumor	+++	+++	+++	+	++
Myoclonus	++	++	++	−	++
Paralysis	+++	++	+++	+	++
Astasia	+++	++	+++	−	+

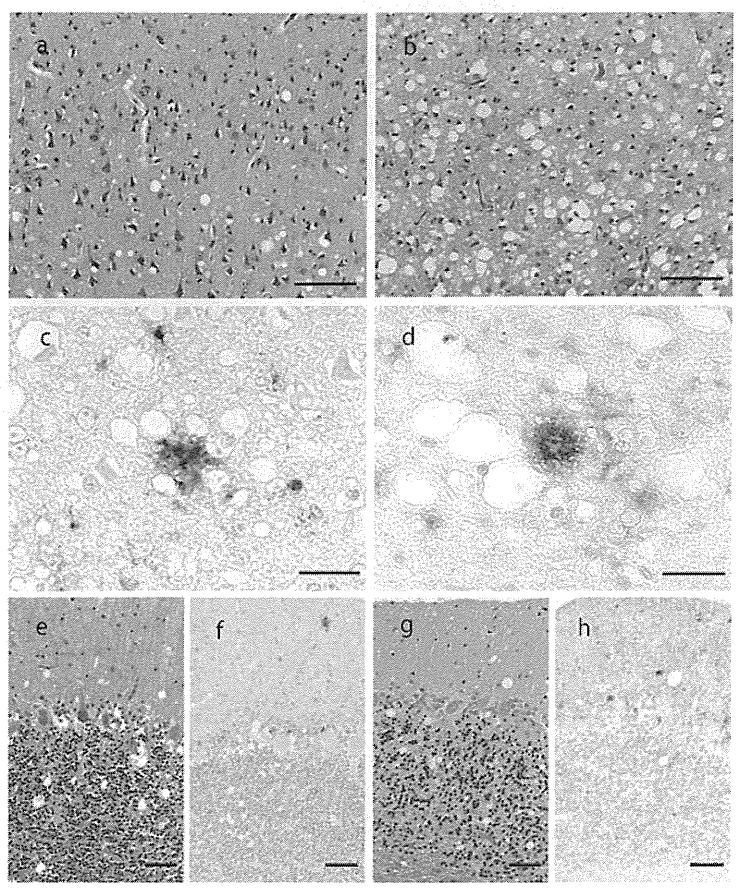

Fig. 1. Histology and immunohistochemistry (IHC) of PrP^{Sc} in the brains of macaques transmitted with BSE. Parts a, b, e, and g represent HE staining of the cerebral (a, b) or cerebellar (e, g) cortex of primary transmitted (a, e) or secondary BSE transmitted monkeys, respectively. Parts c, d, f, and h represent IHC of PrP^{Sc} in the cerebral (c, d) and cerebellar (f, h) cortex of primary (c, f) or secondary (d, h) BSE transmitted monkeys. Bar = 100 μm (a, b, e, f, g, and h); 50 μm (c and d).

although onset of the disease was delayed to 45 mpi, which is around 1.5 years longer than for the other monkeys.

In the secondary transmission experiment, a brain homogenate of a euthanized monkey (#7) was prepared and inoculated into two monkeys. They developed symptoms similar to those observed during the first (interspecies) transmission experiment, although the incubation period and duration of the disease were markedly shortened to 16 months and 6 months, respectively, thereby indicating adaptation of the prion to monkeys.

Histopathological analysis and IHC of the CNS of monkeys: The results of histopathological analysis and IHC of the monkey brains are shown in Fig. 1. Severe spongiform changes and the accumulation of PrP^{Sc} in the brain were detected in all monkeys. Vacuolization was more marked at the cortex of the frontal and temporal lobes (Figs. 1a and b) than in the occipital lobe and cerebellum (data not shown). Florid plaques, a dense core of PrP^{Sc} surrounded by vacuoles, were located at the cerebral cortex (Figs. 1c and d). The deposition of PrP^{Sc} with form of small plaques or coarse granules or fine glanules was found in the molecular or granular layer of the cerebellum, respectively (Figs. 1f and h), whereas fine granular deposits of PrP^{Sc} were observed in the brain of BSE cattle (19). Granular deposition of PrP^{Sc} was also found in the gray matter of the spinal cord, with mild spongiform changes. A small but consistent accumulation of PrP^{Sc} was found in the ganglionic cell layer, inner plexiform layer, and outer plexiform layer of the retina, and in the ganglionic and satellite cells of the trigeminal and dorsal root ganglia without vacuolation (data not shown).

Both spongiform changes (Figs. 1a and b) and the numbers of florid plaques of PrP^{Sc} became severe in the brains of the secondary transmission experiment.

WB and distribution of PrP^{Sc}: The WB results for PrP^{Sc} propagated in monkey brain are shown in Fig. 2a. The glycoform profiles of PrP^{Sc} propagated in each monkey brain are apparently indistinguishable from each other and are likely identical to that of PrP^{Sc} accumulated in the cattle brain. The glycoform profiles of PrP^{Sc} prepared from various brain regions and the spinal cord of monkey #7 were also identical, as shown in Fig. 2b. To explore the occurrence of PrP^{Sc} outside the CNS, homogenates of peripheral nerves (vagus, median, intercostal, sympathetic, sciatic and celiac plexus), lymphatic tissues (spleen, tonsil, and distal ileum), and lymph nodes (inguinal, axillary, submandibular, deep cervices, mesenteric, subiliac, and hilar) were prepared and analyzed by WB. PrP^{Sc} was detected in several peripheral nerves and the submandibular lymph node of

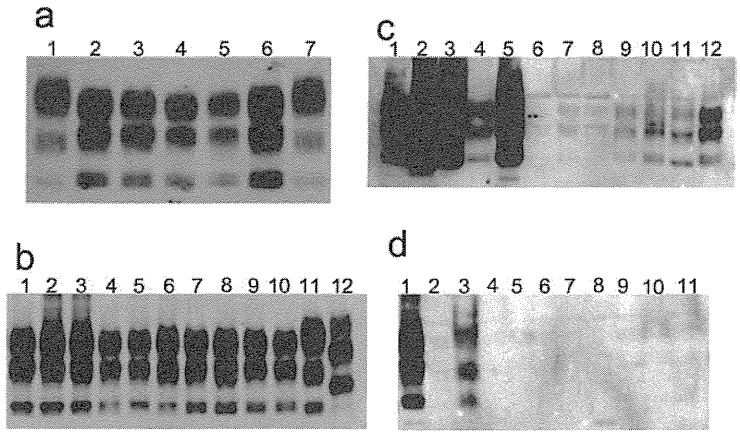

Fig. 2. Distribution of PrP^{Sc}, as analyzed by Western blotting. (a) Western blot of PrP^{Sc} of bovine and macaques. Lanes 1 and 7 represent bovine PrP^{Sc} (100 μg tissue equivalent) of C-BSE; lanes 2–6 represent PrP^{Sc} propagated in monkey brain (12.5 μg tissue equivalent). Samples were prepared from the thalamic region of primary transmitted monkeys: #7 (lane 2), #10 (lane 3), and #11 (lane 4), or secondary transmitted monkeys: #16 (lane 5) and #17 (lane 6). (b) PrP^{Sc} propagated in the CNS of monkey #7 (primary transmission). Lane 1, frontal lobe (8.3); lane 2, parietal lobe (8.3); lane 3, temporal lobe (8.3); lane 4, occipital lobe (19.6); lane 5, thalamus (33.2); lane 6, cerebellum; lane 7, midbrain (14.6); lane 8, pons (22.2); lane 9, medulla oblongata (44.4); lane 10, spinal cord (208); lane 11, bovine PrP^{Sc} (50); lane 12, human PrP^{Sc} (CJD type 1 [40]). Figures in parentheses represent the amounts of tissue equivalents (μg) applied to the well. (c) PrP^{Sc} propagated in the peripheral nerves of monkey #7 (primary transmission). Lane 1, PrP^{Sc} in brain (positive control 0.015); lane 2, olfactory bulb (0.5); lane 3, optic nerve (5); lane 4, retina (0.5); lane 5, trigeminal nerve (0.5); lane 6, celiac ganglia (5); lanes 7 and 8, vagus nerves (5); lane 9, median nerve (5); lane 10, intercostal nerve (5); lane 11, sympathetic nerve (5); lane 12, sciatic nerve (5). Figures in parentheses represent the amounts of tissue equivalents (mg) applied to the well. (d) Extra-neuronal PrP^{Sc} of monkey #7 (primary transmission). Lane 1, brain (positive control 0.008); lane 2, mesenteric lymph node (7.5); lane 3, submandibular lymph node (7.5); lane 4, axillary lymph node (7.5); lane 5, inguinal lymph node (7.5); lane 6, hilar lymph node (7.5); lane 7, thymus (7.5); lane 8, distal ileum (7.5); lane 9, tonsil (7.5); lane 10, spleen (7.5); lane 11, adrenal grand (7.5). Figures in parentheses represent the amounts of tissue equivalents (mg) applied to the well.

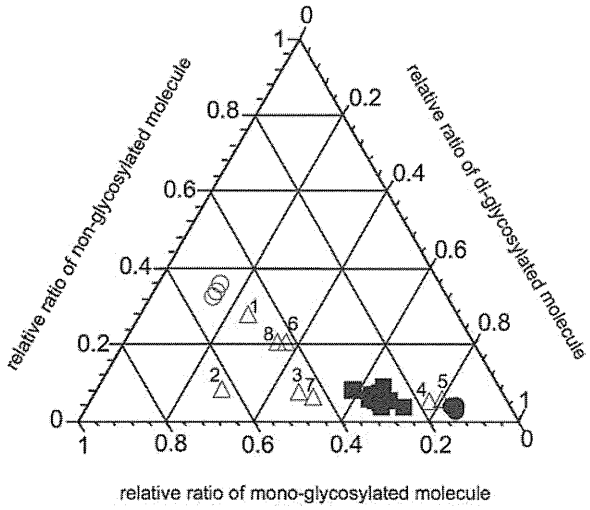

Fig. 3. Comparison of the glycoform profiles of PrP^{Sc} propagated in monkey #7. ○, sporadic CJD MM1; ●, BSE; ■, PrP^{Sc} in brain and spinal cord; △: PrP^{Sc} in 1, sympathetic nerve; 2, trigeminal nerve; 3, sciatic nerve; 4, optic nerve; 5, retina; 6, submandibular lymph node; 7, median nerve; 8, vagus nerve.

monkey #7, as demonstrated in Figs. 2c and d. The sciatic nerve of monkey #10 was also positive for PrP^{Sc}. Interestingly, the peripheral PrP^{Sc} had an apparently different glycoform profile from that of PrP^{Sc} purified from the CNS, as shown in Fig. 3. The amount of PrP^{Sc} in the peripheral nerves and lymphatic tissues/lymph nodes of other monkeys were below the detection limit of WB analysis. Considering the sensitivity of WB analysis (as low as 1 μg brain tissue, see Materials and Methods) and the amount of tissue (5 mg/lane) used in this experiment, the amount of PrP^{Sc} in the peripheral nerves is likely to be lower than 1/1,000 of that in brain tissue.

DISCUSSION

A non-human primate model has been shown to be a suitable model for humans and has often been applied to preclinical trials of compounds or biologicals for potential human use. With regard to a human vCJD model, as described herein, non-human primates are invaluable because they have been shown to develop florid plaques in the brain, as observed in vCJD patients, and the molecular signature of the prion by WB analysis is similar to those of vCJD patients and BSE-affected cattle. Such pathological features are difficult to reproduce in inbred mice.

We successfully performed experimental transmission of BSE to cynomolgus macaques by the intracerebral inoculation of brain homogenates of asymptomatic BSE cattle detected in Japan. The clinical symptoms, brain pathology, and characteristics of PrP^{Sc} observed in this study were essentially the same as those obtained in earlier European BSE transmission experiments in macaques (22,23). These results strongly indicate that the BSE prion used in the present study, an isolate from the BSE/JP6 cow, is of the same strain as the BSE prion prevailing in European countries, thereby supporting the notion that the agent spread from Europe to Japan.

PrP^{Sc} has frequently been detected in the lymphoid follicles of lymphatic tissues, such as the tonsils and appendix of vCJD patients and orally transmitted BSE monkeys (24–26), whereas in this study we were unable to detect PrP^{Sc} in these organs even when tissues were collected from monkeys at a terminal stage of the illness. Most of the lymph nodes (inguinal, axillary, submandibular, deep cervical, mesenteric, subiliac, and hilar) were also negative for PrP^{Sc}, and we were barely able to detect PrP^{Sc} in the submandibular lymph nodes of monkey #7 by WB. These findings indicate the presence of small amounts of PrP^{Sc} in other lymph nodes and possibly in lymphatic tissues and organs. However, the amounts of PrP^{Sc} in lymphatic tissues or lymph nodes might be dependent on the invasion route of the infective agent. We are currently performing parallel experiments in which we directly introduced 1 ml of 10% brain homogenates two times with a week interval into the stomach of three monkeys using a catheter. Even though the amount of inoculum used in this oral-challenge experiment is 10 times higher than that used for the intercerebral transmission experiment, to date none of the monkeys have developed abnormal neuronal signs 7.5 years post-inoculation.

PrP^{Sc} was uniformly detected in retina, trigeminal ganglia, and dorsal root ganglia of monkeys by both IHC and WB. The amount of PrP^{Sc} in peripheral nerves decreased dramatically, with the exception of the CNS, probably due to centrifugal distribution from the brain.

The glycoform profiles of PrP^{Sc} propagated in the CNS of monkeys were almost identical, and were similar, if not identical, to the PrP^{Sc} glycoforms in cattle brain, although with higher ratios of monoglycosylated molecules being present in the PrP^{Sc} of monkeys compared with the PrP^{Sc} in cattle brain. In addition, the diversity of glycoform profiles among PrP^{Sc} propagated in the peripheral nerves and lymph nodes of monkey #7 was evident (Fig. 3). Further characterization of this peripheral PrP^{Sc} was difficult due to the limited amounts of samples available, although it is worth noting that alterations occur in the biochemical characteristics of BSE prions during interspecies transmission (27–30).

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Conflict of interest None to declare.

REFERENCES

1. Prusiner, S.B. (2004): An introduction to prion biology and diseases. p. 1–87. *In* S. B. Prusiner (ed.), Prion Biology and Diseases. 2nd ed. (ISBN 0–87969–693–1). Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
2. Prusiner, S.B. (1982): Novel proteinaceous infectious particles cause scrapie. Science, 216, 136–144.
3. Prusiner, S.B. (1991): Molecular Biology of Prion Diseases. Science, 252, 1515–1522.
4. Chesebro, B., Race, R., Wehrly, K., et al. (1985): Identification of scrapie prion-specific mRNA in scrapie-infected and uninfected brain. Nature, 315, 331–333.
5. Oesch, B., Westaway, D., Wälchli, M., et al. (1985): A cellular gene encodes scrapie PrP 27–30 protein. Cell, 40, 735–746.
6. Will, R.G., Ironside, J.W., Zeidler, M., et al. (1996): A new variant of Creutzfeldt-Jakob disease in the UK. Lancet, 347, 921–925.
7. Wells, G.A.H., Scott, A.C., Johnson, C.T., et al. (1987): A novel progressive spongiform encephalopathy in cattle. Vet. Rec., 121, 419–420.
8. Collinge, J., Sidle, K.C.L., Meads, J., et al. (1996): Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature, 383, 685–690.
9. Hill, A.F., Desbruslais. M., Joiner, S., et al. (1997): The same prion strain causes vCJD and BSE. Nature, 389, 448–450.
10. Bruce, M.E., Will, R.G., Ironside, J.W., et al. (1997): Transmission to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature, 389, 498–501.
11. The National Creutzfeldt-Jakob Disease Surveillance Unit (NCJDSU), The University of Edinburgh. Variant Creutzfeldt-Jakob Disease Current Data (OCTOBER 2010). Online at 〈http://www.cjd.ed.ac.uk/vcjdworld.htm〉.
12. Ghani, A.C., Ferfuson, N.M., Donnelly, C.A. et al. (2000): Predicted vCJD mortality in Great Britain. Nature, 406, 583–584.
13. Valleron, A.-J., Boelle, P.-Y., Will, R., et al. (2001): Estimation of epidemic size and incubation time based on age characteristics of vCJD in the United Kingdom. Science, 294, 1726–1728.
14. Clarke, P. and Ghani, A.C., (2005): Projections of the future course of the primary vCJD in the UK: inclusion of subclincal infection and the possibility of wider genetic susceptibility. J. R. Soc. Interface, 2, 19–31.
15. Hilton, D.A., Ghani, A.C., Conyers, L., et al. (2002): Accumulation of prion protein in tonsil and appendix: review of tissue samples. Br. Med. J., 325, 633–634.
16. Hill, A.F., Butterworth, R.J., Joiner, S., et al. (1999): Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. Lancet, 353, 183–189.
17. Hilton, D.A., Fathers, E., Edwards, P., et al. (1998): Prion immunoreactivity in appendix before onset of variant Creutzfeldt-Jakob disease. Lancet, 352, 703–704.
18. Yamanouchi, K. and Yoshikawa, Y. (2007): Bovine spongiform encephalopathy (BSE) safety measures in Japan. J. Vet. Med. Sci., 69, 1–6.
19. Iwata, N., Sato, Y., Higuchi, Y., et al. (2006): Distribution of PrP^{Sc} in cattle with bovine spongiform encephalopathy slaughtered at abattoirs in Japan. Jpn. J. Infect. Dis., 59, 100–107.
20. Bencsik, A.A., Debeer, S.O.S. and Baron, T.G.M. (2005): An alternative pretreatment procedure in animal transmissible spongiform encephalopathies diagnosis using PrPsc immunohistochemistry. J. Histochem. Cytochem., 53, 1199–1202.
21. Takahashi, H., Takahashi, R., Hasegawa, H., et al. (1999): Characterization of antibodies raised against bovine PrP-peptides. J. Neurovirol., 5, 300–307.
22. Lasmézas, C.I., Deslys, J.-P., Demaimay. R., et al. (1996): BSE transmission to macaques. Nature, 381, 743–744.
23. Lasmézas, C.I, Fournier, J.-G., Nouvel, V., et al. (2001): Adaptation of the bovine spongiform encephalopathy agent to primates and comparison with Creutzfeldt-Jakob disease: implications for human health. Proc. Natl. Acad. Sci. USA, 98, 4142–4147.
24. Herzog, C., Salès. N., Etchegaray, N., et al. (2004): Tissue distribution of bovine spongiform encephalopathy agent in primates after intravenous or oral infection. Lancet, 363, 422–428.
25. Herzog, C., Rivière, J., Lescoutra-Etchegaray, N., et al. (2005): PrP^{TSE} distribution in a primate model of variant, sporadic, and iatrogenic Creutzfeldt-Jakob disease. J. Virol., 79, 14339–14345.
26. Lasmézas, C.I., Comoy, E., Hawkins, S., et al. (2005): Risk of oral infection with bovine spongiform encephalopathy agent in primates. Lancet, 365, 781–783.
27. Asante, E.A., Linehan, J.M., Desbruslais, M., et al. (2002): BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. EMBO J., 21, 6358–6366.
28. Capobianco, R., Casalone, C., Suardi, S., et al. (2007): Conversion of the BASE prion strain into the BSE strain: the origin of BSE? PLoS Pathog., 3, e31 (doi:10.1371/journal.ppat.0030031).
29. Béringue, V., Andréoletti, O., Le Dur, A., et al. (2007): A bovine prion acquires an epidemic bovine spongiform encephalopathy strain-like phenotype on interspecies transmission. J. Neurosci., 27, 6965–6971.
30. Yokoyama, T., Masujin, K., Iwamaru, Y., et al. (2009): Alteration of the biological and biochemical characteristics of bovine spongiform encephalopathy prions during interspecies transmission in transgenic mice model. J. Gen. Virol., 90, 261–268.