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Research article

Development of a quick bioassay for the evaluation of transmission properties of acquired prion diseases

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ABSTRACT

Evaluation of transmission properties is important for the differential diagnosis of a subgroup of acquired Creutzfeldt-Jakob disease (CJD) with methionine homozygosity at polymorphic codon 129 of the *PRNP* gene, an intermediate type abnormal prion protein (PrP), and kuru plaques, denoted as acquired CJD-MMiK. The present study aimed to develop a quick evaluation system of the transmission properties of acquired CJD-MMiK. In the PrP-humanized mice intraperitoneally inoculated with brain homogenates from an acquired CJD-MMiK patient, accumulation of abnormal PrP was observed in follicular dendritic cells of the spleen at 75 days post-inoculation. The transmission properties of acquired CJD-MMiK were quite different from those of sporadic CJD with the same *PRNP* codon 129 genotype. Moreover, even at 14 days post-inoculation, the characteristic transmission properties of acquired CJD-MMiK could be detected. These findings suggest that the bioassay using follicular dendritic cells of the spleen, named as a FDC assay, can be an easy, time-saving, and useful method to distinguish acquired CJD-MMiK from sporadic CJD.

1. Introduction

Prion diseases are fatal neurodegenerative disorders including Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome, fatal familial insomnia, and kuru. The central event in the pathogenesis of prion diseases is a conformational conversion of the normal cellular isoform of prion protein (PrP^{C}) into an abnormal misfolded isoform (PrP^{Sc}) [1]. The conformational conversion of PrP^{C} occurs spontaneously in sporadic CJD (sCJD), due to pathogenic mutations of the *PRNP* gene in genetic CJD, Gerstmann-Sträussler-Scheinker syndrome, and fatal familial insomnia, or due to infection with exogenous PrP^{Sc} in acquired CJD, variant CJD, and kuru.

Acquired CJD is caused by transmission of PrP^{Sc} from individuals undiagnosed as CJD *via* cadaveric dura matter grafts, pituitary hormone, corneal grafts, and contaminated neurosurgical instruments [2]. More than 60% of dura matter graft-associated CJD (dCJD) cases have been reported in Japan. Although most of the Japanese dCJD patients show the same *PRNP* genotype, methionine homozygosity at polymorphic codon 129 (129M/M), they can be divided into two subgroups based on the distinctive clinicopathological features [3,4]. One subgroup designated as non-plaque-type dCJD (np-dCJD) accounts for approximately 70% of total dCJD cases, while the other subgroup designated as plaque-type dCJD (p-dCJD) accounts for approximately 30% of total cases [4]. In sCJD patients, the clinicopathological features are determined by the genotype (methionine (M) or valine (V)) at codon 129 of the *PRNP* gene and the type (1 or 2) of PrP^{Sc} accumulating in the brain [5–8]. The clinicopathological features of np-dCJD patients are identical to those of sCJD-MM/MV1 patients. In contrast, p-dCJD patients show unique phenotypic features such as slow progression of disease, absence or late occurrence of periodic sharp wave complex in electroencephalogram, PrP^{Sc} amyloid plaques (kuru plaques), and accumulation of unusual type of PrP^{Sc} which is intermediate in size (20 kDa) between types 1 and 2, and thus named as intermediate type (type i) [9–11]. These clinicopathological features of p-dCJD patients

Abbreviations: PrP, prion protein; PrP^{C}, normal cellular isoform of PrP; PrP^{Sc}, abnormal misfolded isoform of PrP; CJD, Creutzfeldt Jakob disease; sCJD, sporadic CJD; dCJD, dura mater graft-associated CJD; p-dCJD, plaque-type dCJD; np-dCJD, non-plaque-type dCJD; 129M/M, methionine homozygosity at codon 129 of the PrP gene; 129V/V, valine homozygosity at codon 129 of the PrP gene; DPI, days post-inoculation; MMiK, the combination of the 129M/M genotype, intermediate type PrP^{Sc} and kuru plaques

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resemble those of sCJD-VV2 or −MV2 patients except for the type of PrP^{Sc} [12]. We reported previously that the two subgroups of dCJD are distinguishable also by their transmission properties [10,13,14]. After intracerebral challenge with brain materials from np-dCJD patients, transgenic mice and knock-in mice expressing human PrP with the 129M/M genotype showed short incubation periods, diffuse synaptic-type PrP^{Sc} deposition, and type 1 PrP^{Sc} accumulation in the brain. These transmission properties were identical to those of the sCJD-MM/MV1 subtype. Meanwhile, after intracerebral challenge with brain materials from p-dCJD patients, transgenic mice and knock-in mice expressing human PrP with the 129V/V genotype showed shorter incubation periods than the PrP-humanized mice with the 129M/M genotype despite the mismatched codon 129 genotype and produced type 2 PrP^{Sc} in the brain. These transmission properties were similar to those of the sCJD-VV2 or −MV2 subtypes. The similarities in clinicopathological features and transmission properties suggested that the causative agents of np-dCJD and p-dCJD were PrP^{Sc} from sCJD-MM/MV1 patients and PrP^{Sc} from sCJD-VV2 or –MV2 patients, respectively [11].

The distinctive pathological and biochemical features of p-dCJD help us to identify acquired CJD cases caused by transmission of sCJD-VV2 or −MV2, designated as acquired CJD-MMiK (the 129M/M genotype, type i PrP^{Sc}, and kuru plaques). In fact, we have identified two acquired CJD cases using these pathological and biochemical criteria [15]. However, to verify that the identified CJD cases certainly represent acquired CJD and not a very rare variation of sCJD, it is important to confirm the unique transmission properties of acquired CJD-MMiK, *i.e.*, the high susceptibility of the 129V/V mice and the shift of PrP^{Sc} type from type i to type 2 in the inoculated 129V/V mice. Assessment of the transmission properties after intracerebral inoculation is reliable and still the gold standard. However, intracerebral transmission requires a very long incubation period of over 600 days and thus hampers rapid identification of acquired CJD cases. To overcome this problem, the present study aimed to develop a quick bioassay system for the evaluation of the transmission properties of acquired CJD-MMiK. After peripheral exposure, PrP^{Sc} replicates first on follicular dendritic cells (FDCs) in the lymphoid tissues before spreading to the brain [16]. We reported previously that knock-in mice expressing human/mouse chimeric PrP showed PrP^{Sc} accumulation in FDCs of the spleen by 75 days post-inoculation (DPI) after intraperitoneal challenge with brain materials from CJD patients [17]. In addition, transgenic mice carrying bovine PrP also showed PrP^{Sc} accumulation in FDCs of the spleen at 75 DPI after intraperitoneal challenge with bovine spongiform encephalopathy materials [18]. Assuming that this bioassay system, named as a FDC assay, enables us to rapidly and easily identify acquired CJD-MMiK, we have performed FDC assays of various CJD isolates.

2. Materials and methods

2.1. Transmission experiments

Production of knock-in mice expressing human PrP with the 129M/M or 129V/V genotype (Ki-Hu129M/M or Ki-Hu129V/V) has been reported previously [19]. The human brain tissues were obtained at autopsy after receiving written informed consent for research use. The diagnosis and classification of the CJD patients were performed by neuropathological examination, PrP^{Sc} immunohistochemistry, western blot analysis of PrP^{Sc}, and sequence analysis of the *PRNP* gene as described [20,21]. According to Parchi's classification [2,5], the sCJD cases included in the present study were classified as follows: MM1, one case; MM2 cortical (MM2C), one case; MM2 thalamic (MM2T), one case; VV2, one case. In addition, brain tissues from one np-dCJD case and one p-dCJD case were also included. These patients showed typical phenotypes of each CJD subgroup in the clinicopathological and biochemical examinations. Brain homogenates (10%) were prepared in sterile phosphate-buffered saline using glass homogenizers, and 50 μl of the homogenates were intraperitoneally inoculated into groups of 6 or 7 mice of each line (Ki-Hu129M/M and Ki-Hu129V/V). The inoculated mice were sacrificed at 3, 7, 14, 28, or 75 DPI. Half of the spleen was fixed in 10% buffered formalin for immunohistochemistry, and the other half was immediately frozen for western blotting.

2.2. Immunohistochemistry

Formalin-fixed mouse spleen tissues were treated with 60% formic acid (Wako Pure Chemical Industries) for 1 h at room temperature to inactivate the infectivity and were embedded in paraffin. The embedded tissues were sectioned at a thickness of 5 μm. After deparaffinization with xylene and rehydration with a graded ethanol series, the tissue sections were treated with methanol containing 0.3% hydrogen peroxide for 15 min at room temperature to inhibit endogenous peroxidase activity. Antigens were retrieved by autoclaving in 1.3 mM hydrochloric acid solution for 10 min at 121 °C [20]. Then, the tissue sections were incubated with anti-PrP polyclonal antibody PrP-N [22] overnight at 4 °C. After washing with phosphate-buffered saline, the tissue sections were incubated with goat anti-rabbit immunoglobulin polyclonal antibody labeled with a peroxidase-conjugated dextran polymer, EnVsion+ (Dako) for 1 h at room temperature. Then, the tissue sections were washed with phosphate-buffered saline, and the reactions were visualized with 3-3′-diaminobenzidine substrates (Dojindo Molecular Technologies). Subsequently, the tissue sections were counterstained with hematoxylin.

2.3. Western blotting

Spleen tissues were homogenized in 2 ml of lysis buffer (100 mM Tris-HCl pH 8.0, 10 mM NaCl, 10 mM $MgCl_2$, 2% Triton X-100, and 25 units/ml DNase I (Takara Bio)) and digested with collagenase (1 mg/200 mg tissue) (Wako Pure Chemical Industries) overnight at room temperature. Collagenase digestion disrupts the connective tissue and improves the accessibility of detergents and/or proteinase K (PK) to PrP^{Sc} [23]. The digested homogenates were ultracentrifuged at 453,000*g* for 30 min at 4 °C, and the pellets were resuspended and sonicated in 870 μl of PK-digestion buffer (100 mM Tris-HCl pH 8.0 and 5% Sarkosyl (Sigma-Aldrich)). The resuspended samples were centrifuged at 10,000*g* for 3 min to remove the cell debris, and the supernatants (800 μl) were digested with PK (4 μg/200 mg tissue) (Wako Pure Chemical Industries) for 1 h at 37 °C. It has been reported that these conditions for PK-digestion were sufficient for the complete digestion of normal PrP^{C}, and that higher PK concentrations caused unfavorable degradation of PrP^{Sc} [24]. The PK-digested proteins were precipitated by adding 200 μl of 99.5% ethanol and ultracentrifugation at 135,000*g* for 30 min at 4 °C. The pellets were resuspended in 400 μl/200 mg tissue of Laemmli's sample buffer (60 mM Tris-HCl pH 6.8, 5% glycerol, 2% SDS, and 0.01% bromophenol blue). Protein samples were subjected to SDS-PAGE using 13.5% Bis-tris long gels of 15 cm length and western blotting [19]. The anti-PrP monoclonal antibody 3F4 (Signet) was used as the primary antibody, and anti-mouse EnVision+ (Dako) was used as the secondary antibody. The blots were visualized with enhanced chemiluminescence western blotting detection reagent, ECL select (GE Healthcare), and images were obtained by imaging device ImageQuant LAS 4000 mini (GE Healthcare).

2.4. Ethical statement

This study was approved by the Institutional Ethics Committee of Hokkaido University Graduate School of Veterinary Medicine. All experiments using human materials were in compliance with the Helsinki Declaration. Animal experiments were performed in strict accordance with the Regulations for Animal Experiments and Related Activities at Hokkaido University. The protocols for animal experiments were approved by the Institutional Animal Care and Use Committees of