Compared to the regular PK-resistant core of PrP^{Sc} that is produced by PK digestion without GdnHCl treatment, the infectivity of the N-terminally truncated PK-resistant PrP^{Sc} was extremely low despite the C-terminal region existed as PK-resistant fragments (Table 2). Since we have not had a dose-incubation standard curve for the Chandler strain in Jcl:ICR mice, we cannot estimate the exact reduction rate. However, the attack rate and the survival time suggested that the infectivity decreased to nearly the detection limit in the bioassay. This provides direct evidence that the aa 81-137 of PK-resistant PrP^{Sc} is critical for prion infectivity, although evidence for other prion strains remains to be elucidated. However, PK-treatment alone reduced the infectivity of the Chandler strain (159 and 170 days without and with PK-treatment, respectively, in Table 2), indicating that there is the PK-sensitive PrP^{Sc} fraction possessing prion infectivity in the brain homogenates of the Chandler strain-infected mice. Our results clearly showed that the aa 81-137 of the PK-resistant core of the Chandler PrP^{Sc} is important for the infectivity, however, it remains unclear whether the same is applicable to the infectivity of the PK-sensitive PrP^{Sc} fraction.

Compared to the removal of this region, the denaturation of this region by 3 M GdnHCl treatment appeared less effective in reducing prion infectivity. However, considering the effect of GdnHCl on PrP^{Sc} aggregates, the denaturation itself appears to result in a substantial loss of infectivity (Table 2). The GdnHCl treatment has two expected effects; dissociation of large PrP^{Sc} aggregates into small aggregates and denaturation of the PrP^{Sc} molecules. Hence, without PK digestion, small aggregates consisting of PrP^{Sc} with incompeletely denatured aa 81-137 may remain and infectivity may be observed. Such small PrP^{Sc} aggregates should be PK-sensitive and therefore the infectivity should be diminished after PK digestion (31). Alternatively, this region may have been somewhat refolded after the GdnHCl treatment, which would lead to infectivity.

Several distinct domains of PrP^{C} are reported to be involved in the direct interaction to

PrP^{Sc} (21, 33), whereas binding domains on PrP^{Sc} that are involved in binding to PrP^{C} still remain undetermined. The N-terminally truncated PrP^{Sc} may be useful for the analysis of the binding domain on the PrP^{Sc} molecule to PrP^{C}. Here, we showed an example of the possible biochemical approach of PrP^{Sc} manipulation, in which we directly produced the N-terminally truncated PrP^{Sc} from native PrP^{Sc}. It has been reported that some conditions (*e.g.*, pH) of protease digestion affect the N-terminal truncation of the PK-resistant core of PrP^{Sc} (24). Thus, further investigation of region-specific denaturation and proteolysis may be useful not only for the analysis of prion strains but also for the manipulation of PrP^{Sc}.

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FIGURE LEGENDS

Figure 1. Conformational stability of PrP^{Sc} of various prion strains. (A) Immunoblots for conformational-stability assay. Brain homogenates from prion-infected mice (indicated on the left) were treated with 0 - 4 M GdnHCl (indicated on the top) followed by PK digestion. PrP^{Sc} was detected by either pAb B103 (left colums) or mAb 44B1 (right column). Independent assays of each strain were carried out at least 3 times for mAb 44B1 (indicated in parentheses), and based on quantitative results of the blot probed with mAb 44B1, the denaturation curves were plotted using a non-linear least-squares fit. Half-maximum GdnHCl concentrations, $[GdnHCl]_{1/2}$, are indicated for each graph (mean ± SD). Numbers at the top-right corner in the blots probed with pAb B103 represent the $[GdnHCl]_{1/2}$ values (M). (B) Molecular weights of PrP^{Sc}. Brain homogenates from prion-infected mice (indicated on the top) were treated with PK and immunoblot was probed with pAb B103 (left). To compare the molecular weight of PK-resistant core of PrP^{Sc} more precisely, PK-treated samples were further treated with PNGasae F (right).

Figure 2. Region-dependent conformational stability of PrP^{Sc} of the Chandler strain. Brain homogenates from mice infected with the Chandler (left) and the Obihiro (right) strains were subjected to the conformational-stability assay and immunoblots were probed with various anti-PrP antibodies indicated on the left. Epitopes for antibodies are indicated in parentheses. Due to the relatively weak reactivity, 5-times sample volumes were loaded for mAb 118. Numbers at the top-right corner in each blot represent the $[GdnHCl]_{1/2}$ values (M).

Figure 3. Region-dependent conformational stability of PrP^{Sc} in cells persistsently

infected with the Chandler strain. PrP^{Sc}-enriched fractions obtained from ScN2a-5 cells were subjected to the conformational stability assay. Antibodies used are indicated on the left.

Figure 4. Region-dependent conformational stability of the Chandler PrP^{Sc} in mice with different genetic backgrounds. Brain homogenates from Jcl:ICR (*Prnp*$^{a/a}$), C57BL/6J (*Prnp*$^{a/a}$), and I/LnJ (*Prnp*$^{b/b}$) mice infected with the Chnadler strain were subjected to the conformational-stability assay. Antibodies used and their epitopes (in parentheses) are indicated. Numbers at the top-right corner in each blot represent the $[GdnHCl]_{1/2}$ values (M).

Figure 5. Region-specific denaturation or removal of PrP^{Sc} in inoculums for bioassay. (A) Confirmation of region-specific denaturation. Brains of mice infected with the Chandler strain were treated with various concentrations of GdnHCl (without PK treatment) and the fraction containing PrP^{Sc} was recovered by ultracentrigation. Small aliquots of the inoculums were treated with PK and analyzed by immunoblotting with mAb 44B1. (B) Confirmation of removal of the aa 81-137. Brain homogenates from mice infected with the Chandler and the Obihiro strains were treated with 0 or 3 M GdnHCl and followed by PK digestion. After terminating proteolysis, samples were ultracentrifuged to collect the fraction containing PrP^{Sc}. Small aliquots of the inoculums were analyzed by immunobloting with mAb 44B1. Equal brain tissues equivalent was loaded in each lane.

Figure 6. Schematic representation of region-specific denaturation of the Chandler PrP^{Sc}. PK-resistant core of the Chandler PrP^{Sc} (from ~aa 81 to 231) were depicted with the

592 locations of two β-strands (β1 and β2), three α-helices (α1 to α3), two N-glycosylation sites
593 (CHO), and an intramolecular disulfide bond (S-S). The locations of epitopes were
594 indicated with thick lines with aa numbers (in parentheses). The epitope for mAb 44B1 that
595 recognizes discontinous epitope was indicated with dashed line, while those for other
596 antibodies that recognize linear epitope were indicated with solid lines. The region I
597 indicated above (aa 81- 90) was denatured almost completely by up to 2 M GdnHCl treatment,
598 and the removal of this region generates the 1-2 kDa smaller PK-resistant PrP^{Sc}. The region
599 II (aa 90- 137) was denatured almost completely by up to 3 M GdnHCl treatment, and the
600 removal of the regions I and II consequently generates the 6-7 kDa smaller PK-resistant PrP^{Sc}
601 (region III, aa 137-C-terminus) that is highly resistant to denaturation but lacks prion
602 infectivity.

603 Table 1. Conformational stabilities and incubation periods of prion strains

Prions	Mouse strain for propagation	Number of serial passage[a]	$[GdnHCl]_{1/2}$ (M) pAb B103	$[GdnHCl]_{1/2}$ (M) mAbs 44B1[f] /31C6[g]	Incubation periods (days, mean ± SD)	Number of mice[h]
G1	slc:ICR	4[b]	2.1	2.1 ± 0.1[f]	326 ± 53	5
Obihioro	Jcl:ICR	>5	2.3	2.0 ± 0.0[f]	153 ± 7	24
Chandler	Jcl:ICR	>5	1.8	3.2 ± 0.2*[f]	150 ± 8	20
	I/LnJ	2[c]	2.2	>3.5[g]	227 ± 7	4
	C57BL/6J	3[c]	2.3	3.5[g]	153 ± 6	6
22L	Jcl:ICR	2[d]	1.5	1.7 ± 0.0**[f]	144 ± 3	5
Fukuoka-1	Jcl:ICR	2[d]	2.1	2.0 ± 0.0[f]	146 ± 8	8
KUS-m	RIII/J	3[e]	2.4	2.5 ± 0.2*[f]	165 ± 11	6
TE-m	C57BL/6J	3[e]	2.2	2.6 ± 0.2*[f]	168 ± 4	6

604 [a] History (number) of serial passage in mice listed on the left.

605 [b] Source of prion: experimental sheep scrapie G1.

606 [c] Chandler strain passaged in Jcl:ICR mice were then passaged in I/LnJ or C57BL/6J mice.

607 [d] Source of prions: the 22L and Fukuoka-1 strains passaged in mice carrying $Prnp^{a/a}$ but

608 different from Jcl:ICR mice.

609 [e] Source of prions: BSE field cases KUS and TE.

610 [f] The $[GdnHCl]_{1/2}$ values were estimated from the denaturation curves plotted by blots probed

611 with mAb 44B1 (at least three independent asssays). *, higher than G1 ($p < 0.05$); **, lower

612 than G1 ($p < 0.05$).

613 [g] The $[GdnHCl]_{1/2}$ values were estimated from the denaturation curves plotted by blots probed

614 with mAb 31C6.

615 [h] Number of mice used for the calculation of incubation period.

Table 2. Effect of GdnHCl treatment and PK digestion on prion infectivity

Strain	GdnHCl (M)	PK	Mice[a] (n/N)	Survival time (Mean ± SD) (dpi)
Chandler	0	–	4/4	159 ± 14
	1	–	5/5	150 ± 9
	1.5	–	7/7	165 ± 12
	2	–	4/4	176 ± 12
	3	–	5/5	207 ± 25
	0	+	6/6	170 ± 11
	3	+	2/5[b]	234, 236, >365
Obihiro	0	+	5/5	152 ± 7
	3	+	5/5	186 ± 11

[a] n, number of mice which showed typical clinical manifestations of scrapie and/or were positive for PrP^{Sc} in immunoblotting; N, number of mice used in bioassay.

[b] Two mice showed typical clinical manifestations and were positive for PrP^{Sc} (at 234 and 236 dpi), one mouse was found died without any symptoms at 336 dpi and was negative for PrP^{Sc}. Remaining two mice were still alive without any symptoms (>365 days).

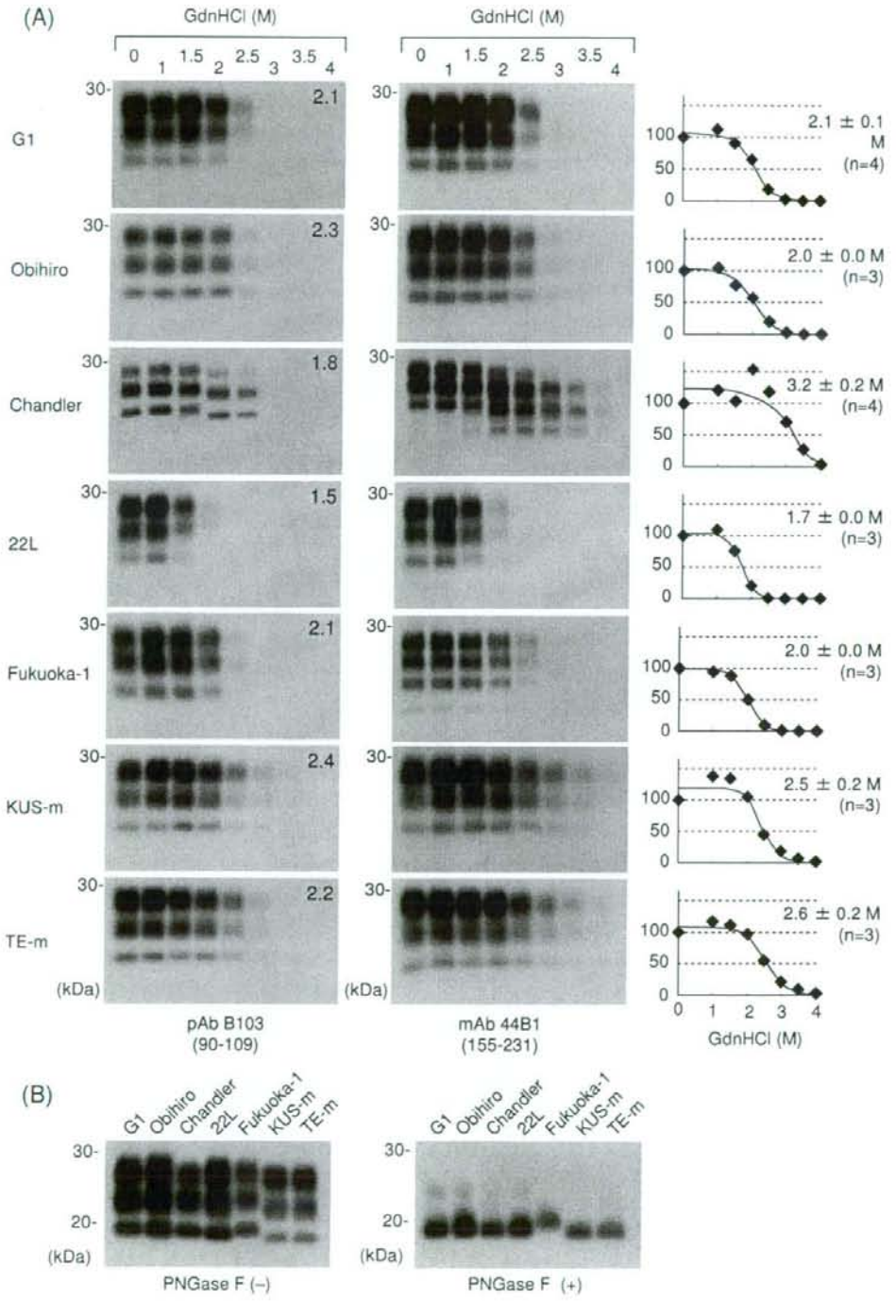
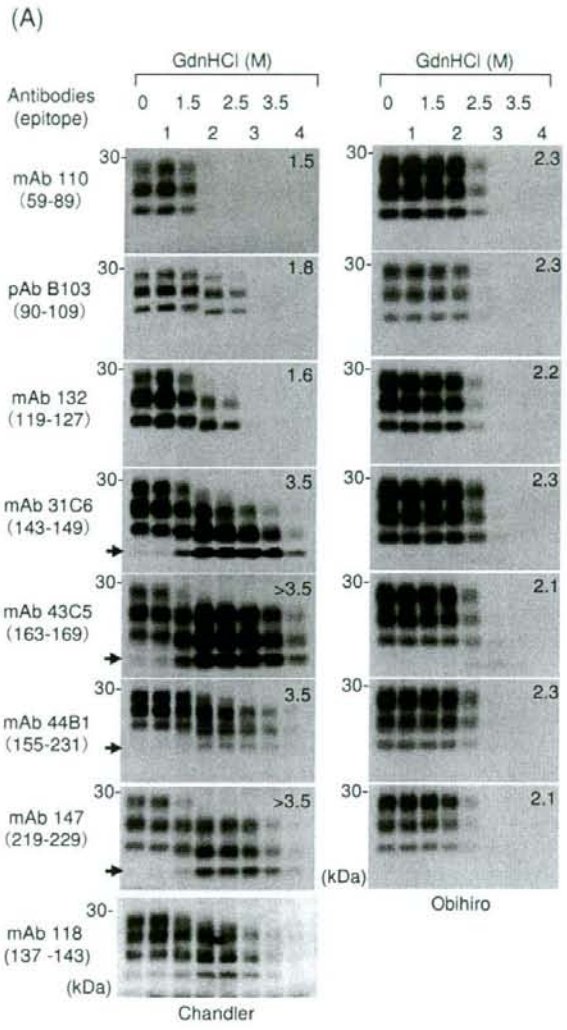


Fig. 1 Shindoh et al.,



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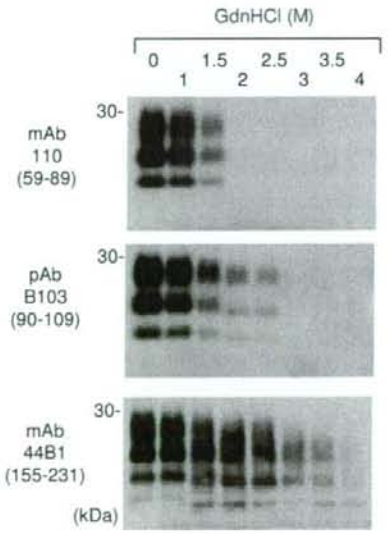

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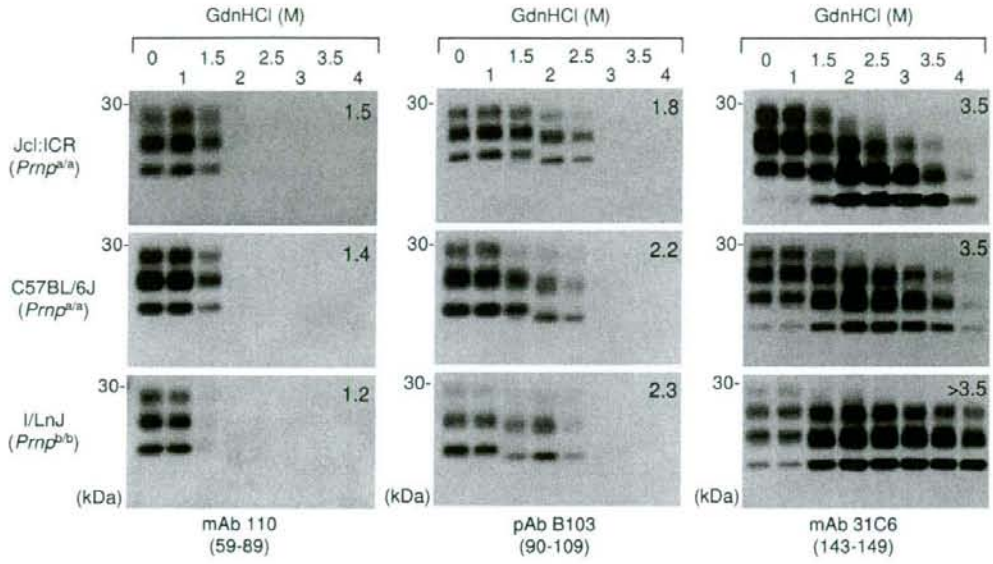

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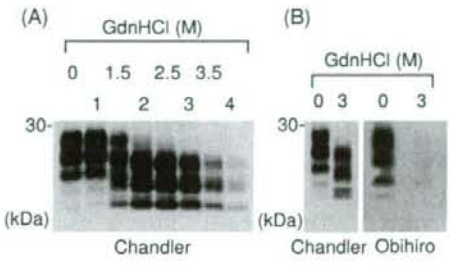

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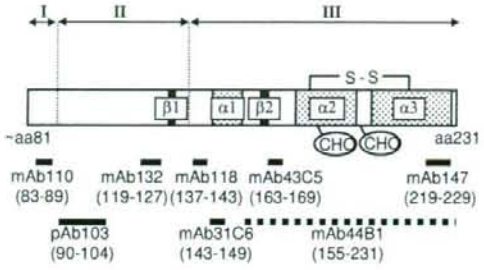

Fig. 6 Shindoh et al.,

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Bone marrow stroma cells are susceptible to prion infection

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ABSTRACT

Abnormal protease-resistant prion protein (PrP-res) is the only surrogate biochemical marker for prion diseases, and a sensitive technique to detect PrP-res in blood or tissues is urgently needed. Primary cultured bone marrow stromal cells (MSCs) expressed PrP and were capable of supporting stable human prion infection. Using a mouse-adapted BSE strain, we demonstrated that PrP-res can be detected in expanded MSCs. We then analyzed the bone marrow cells collected at autopsy from two individuals with sporadic Creutzfeldt-Jakob disease (CJD), and, in both cases, cultured MSCs were positive for PrP-res. These data would suggest that ex vivo MSC expansion accompanied by PrP-res analysis could be a helpful tool in the definitive diagnosis of prion disease at an earlier stage in the disease process than is currently possible, and with considerably less distress to the patient.

Creutzfeldt-Jakob disease (CJD) in man is thought to be caused by an "infectious protein particle", termed prion [1]. Accumulation of the disease-associated form of prion protein (PrP^{Sc}) and infectivity are seen mainly in the central nervous system (CNS), but are not limited to the CNS. Infectivity in the blood of sporadic CJD patients and experimentally infected animals has been reported [2], and recently it has been shown that variant CJD (vCJD) can be transmitted by transfusion [3,4]. The problem, however, is that detection of PrP^{Sc} in blood is extremely difficult and no reliable test exists [5]. For this reason, we instead focused on bone marrow stroma cells (MSCs) [6,7], which possess multipotential stem cell-like characteristics, and investigated whether or not they were susceptible to TSE agents. Ex vivo cultured MSCs expressed PrP^{C} and were susceptible to a CJD agent. In addition, we were able to detect PrP^{Sc} in MSCs isolated from both infected animals and sporadic CJD patients. These results suggest that bone marrow biopsy followed by ex vivo expansion of MSCs could form the basis of a new diagnostic test for TSEs.

Materials and methods

Isolation and culture of MSCs. Adult male Wistar rats, 8 weeks old, were killed and the femurs and tibias were dissected out. Isolation of the bone marrow was performed according to the method described by Azizi et al. [7]. The ends of the bones were cut and the marrow was extruded with 5 ml of alpha-MEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml kanamycin, or 100 U/ml penicillin and 100 μg/ml streptomycin. The bone marrow cells were incubated at 37 °C with 95% humidity and 5% CO_2 for 48 h, and the non-adherent cells removed by replacing the medium. Adherent cells were subcultured several times, and used for the transmission studies. The cells were subjected to a neuronal differentiation study to confirm that they were stromal cells (MSC), according to Dezawa's method [8,9]. Additionally, bone marrow stromal cells isolated from mice, hamsters and cows were cultured in the same way as for rat MSCs. Normal human MSCs were purchased from Cambrex Bio Science Walkersville, Inc. In the case of CJD patients, informed consent was obtained from the patient's family, and the investigation protocol was approved by the Ethics Committee of Nagasaki University Hospital (ID 06012755).

Animal TSE models. To establish a rat TSE model, 10% (w/w) homogenates of a brain taken from a Gerstmann-Sträussler-Scheinker syndrome (GSS) patient carrying the P102L mutation in PRNP [10] were prepared with sterile PBS. We first inoculated the homogenate into Wistar rats (3 weeks old) and NZW mice (4 weeks old). Although all the rats ($n = 6$) remained free of any neurological signs and were healthy until 2 years post inoculation, the NZW mice developed typical mouse TSE after around 230 days. The mouse brain homogenate was then inoculated into Wistar rats, and those rats developed disease at about 330 days. Accumulation of PrP^{Sc} in the affected brains was confirmed by Western blotting (data not shown), and by histology, in which common characteristics of prion disease such as spongiform change, neuronal loss, and gliosis were observed, and accumulated PrP^{Sc} was stained diffusely

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