FIG. 3. Relative PK resistance of PrP^{Sc} generated in mouse brain. (a) PrP^{Sc}-containing fractions prepared from mouse brains were treated with various concentrations of PK (0 to 1,000 μg/ml, indicated above the photo) at 37°C for 2 h. Molecular mass markers are indicated. PrP was detected by immunoblotting as in Fig. 2. (b) The same PrP^{Sc}-containing fractions as used in panel a were treated with PK (200 μg/ml) at 37°C for various times (0 to 16 h, indicated above the photo).

United Kingdom based on transmissibility to mice (8, 11), because some isolates were essentially nontransmissible whereas others were readily transmissible to PrP$^{A/A}$ mice with incubation periods of >350 days. However, to our knowledge, sheep scrapie which successfully transmits to mice with an average incubation period of ~230 days, like A1 and B3, has an extremely short incubation period in primary transmission.

TSE agents have been characterized by transmissibility to experimental animals and distribution of neuropathological lesions (5, 11). In addition, biochemical characterization of PrP^{Sc} seems to be useful for discriminating between TSE strains. For example, two hamster-adapted transmissible mink encephalopathy strains, "Hyper" and "Drowsy," can be distinguished from each other not only by incubation period and neuropathology but also by the relative PK resistance and molecular mass of PrP^{Sc} (2, 3). Here we found that relative PK resistance of PrP^{Sc} is likely to be useful for the distinction of sheep scrapie strains to some extent, as the samples we analyzed could be divided into two groups based on relative PK resistance. Recently, the ratio of glycosylated PrP^{Sc} bands was used to distinguish vCJD from other types of CJD (10), and sheep scrapie can be divided into several groups by using this glycoform typing of PrP^{Sc} (15). In general, strain typing approaches using a combination of different properties provide a

more reliable means of strain differentiation. Analyses of biochemical properties of PrP^{Sc} are less time-consuming than bioassays in mice, and therefore, it is advantageous to use a combination of several biochemical properties such as relative PK resistance, molecular mass, and glycoform typing of PrP^{Sc}, to achieve more definitive strain typing.

A recent study using 10 sheep with scrapie in the United States showed a perfect correlation between the detection of PrP^{Sc} in sheep brain and transmissibility to mice (26). In contrast, it is well-known that some sheep scrapie strains are difficult to transmit to mice (4, 8, 11). In this study, we showed that scrapie strains classified into groups 1 and 2 differed in their transmissibility to mice but were indistinguishable by the relative PK resistance of PrP^{Sc} (Table 3). The discrepancy between the presence of PrP^{Sc} and the transmissibility to mice may imply that factors other than PrP^{Sc} are involved in determining the infectivity or that PrP^{Sc} is not the molecule responsible for infectivity (21). Alternatively, strain-specific conformations of PrP^{Sc} have been shown (9, 28), and so it is also conceivable that subtle differences in biochemical and/or biophysical properties of PrP^{Sc} which cannot be detected by PK digestion may influence the transmissibility to mice. It is also possible that the sheep PrP genotype may explain the discrepancy. It has not yet been clarified whether amino acid sequences of sheep PrP^{Sc} influence transmission to mice. Here we showed that scrapie occurring in sheep homozygous for PrP^{MARQ} transmitted to mice (e.g., SB, Y2, Y5, A1, and G1). This indicates that sheep PrP^{Sc} composed of the product of the PrP^{MARQ} allele could initiate the accumulation of PrP^{Sc} in mice by using mouse PrP^{C} as a substrate. However, there are differences in the incubation periods for A1 between these sheep and other sheep possessing the PrP$^{MARQ/MARQ}$ genotype (Table 2), and more recently, it was reported that one scrapie case occurring in PrP$^{ARQ/ARQ}$ sheep (amino acid polymorphisms at codon 112 was unavailable) was virtually nontransmissible to mice (8), suggesting the amino acid sequence of sheep PrP^{Sc} is not the sole determinant of the transmissibility to mice.

Among the sheep with scrapie used here, S1, S2, and S3 differed in clinical course from other sheep by death at a young age and hypersensitivity. The simple explanation for this is that the strain(s) infecting these sheep differs from those of the other scrapie cases, and indeed, these agents obviously differed from the others in transmissibility to mice. Scrapie strains adapted to rodents exhibit strain-specific clinical symptoms, neuropathological lesions, and incubation periods (3, 5, 6). However, it is unclear whether a given sheep scrapie strain determines strain-specific clinical and/or clinico-pathological features. As described in Materials and Methods, these sheep have an immediate blood relationship and were kept on the same ranch, and so other factors such as genetic background, breeds, route of infection, and environment may be involved in defining the type of disease in these particular cases.

A particular type of scrapie agent prevalent in the United Kingdom is believed to have initially caused BSE, and later on, this agent passed to felids and human beings through cattle (4, 7, 13). The diversity of field scrapie isolates in Japan is similar to that reported in the United Kingdom at least in terms of primary transmission to mice (11). Therefore, at present, we cannot exclude the possibility of the presence of a scrapie

strain in Japan that possesses properties similar to the BSE agent. Strain typing using two mouse strains, RIII and C57BL, carrying the PrP$^{A/A}$ allotype appears to be one of the methods to distinguish BSE and BSE-related TSE agents from sheep scrapie (4, 8). Further analysis will be required to investigate whether sheep scrapie strains in Japan have the potential risk to create new epidemics like the BSE agent.

ACKNOWLEDGMENTS

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Quantitative Analysis of Prion Protein by Immunoblotting

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Transmissible spongiform encephalopathy (TSE) is a neurodegenerative disease characterized by spongiform degeneration and accumulation of an infectious isoform (PrP^{Sc}) of the prion protein in the central nervous system. PrP^{Sc} originates from a ubiquitous cellular prion protein (PrP^{C}). We attempted to develop an easy method of quantitative analysis of PrP by immunoblotting based on densitometry data for PrP bands in immunoblots. Both PrP^{C} and PrP^{Sc} yield three bands in immunoblots, and they correspond to PrP molecules carrying two, one, and no Asn-linked sugar chains. We used bovine PrP^{C} as a model protein in the immunoblotting study. We removed the Asn-linked sugar chains from the PrP molecules with N-glycanase to convert all three glycoforms of PrP into a single band of the deglycosylated form and determined the PrP by densitometry calibrated with recombinant bovine PrP.

Key words —— prion protein, immunoblotting, quantification, bovine, PrP

INTRODUCTION

Transmissible spongiform encephalopathy (TSE) is a neurodegenerative disease characterized by spongiform degeneration and accumulation of an abnormal prion protein (PrP^{Sc}) in the central nervous

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system.[1] PrP^{Sc} is an infectious isoform of the prion protein (PrP) that originates from a normal isoform, ubiquitous cellular prion protein (PrP^{C}), and promotes conversion of PrP^{C} into PrP^{Sc}. Although the two isoforms have the same amino acid sequence, PrP^{Sc} differs significantly from PrP^{C} in the following respects. It has β-sheet-rich secondary structures, is resistant to proteinase K digestion, and forms aggregates that are not solubilized with nonionic detergents.

Detection of PrP^{Sc} by immunodetection with anti-PrP antibodies by ELISA, Western blotting, and immunohistochemistry has been used to diagnose TSE,[2] but since no antibodies discriminate between abnormal and normal isoforms of PrP, with the minor exception of those[3] that react with PrP^{C} and not with PrP^{Sc}, detection of PrP^{Sc} requires removal of PrP^{C} by proteinase K digestion. Moreover, aggregates of PrP^{Sc} require denaturation and depolymerization of PrP^{Sc} to expose antigenic epitopes to antibodies on the outside. ELISA and immunoblotting following proteinase K digestion and denaturation steps have been applied to the diagnosis of bovine spongiform encephalopathy (BSE) in the EU[4] and Japan. Immunoblotting has the advantage of a lower rate of false-positive results over ELISA, because the remaining PrP^{C}, which interferes with the detection of protease-resistant PrP^{Sc} by ELISA, can be distinguished by its size from PrP^{Sc} when immunoblotted. However, it has the disadvantage of quantitative determination by immunoblotting being more labor-intensive than ELISA. Quantification of immunoblots usually employs endpoint dilutions where the target protein band disappears to quantitate the target protein in a solution.

In this study, we attempted to develop an easy method of quantitative analysis of PrP by immunoblotting based on densitometry data for PrP bands on chemiluminescence films. Immunoblotting of both PrP^{C} and PrP^{Sc} yield three bands, which correspond to PrP molecules carrying two, one, and no Asn-linked sugar chains. We used bovine PrP^{C} as a model protein in the immunoblotting study. N-glycanase was used to remove the Asn-linked sugar chains from PrP molecules and convert the three glycoforms of PrP into a single band of the deglycosylated form, and PrP was then determined by densitometry calibrated with recombinant bovine PrP (rBoPrP). This method succeeded in quantitating PrP^{C} in bovine brain homogenates.

MATERIALS AND METHODS

Anti-PrP Antibodies —— Two different anti-PrP monoclonal antibodies were used to detect PrP^C by immunoblotting: BSPX-54[5] and 6H4. BSPX-54 was prepared from the supernatant of a hybridoma culture by ammonium sulfate precipitation and Protein-A column chromatography. 6H4 was purchased from Prionics AG (Zürich, Switzerland).

Preparation of Bovine Brain Homogenates —— Brains from healthy Holstein cattle were used. Fragments of cerebral cortex were homogenized in a 6- to 9-fold volume of homogenizing buffer (0.32 M sucrose, 50 mM Tris–HCl (pH7.2), 1 mM EDTA, 1 mM PMSF), and after cleaning the homogenate by centrifugation at $1000 \times g$ for 10 min, the supernatant was used as the homogenate. The pellets were resuspended in suspension buffer (50 mM Tris–HCl (pH7.2), 1 mM EDTA, 1 mM PMSF) and centrifuged at $10000 \times g$ for 20 min, and after suspending the pellets in the suspension buffer, the suspension was used as the P2 fraction.

Preparation of Recombinant Bovine Prion Protein —— cDNA coding bovine PrP^C (BoPrP^C) was prepared from the cerebral cortex of Holstein cattle by the RT-PCR method as described by Yoshimoto et al.[6] The sense primer was: 5′-ACGGAATTCCA-TATGTCATCATGGTGAAAAGCCACATAGG-3′, and the antisense primer was: 5′-TTATCTCGAGG-CGGCCGCAGGAAGGTTGCCCCTATCCTACT-AT-3′. The cDNA was cloned in pBlueScript II SK (+) vector (Stratagene, CA, U.S.A.). The sequence was identical to that of pPCJY1 (Accession # D10613) reported by Yoshimoto et al.[6] The insert DNA for expression plasmids in *Escherichia coli* was prepared by PCR with the cloned cDNA as a template. The sense primer was: 5′-CTTGGACCAT-ATGTCCAAGAAGCGTCCGAAACCTGGAGG-A-3′, and the antisense primer was: 5′-TCCCA-AGCTTCTCATGCCCCTCGTTGGTAATAAGCC-TGG-3′. The insert DNA, which codes MetSer-BoPrP (25-241), molecular weight 23.7 kDa, was cloned between the *Nde*I and *Hind*III sites in pET23b vector (Novagen, WI, U.S.A.). The resulting plasmid was transfected into *E. coli* BL21(DE3)pLysS (Novagen), and rBoPrP was produced in *E. coli* in the presence of 1 mM IPTG. rBoPrP was solubilized from inclusion bodies with 8 M urea in 50 mM Tris–HCl (pH8.0) containing 1 mM PMSF. After dilution of the urea concentration to 6 M and addition of 0.05% sodium azide, the solution was allowed to stand in a refrigerator for 1 month to permit disul-

fide bonds to form in PrP molecules by slow air oxidation of cysteine residues. The oxidized protein was purified by Cu^{2+}-chelating column chromatography (HiTrap Chelating column, 5 ml, Amersham Pharmacia-Biotech, Buckinghamshire, UK). After charging the column with copper ion (Cu^{2+}), it was equilibrated with the elution buffer (50 mM Tris–HCl (pH8.0), 6 M urea, 0.02% sodium azide). The sample was then loaded onto the column and eluted with 0, 25, 50, 100 and 300 mM imidazole and 50 mM EDTA in the elution buffer. rBoPrP was collected mainly in the 100 mM imidazole fraction. The fraction was dialyzed in 3 M, 1 M, and 0 M urea in 50 mM Tris–HCl (pH8.0). After removing the insoluble proteins, the dialysate was concentrated to about one tenth to one twentieth. The protein concentration was determined with a BCA reagent kit (Pierce, IL, U.S.A.). The purity of the refolded soluble rBoPrP was confirmed by SDS-PAGE/silver staining, and the rBoPrP was used as the standard protein.

Immunoblot Analysis of PrP^C in Bovine Brain —— The brain homogenate and P2 fraction were treated with *N*-glycanase (PNGase F, peptide: *N*-glycosidase F; New England Biolabs, MA, U.S.A.) according to the manufactures instructions. The samples were subjected to SDS-PAGE and transferred to a nitrocellulose or PVDF membrane. PrP was detected with anti-PrP monoclonal antibody as the first antibody, HRP-labeled anti-mouse IgG antibody as the second antibody and ECL Plus (Amersham Pharmacia Biotech) as the substrate for chemiluminescence detection. The immunoreactive bands were visualized on film. The intensity of the bands was quantified by computer-assisted densitometry of film, and calibrated with rBoPrP for each membrane.

RESULTS AND DISCUSSION

Immunoblot Analysis of PrP^C in Bovine Brain with Anti-PrP Antibodies

PrP^C in the bovine brain P2 fraction was analyzed by immunoblotting with the two anti-PrP monoclonal antibodies, BSPX54 and 6H4. PrP^C yielded three bands, which corresponded to PrP molecules carrying two, one, and no Asn-linked sugar chains. The relative intensity of the three bands on the immunoblots varied with the antibody (Fig. 1), suggesting that the glycoforms of PrP^C affect the antibody's recognition to the epitopes on PrP
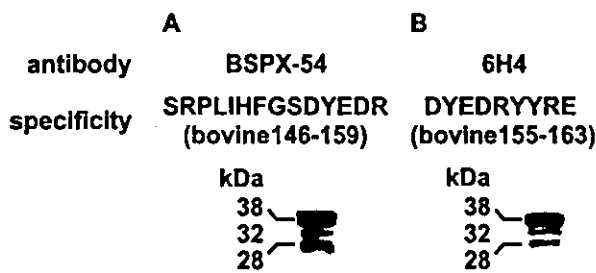
Fig. 1. Reactivity of Anti-PrP Monoclonal Antibodies with the Bovine Brain P2 Fraction.

The P2 fraction was subjected to SDS-PAGE and transferred to a PVDF membrane. PrP was detected with anti-PrP monoclonal antibody BSPX-54 (A) and 6H4 (B). Specificity for BSPX-54 means the antigen peptide sequence used to immunize mice. Specificity for 6H4 means the sequence recognized by the antibody.
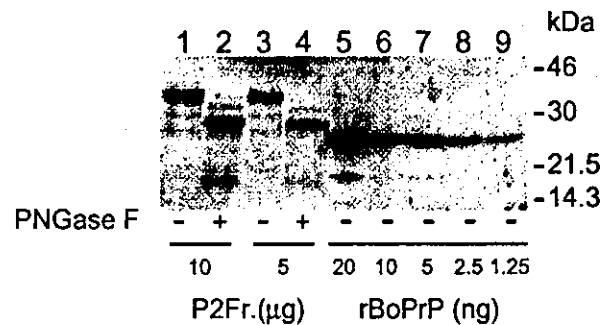

Fig. 2. Determination of PrP^c in the Bovine P2 Fraction by Immunoblotting.

The P2 fraction was treated with N-glycanase (PNGase F +), subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. The samples were analyzed by immunoblotting with anti-PrP monoclonal antibody BSPX-54. The intensity of the bands was quantified by calibration with rBoPrP. The values under the lanes are the amounts of rBoPrP and the amounts of total protein in the P2 fraction (P2Fr.) before N-glycanase treatment.

molecules. The glycoform patterns also vary with the infectious PrP strain[7] and the brain region,[8] making it necessary to use PrP carrying Asn-linked sugar chains as a calibration standard protein for quantitative determination of PrP by immunoassay. However, it is difficult to prepare large amounts of PrP with sugar chains from brains.

N-Glycanase converted the three bands of PrP^c into a single band of the deglycosylated form in the immunoblots (Fig. 2). PrP^c in brain has two or fewer N-glycans and one glycophosphatidylinositol (GPI) anchor, whereas rBoPrP has nor N-glycans or GPI anchor. Consequently, the molecular weight of the rBoPrP (apparent molecular weight 25 kDa, Fig. 2 lanes 5–9) was lower than that of the unglycosylated PrP^c (apparently 27 kDa, the third band from the top in lane 1). The deglycosylated PrP (lane 2) has a slightly higher molecular weight (apparently 29 kDa) than the unglycosylated PrP^c. The reason for this is unclear.

We compared the intensity of the PrP^c bands in the bovine brain P2 fraction before and after deglycosylation with N-glycanase. With the intensity of the band corresponding to deglycosylated PrP^c set equal to 100%, the intensity of the bands of the untreated diglycosylated, monoglycosylated, and unglycosylated PrP^c was $40.0 \pm 12.5\%$, $10.7 \pm 6.6\%$ and $17.1 \pm 15.2\%$ (mean $\pm$ S.E. derived from three independent experiments), respectively. The total intensity of the three bands was $67.9 \pm 13.7\%$. The fact that the sum of the intensities is less than 100% suggests inaccurate quantification of PrP without deglycosylation.

N-terminally truncated 18 kDa PrP^c is found in normal human and mouse brain after treatment with N-glycanase,[9,10] and the 18 kDa band was found in

the N-glycanase treated P2 fraction from bovine brain. Since the ratio of the intensity of the 18 kDa band to that of the deglycosylated PrP^c band (29 kDa) was only 3–7%, not measuring the 18 kDa band has little influence on the accuracy of quantitative determination of PrP^c.

To avoid the problems described above, we quantified deglycosylated PrP^c with N-glycanase by using recombinant PrP derived from $E.\ coli$ as a calibration standard protein.

Quantitative Analysis of PrP^c in Bovine Brain

The amounts of PrP^c in healthy bovine brain (cerebral cortex) homogenate and the P2 fraction were determined by immunoblotting with anti-PrP antibody BSPX-54 using the densitometry data for the bands. Deglycosylated PrP^c was quantified with N-glycanase. The immunoblot pattern of the P2 fraction is shown in Fig. 2. The band intensity of rBoPrP on the immunoblots depended on the amount of rBoPrP in the range between 1 to 20 ng/lane on the linear dose-response curve (data not shown). The amount of PrP^c in the P2 fraction was calculated according to the calibration curve of rBoPrP. The amount of PrP^c in the brain homogenate was also determined by similar procedures. The results are shown in Table 1. The P2 fraction, which is rich in synaptosomes, contained a higher amount of PrP^c than the homogenate, consistent with PrP^c being concentrated primarily in the synaptic fields of neurons.[11] The amount of PrP^c in bovine brain homogenate measured by immunoblotting (0.4 μg/mg of total protein) was similar to the amount measured

Table 1. PrP^C in Bovine Brain Homogenate and the P2 Fraction

Sample	PrP^C (μg/mg of total protein)$^{a)}$	
	Immunoblotting$^{b)}$	Competitive ELISA$^{c)}$
Homogenate	0.4 ± 0.1	0.3 ± 0.1
P2 fraction	0.9 ± 0.1	1.0 ± 0.1

a) The amount of PrP^C is expressed as rBoPrP equivalents. The values are mean ± S.E. ($n = 3$). b) PrP^C was determined by immunoblotting with anti-PrP monoclonal antibody BSPX54 as the first antibody. c) Unpublished data. PrP^C was determined by ELISA with the anti-PrP polyclonal antibody as the first antibody and rBoPrP as competitor.

by competitive ELISA (0.3 μg/mg of total protein) (unpublished data). Our results are consistent with a report[12] that the amount of PrP^C detected in healthy bovine brain (thalamus) homogenate by dot blotting was about 0.2 μg/mg of the total protein in the homogenate, although the amount of PrP^C varies with the anatomic site in the brain.

In this study, we attempted to develop an easy quantitative method for analysis of PrP by immunoblotting. Some ELISA and immunoblotting systems are used to detect PrP^{Sc} in bovine brain and medulla oblongata to prevent material from cattle with BSE from entering the market. No diagnostic antibodies distinguish PrP^{Sc} from PrP^C. Some antibodies recognize denatured PrP. In other words, they do not discriminate between PrP^C and PrP^{Sc} after denaturation. Other antibodies recognize similar 3D structures in epitopes on both PrP^C and PrP^{Sc}. We therefore think that the N-glycanase treatment of PrP^C used in this study can be applied to the detection of PrP^{Sc}.

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G$_1$-Dependent Prion Protein Expression in Human Glioblastoma Cell Line T98G

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Human glioblastoma cell line T98G produced a cellular form of prion protein (PrP^C), and we confirmed expression of PrP mRNA by RT-PCR. Immunoblot analysis of whole cell lysate revealed one major (35 kDa) and two faint bands (31, 25 kDa) that reacted with monoclonal anti-human PrP antibody 3F4. Cells treated with tunicamycin produced only a 25 kDa band, representing a deglycosylated form of PrP. Similarly, peptide: N-glycosidase F treatment of whole cell lysate altered the Asn-linked form to the deglycosylated form. When T98G cells were cultured for a longer period, the amount of PrP^C per cell increased on Day 4 to 16 in a time-dependent manner. When the cells were cultured at high cell-density, the cells on Day 4 produced the same amount of PrP^C as those on Day 16 of the usual culture. Moreover, in a serum-free medium, cells cultured at a low cell-density produced the same amount of PrP^C as those cultured at the high cell-density. These results demonstrate that PrP^C production in T98G cells was dependent on the phase of the cell cycle, probably the G$_1$ phase.

Key words   cellular prion protein; T98G cell; cell density; cell cycle

Prion diseases, including Creutzfeldt–Jakob disease, kuru, Gerstmann–Sträussler–Scheinker syndrome, and fatal familial insomnia known as transmissible spongiform encephalopathies are disorders of the central nervous system[1] and present as infectious, genetic, and sporadic disorders.[2] These fatal diseases are characterized by the formation and accumulation of an abnormal prion protein (PrP^{Sc} or PrP^{res}) in the brain. PrP^{Sc}, which is derived from a cellular prion protein (PrP^C or PrP^{sen}) that is easily solubilized and degraded by proteinase K, exists as an insoluble aggregate that is resistant to proteinase K digestion. There are some differences in the secondary structure between the two isoforms: PrP^C is rich in α-helical structure whereas PrP^{Sc} is rich in β-sheet structure. The conversion mechanism from PrP^C to PrP^{Sc}, however, remains unclear.

PrP mRNA is constitutively expressed in the central nervous system and in several peripheral tissues such as lymphoid organs[3—5] and muscle.[6] PrP^C encoded by *PRNP* is a glycoprotein anchored to the cell surface by a glycosylphosphatidylinositol (GPI) moiety.[7] In scrapie-infected neuroblastoma ScN2a cells, PrP^{Sc} is found primarily inside infected cells where it appears to accumulate, while PrP^C is a surface protein that is degraded with a half-life of several hours.[8,9] It has been proposed that PrP^{Sc} is converted from PrP^C on the cell surface or in the endocytic cellular compartments,[10] but the trafficking pathway and cellular localization of PrP^C have not been fully investigated.

PrP^C may play a role in neural differentiation,[11] lymphocyte proliferation,[12] or cell adhesion.[13] PrP^C does not cause neurodegeneration, but a synthetic PrP peptide (consisting of residues 106—126) is toxic to neurons that express PrP^C.[14—16] In culture, the neurotoxic PrP peptide enhances proliferation of microglia but not astrocytes.[17]

Transgenic mice expressing a high number of wild-type PrP transgenes eventually develop truncal ataxia, hind-limb paralysis, and tremors after a long time incubation.[18] Although mice defective for the PrP gene (PrP$^{-/-}$) display normal early development, aged mice show progressive ataxia due to deletion of Purkinje cells in the cerebellum.[19,20] Research indicates that PrP mRNA levels increase in response to neurotrophic factors,[21] cytokines,[22,23] and hormones.[18,24] Migration inhibitory factor-related protein (MRP8) fragment stimulates PrP^C expression, and platelet derived growth factor has an opposite effect, acting as a suppressor in human fibroblasts.[25] Another study showed that M17 human neuroblastoma cells produce PrP^C when long neurites are formed in response to addition of retinoic acid.[26] The regulation of PrP gene expression, however, remains elusive.

The currently available cell lines are relatively low in expression level of PrP^C to clarify its function. We examined PrP^C expression in several cell lines and found that human glioblastoma cell line T98G highly expresses endogenous PrP^C. We used the cell line to investigate the mode of PrP^C expression, which will contribute to the development of therapies of prion diseases.

MATERIALS AND METHODS

Materials   Primer sets for human PrP^C (5'GGCAGT-GACTATGAGGACCGTTAC3' and 5'GGCTTGACCAGC-ATCTCAGGTCTA3'; the expected product size 528 bp)[23] or for human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) (5'CCATGTTCGTCATGGGTGTGAACCA3' and 5'GCCAGTAGAGGCAGGGATGATGTTC3'; the expected product size 251 bp)[23] were chemically synthesized. Peptide homologous to the C-terminus of human PrP (residues 214—230, CITQYERESQAYYQRGS) was chemically synthesized and was >95% pure; analysis by HPLC revealed a single peak. The molecular weight was confirmed by liquid secondary-ion mass spectrometry and matrix-assisted laser desorption/ionization mass spectrometry. Lyophilized peptide was dissolved in 0.1% trifluoroacetic

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acid and stored at −80 °C under N_2 gas until used. Peptide: N-glycosidase F (PNGase F) was purchased from New England Biolabs (Beverly, MA, U.S.A.), RPMI 1640 from Nissui Pharmaceutical (Tokyo, Japan). BCA protein assay and SuperSignal West Femto Maximum Sensitivity Substrate were from Pierce (Rockford, IL, U.S.A.). Oligo-dT primer and Hybond-P PVDF membrane were purchased from Amersham PLC (Buckinghamshire, U.K.). Fetal calf serum (FCS) and [methyl-^{3}H]thymidine (740 GBq/mmol) were purchased from ICN Pharmaceuticals (Costa Mesa, CA, U.S.A.). Anti-human PrP monoclonal antibody 3F4 was purchased from Senetek PLC (Napa, CA, U.S.A.). Anti-human PrP monoclonal antibody 6H4 was purchased from Prionics AG (Zürich, Switzerland). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated rabbit anti-chicken IgG, bovine serum albumin (BSA), 3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS), and tunicamycin were purchased from Sigma (St. Louis, MO, U.S.A.). Affi-gel Protein A agarose was purchased from Bio-Rad (Hercules, CA, U.S.A.), complete Freund's adjuvant (FCA) was from Difco (Detroit, MI, U.S.A.), vanadyl ribonucleoside complex (VRC) and SuperScript II reverse transcriptase from Life technologies (Rockville, MD, U.S.A), KOD DNA polymerase from Toyobo (Osaka, Japan), DNase-I from Takara (Tokyo, Japan).

Preparation of Antibodies Preparation and purification of rabbit polyclonal antibodies against human PrP peptide residues 214—230 were carried out as previously described[27] with slight modification. Briefly, peptide (3.2 µmole) was reacted with MBS (3.2 µmole) and BSA (0.3 µmole), and then used as an immunogen. Three rabbits were immunized with 6 subcutaneous injections of 0.5 mg of the immunogen with FCA at 21- to 28-day intervals. The antibody activity (more than 95%) of the antisera was recovered in the IgG fraction when separated on a Protein A column, and finally one polyclonal antibody specific for human PrP, HPC2, was obtained. Preparation of chicken monoclonal antibody HUC2-13 (IgG) against human PrP peptide residues 25—49 was as reported previously.[28] The hybridoma was cultured to obtain the supernatants containing HUC2-13.

Cell Culture The human glioblastoma cell line T98G (JCRB9041) was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). T98G cells were cultured at 37 °C in monolayers in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 60 µg kanamycin/ml, and 10 mM Hepes/NaOH. The medium was changed every 4 d. Usually, the cells were replated at 5.0×10^5 cells per 9-cm dish (55 cm^2). For high cell-density cultures, the cells were replated at 8.0×10^6 cells per 9-cm dish.

RNA Extraction Extraction of total RNA from the cells was carried out according to the method[29] with slight modification. Briefly, T98G cells (1.5×10^7 cells) were resuspended in 250 µl of lysis buffer (10 mM Tris–HCl, pH 7.8, 150 mM NaCl, 10 mM VRC). After incubation for 1 min on ice, 28 µl of NP-40 was added and the suspension mixed briefly. Insoluble material was eliminated following centrifugation at $19000 \times g$ for 2 min at 4 °C, and then 250 µl of preparation buffer (40 mM Tris–HCl, pH 7.8, 40 mM EDTA, 700 mM NaCl, 2% SDS) was added to the supernatant. RNA

was extracted from the supernatant with phenol–chloroform–isoamylalcohol and chloroform–isoamylalcohol.

RT-PCR Analysis RT-PCR analysis was performed according to the method[23] with slight modification. Briefly, 5 µg of total RNA was treated with DNase-I for 15 min at room temperature. After addition of oligo-dT primer and SuperScript II reverse transcriptase, and 20 µl (2.5 µg total RNA) was incubated at 42 °C for 60 min to synthesize cDNA. Subsequently, 10 µl of cDNA solution was subjected to PCR in a total volume of 50 µl that included 1×KOD buffer, 0.2 mM dNTPs, 1 mM MgCl$_2$, 1 µl KOD DNA polymerase, and 50 pmol sense and antisense primers. The amplification program was as follows: denaturation at 98 °C for 15 s, annealing at 65 °C for 5 s, and elongation at 74 °C for 40 s for 40 cycles. Final elongation was performed at 74 °C for 1 min. PCR was carried out in a GeneAmp PCR system 2400 (Applied Biosystems; Foster city, CA, U.S.A.). PCR products were electrophoresed in 2% agarose gel and checked by ethidium bromide staining.

Preparation of Whole Cell Lysate T98G cells were maintained in a 9-cm dish in 10 ml medium. At the indicated times, the cells were washed twice with ice-cold phosphate buffered saline (PBS) and scraped into lysis buffer (1.8×10^4 cells/µl; 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% NP-40, 10 mM NaF, 1 mM EDTA, 0.5 mM Na$_3$VO$_3$, 10 mM tetrasodium pyrophosphate, aprotinin 90 KIU/ml, 20 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride). After sonication, insoluble material was eliminated by centrifugation at $14000 \times g$ for 15 min at 4 °C to yield whole cell lysate. Protein concentration was determined by the BCA protein assay. To remove Asn-linked oligosaccharides, aliquots of whole cell lysate were treated with PNGase F as follows (Chen et al. 1995)[26]: the lysate (50 µg protein) was denatured by boiling for 10 min in 0.5% SDS, 1% 2-mercaptoethanol. After addition of NP-40 to 1%, the lysate was incubated at 37 °C for 2 h with 0.15 IUB milliunit of PNGase F in 50 mM phosphate buffer, pH 7.5.

Immunoblotting Usually, 50 µg total protein prepared from approximately 1.7×10^5 cells was subjected to SDS-gel electrophoresis. Briefly, aliquots of whole cell lysate treated with or without PNGase F were mixed with 2× electrophoresis sample buffer. After boiling for 10 min, the samples were electrophoresed on 12.5% acrylamide gel and the proteins were transferred onto PVDF membranes. The membranes were blocked with 0.5% casein in PBS (casein-PBS) and incubated with anti-prion antibodies in casein-PBS. Immunoreactive bands were visualized using HRP-conjugated anti-IgG and SuperSignal West Femto Maximum Sensitivity Substrate, according to the manufacturer's instructions. Quantitative analysis of the 25 kDa deglycosylated form of PrP^C was performed by computer-assisted densitometry.

Determination of DNA Synthesis The [^{3}H]thymidine incorporation assay was carried out as previously described[30] with slight modification. Briefly, T98G cells in a 9-cm dish were maintained at 37 °C in medium containing 10% FCS or serum-free medium containing BSA (0.1 mg/ml). [^{3}H]Thymidine (37 KBq/dish) was added for the last 6 h of incubation. The cells were trypsin-treated, suspended in distilled water, and trapped onto a GA-100 glass filter (Advantec Toyo, Tokyo, Japan). The radioactivity collected on each filter was determined with a scintillation counter (LSC-5101;
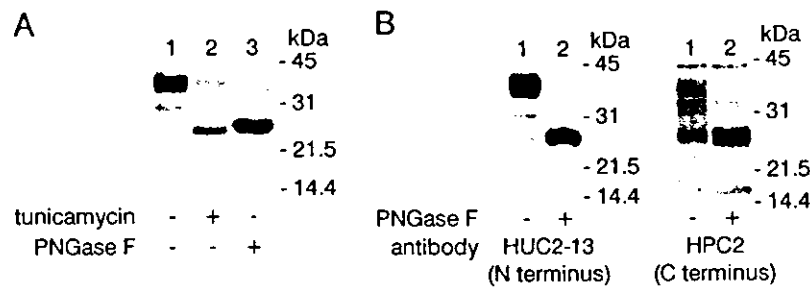
Fig. 1. T98G Cells Produced a Glycosylated Form of PrP^C

A: T98G cells were incubated with 10% FCS-RPMI 1640 for 12 d. They were then treated with (lane 2) or without (lanes 1, 3) tunicamycin (0.1 μg/ml). Whole cell lysates were prepared after 4 d incubation. Fifty micrograms of whole cell lysates were treated with (lane 3) or without (lanes 1, 2) PNGase F to remove Asn-linked oligosaccharides. After incubation for 2 h at 37 °C, the lysates were boiled for 10 min and subjected to immunoblotting with 3F4 antibody as described under Materials and Methods. B: T98G cells were incubated with 10% FCS-RPMI 1640 for 16 d and the lysates were prepared. Fifty micrograms of whole cell lysates were treated with (lane 2) or without (lane 1) PNGase F. After incubation for 2 h at 37 °C, the lysates were boiled for 10 min and subjected to immunoblotting with HUC2-13 (left panel) and HCP2 (right panel) antibody as described under Materials and Methods.

Aloka, Tokyo, Japan).

RESULTS

Human Glioblastoma Cell Line T98G Produced PrP^C

To estimate the production of endogenous PrP^C, we analyzed whole cell lysates of T98G cells by immunoblotting with anti-PrP antibodies. The lysate from Day 16 T98G cells revealed one major band (35 kDa) and two faint bands (31, 25 kDa) that reacted with mouse anti-human PrP monoclonal antibody 3F4 (Fig. 1A, lane 1). When T98G cells were treated with tunicamycin (0.1 μg/ml), a glycosylation inhibitor, for 4 d prior to harvest, the fully glycosylated (35 kDa) and partially glycosylated (31 kDa) forms were replaced by an unglycosylated (25 kDa) form (Fig. 1A, lane 2). Inhibition of glycosylation was confirmed by N-glycosidase digestion. Treatment of the Day 16 T98G cell lysate with PNGase F replaced the 35 and 31 kDa bands with a 25 kDa band (Fig. 1A, lane 3), suggesting that the Asn-linked forms were converted to the deglycosylated form. To determine if the T98G cells produced a truncated form of prion protein, we estimated the length of PrP^C by immunoblotting with chicken monoclonal anti-N terminus PrP antibody HUC2-13 and rabbit polyclonal anti-C terminus PrP antibody HPC2. As shown in Fig. 1B, left panel, HUC2-13 detected PrP^C of the same molecular weight that 3F4 antibody detected. On the other hand, HPC2 showed a different recognition pattern, which included a very intense 25 kDa band and faint 35 and 31 kDa bands (Fig. 1B, right panel), suggesting that the presence of Asn-linked oligosaccharides at the C terminus of PrP^C interfered with epitope recognition by HPC2. Moreover, HPC2 recognized an additional 18 kDa band, indicating that the 18 kDa band was truncated at the N terminus. This agrees with the report that anti-C terminus antibody recognizes an 18 kDa fragment in M17 human neuroblastoma cells[26] and in mouse brain.[31] We also detected constitutive expression of PrP^C mRNA in T98G cells on Day 16 (Fig. 2). These results established that T98G cells produced the glycosylated form of PrP^C.

PrP^C Increased in a Time-Dependent Manner

We next examined the correlation between PrP^C production and T98G cell growth. The growth curve (Fig. 3A) and phase-contrast microscopic examination (Fig. 5A, photos I and III) revealed that T98G cells were confluent on Days 12 to 16. In
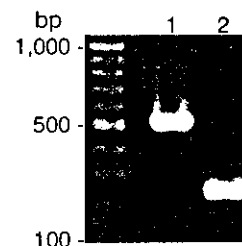

Fig. 2. T98G Cells Expressed PrP mRNA

T98G cells were incubated with 10% FCS-RPMI 1640 for 16 d and total RNA was prepared. Five micrograms of total RNA was treated with DNase-I, followed by RT-PCR using oligo dT primer. The resulting cDNA was amplified by PCR using human PrP primer set (lane 1) and human GAPDH primer set (lane 2). PCR products were separated on a 2% agarose gel and visualized with ethidium bromide. The DNA size marker is shown on the left.

culture, the 35, 31, and 25 kDa band intensities increased on Days 4 to 16 (Fig. 3B, left panel). To estimate the amount of immunoreactive PrP^C, we digested the T98G cell lysate with PNGase F to produce the deglycosylated form of PrP^C. The 25 kDa band which represents the deglycosylated increased in a time-dependent manner (Fig. 3B, right panel). The PrP^C content on Day 16 was approximately twice that on Day 4 (Fig. 3C).

Production of Endogenous PrP^C was Dependent on Cell-Density

T98G cells cultured for 4 d at low cell-density produced less PrP^C than those cultured for 16 d at high cell-density (Fig. 4, lanes 2 and 3). Quantitative analysis of the 25 kDa deglycosylated form of PrP^C from Fig. 4 (right panel) reveal that the amount of deglycosylated 25 kDa of Day 4 was almost half that of Day 16 (Table 1). The cells were almost confluent on Day 4 when cultured at a high cell-density (1.7×10^5 cells/cm^2), but the amount of deglycosylated 25 kDa they produced was almost the same as that produced by Day 16 cells (Table 1), demonstrating that PrP^C production was dependent on cell-density.

T98G Cells in Serum-free Cultures Produced a Large Amount of Endogenous PrP^C

T98G cells are arrested in G$_1$ phase under conditions of high cell-density or low serum concentration,[32] so we examined how plating density and/or cell proliferation affect PrP^C production. To address the effect of density, we compared two culture conditions with similar average cell-densities but distinct cell proliferation rates: one with medium containing 10% FCS and the other
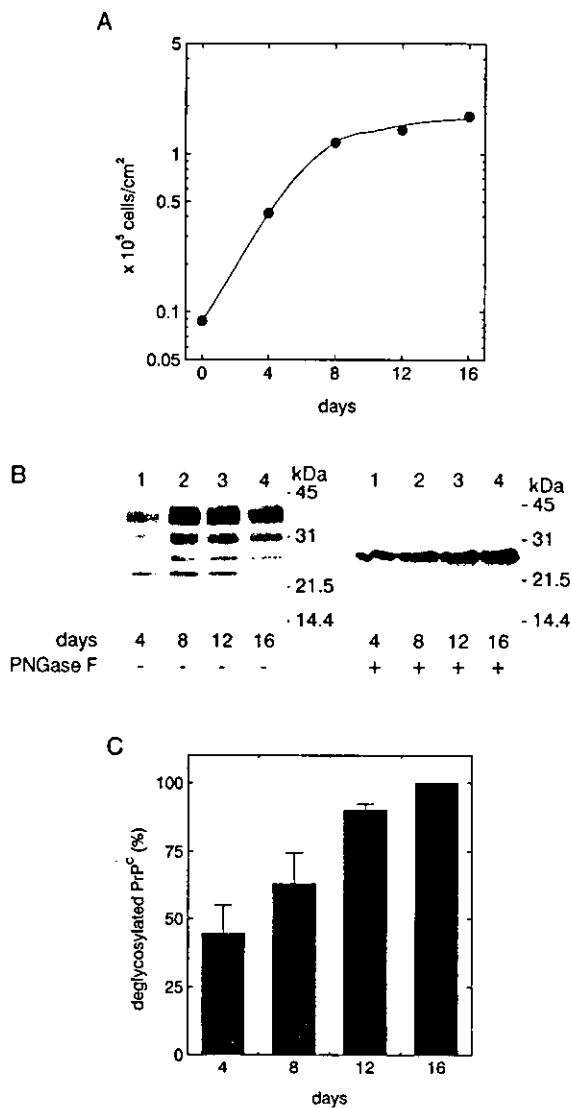
A

Table 1. Effect of Cell-Density and Conditioned Medium on PrP^C Production in T98G Cells

Incubation time (d)	Cell-density ($\times 10^5$ cells/cm^2)	Deglycosylated PrP^C (%)
4	1.7	$95.5 \pm 16.0^{a)}$
4	0.7	48.6 ± 1.3
16	1.6	100.0 ± 0.0

a) The integrated intensity of the deglycosylated 25 kDa bands were normalized for intensity on Day 16 as 100%. Results are means±ranges from two independent experiments.

B

Fig. 4. Effect of Cell-Density on Endogenous PrP^C Production in T98G Cells

T98G cells were plated at two different densities (high and low). After 4 (lanes 1 and 2) or 16 (lane 3) days incubation, cells were harvested. Fifty micrograms of whole cell lysates were treated with (right panel) or without (left panel) PNGase F. After incubation for 2 h at 37 °C, the lysates were subjected to immunoblot with 3F4 antibody as described under Materials and Methods.

C

Fig. 3. Endogenous PrP^C Production in T98G Cells Increased in a Time-Dependent Manner

T98G cells were incubated with 10% FCS-RPMI 1640 in a 9-cm dish. The cells were harvested on Day 4, 8, 12, and 16. A: Growth curve of T98G cells. The cells were dissociated and counted by hemocytometer. On the panel is shown the plot of the cell density versus incubation time. B: Fifty micrograms of whole cell lysates were treated with (right panel) or without (left panel) PNGase F. After incubation for 2 h at 37 °C, the lysates were subjected to immunoblot with 3F4 antibody as described under Materials and Methods. C: Densitometric quantitation of the PrP^C generated by measuring the 25 kDa deglycosylated form. Quantitative analysis of the 25 kDa deglycosylated form of PrP^C shown in B (right panel) were performed by computer-assisted densitometry. The integrated intensity of each bands was percentage of the intensity on Day 16. On the panel is shown the bar of integrated intensity versus incubation time. Bars are means±ranges from two independent experiments.

with serum-free medium. Day 3 T98G cell cultures were incubated for another 5 d in serum-free medium to shift them to the quiescent phase at low cell-density. As shown in Fig. 5, morphology (A) and cell-density (B) of the serum-free cultures (II) were almost the same as those of the log-phase cultures (III) in medium containing 10% FCS for 4 d. Compared with the log-phase cultures (Fig. 5C, III), the serum-free cultures (Fig. 5C, II) showed dramatically reduced [^{3}H]thymidine incorporation, similar to that of the usual Day 16 confluent cultures (Fig. 5C, I). While PrP^C production in log-phase cultures at low cell-density (Fig. 5D, III) was lower than in confluent cultures (Fig. 5D, I), it was higher in serum-free

cultures even at low cell-density (Fig. 5D, II). Moreover, the deglycosylated 25 kDa bands of the serum-free cultures (Fig. 5E, II) were almost equal to those of the confluent cultures (Fig. 5E, I). Taken together, these results suggest that T98G cells produced PrP^C at the G$_1$ phase of cell cycle.

DISCUSSION

Conversion of PrP^C to PrP^{Sc} is an important process of human prion disease. Host PrP^C is necessary for the neurotoxic effect of PrP^{Sc} in vivo.[14,15] Knowing the function of PrP^C would be helpful in establishing therapies. Although a variety of functions have been proposed for the protein, its precise role is still unclear. Several neuronal cell lines that can be persistently infected with scrapie, such as mouse N2a neuroblastoma cells,[33] have been used to study PrP^{Sc} generation and trafficking,[9] but there are no lines in which to study the physiological role of PrP^C, probably due to the relatively low level of endogenous PrP^C production.

In the present study, we showed that human glioblastoma cell line T98G constitutively expressed PrP^C mRNA. Quantitative immunoblot analysis of the 25 kDa deglycosylated form of PrP^C revealed that these cells produced the endogenous form of PrP^C. Although human astrocytoma cell line U373MG constitutively expresses a high level of PrP^C mRNA,[23] T98G cells produced relatively higher levels (assessed by quantitative immunoblot analysis; data not shown). Furthermore, by using anti-N and anti-C terminus antibodies, we demonstrated that the C terminal fragment of PrP was present. This observation agrees with previous studies showing that in human brain and in neuroblastoma cells, PrP^C undergoes proteolytic cleavage in a potentially pathogenic re-

A

B

C

D

whole cell lysate   I   II   III   I   II   III
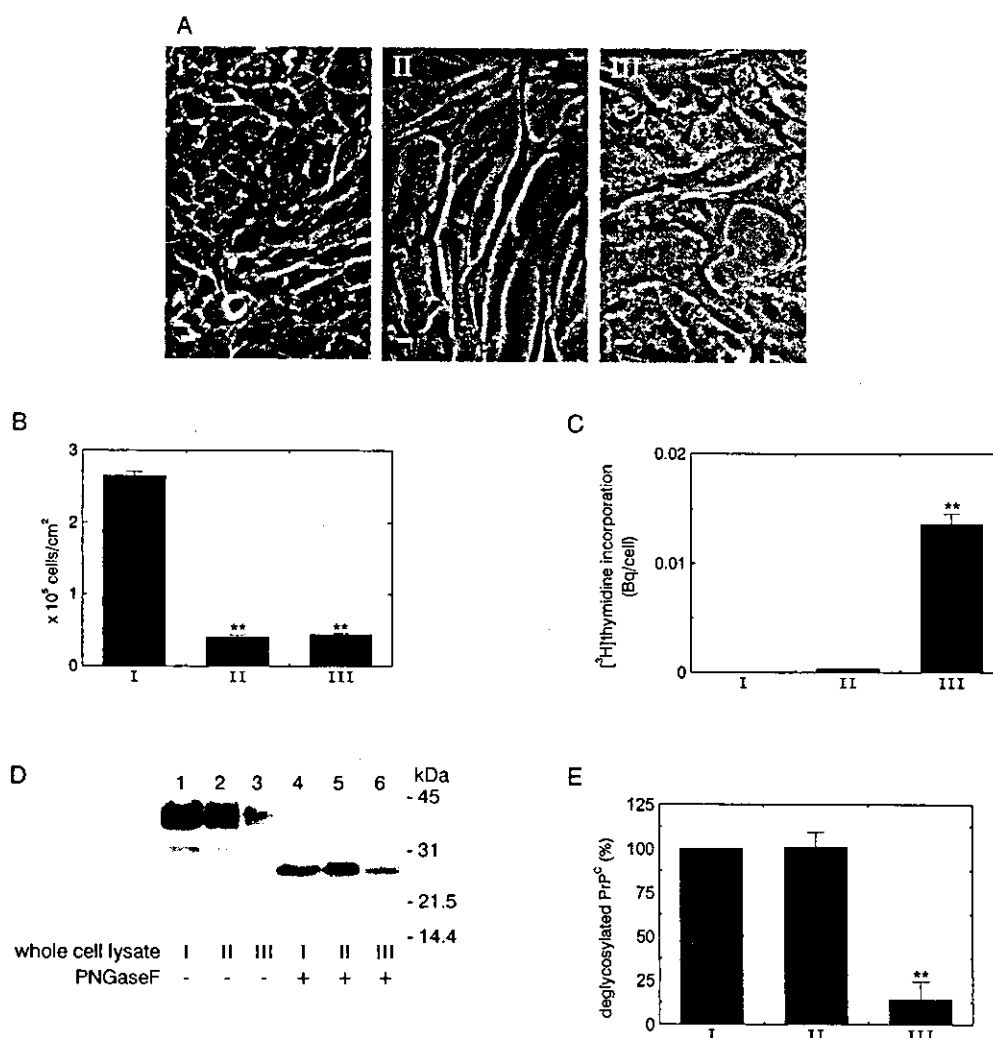PNGaseF   -   -   -   +   +   +

E

Fig. 5.   High-Level Expression of Endogenous PrP^C in Quiescent T98G Cells

T98G cells were incubated with 10% FCS-RPMI 1640 under the following conditions. (I) The cells were cultured for 16 d. (II) The cells were cultured for 3 d. After the medium was changed to serum-free medium containing BSA (0.1 mg/ml), the cells were incubated for another 5 d. (III) The cells were cultured for 4 d. A: Morphology of the cells photographed by phase-contrast microscopy. Scale bars, 10 μm. B: Cell-density of cultures. The cells were dissociated and counted by hemocytometer. On the panel is shown the bar of cell-density. Results are means±S.E.M. from triplicate dishes. **$p<0.01$ compared with (I) (Dunnett's test). C: Cell proliferation was determined by [^{3}H]thymidine incorporation assay. The cultures were pulsed with [^{3}H]thymidine for the last 6 h. Results are means±S.E.M. from triplicate dishes. **$p<0.01$ compared with (I) (Dunnett's test). D: Fifty micrograms of whole cell lysate was treated with (lanes 4—6) or without (lanes 1—3) PNGase F. After incubation for 2 h at 37 °C, the lysates were subjected to immunoblot with 6H4 antibody as described in Materials and Methods. E: Densitometric quantitation of the PrP^C generated via measurment of the 25 kDa deglycosylated form. Quantitative analysis of the 25 kDa deglycosylated form of PrP^C shown in D (lanes 4, 5, and 6) was performed using computer-assisted densitometry. The integrated intensity of each bands was the percentage of the intensity on Day 16 (lane 4). Bars are means ranges from three independent experiments. **$p<0.01$ compared with (I) (Dunnett's test).

gion.[26,34] The mechanism of that cleavage and the physiological role of the C terminal fragment are unknown; T98G cells might provide a useful model for studying the proteolytic cleavage site.

In this study, we observed that endogenous PrP^C production varied directly with cell-density. T98G cells are like normal cells in that they become arrested in G$_1$ phase under stationary phase conditions,[32] and we have shown that T98G cells arresting at G$_1$ phase at low cell-density in serum-free medium produce a high level of endogenous PrP^C. In primary cultures of periodontal ligament cells, up-regulation of PrP mRNA may be relevant to cell growth arrest and differentiation.[25] More recently it has been reported that the PRNP gene is transcriptionally activated in the G$_1$ phase in confluent and terminally differentiated viral-transformed mouse spleen hematopoietic cells.[35] Mouse neuroblastoma cell line N2a and ScNa also produce PrP in a density-depend

manner.[36] The results we present here also demonstrate a correlation between endogenous PrP^C production and cell growth.

Recently, it has been reported that the caveolin-1-dependent coupling of PrP^C to tyrosine kinase Fyn was observed mainly at neurites of murine 1C11 neuronal cells.[37] PrP^C is a GPI-anchored cell-surface protein and might be a signal transduction protein. It would be interesting to investigate whether the signal coming from PrP^C through Fyn is coupled to the cell cycle.

Immunoblotting with anti-PrP antibodies is the most common procedures for identifying PrP^{Sc} in prion diseases. In this study, we used 3 monoclonal antibodies (3F4, 6H4, and HUC2-13) and 1 polyclonal antibody (HPC2) to detect PrP^C. These are directed to distinct epitopes throughout PrP^C; HUC2-13 reacts with the N terminus, 3F4 and 6H4 react with the mid-region, and HPC2 reacts with the C terminus.

HPC2 showed a different reactivity pattern; it reacted strongly with the deglycosylated form of PrP^C but weakly with the glycosylated form. Other antibodies also react differently with the PrP^C bearing heterogeneous Asn-linked oligosaccharides. We have shown that quantitative analysis of the 25 kDa deglycosylated form of PrP^C treatment with PN-Gase F was helpful in obtaining precise measurements of PrP content. These methods will be useful in comparing the PrP^C production in cultured cells.

Prion diseases are characterized by gliosis, loss of neurons, and formation of amyloid plaques.[2] PrP^{Sc} accumulates in white matter and in glia cytoplasm.[38,39] In addition, PrP mRNA is expressed not only in neurons but also in glia.[40] Therefore, more evidence is needed from cell culture models to clarify the role of glia cells in prion disease.

In conclusion, cell cycle arrests in G_1 phase induced endogenous PrP^C production in human glioblastoma cell line T98G. T98G cells should be useful for studying the cellular function and molecular mechanisms of action of PrP^C.

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Biological and Biochemical Characterization of Sheep Scrapie in Japan

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Due to the apparent absence of an agent-specific nucleic acid genome, scrapie strains cannot be classified by genome characterization, which is commonly used for the classification of many viruses. However, scrapie strains can be distinguished to some extent by biological properties such as transmissibility to experimental animals and distribution of neuropathological lesions and by biochemical properties such as the molecular mass and relative protease-resistance of the disease-specific isoform of prion protein (PrP^{Sc}). In order to preliminarily characterize the scrapie strains that are prevalent in Japan, we analyzed the transmissibility of sheep scrapie isolates to mice and the relative proteinase K (PK) resistance of the corresponding PrP^{Sc}. The results indicate that Japanese scrapie strains can be divided into at least three groups based on biological and biochemical properties. The first group includes isolates which cause disease in mice with an incubation period of ~400 days and possess PrP^{Sc} with relatively high PK resistance. Isolates of the second group contain PrP^{Sc} that is highly resistant to PK digestion but transmit poorly to mice. The final group consists of isolates that cause disease in mice with an incubation period of less than 300 days and are associated with PrP^{Sc} with reduced PK resistance. Sheep scrapie has occurred sporadically in Japan since1982, with only ~60 officially reported cases so far. However, the diversity of scrapie strains in the field suggested by our data raises the concern that a scrapie strain similar to the parental agent of bovine spongiform encephalopathy could exist or emerge in Japan. Thus, continuous surveillance for scrapie will be required to prevent the further spread of scrapie, not only among the sheep population but also to other species, and to eliminate any potential risk of sheep scrapie to public health.

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases, which include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE), and Creutzfeldt-Jakob disease (CJD) in humans. Scrapie has existed for more than two centuries, while BSE was first recognized in 1985, followed by a BSE epidemic in the United Kingdom (31). Epidemiological studies suggested that BSE was primarily caused by feeding meat and bone meal (MBM) contaminated with scrapie agent to cattle (32). Once BSE appeared, the causative agent spread through the cattle population by the use of BSE agent-contaminated MBM. The appearance of feline spongiform encephalopathy (FSE) in domestic and captive cats (34, 35) and, more recently, variant CJD (vCJD) in humans in 1996 (33), has raised a global concern for the spread of the BSE agent to other species via the food chain.

It is known that there are biologically distinguishable sheep scrapie strains in the United Kingdom (8, 11); however, BSE isolates studied so far appear to have comparatively uniform characteristics (4). The biological and biochemical properties of the BSE agent are very similar to those of the agents of vCJD and FSE, providing strong evidence that cross-species transmission of BSE to humans and felids resulted in vCJD and FSE, respectively (7, 13). Thus, it is now probable that a

particular scrapie strain, which possessed enhanced resistance to heat inactivation, survived the rendering process and was transmitted to cattle via contaminated MBM. Thereafter, the agent passed into humans, possibly via the food chain. Alternatively, a particular strain capable of infecting both cattle and humans might have been selected and amplified during the transmission of the scrapie agent to cattle.

One of the characteristics of TSEs is an accumulation of a protease-resistant, abnormal isoform of a host protein, PrP^{Sc}, which is specifically detected in the central nervous system and lymphoid tissues of scrapie-affected animals. PrP^{Sc} is post-translationally generated from the host-encoded sialoglycoprotein, prion protein (PrP^{C}). PrP^{C} and PrP^{Sc} have the same primary structure (14) but different conformations as detected by analysis of biophysical properties (24, 27) and biochemical properties, such as resistance to protease digestion and solubility in nonionic detergent (23). The presence of PrP^{Sc} usually correlates with scrapie infectivity. Thus, PrP^{Sc} is thought to be one of the major components of the scrapie agent, and so detection of PrP^{Sc} is often considered an indication of the presence of infectivity. Although the exact nature of the scrapie agent is still controversial, the failure to find an agent-specific genome to date prevents the use of nucleic acid sequencing for strain characterization, a method commonly used for strain typing of viruses and bacteria (1, 22). However, TSE agents can be distinguished to some extent by incubation periods and distribution of neuropathological lesions on transmission to experimental animals (5, 6), as well as biochemical properties, such as relative protease resistance and/or molec-

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ular mass of PrP^{Sc} (2, 3, 25, 30) and differences in the ratio of glycosylated PrP^{Sc} bands (10).

There is a sporadic occurrence of scrapie in Japan, and the existence of BSE was disclosed in September 2001. Since BSE is thought to originate from sheep scrapie, surveillance of scrapie-positive sheep and characterization of prevalent scrapie strains in the field are required for the prediction and elimination of a potential risk of scrapie to public health. In order to attempt to characterize scrapie strains present in Japan, we analyzed several isolates for their transmissibility to mice and the biochemical properties of the associated PrP^{Sc}. The results showed that at least three different strains of scrapie agent exist in Japan.

MATERIALS AND METHODS

Sheep with scrapie. Eight naturally occurring sheep scrapie isolates (KH2, KU, SB, Y2, Y5, S1, S2, and S3) collected from 1987 to 1996 and three first-passage isolates from experimental sheep scrapie infections (A1, B3, and G1) were used in this study. As a negative control, one sheep (S4), which was defined as negative for scrapie by the absence of both neuronal vacuolation in histopathological examination and detectable PrP^{Sc} in central nervous system and lymphoid tissues in immunoblot analysis, was also used. Sheep S1, S2, S3, and S4 were kept on ranch S. Sheep S1, S2, and S3 had been born from two ewes which showed neurological dysfunction several months after delivery and were diagnosed with scrapie by clinical and/or histopathological methods. The sheep S1, S2, and S3 shared the same sire. However, there were no disease-associated mutations in the PrP genes of these sheep, and thus the sheep S1, S2, and S3 were tentatively grouped as cases of endemic scrapie. Sheep KH2, KU, and SB, each from independent ranches, and sheep Y2 and Y5 from ranch Y, were grouped as sporadic cases because there is no immediate blood relationship among these sheep. Sheep A1 and B3 with experimental scrapie received the same brain homogenate of a scrapie-affected sheep that was not included in the natural scrapie group used here, and the results of experimental transmission of this sheep scrapie isolate to mice were reported elsewhere (29). Sheep G1 was inoculated intravenously with a 10% brain homogenate of scrapie-affected sheep Y4 from the Y ranch. Sheep Y2, Y4, and Y5 were grown on the same farm and developed scrapie within 6 months, but they were born to different ewes.

PrP genotyping of sheep. PrP genotyping of sheep was performed as described previously (18). Amino acid polymorphisms at codons 112 Met/Thr, 136 Ala/Val, 154 Arg/His, and 171 Gln/Arg/His were basically used for distinction of the PrP genotype.

Bioassay. Twenty microliters of 10% brain homogenates (in phosphate-buffered saline) from scrapie-affected or scrapie-negative sheep were inoculated intracerebrally into 4-week-old female ICR mice (PrP allotype PrP$^{A/A}$; PrP^A encodes PrP with codons 108 Leu and 189 Thr). In some cases I/LnJ mice (PrP allotype PrP$^{B/B}$; PrP^B encodes PrP with codons 108 Phe and 189 Val) were also used. When mice showed clinical symptoms of the terminal stage of scrapie, mice were sacrificed under anesthesia and brains were removed and processed for the detection of PrP^{Sc}. The brains of mice that died of unknown causes were also checked for the presence of PrP^{Sc}.

Sample preparation and PK digestion. Preparation of brain samples for proteinase K (PK) digestion was carried out as described elsewhere with slight modifications (12). Brains were homogenized with 7 volumes (wt/vol) of 10 mM Tris-HCl (pH 7.5) and 7.5 mM MgCl$_2$, and the homogenates were incubated at 37°C for 1 h with DNase I (40 µg/100 mg tissue). After adding 20% Sarkosyl to a final concentration of 5%, the homogenates were kept at room temperature (RT) for 30 min and then centrifuged at 12,000 × g at RT for 5 min. The resulting supernatants were transferred to new tubes, and then solid NaCl was added to give a final concentration of 10%. After a 16-h incubation at 4°C with continuous rotation, the homogenates were centrifuged at 16,000 × g, 4°C and, for 40 min, and the resulting pellet was resuspended in 10 mM Tris-HCl (pH 7.5). This suspension was subjected to PK digestion at various PK concentrations and times as indicated in each experiment. Phenylmethylsulfonyl fluoride (PMSF) was added to stop the digestion (final conc. 1 mM), and then the reaction mixture was adjusted to 4% sodium dodecyl sulfate (SDS) and boiled for 5 min. The proteins were precipitated with 10 volumes of ice-cold methanol, and the final pellet was dissolved by SDS-polyacrylamide gel electrophoresis in sample buffer (4% SDS, 5% 2-mercaptoethanol, 5% glycerol, 0.01% bromophenol blue, 62.5 mM Tris-HCl [pH 6.8]).

Detection of PrP^{Sc}. (i) Immunoblot analysis. Immunoblot analysis was carried out as described previously with B-103 rabbit serum (16). Densitometric analysis of X-ray film was performed with a Lane & Spot Analyzer (Atto, Tokyo, Japan).

(ii) Dot blot analysis. The 10% brain homogenates of sheep with scrapie were mixed with an equal volume of cold lysis buffer [0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM EDTA, 100 mM NaCl, 50 mM Tris-HCl (pH 7.4)], diluted serially in twofold steps and blotted onto a nitrocellulose membrane. The membrane was dried and then washed with Tris-buffered saline containing 0.1% Tween 20 (TBST). The membrane was treated with PK (25 µg/ml) in TBST at 37°C for 1 h, and the reaction was stopped by adding PMSF to a final concentration of 2 mM. After washing with TBST, the membrane was treated with 3 M guanidinium isothiocyanate for 20 min at RT and washed with TBST again. Immunostaining of the membrane was then performed as described for the immunoblot analysis.

RESULTS

Sheep with scrapie. Characteristics of the sheep used in this study are summarized in Table 1. The major symptoms of all scrapie cases used here were ataxia and, at the terminal stage, astasia. No sheep except for KU showed obvious loss of fleece, which is thought to be caused by pruritus. The sheep S1, S2, and S3, which were tentatively grouped as cases of endemic scrapie (see Materials and Methods), differed from other sheep with scrapie. They showed hypersensitivity at the onset of disease and died of scrapie at a considerably young age (average, 16.3 months old) compared to sheep with scrapie grouped as sporadic cases here (average, 42.0 months old) or those described by others (2 to 6 years old [11]).

Transmissibility of sheep scrapie to mice. Transmission of 11 sheep scrapie samples to mice is shown in Table 2. Eight of 11 successfully transmitted to ICR mice (PrP allotype, PrP$^{A/A}$), with average incubation periods from 229 to 451 days postinfection (dpi). These eight sheep scrapie samples seem to be divided into two groups based on the incubation periods; one includes A1 and B3, which transmitted to mice with incubation periods of around 230 dpi, and the other includes KH2, KU, SB, Y2, Y5, and G1, which caused disease in mice with incubation periods of ~386 to 451 dpi. There is no significant difference among the incubation periods of KH2-, KU-, SB-, Y2-, and Y5-inoculated mice ($P > 0.05$ in t test). However, the incubation period of G1-inoculated mice was somewhat shorter than that of the others, with a statistically significant difference between the incubation period of G1-inoculated mice and that of KH2-, SB-, and Y2-inoculated mice ($P < 0.05$ in t test). The sheep A1 and B3 received the same brain homogenate from a naturally occurring sheep scrapie isolate by intravenous injection. The transmission of this parental isolate of A1 and B3 directly to mice was achieved within 271 to 307 dpi (29), consistent with the incubation periods observed in A1- and B3-inoculated mice. Thus, the first-pass scrapie samples A1 and B3 and their parental isolate all possessed the ability to transmit to mice with relatively short incubation periods.

In contrast, the S1, S2, and S3 scrapie isolates were virtually nontransmissible to ICR mice (Table 2). One mouse inoculated with the brain homogenate of sheep S1 died at 359 dpi without typical symptoms of scrapie but was positive for PrP^{Sc} in the brain. We cannot confirm whether this one case is due to actual transmission or contamination with a mouse-adapted scrapie agent. Several ICR mice inoculated with the brain homogenates of S2 or S3 sheep died without typical symptoms

TABLE 1. Sheep used in this study

Sheep	Breed[a] (age [mo])[b]	PrP genotype[c]	Symptom(s)
With scrapie			
Sporadic			
KH2	S (48)	MARQ/MARR	NA[d]
KU	S (52)	MARQ/TARQ	Ataxia, subtle loss of fleece
SB	S (34)	MARQ/MARQ	Ataxia, debilitation
Y2	S (35)	MARQ/MARQ	Ataxia, debilitation
Y5	S (41)	MARQ/MARQ	Ataxia, debilitation
Endemic			
S1	S × C (12)	MARQ/MARH	Ataxia, hypersensitivity
S2	S × C (21)	MARQ/MARH	Ataxia, hypersensitivity
S3	S × C (16)	MARQ/MVRQ	Ataxia, hypersensitivity
Exptl			
A1	S (27)	MARQ/MARQ	Ataxia
B3	S (21)	MARQ/TARQ	Ataxia
G1	S (26)	MARQ/MARQ	Ataxia, debilitation
Healthy (S4)	S × C (20)	MARQ/MARQ	

[a] Abbreviations: S, Suffolk; C, Corriedale; S × C, F₁ of Suffolk and Corriedale.
[b] Age of the sheep with scrapie grouped as sporadic or endemic indicates the age at death (months old), while that of the experimental group indicates time to death after inoculation (months postinoculation).
[c] The nomenclature of PrP genotypes was described elsewhere (18).
[d] NA, no record of clinical symptoms was available.

TABLE 2. Transmissibility of sheep scrapie to mice

Sheep	Mouse strain	Dead mice (n/N)[a]	PrP^Sc-positive mice (n'/N')[b]	Mean time to death ± SD (day)
Scrapie				
Sporadic				
KH2	ICR	6/6	5/5	417 ± 29
KH2	I/LnJ	5/5	3/3	274, 275, 275, 275, 307[d]
KU	ICR	7/7	7/7	394 ± 672
SB	ICR	9/9	6/7	231, 451 ± 39[e]
Y2	ICR	5/5	4/4	418 ± 42
Y5	ICR	5/5	5/5	427 ± 15
Endemic				
S1	ICR	1/6	1/6	359, >462[f]
S2	ICR	5/6	0/6	301, 400, 430, 478, 536, >620
S3	ICR	2/7	0/7	222, 413, >620
S3	I/LnJ	5/5	0/5	188, 200, 270, 363, 603[g]
Exptl				
A1	ICR	6/6	6/6	229 ± 12
B3	ICR	7/7	7/7	236 ± 14
G1	ICR	9/9	8/9	190, 386 ± 20[h]
Healthy (S4)	ICR	0/4	ND[c]	>620

[a] Abbreviations: n, number of animals which died of any cause during the observation period; N, number of animals which received brain homogenates.
[b] Abbreviations: n', number of PrP^Sc-positive mice; N', number of mice used for the examination of PrP^Sc.
[c] ND, not determined.
[d] All the mice died of wounds received in violent fights without any symptoms of scrapie. The three mice which died at 275 dpi were found to be positive for PrP^Sc.
[e] One mouse which died at 231 dpi was negative for PrP^Sc. The mean and SD for time to death were calculated from data for the remaining eight mice.
[f] One mouse which died at 359 dpi did not show scrapie symptoms but was found to be positive for PrP^Sc.
[g] Four mice died at 188, 200, 270, and 363 dpi due to fights.
[h] One mouse which died at 190 dpi was negative for PrP^Sc. The mean and SD for time to death was calculated from data for the remaining eight mice.

of scrapie during the observation period but were also negative for PrP^Sc. Therefore, it is obvious that S1, S2, and S3 differ from the other scrapie isolates used here. Furthermore, isolate S3 did not transmit to I/LnJ mice (PrP allotype, PrP^B/B), whereas transmission of isolate KH2 to these mice was confirmed by the detection of PrP^Sc at 275 dpi. The sheep S1, S2, and S3 were born on the same farm and in the same parturient season from two ewes that developed scrapie several months after delivery, and thus the sheep were potentially infected with the same agent. One explanation for the failure of the transmission of these isolates to mice is that the brain homogenates may possess a relatively low degree of infectivity. However, dot blot analysis of serial dilutions of brain homogenates revealed that variations in the amount of PrP^Sc in the homogenates appear to be less than fourfold among the samples tested (Fig. 1), suggesting that the lack of transmission was not due to reduced degrees of infectivity. Therefore, we conclude that the sheep scrapie isolates S1, S2, and S3 have an extremely low, if any, transmissibility to mice.

PK resistance of sheep PrP^Sc. Since some TSE strains are known to differ in the relative PK resistance of PrP^Sc (3), we analyzed the PK resistance of PrP^Sc in the brains of sheep with scrapie in order to identify any biochemical differences among sheep scrapie isolates. Partially purified PrP^Sc-containing fractions without PK treatment were first analyzed by immunoblot, and following densitometric analysis to allow normalization of the amount of PrP^Sc among the samples, the fractions were digested with PK at 0, 8, 40, 200, and 1,000 μg/ml for 2 h (Fig. 2a). The bands of PrP^Sc were clearly detected after PK treatment at 40 μg/ml in KH2 and S2, while PrP^Sc in A1 was undetectable under the same digest conditions. To confirm this difference, the same fractions were treated with PK at 40 μg/ml for various time periods (Fig. 2b). Consistent with the result in

Fig. 2a, the PrP^Sc bands in the A1 fraction were virtually eliminated by a 2-h treatment with 40-μg/ml of PK, while the PrP^Sc bands in the KH2 and S2 fractions were readily detected after the same treatment. The PrP^Sc of B3 exhibited the same PK resistance as A1, while KU, SB, Y2, Y5, S1, and S3 showed levels of PK resistance similar to KH2 and S2 (data not shown). Thus, the sheep scrapie isolates used here appeared to be
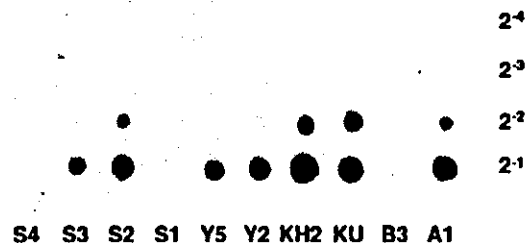

FIG. 1. Dot blot analysis of sheep brain homogenates for the presence of PrP^Sc. Serial twofold dilutions of sheep brain homogenates used for inoculation into mice were dotted onto a nitrocellulose membrane and stained with B-103 anti-PrP synthetic peptide rabbit serum and ECL Western blot detection reagent (Amersham). The sheep are indicated at the bottom and dilutions are on the right. In this dot blot analysis, S1 appears to be negative for PrP^Sc; however, PrP^Sc was detected in the immunoblot analysis (data not shown).
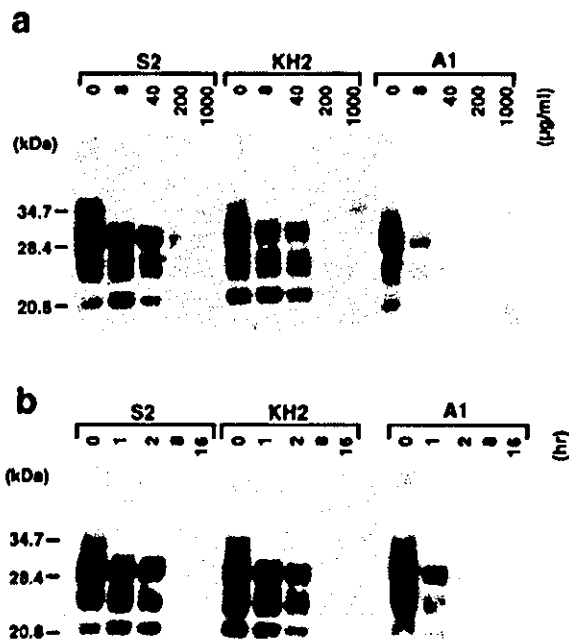
a

b

FIG. 2. Relative PK resistance of sheep PrP^{Sc}. (a) PrP^{Sc}-containing fractions prepared from sheep brains were treated with various concentrations of PK (0 to 1,000 μg/ml, indicated above the photo) at 37°C for 2 h. Molecular mass markers are indicated. PrP was detected by immunoblotting with PrP-specific antibody and ECL. (b) The same PrP^{Sc}-containing fractions as used in panel a were treated with PK (200 μg/ml) at 37°C for various times (0 to 16 h, indicated above the photo).

TABLE 3. Grouping of sheep scrapie samples based on transmissibility to mice and relative PK resistance of PrP^{Sc}

Sheep	PK resistance[a] (Sheep PrP^{Sc})	Transmissibility to ICR mice (mean ± SD [day])	PK resistance[b] (Mouse PrP^{Sc})
Group 1			
KH2	H	Yes (417 ± 29)	H
KU	H	Yes (394 ± 67)	H
SB	H	Yes (451 ± 39)	ND[c]
Y2	H	Yes (418 ± 42)	ND
Y5	H	Yes (427 ± 15)	H
G1	H	Yes (386 ± 20)	ND
Group 2			
S1	H	No	NA[d]
S2	H	No	NA
S3	H	No	NA
Group 3			
A1	L	Yes (229 ± 12)	L
B3	L	Yes (236 ± 14)	L

[a] H indicates PrP^{Sc} that is resistant to a treatment with PK (40 μg/ml) for 2 h, while L indicates PrP^{Sc} that is sensitive to the same treatment.
[b] H indicates PrP^{Sc} that is resistant to a treatment with PK (200 μg/ml) for 1 h, while L indicates PrP^{Sc} that is sensitive to the same treatment.
[c] ND, not determined.
[d] NA, not available.

divided into two groups based on the relative PK resistance of PrP^{Sc}. One possesses PrP^{Sc} that is highly resistant to PK digestion, as defined by resistance to treatment with PK at 40 μg/ml for 2 h, and the other possesses PrP^{Sc} of relatively low PK resistance and is completely degraded under these conditions. Together, the mouse transmission and PrP^{Sc} PK resistance data can be used to further divide the sheep scrapie isolates into three groups (Table 3). The first group includes KH2, KU, SB, Y2, Y5, and G1, which are transmissible to ICR mice with incubation periods of ~400 days and contain PrP^{Sc} with relatively high PK resistance. The second group includes S1, S2, and S3, which transmit poorly to mice but possess PrP^{Sc} with a relative PK resistance similar to the first group. The last group includes A1, B3, and probably their parental isolate, which are transmissible to ICR mice with incubation periods of ~230 days and possess PrP^{Sc} with relatively low PK resistance.

PK resistance of PrP^{Sc} in mice inoculated with sheep scrapie. Sheep scrapie isolates which transmitted to ICR mice segregated into two groups based on incubation periods (Table 2), and interestingly, the grouping of sheep scrapie isolates by relative PK resistance of PrP^{Sc} in sheep brains appears to coincide with the grouping by incubation periods (Table 3). To address the question of whether the phenotype of relative PK resistance of PrP^{Sc} in sheep brain is conserved in infected mice, the relative PK resistance of PrP^{Sc} in the mouse brains was also examined (Fig. 3). PrP^{Sc} in the brain of a mouse inoculated with KH2 was resistant to treatment with PK at 200 μg/ml for 1 h, while PrP^{Sc} in the brain of a mouse inoculated with A1 was

sensitive to this condition (Fig. 3a). This difference in the relative PK resistance was also confirmed by the experiments in Fig. 3b, in which the samples were treated with PK at 200 μg/ml for the various time periods indicated. PrP^{Sc} in the brains of mice inoculated with KU and Y5 showed the same PK resistance as that of a KH2-inoculated mouse, whereas PrP^{Sc} in the brain of a mouse inoculated with B3 showed the same PK resistance as that of an A1-inoculated mouse (data not shown). Therefore, the relative PK resistance of PrP^{Sc} generated in the brains of mice was similar to that of PrP^{Sc} in the inoculum of the corresponding sheep.

DISCUSSION

We were interested in characterizing the scrapie strains prevalent in Japan. In the absence of known biological clones (which would take many years to develop), we attempted to gain information about the nature of these strains by examining the biological and biochemical properties of a panel of isolates and showed that at least three types of scrapie strains exist in Japan. It is thought that the scrapie agent entered into Japan along with imported sheep in the1970s (17, 29). Since the recognition of the first scrapie case early in the1980s, ~60 scrapie cases have been officially reported so far. Thus, our data indicate the possibility that the primary scrapie strain has already exhibited considerable variation during the past quarter century. However, it is unknown if the current repertoire of scrapie strains is derived from a single parental strain that has been changing during its spread through the sheep population, similar to the mutation-like change found on serial transmission of the scrapie agent in experimental animals (20). Alternatively, multiple infection is likely to occur in natural scrapie (19), so that it is also possible that multiple strains have entered into Japan with imported sheep. The diversity of sheep scrapie strains in Japan seems similar to that described in the
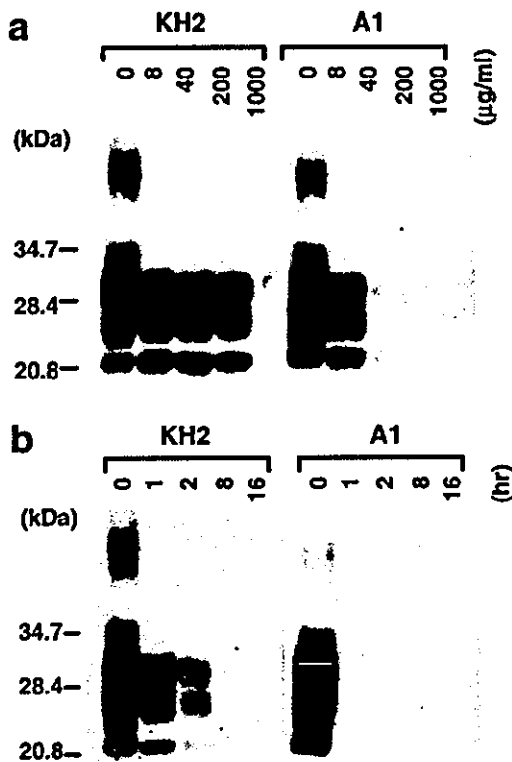
FIG. 3. Relative PK resistance of PrP^{Sc} generated in mouse brain. (a) PrP^{Sc}-containing fractions prepared from mouse brains were treated with various concentrations of PK (0 to 1,000 μg/ml, indicated above the photo) at 37°C for 2 h. Molecular mass markers are indicated. PrP was detected by immunoblotting as in Fig. 2. (b) The same PrP^{Sc}-containing fractions as used in panel a were treated with PK (200 μg/ml) at 37°C for various times (0 to 16 h, indicated above the photo).

United Kingdom based on transmissibility to mice (8, 11), because some isolates were essentially nontransmissible whereas others were readily transmissible to PrP$^{A/A}$ mice with incubation periods of >350 days. However, to our knowledge, sheep scrapie which successfully transmits to mice with an average incubation period of ~230 days, like A1 and B3, has an extremely short incubation period in primary transmission.

TSE agents have been characterized by transmissibility to experimental animals and distribution of neuropathological lesions (5, 11). In addition, biochemical characterization of PrP^{Sc} seems to be useful for discriminating between TSE strains. For example, two hamster-adapted transmissible mink encephalopathy strains, "Hyper" and "Drowsy," can be distinguished from each other not only by incubation period and neuropathology but also by the relative PK resistance and molecular mass of PrP^{Sc} (2, 3). Here we found that relative PK resistance of PrP^{Sc} is likely to be useful for the distinction of sheep scrapie strains to some extent, as the samples we analyzed could be divided into two groups based on relative PK resistance. Recently, the ratio of glycosylated PrP^{Sc} bands was used to distinguish vCJD from other types of CJD (10), and sheep scrapie can be divided into several groups by using this glycoform typing of PrP^{Sc} (15). In general, strain typing approaches using a combination of different properties provide a

more reliable means of strain differentiation. Analyses of biochemical properties of PrP^{Sc} are less time-consuming than bioassays in mice, and therefore, it is advantageous to use a combination of several biochemical properties such as relative PK resistance, molecular mass, and glycoform typing of PrP^{Sc}, to achieve more definitive strain typing.

A recent study using 10 sheep with scrapie in the United States showed a perfect correlation between the detection of PrP^{Sc} in sheep brain and transmissibility to mice (26). In contrast, it is well-known that some sheep scrapie strains are difficult to transmit to mice (4, 8, 11). In this study, we showed that scrapie strains classified into groups 1 and 2 differed in their transmissibility to mice but were indistinguishable by the relative PK resistance of PrP^{Sc} (Table 3). The discrepancy between the presence of PrP^{Sc} and the transmissibility to mice may imply that factors other than PrP^{Sc} are involved in determining the infectivity or that PrP^{Sc} is not the molecule responsible for infectivity (21). Alternatively, strain-specific conformations of PrP^{Sc} have been shown (9, 28), and so it is also conceivable that subtle differences in biochemical and/or biophysical properties of PrP^{Sc} which cannot be detected by PK digestion may influence the transmissibility to mice. It is also possible that the sheep PrP genotype may explain the discrepancy. It has not yet been clarified whether amino acid sequences of sheep PrP^{Sc} influence transmission to mice. Here we showed that scrapie occurring in sheep homozygous for PrP^{MARQ} transmitted to mice (e.g., SB, Y2, Y5, A1, and G1). This indicates that sheep PrP^{Sc} composed of the product of the PrP^{MARQ} allele could initiate the accumulation of PrP^{Sc} in mice by using mouse PrP^C as a substrate. However, there are differences in the incubation periods for A1 between these sheep and other sheep possessing the PrP$^{MARQ/MARQ}$ genotype (Table 2), and more recently, it was reported that one scrapie case occurring in PrP$^{ARQ/ARQ}$ sheep (amino acid polymorphisms at codon 112 was unavailable) was virtually nontransmissible to mice (8), suggesting the amino acid sequence of sheep PrP^{Sc} is not the sole determinant of the transmissibility to mice.

Among the sheep with scrapie used here, S1, S2, and S3 differed in clinical course from other sheep by death at a young age and hypersensitivity. The simple explanation for this is that the strain(s) infecting these sheep differs from those of the other scrapie cases, and indeed, these agents obviously differed from the others in transmissibility to mice. Scrapie strains adapted to rodents exhibit strain-specific clinical symptoms, neuropathological lesions, and incubation periods (3, 5, 6). However, it is unclear whether a given sheep scrapie strain determines strain-specific clinical and/or clinico-pathological features. As described in Materials and Methods, these sheep have an immediate blood relationship and were kept on the same ranch, and so other factors such as genetic background, breeds, route of infection, and environment may be involved in defining the type of disease in these particular cases.

A particular type of scrapie agent prevalent in the United Kingdom is believed to have initially caused BSE, and later on, this agent passed to felids and human beings through cattle (4, 7, 13). The diversity of field scrapie isolates in Japan is similar to that reported in the United Kingdom at least in terms of primary transmission to mice (11). Therefore, at present, we cannot exclude the possibility of the presence of a scrapie

strain in Japan that possesses properties similar to the BSE agent. Strain typing using two mouse strains, RIII and C57BL, carrying the PrP$^{A/A}$ allotype appears to be one of the methods to distinguish BSE and BSE-related TSE agents from sheep scrapie (4, 8). Further analysis will be required to investigate whether sheep scrapie strains in Japan have the potential risk to create new epidemics like the BSE agent.

ACKNOWLEDGMENTS

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Follicular dendritic cell of the knock-in mouse provides a new bioassay for human prions

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Abstract

Infectious prion diseases initiate infection within lymphoid organs where prion infectivity accumulates during the early stages of peripheral infection. In a mouse-adapted prion infection, an abnormal isoform (PrP^{Sc}) of prion protein (PrP) accumulates in follicular dendritic cells within lymphoid organs. Human prions, however, did not cause an accumulation of PrP^{Sc} in the wild type mice. Here, we report that knock-in mouse expressing humanized chimeric PrP demonstrated PrP^{Sc} accumulations in follicular dendritic cells following human prion infections, including variant Creutzfeldt–Jakob disease. The accumulated PrP^{Sc} consisted of recombinant PrP, but not of the inoculated human PrP. These accumulations were detectable in the spleens of all mice examined 30 days post-inoculation. Infectivity of the spleen was also evident. Conversion of humanized PrP in the spleen provides a rapid and sensitive bioassay method to uncover the infectivity of human prions. This model should facilitate the prevention of infectious prion diseases. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Creuzfeldt–Jakob disease; Prion protein; Knock-in mouse; Follicular dendritic cell; Conversion

Creutzfeldt–Jakob disease (CJD), scrapie, and bovine spongiform encephalopathy (BSE) are transmissible neurodegenerative diseases. Attempts to isolate the agent of scrapie have led to the discovery of a protein, designated prion protein (PrP) [1]. The protease-resistant isoform of PrP (PrP^{Sc}) is implicated in the pathogenesis and transmission of scrapie and CJD [2]. The infectious agent of both CJD and scrapie replicates within lymphoid organs prior to the involvement of the central nervous system [3,4]. Accordingly, at the early stage, PrP^{Sc} can be detected by Western blotting in lymphoid organs [5–7] where follicular dendritic cells

(FDCs) were identified as the sites of PrP^{Sc} accumulations [8].

Sequential transmission experiments revealed that PrP^{Sc} accumulation within FDC was detectable in the early stages of mouse prion infection [9,10]. The preclinical diagnostic value of FDC has been confirmed by examination of the tonsilar tissues of the scrapie-infected sheep [11,12] and the appendix tissues from a patient prior to the onset of variant CJD (vCJD) [13]. Thus, PrP^{Sc} detection in FDC of a mouse model might be a rapid bioassay system for human prions. However, the accumulation of PrP^{Sc} in the FDC did not occur in NZW mice inoculated with materials from human CJD, suggesting that there is a species barrier working in the lymphoreticular system between humans and mouse

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[14]. We initiated present study in an attempt to attest our hypothesis that mouse model of humanized PrP serves to overcome the species barrier. We report herein that the FDC of humanized knock-in mice provides a sensitive and rapid bioassay system for human prions including those from vCJD.

Materials and methods

Production of transgenic mice and knock-in mice. The transgenic construct was composed of three segments isolated or constructed separately: a 5 kilo-base pairs (kb) 5′ fragment, a 13 kb intermediate BamHI fragment, and a 6.5 kb 3′ BamHI/SalI fragment (Fig. 1). The 3′ BamHI/SalI fragment was constructed as follows; a BamHI/SalI fragment of 6.5 kb encompassing exon 3 of the PrP gene was isolated from the 129/SvJ mouse. Genomic DNA was then isolated from the peripheral blood lymphocytes of a Japanese male possessing the codon 129 Val/Met allele. A segment, corresponding to positions 106–817 relative to the start of the human PrP open reading frame (ORF), was amplified by polymerase chain reaction (PCR). The 5′ primer was designed to incorporate a SmaI site at position 115 [15]. Two types of PCR product, one containing a guanine at position 385 for 129Val and another possessing an adenine at position 385 for 129Met, were isolated. The SmaI/BstEII fragment, subcloned from the PCR products, was utilized to replace the corresponding sequence in the 6.5 kb BamHI/SalI mouse genomic fragment. Thus, the ORF has a chimeric human/mouse PrP gene, containing either a Met (designated ChM) or a Val (designated ChV) at codon 129. The intermediate BamHI fragment, isolated from the genomic DNA of I/Ln mice, encompassed exons 1 and 2 of the PrP gene. The fragment was 7 kb shorter than the corresponding segment in the Prnp* allele of the 129/SvJ mouse, due to a deletion in intron 2. The 5 kb 5′ fragment was derived from the Prnp* allele of the 129/SvJ mouse. The entire construct comprised 24.5 kb. Transgenic mice were generated as previously described [16]. Transgenic mice with an ablated background (Prnp$^{0/0}$) were produced by repeated backcrosses with PrP knockout mice [17].

The knock-in construct, reported previously [15], contained minor modifications. The ORF was replaced with the ChM construct. The selection marker for homologous recombination was altered from loxp-MCIneo-PGKgpt-loxp gene to loxp-PGKneo-loxp gene. The homologous recombination, germ-line transmission, and Cre-induced recombination were described previously [15].

Transmission experiments. Human brain specimens were collected from either patients with sporadic, inherited, or infectious prion diseases or patients with Alzheimer's disease. The genotyping of patients was performed by PCR-direct sequencing [18]. Human brain homogenates (10%) were prepared in phosphate buffer by repeated extraction with a sterile disposable homogenizer. Twenty µl of homogenate samples was inoculated intracerebrally. Mice were examined daily following the appearance of central nervous system dysfunction. Histopathology was performed to confirm clinical diagnosis. For the FDC bioassay, 50 µl of homogenate samples was inoculated intraperitoneally. Mice were sacrificed at 14, 30, 45, 60, 75, or 150 days post-inoculation, or after the onset of disease.

Immunohistochemistry. Mouse tissues were immersion-fixed in 10% buffered formalin, then treated with 60% formic acid before embedding in paraffin. Tissue sections were processed for PrP immunohistochemistry by hydrolytic autoclaving pretreatment [19]. The 3F4 monoclonal antibody, the TNT#71 monoclonal antibody [20], and PrP-N antiserum [21] were used as the primary antibodies. Goat anti-mouse or anti-rabbit immunoglobulins labeled with the peroxidase-conjugated dextran polymer, Envision (Dako, Glostrup, Denmark), was used as a secondary antibody.

Splenic tissues were isolated from either Tg-ChV#12 (Prnp$^{0/0}$) or Ki-ChM (Prnp$^{ChM/ChM}$) mice. Thirty µm-thick cryostat sections was fixed for 10 min with 10% formalin. The sections were immunolabeled with PrP-N antiserum. Subsequent steps in the immunofluorescent analysis were described previously [8].

Western immunoblots. PrP^{Sc} was extracted from either spleen or brain with collagenase treatment as previously described [22]. PrP^{C} was measured in the membrane fractions of splenic tissues isolated from either Tg-ChV#12 or Ki-ChM mice [19]. Enhanced chemiluminescent detection (Amersham Pharmacia Biotech, Buckinghamshire, England) was used to visualize Western blots. Anti-PrP antiserum (anti-PrP-N or anti-APC) was used as the primary antiserum [21,23].

Results

Susceptibility to human prions in transgenic mice or knock-in mice

We constructed a chimeric human/mouse PrP gene, designated ChM or ChV. Another chimeric transgene (MHu2M), showing N-terminal and C-terminal chimerism, was reported as a sensitive transgenic mice for human prions [24]. We made ChM or ChV construction demonstrating only C-terminal chimerism. We created transgenic mice (Tg-ChM or Tg-ChV) expressing either ChM or ChV, and knock-in mice (Ki-ChM) expressing ChM. The transgenic lines utilized in this study are designated Tg-ChM#30, Tg-ChV#12, and Tg-ChV #21. The relative expression levels of the recombinant PrP in transgenic mice were determined by the Western blot analysis comparing with the wild-type mice (Table 1).

To measure the transmission of human prions, brain homogenates were prepared from a patient with sporadic CJD (sCJD), iatrogenic CJD (iCJD), or familial
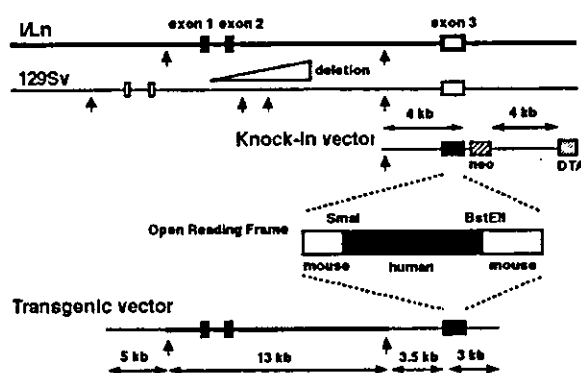

Fig. 1. Genomic structure of prion protein gene, transgenic vector and knock-in vector. The knock-in vector is composed of the 129/Sv mouse genome, with differences in the open reading frame of PrP and the addition of both the loxP-neo-loxP and DTA (diphtheria toxin) genes. The transgenic vector is comprised of the 129/Sv and I/Ln genome, with changes in the open reading frame of exon 3. The open reading frame of ChM or ChV construct was chimeric between SmaI and BstEII site. The ChM or ChV PrP contains the human PrP sequence between codons 23 and 188 and the mouse PrP sequence between codons 189 and 231 after post-translational modifications. Arrow heads show a BamHI site.