resistance to prion replication. However, other prion strains may lead to a more efficient infection in HpL3-4-PrP cells. Therefore, to completely confirm these results, further study using other prion strains is necessary. Experiments which determine whether HpL3-4 cells expressing mammalian species of PrP other than mouse PrP are infected with the corresponding prion species are of interest. We are presently devising a system for the investigation of BSE prion and 263K prion in HpL3-4 cells expressing bovine PrP and hamster PrP, respectively. It is also of interest whether CJD prion infects HpL3-4 cells expressing human PrP.

Additionally, it is also of interest whether by inoculation of HpL3-4 cells expressing PrP with a mutation causing GSS, familial CJD, or FFI (or the corresponding amino acid position in each animal PrP) into mice or hamsters induces prion diseases. Such a study may lead to the production of artificial prions with amino acid changes. Furthermore, it is also of interest whether HpL3-4 cells expressing PrP with such mutations have altered functions of PrP. It will also be interesting to examine whether artificial prions are eliminated by the transfer of various genes. HpL3-4 cells may also be used to study the effect of PrP polymorphisms on the conversion of PrP^{C} to PrP^{Sc}. Additionally, HpL3-4 cells may be useful for examining the species barrier; differences in amino acid sequence between the host PrP^{C} and inoculated PrP^{Sc} influence incubation time (66). Taken together, this experimental system using HpL3-4 cells is advantageous in terms of prion infection studies, because the newly formed PrP^{Sc} is entirely derived from PrP, and thus suited for testing multiple artificial PrP^{C} for their capacity to convert to PrP^{Sc}. Therefore, this *in vitro* model for studying the influence of amino acid sequence changes can be absolutely assayed by an animal bioassay. We want to emphasize that PrP-deficient cells might be a useful tool for a broad range of studies on prion biology such as the search for a candidate for

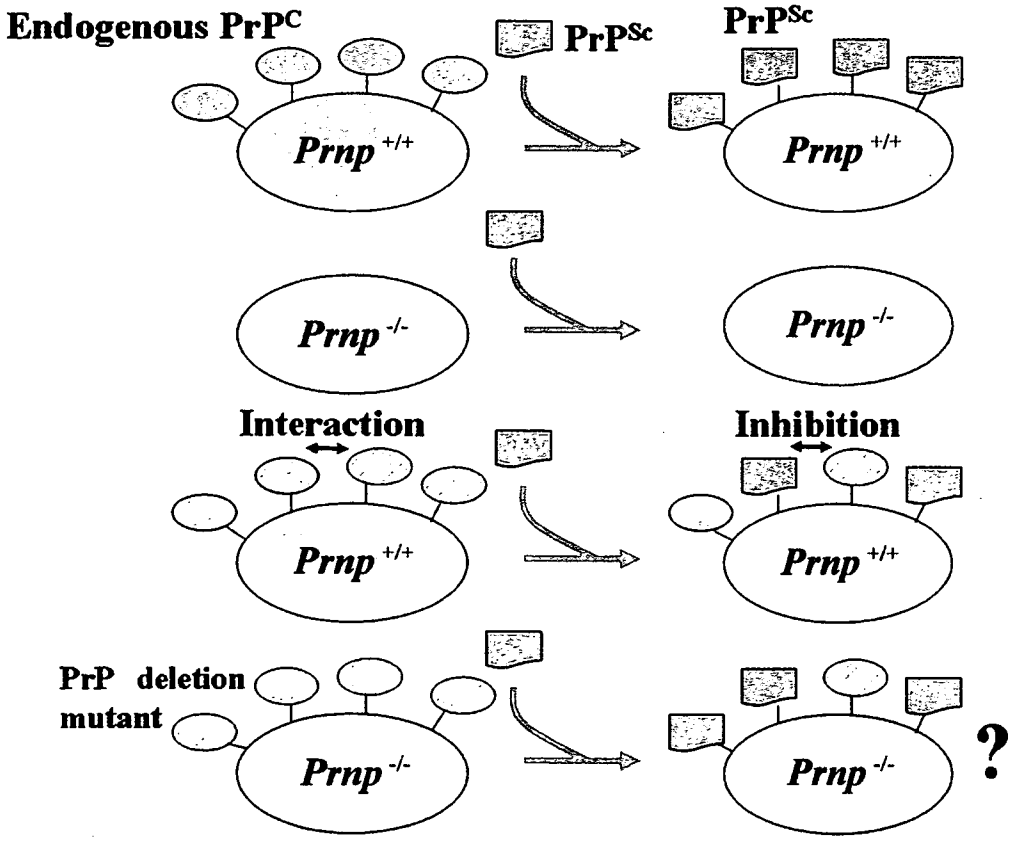

Fig. 5. Usefulness of prion protein gene (*Prnp*)-deficient neuronal cells for the study of prion replication. In cell culture, the effect of deletion and mutation of prion protein (PrP) on prion infection and the abnormal isoform of prion protein (PrP^{Sc}) accumulation has been hampered by the concomitant expression of the endogenous cellular isoform of prion protein (PrP^{C}) when $Prnp^{+/+}$ cells are used. Analysis of the domain of PrP^{C} provides useful information about prion pathogenicity. The domain of PrP^{C} required for prion proliferation was identified by transfecting various PrP deletion mutants into *Prnp*-knockout ($Prnp^{-/-}$) cell lines lacking endogenous PrP^{C} following prion infection.

therapy, mechanism of host cell tropism, and strain characteristics and PrP^{Sc} formation.

Although most cell lines and primary cells express PrP^{C} (58), some including both neuronal and non-neuronal cells, are susceptible to prion infections (68). Therefore, PrP^{C} appears not to be the sole factor determining susceptibility to infection. Other cellular factors may be involved in an efficient infection. In other cases, some conditions may decrease or inhibit the loss of host factors for susceptibility to prion infection (4, 26, 78). Furthermore, most cell lines have prion strain-specificity, indicating that the matching between the host cell and prion strain is restricted.

Conclusions

In order to facilitate and encourage progress in prion biology, the development of immortalized cell lines is important. We have established the immortalized *Prnp*$^{-/-}$ neuronal cell lines HpL3-4, HpL2-1, HpL4-2, and HpL3-2. The characteristics of HpL cell lines represent those of the neuronal precursor cell lineage. Recently, we have established the *Prnp*$^{-/-}$ glial cell lines GpL1 and GpL2 and the *Prnp*$^{-/-}$ neuronal cell lines ZpL1 and ZpL2. Several independent groups have also established *Prnp*$^{-/-}$ neuronal and fibroblastic cell lines. The availability of these *Prnp*$^{-/-}$ cell lines makes it possible to examine which gene products can be replaced by gene transfection to elucidate the functions of PrP. Therefore, the discoveries made using gene transfer approaches with HpL3-4 cell lines should provide valuable new insights into the treatment of prion diseases. Accompanying PrP expression, many genes and proteins may become expressed. By microarray analyses, global changes induced by PrP expression in *Prnp*-deficient could be explored. No doubt within the next few years, much more information will be obtained using this model system in which bulk biochemical approaches as well as single cell studies can be carried out. As the available types of *Prnp*$^{-/-}$ cell lines are limited, the establishment of various types of *Prnp*$^{-/-}$ cell lines will be important. For example, leukocytes and follicular dendritic cells, microglial cells, and Sertoli cells are very interesting for the estab-

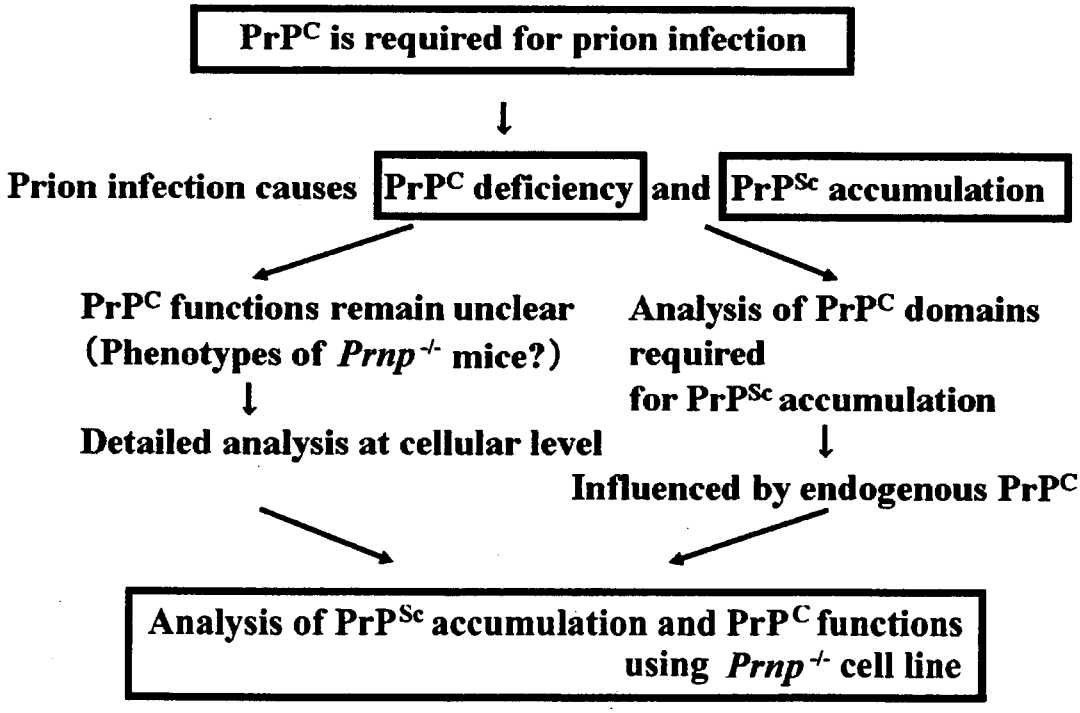

Fig. 6. Schematic presentation of why prion protein gene (*Prnp*)-deficient neuronal cell lines are useful for studying prion replication. The cellular isoform of prion protein (PrP^{C}) is required for prion infection. Prion infection causes the abnormal isoform of prion protein (PrP^{Sc}) to accumulate and PrP^{C} deficiency. PrP^{C} functions remain unclear, because phenotypes of *Prnp*-knockout mice have been ambiguous. Therefore, further detailed analysis using cell lines is necessary. Analysis of PrP^{C} domains at cellular levels provides useful information about mechanisms of PrP^{Sc} replication. Concomitant expression of endogenous PrP^{C} inhibits PrP^{Sc} replication. Therefore, information obtained from analyses of PrP^{Sc} replication and PrP^{C} functions using *Prnp*$^{-/-}$ cell lines will be helpful to answer such questions.

lishment of cell lines. As all tissues express the PrP gene at the mRNA level (58), embryonic stem (ES) cells may also express PrP^C. ES cells can differentiate into many different kinds of cell types. Therefore, the establishment of *Prnp*-deficient ES cells would open the opportunity for detailed study of PrP functions in differentiation. Cocultures of PrP-deficient neuronal cells expressing PrP or not and PrP-deficient glial cells expressing PrP or not could provide insight into the role of the interaction between two cell groups in prion infections, and electrical coupling and the exchange of secreted proteins could be studied in detail using such an *in vitro* system. The electrophysical analysis of PrP-deficient cells is still in its early days (16, 19). Using this system, which allows for a combination of biochemical and single cell approaches, functional relationships could be made between PrP and electrical potential resulting from its underlying ion channels. PrP^C deficiency and PrP^{Sc} accumulation are important events in prion diseases, and PrP-deficient cell lines are useful for studying both (Fig. 6). So far, only limited information about PrP functions and prion infection mechanisms using $Prnp^{-/-}$ cell lines is available. However, considering the many advantages of $Prnp^{-/-}$ cell lines, it should be possible to elucidate such topics. When combined with studies *in vivo* using PrP-knockout mice and transgenic mice, we are able to obtain much greater insight into prion biology.

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Characterization of Prion Susceptibility in Neuro2a Mouse Neuroblastoma Cell Subclones

Masahide Uryu[1], Ayako Karino[2], Yukiko Kamihara[1], and Motohiro Horiuchi*[,1]

[1]*Laboratory of Prion Diseases, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060–0818, Japan, and* [2]*Department of Veterinary Public Health, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan*

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Abstract: **In this study, we established Neuro2a (N2a) neuroblastoma subclones and characterized their susceptibility to prion infection. The N2a cells were treated with brain homogenates from mice infected with mouse prion strain Chandler. Of 31 N2a subclones, 19 were susceptible to prion as those cells became positive for abnormal isoform of prion protein (PrP^{Sc}) for up to 9 serial passages, and the remaining 12 subclones were classified as unsusceptible. The susceptible N2a subclones expressed cellular prion protein (PrP^{C}) at levels similar to the parental N2a cells. In contrast, there was a variation in PrP^{C} expression in unsusceptible N2a subclones. For example, subclone N2a-1 expressed PrP^{C} at the same level as the parental N2a cells and prion-susceptible subclones, whereas subclone N2a-24 expressed much lower levels of PrP mRNA and PrP^{C} than the parental N2a cells. There was no difference in the binding of PrP^{Sc} to prion-susceptible and unsusceptible N2a subclones regardless of their PrP^{C} expression level, suggesting that the binding of PrP^{Sc} to cells is not a major determinant for prion susceptibility. Stable expression of PrP^{C} did not confer susceptibility to prion in unsusceptible subclones. Furthermore, the existence of prion-unsusceptible N2a subclones that expressed PrP^{C} at levels similar to prion-susceptible subclones, indicated that a host factor(s) other than PrP^{C} and/or specific cellular microenvironments are required for the propagation of prion in N2a cells. The prion-susceptible and -unsusceptible N2a subclones established in this study should be useful for identifying the host factor(s) involved in the prion propagation.**

Key words: **Neuro2a, Prion, PrP, Susceptibility**

Transmissible spongiform encephalopathies, so-called prion diseases, are fatal neurodegenerative disorders that include scrapie in sheep and goats, bovine spongiform encephalopathy, and Creutzfeldt-Jakob disease in humans. The major component of causative agent of prion diseases, prion, is thought to be an abnormal isoform of prion protein (PrP^{Sc}). PrP^{Sc} is generated from a normal cellular prion protein (PrP^{C}) by certain post-translational modifications and the process in the conversion of PrP^{C} to PrP^{Sc} is considered to be a central event in pathogenesis of prion diseases (31). PrP^{C} is a sialo-glycoprotein expressed on the cell surface as a glycosyl-phosphatidylinositol anchoring protein (36). Cell biological studies have revealed that mature PrP^{C} on the cell surface acts as a substrate for the PrP^{Sc} biosynthesis, and formation of PrP^{Sc} takes place either at the cell membrane or during the endocytic pathway (3, 8, 37). Depletion of cholesterol inhibits PrP^{Sc} formation in prion-infected cells (2, 38), and the co-existence of PrP^{C} and PrP^{Sc} in lipid rafts or caveolae-like domains suggests that cholesterol- and sphingolipid-enriched membrane microdomains are sites for the interaction between PrP^{C} and PrP^{Sc} (29, 41).

Although PrP^{C} is essential for the propagation of prion and the development of prion diseases (6), other host factors are thought to be involved in the PrP^{Sc} formation, i.e., prion replication. Studies using chimeric

**Address correspondence to* Dr. Motohiro Horiuchi, Laboratory of Prion Diseases, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo, Hokkaido 060–0818, Japan. Fax: +81–11–706–5293. E-mail: horiuchi@vetmed.hokudai.ac.jp

Abbreviations: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HS, heparan sulfate; LRP/LR, laminin receptor precursor/laminin receptor; MAb, monoclonal antibody; N2a, Neuro2a; PBS, phosphate-buffered saline; PrP, prion protein; PrP^{C}, cellular prion protein; PrP^{Sc}, abnormal isoform of prion protein; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WB, Western blotting.

and point mutants of PrP have suggested that a host factor designated Protein X is required for prion propagation (18, 40), although its identity remains unknown. To date, Bcl-2 (21), laminin receptor precursor/laminin receptor (LRP/LR) (32), neural cell adhesion molecule (35), and several other proteins have been identified as possible counterparts of PrP^C. Plasminogen has been reported to bind PrP^{Sc} (11). In addition, a reduction in the level of LRP/LR inhibits PrP^{Sc} formation in prion-infected cells, suggesting that the LRP/LR has a direct or indirect role in prion propagation (22). The biological significance of other proteins in prion propagation remains unclear (34, 35).

Recently, cysteine proteases, such as calpain, and cathepsin B and L were reported to modulate PrP^{Sc} formation (23, 42). Furthermore, inhibitors of c-Abl tyrosine kinase and mitogen-activated protein kinase kinase are reported to accelerate PrP^{Sc} degradation in prion-infected cells (10, 30). Thus, changes in the cellular microenvironment, by interfering with cellular signaling, may also affect prion propagation. These findings suggest the involvement of other host factors for prion propagation. Identification of such host factors and cellular microenvironments involved in prion propagation is of great interest not only for understanding the basic mechanisms of prion propagation but also for finding new therapeutic targets.

Comparative analyses between permissive and non-permissive conditions for prion propagation will facilitate the identification of the host factors involved in prion propagation. In the present study, we established subclones of mouse Neuro2a (N2a) neuroblastoma cells and analyzed their susceptibility to prion. The prion-susceptible and -unsusceptible subclones established in this study should be useful for identifying host factors involved in prion replication.

Materials and Methods

Cell culture and cloning of the cells. N2a cell line (American Type Culture Collection CCL-131, 58th passage at the purchase) was grown in Dulbecco's modified Eagle's medium with high glucose (DMEM; ICN Biomedicals), 10% fetal bovine serum (FBS), and non-essential amino acids. N2a subclones were obtained by limiting dilution.

Inoculation of prion to N2a cells. Mouse prion strain Chandler was propagated in ICR mice (CLEA Japan, Inc.). The brains of mice at the terminal stage of the disease were homogenized in phosphate-buffered saline (PBS) at 10% (w/w) and the homogenates were stored at −30 C until use. The brain homogenate was diluted to 2% with the medium, and 500 μl was added to N2a cells in 60-mm dishes containing 1 ml of medium. After 24 hr, the medium was refreshed, and cells were serially passaged every 3 to 4 days at a 1:10 dilution.

Detection of PrP^{Sc}. Preparation and detection of PrP^{Sc} in the prion-infected cells were carried out as described previously (19, 20) with slight modifications. Cells were lysed in lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM EDTA), and small aliquots of the lysates were stored for the determination of the protein concentration. The remaining lysates were digested with 20 μg/ml proteinase K at 37 C for 20 min. The digestion was stopped by the addition of Pefabloc (Roche) to 5 mM. The mixture was adjusted to 0.3% phosphotungstic acid by addition of a 5% solution and then incubated for 30 min at 37 C with constant rotation. PrP^{Sc} was then collected by centrifugation at 20,000 × *g* for 20 min and subjected to SDS-PAGE followed by Western blotting (WB). Blots were probed with monoclonal antibody (mAb) 31C6 (20) and horseradish peroxidase-conjugated sheep $F(ab')_2$ fragment of anti-mouse IgG (Amersham Bioscience). The specific bands were visualized with ECL Western Blotting Detection Reagents (Amersham Bioscience) and a LAS-3000 chemiluminescence image analyzer (Fujifilm). Quantitative analyses of the blots were carried out with Image Reader LAS-3000 version 1.11 (Fujifilm).

Flow cytometry. Flow cytometric analysis was performed as described previously (19).

PrP^{Sc} binding assay. Cells were seeded at 2.5×10^4 cells/well in 6-well plates and grown for 48 hr. Cells were then fed with 500 μl of the fresh medium and inoculated with 250 μl of 0.4 to 2% prion-infected mouse brain homogenate diluted with medium, and kept for 3 hr at either 37 C or on ice with occasional tilting. After the incubation, cells were washed with PBS three times, and bound PrP^{Sc} was detected as described in detection of PrP^{Sc}.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated from the cells with TRIzol Reagent (Invitrogen Life Technologies). First strand cDNA was synthesized from the total RNA using a First-Strand Synthesis Kit (Amersham Biosciences) according to the manufacturer's instructions. Real-time *Taq*Man PCR assays were performed to determine the relative quantity of mouse PrP gene expression. Amplification reaction mixtures contained template cDNA, 1X pre-designed set of primers and a *Taq*Man probe targeting the boundary between exons 1 and 2 of the PrP gene (*Taq*Man Gene Expression Assays No. Mm-00448389), and 1X *Taq*-

Man Universal PCR Master Mix (Applied Biosystems) in a final reaction volume of 20 μl. The amplification profile was monitored with an ABI PRISM 7900HT (Applied Biosystems), and the relative quantity was determined by the standard curve method (1) using SDS Plate Utility version 2.1 (Applied Biosystems). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was monitored as an endogenous control using *Taq*Man Rodent-GAPDH Control Reagents (Applied Biosystems).

Cellular cholesterol content of N2a subclones. Cells were seeded in 6-well plates in DMEM containing 10% FBS. After 48 hr, the medium was changed to Opti-MEM (Gibco) and cells were kept for additional 24 hr. Then, cells were washed three times with PBS and lysed with PBS containing 0.1% Triton X-100. The lysates were frozen at −30 C until use. The lysates were clarified by centrifugation at 20,000 × *g* for 15 min at 4 C, and the resulting supernatants were assayed for cholesterol using an Amplex Red Cholesterol Assay Kit (Molecular Probes) according to the manufacturer's instructions. Fluorescence was measured with a fluorescence microplate reader ARVO-SX (Wallac) using excitation at 560 nm and detection at 580 nm.

Stable expression of $MoPrP^C$. Eukaryotic expression vector, pRc/EF-MoPrP (M. H. and A. K. manuscript in preparation), which contains a mouse PrP cDNA expression unit driven by peptide chain elongation factor 1α promoter (27) along with the bacterial aminoglycoside phosphotransferase gene (G418 resistant gene) expression unit, was introduced into N2a cells with FuGENE 6 (Roche). The transfected cells were cultured in the presence of 0.3 mg/ml G418 (Gibco), and G418-resistant cells were selected. The cells were stained with anti-PrP mAb as described for the flow cytometric analysis (19), and cell sorting was performed using an EPICS ALTRA flow cytometer (Beckman Coulter). The cells with fluorescence intensities ranging from 100 to 500 were recovered and cultured with DMEM. Cells passaged more than 3 times were used for prion infection experiments.

Results

Prion-Susceptibility of N2a Subclones

We isolated 31 N2a subclones by limiting dilution and examined them for susceptibility to prion. The N2a subclones were inoculated with scrapie Chandler strain-infected mouse brain homogenates, and prion-susceptibility was determined by the presence of PrP^{Sc} during nine passages after inoculation. Figure 1 shows the representative results for PrP^{Sc} detection in the N2a subclones at the third, sixth, and ninth passages after inoculation. Of 31 N2a subclones, 19 (N2a-2, -3, -5, -6, -7, -17, -21, -22, and -25 in Fig.1) were judged to be prion-susceptible, and the remaining 12 subclones (N2a-1, -4,

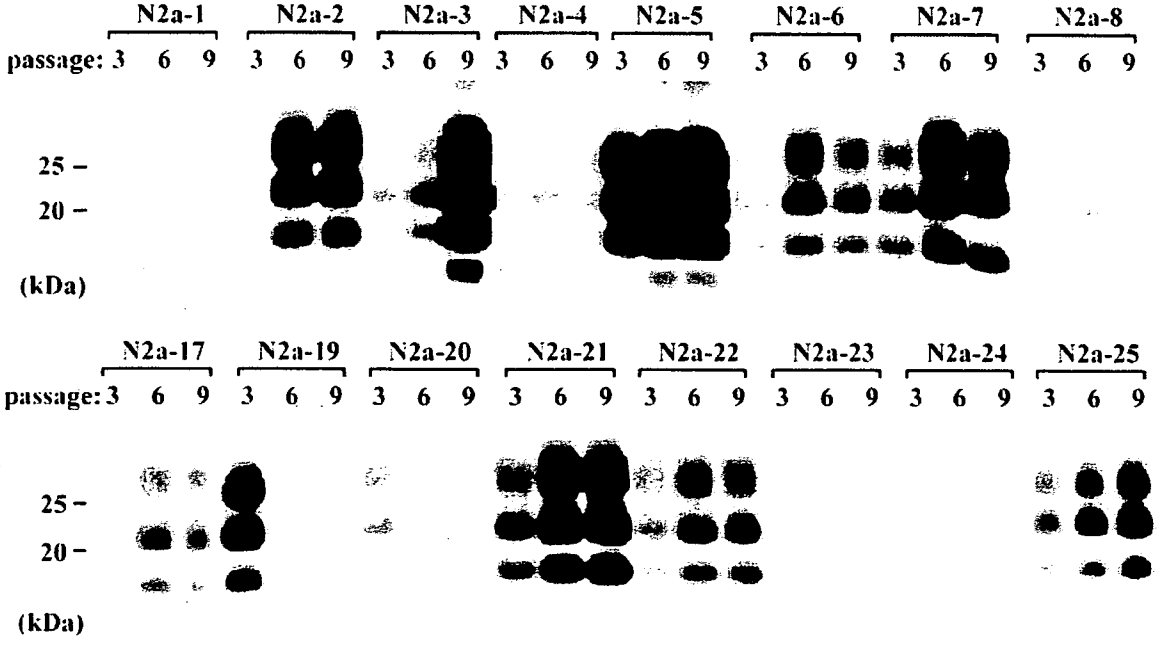


Fig. 1. Detection of PrP^{Sc} in N2a subclones inoculated with prion. N2a subclones were inoculated with 2% brain homogenate from mice infected with Chandler strain, and then consecutively passaged up to nine times. The presence of PrP^{Sc} was examined at the third, sixth, and ninth passages by WB. PrP^{Sc}-enriched sample derived from 0.1 mg of the cell lysates was loaded on each lane, and PrP^{Sc} was detected with mAb 31C6. Results of representative N2a subclones are indicated. Molecular markers are indicated on the left.

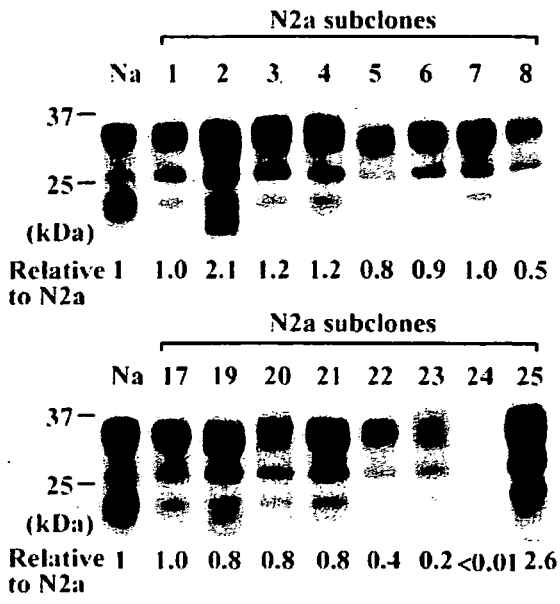


Fig. 2. Expression of PrP^C in N2a subclones. Ten micrograms of cell lysates were loaded on each lane, and PrP^C was detected with mAb 31C6. Na indicates parental N2a cells, and the numbers on the top of the images indicate the number of N2a subclones. Results of representative N2a subclones are indicated. The luminescence intensities were quantified with a LAS-3000 chemiluminescence image analyzer, and the numbers below the images indicate the mean PrP^C expression relative to that in the parental N2a cells (n = 2).

-8, -19, -20, -23, and -24 in Fig. 1) were classified as unsusceptible because they were negative for PrP^{Sc} at all passages examined. Subclones N2a-3 and -5, which showed intense PrP^{Sc} bands at the ninth passage (Fig. 1), were positive for PrP^{Sc} for more than 30 serial passages (data not shown). Thus, we used N2a-3 and -5 as representative prion-susceptible N2a subclones in the following experiments.

Expression of PrP^C and PrP Gene

Because PrP^C is essential for the propagation of prion and formation of PrP^{Sc} (5–7), we first investigated PrP^C expression in N2a subclones by WB (Fig. 2). As expected, prion-susceptible subclones expressed 0.4- to 2.6-fold as much PrP^C as the parental N2a cells (Fig. 2). Among the prion-susceptible subclones, N2a-22 showed the lowest PrP^C expression; it expressed only 0.4 ± 0.1-fold as much PrP^C as the parental N2a cells. In contrast, PrP^C expression varied among the prion-unsusceptible subclones. For instance, subclones N2a-1, -4, -8, -19, and -20 expressed similar level of PrP^C as the parental N2a cells and susceptible subclones, whereas N2a-23 and -24 had lower PrP^C expression than the parental cells, and actually, subclone N2a-24 expressed only one one-hundredth as much PrP^C as the parental

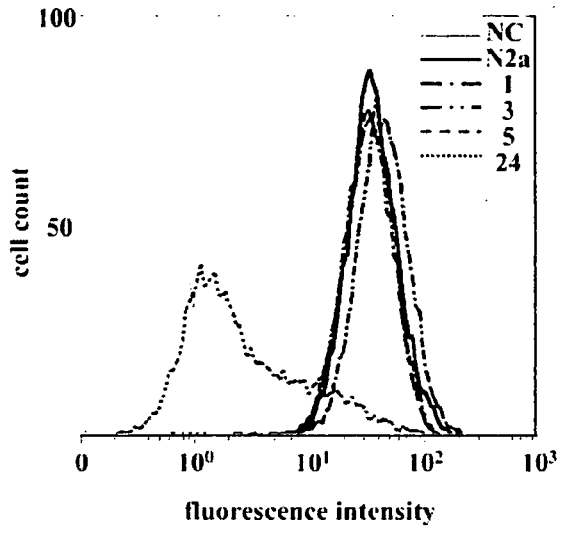


Fig. 3. Cell surface expression of PrP^C in N2a subclones. PrP^C on the cell surface of N2a and subclones N2a-1, -3, -5, and -24 was detected by flow cytometry. NC indicates the fluorescence intensity of N2a cells stained with negative control mAb P1-284 as a primary antibody. The PrP^C expression levels relative to that in parental N2a cells were calculated from mean fluorescence intensities and are shown in Table 1.

Table 1. Levels of PrP^C and PrP mRNA expression in N2a subclones

N2a subclone	Relative to parental N2a cells		
	Total PrP^C [a]	PrPC on cell surface[a]	PrP mRNA[a]
N2a-1	1.0 ± 0.7	1.1 ± 0.06	1.4 ± 0.4
N2a-3	1.2 ± 0.5	1.3 ± 0.07	1.8 ± 0.7
N2a-5	0.8 ± 0.2	1.0 ± 0.06	1.5 ± 0.4
N2a-24	0.01 ± 0.004	0.07 ± 0.006	0.1 ± 0.04

[a] Means ± S.D. (n=3) relative to parental N2a cells.

N2a cells.

Flow cytometric analysis showed that susceptible subclones N2a-3 and -5, and the unsusceptible subclone N2a-1 expressed PrP^C on their cell surfaces, whereas, consistent with the WB analysis, the level of PrP^C on the cell surface of the N2a-24 subclone was less than one-tenth of that in parental N2a cells (Fig. 3, Table 1). Furthermore, quantitative RT-PCR analysis also showed that the expression of the PrP gene in N2a-1, -3, and -5 was 1.4-, 1.8-, and 1.5-fold higher than in the parental N2a cells, respectively, whereas N2a-24 expressed only one-tenth as much PrP mRNA as the parental N2a cells (Table 1). Thus, the low level of PrP^C expression in N2a-24 is probably due to inefficient transcription of the PrP gene. These results indicated that there are two types of prion-unsusceptible N2a subclones: one (e.g., N2a-1) that expresses a level of PrP^C similar to prion-susceptible N2a cells, and another (e.g., N2a-24) that

expresses lower levels of PrP^C than susceptible cells.

Cellular Cholesterol Content of N2a Subclones

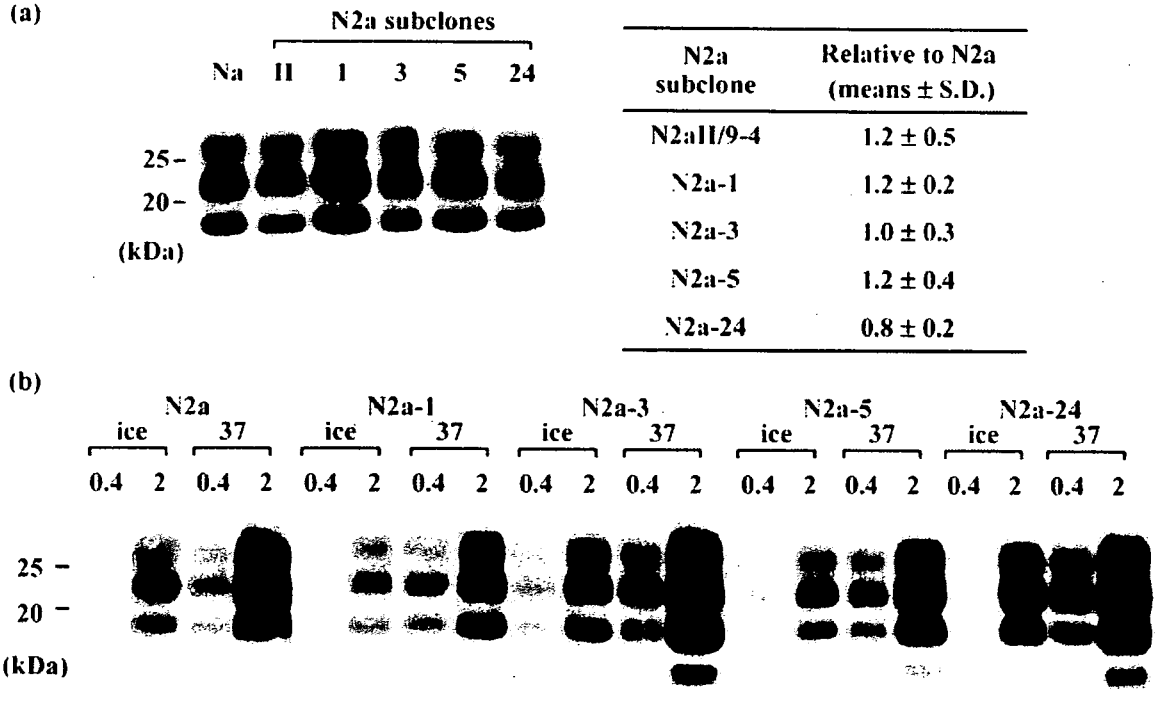
The cellular cholesterol is reported to be important for the accumulation of PrP^{Sc} in prion-infected N2a cells (2, 38). We therefore measured the cellular cholesterol content in representative N2a subclones; however, there was no significant difference in the cellular cholesterol contents between the parental N2a cells (8.3 ± 0.9 μg/mg protein), prion-unsusceptible subclones N2a-1 and -24 (8.6 ± 0.7 and 9.3 ± 0.9 μg/mg protein, respectively) and susceptible subclones N2a-3 and -5 (9.2 ± 0.4 and 7.9 ± 0.9 μg/mg protein, respectively).

Binding of PrP^{Sc} to N2a Subclones

Binding of PrP^{Sc} to the cells is considered to be the initial step in the prion infection after cells are inoculated with prion-infected brain homogenates. Thus, we examined the binding of PrP^{Sc} to N2a subclones to investigate whether the binding step is involved in determining the prion-susceptibility and whether the expression of PrP^C affects PrP^{Sc} binding. Figure 4a shows the representative results for the binding of PrP^{Sc} to N2a subclones at 37 C. PrP^{Sc} bound equally to prion-susceptible (N2a-3 and -5) and unsusceptible N2a subclones (N2a-1 and -24). In addition, there was no significant difference in the amount of bound PrP^{Sc} among N2a subclones and N2aII/9-4 that of stably overexpressed mouse PrP^C (Fig. 5a). In addition, we observed a dose-dependent increase in PrP^{Sc} binding both on ice and at 37 C, regardless of the prion susceptibility or level of PrP^C in the cells (Fig. 4b). The increase of bound PrP^{Sc} at 37 C suggests that a part of bound PrP^{Sc} may be internalized during the incubation. These results revealed that prion susceptibility of these subclones is not determined by the binding and/or uptake of PrP^{Sc} and that PrP^C is not directly involved in the binding and/or uptake of PrP^{Sc}.

Effect of Exogenously Introduced PrP^C on Prion Susceptibility

We speculated that the low level of PrP^C expression in N2a-24 may explain its inability to support prion replication. To examine this possibility, we transfected N2a-1 and -24 cells with the mouse PrP gene expression vector pRc/EF-MoPrP and selected stable transformants in the presence of G418. We also used the PrP^C-overexpressing N2a subclone N2aII/9-4, which is a stable transformant by pRc/EF-MoPrP, as a control for G418-resistant prion-susceptible cells. Quantitative analysis of



N2a subclone	Relative to N2a (means ± S.D.)
N2aII/9-4	1.2 ± 0.5
N2a-1	1.2 ± 0.2
N2a-3	1.0 ± 0.3
N2a-5	1.2 ± 0.4
N2a-24	0.8 ± 0.2

Fig. 4. Binding of PrP^{Sc} to N2a subclones. (a) Representative results for the binding of PrP^{Sc} to N2a cells. The cells were inoculated with 2% brain homogenate from mice infected with Chandler strain and then incubated at 37 C for 3 hr. Bound PrP^{Sc} was detected as described in "Materials and Methods." The binding of PrP^{Sc} relative to that in parental N2a cells is shown in the table on the right. Values in the table are the means ± S.D. from three independent experiments. Na, parental N2a cells; II, N2aII/9-4; 1, 3, 5, and 24, N2a subclone-1, -3, -5, and -24, respectively. (b) Dose- and temperature-dependent binding of PrP^{Sc}. The cells were inoculated with 0.4 and 2% brain homogenate from mice infected with Chandler strain and incubated at 37 C or on ice for 3 hr.

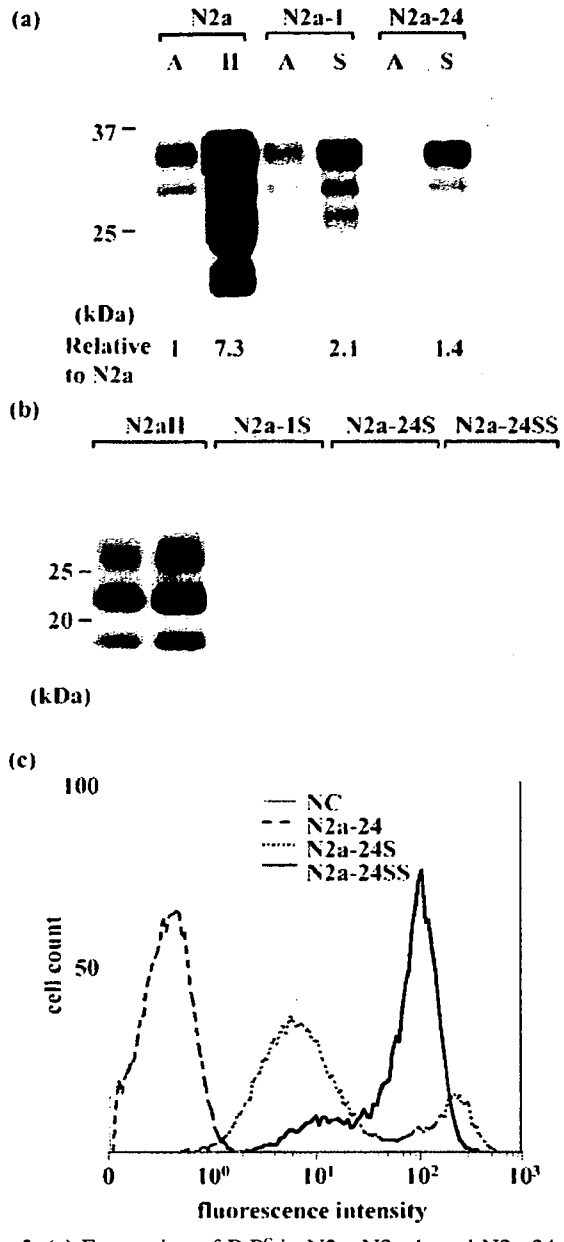


Fig. 5. (a) Expression of PrP^C in N2a, N2a-1, and N2a-24 cells. N2a-1 and N2a-24 subclones were transfected with pRc/EF-MoPrP, and G418-resistant cells were selected. A indicates authentic cells, and S indicates G418-resistant cells. The G418-resistant N2a subclone II/9-4 was used as a control for G418-resistant prion-susceptible cells. Ten micrograms of the cell lysates were loaded on each lane, and PrP^C was detected with mAb 31C6. The luminescence intensities were quantified, and the numbers below the image indicate the amount of PrP^C relative to that in N2a cells. (b) Effect of PrP^C expression on prion susceptibility of subclones N2a-1 and N2a-24. N2aII/9-4, N2a-1S, and N2a-24S cells were inoculated with 2% brain homogenate from mice infected with Chandler strain. PrP^{Sc} was detected after the sixth consecutive passage. The results of duplicate samples are shown. (c) Cell surface expression of PrP^C in N2a-24 cells. PrP^C on the cell surface of N2a-24, 24S, and 24SS (selected by cell sorter) was detected by flow cytometry. NC indicates the fluorescence intensity of N2a-24S cells stained with negative control mAb P1-284 as a primary antibody.

WB revealed that the G418-resistant N2a-1 (N2a-1S) and N2a-24 (N2a-24S) cells expressed 2.1- and 1.4-fold more PrP^C than the parental N2a cells (Fig. 5a). In addition, the G418-resistant N2a subclone N2aII/9-4 expressed 7.3-fold more PrP^C than parental N2a cells. Flow cytometric analysis revealed that 39% of N2a-1S (data not shown) and 32% of N2a-24S cells expressed higher surface level of PrP^C than the corresponding authentic subclones (Fig. 5c). These cells were inoculated with brain homogenate from prion-infected mice and then examined for PrP^{Sc} after six serial passages. PrP^{Sc} was detected in neither N2a-1S nor N2a-24S cells but was detected in N2aII/9-4 cells, suggesting that stable expression of PrP^C did not confer the prion susceptibility to N2a-1 and N2a-24 cells (Fig. 5b). The fact that 32% of G418-resistant N2a-24S cells expressed elevated levels of PrP^C might cause the inefficient prion replication in N2a-24S cells. To exclude this possibility, we collected PrP^C-overexpressing cells from G418-resistant N2a-24S cells by cell sorting. In this cell population (N2a-24SS), 79% of cells expressed elevated cell surface levels of PrP^C (Fig. 5c); however, PrP^{Sc} was not detected in these cells when they were inoculated with prion-infected brain homogenates (data not shown).

Discussion

N2a cells have been reported to be composed of cells with different susceptibilities to prion infection (4, 9). One of the determinants of prion susceptibility is the expression of PrP^C (3–5), but the quantitative relationship between PrP^C expression and prion susceptibility is not well understood. We found considerable variation in the expression of PrP^C in the N2a subclones established in this study. In particular, subclone N2a-24 expressed less than one one-hundredth as much PrP^C as the parental N2a cells. Among the prion-susceptible N2a subclones, N2a-22 showed the lowest expression of PrP^C but still expressed 0.4-fold as much PrP^C as the parental N2a cells, suggesting that a substantial amount of PrP^C is required to support prion propagation in N2a subclones. On the other hand, as represented by subclone N2a-1, some prion-unsusceptible subclones expressed similar levels of PrP^C as the parental N2a cells and other prion-susceptible subclones. These results are consistent with the idea that the expression of PrP^C is a critical factor but they also indicate that other factors and/or cellular microenvironments also determine the susceptibility of N2a cells to prion (4).

Exposing cells to prion-infected materials such as brain homogenates usually starts infection, and many cell lines can bind and internalize exogenous PrP^{Sc} (24, 25, 39). Recently, Hijazi et al. showed that the similar

levels of PrP^{Sc} bind to wild-type Chinese hamster ovary (CHO) cells, which do not express detectable levels of PrP^C, and CHO cells overexpressing PrP^C (13). Magalhaes et al. also showed that uptake of exogenous PrP^{Sc} does not require the presence of endogenous PrP^C (25). In agreement with these observations, our results indicated that the binding of exogenous PrP^{Sc} to the N2a cells was neither related to the prion susceptibility nor the level of PrP^C expression. This also indicated that the binding of PrP^{Sc} observed in this study did not account for the specific binding to PrP^C but binding of PrP^{Sc} to cell surface, although molecules and microenvironments involved in the binding are unclear. Cellular heparan sulfate (HS) has been reported to be involved in the uptake of exogenous PrP^{Sc} following productive prion propagation in cells (14). In addition, the complex of LRP/LR and HS proteoglycan has been suggested to act as a receptor for PrP^{Sc} (12, 15). We did not address the role of LRP/LR and HS proteoglycan in the binding of PrP^{Sc} to N2a subclones; however, when the binding assay was carried out at 37 C, PrP^{Sc} binding to the N2a subclones was increased regardless of the PrP expression level or prion susceptibility, suggesting that the uptake of PrP^{Sc} may not be a major determinant of prion susceptibility in the N2a subclones. A recent report showed that the trafficking of exogenous PrP^{Sc} in mouse septum neuron-derived SN56 cells differed according to the ability of prion to propagation in the cells (25). This suggested that prion susceptibility may be determined by events occurring after the uptake of PrP^{Sc}. Therefore it would be of interest to analyze the fate of PrP^{Sc} after its binding to prion-susceptible and -unsusceptible N2a subclones.

In this study, we obtained a subclone, N2a-24, in which the expression of PrP^C was much lower than in the parental N2a cells and other subclones. We confirmed that the low PrP^C expression was due to inefficient expression of PrP mRNA. To address whether the low level of PrP gene expression is caused by genomic mutations in the regions involved in transcription of the PrP gene, we used long PCR to amplify the 5′- and 3′-flanking regions of exon 1 (nucleotides 6055–12058 in accession number U29186), which contain regions influencing PrP gene expression (17, 33) and carried out a direct sequencing of the amplified products. We did not find any nucleotide differences in this region between authentic N2a cells and subclones N2a-5 and N2a-24. We further analyzed the nucleotide sequences of exon 3 and its flanking regions (nucleotides 27096–30189 in accession number U29186), but again, the sequences were identical in authentic N2a cells and subclones N2a-5 and N2a-24 (data not shown). Thus, the low level of PrP gene expression in N2a-24 was not due to a mutation in the PrP gene but rather was probably due to a deficiency in the cellular machinery used for transcription of the PrP gene. In hepatic stellate cells, which express trace amounts of PrP^C, the expression of PrP^C increased in response to CCl_4-induced hepatic damage (16). In addition, PrP^C expression is increased in peripheral nerves during axon regeneration (28). These studies raised the possibility that the regulation of PrP^C expression plays a role in neural and hepatic regeneration. In addition, PrP gene expression is developmentally regulated (26), but the molecular mechanism controlling PrP gene expression remains unclear. Thus, the low expression of the PrP gene in subclone N2a-24 despite a lack of mutations in the genomic region of the PrP gene, suggests that this subclone will be useful for analyzing the mechanism of PrP gene expression.

Host factors other than PrP^C may be involved in the PrP^{Sc} formation, i.e., prion replication, but little is known so far. A comparison of prion-susceptible and -resistant tissues could help to identify such host factors; however, large differences exist in gene expression profiles between tissues and thus comparison between tissues would complicate the identification of factors influencing prion susceptibility. A fine comparison would be possible, however, if the compared samples have similar biological properties. Hence, the N2a subclones established in this study should be useful for identifying host factors and cellular microenvironments influencing prion replication. Comprehensive comparison of these cells by transcriptomic and proteomic analyses will be useful in this regard and should help to elucidate the molecular mechanism of prion replication.

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NOTE *Virology*

Frequencies of PrP Genotypes in Meat Breeds of Japanese Sheep and Trail of Selective Breeding in Experimental Sheep Flock

Jiro OHARA[1], Tetsuro TOGARI[2], Aya KUROKAWA[1], Junko MAEDA[1], Naotaka ISHIGURO[3], Hidefumi FURUOKA[4] and Motohiro HORIUCHI[1]

[1]*Laboratory of Prion Diseases, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060–0818,* [2]*Poultry and Sheep Research Unit, Hokkaido Animal Research Center, Shintoku 081–0038,*
[3]*Laboratory of Food and Environmental Hygiene, Faculty of Applied Biological Science, Gifu University, Yanagito, Gifu 501–1193 and*
[4]*Department of Pathological Science, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro 080–8555, Japan*

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ABSTRACT. The selection of sheep with scrapie-resistant PrP genotypes is one of the control measures for transmissible spongiform encephalopathies in ruminants. In this study, we investigated the frequencies of PrP genotypes in meat breeds in Japan. The nationwide surveillance revealed that nearly half of the Suffolk sheep, a major meat breed in Japan, carried scrapie-susceptible AQ/AQ and AQ/VQ genotypes. In addition, the VQ haplotype, which confers high susceptibility to scrapie within sheep, was also found in Poll Dorset sheep. A trial of selective breeding using sires with scrapie-resistant PrP genotypes AQ/AR and AR/AR could raise the ratio of scrapie-resistant sheep from less than 50% to 80% within 3 years. However, the use of sires with the AR/AR genotype and the selection of ewes would be required to achieve a higher ratio of scrapie-resistant sheep.
KEY WORDS: prion, scrapie, transmissible spongiform encephalopathy.

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Scrapie is a fatal neurodegenerative disease of sheep and goats, and is classified as a transmissible spongiform encephalopathy (TSE). In the sheep prion protein (PrP) gene, nucleotide polymorphisms causing amino acid substitutions have been reported in more than 20 codons [14]. Among these, polymorphisms at codons 136 (valine [V]/alanine [A]) and 171 (glutamine [Q]/arginine [R]/histidine [H]/lysine [K]) are closely associated with susceptibility to scrapie. The VQ haplotype confers a high susceptibility to the disease, and the wild-type AQ haplotype also confers disease susceptibility while the AR haplotype is associated with resistance to scrapie [16]. Due to the clear genetic susceptibility to the disease, the scrapie susceptibility of sheep and flocks can be estimated by analyzing PrP genotypes.

In Japan, there are around 20,000 sheep, more than half of which are being reared in Hokkaido. Although the sheep population is relatively small in Japan, there are sporadic occurrences of scrapie. In a previous study, we analyzed the PrP genotypes of Japanese Suffolk sheep, although their numbers are limited [18]. It is important to estimate the scrapie susceptibility of sheep flocks for the control of scrapie prevalence; however, the recent and precise frequencies of PrP genotypes in Japanese sheep remain unclear.

Breeding programs that aim to increase the population of scrapie-resistant sheep by eliminating the scrapie-susceptible VQ haplotype and increasing the scrapie-resistant AR haplotype are considered as part of the measures to eradicate scrapie. In European countries including the UK, the Netherlands, France, and Germany, breeding programs have been started [9, 11]. However, in Japan, a breeding program has not been implemented yet.

In order to contribute to the control measures for TSE in Japan, we carried out large-scale PrP genotyping of meat breeds of sheep in Japan. In addition, to estimate the efficacy of selective breeding using sires carrying the scrapie-resistant PrP genotype, we have conducted a trial of selective breeding using an experimental sheep flock since 2002. Here we report the frequencies of sheep PrP genotypes in Japan and the transition of the frequencies of PrP genotypes by selective breeding using sires carrying the AR haplotype.

For the investigation of the frequencies of PrP genotypes in Japan, a total of 880 sheep, including 648 Suffolk, 92 Poll Dorset, 20 Southdown, 44 cross-bred Suffolk and Southdown, and 76 cross-bred Poll Dorset and Southdown sheep, were used in this study. The genomic DNA of 195 Suffolk sheep was obtained from the obex samples that were used for active surveillance of scrapie in sheep, and other DNA samples were obtained from the venous blood collected at the farms. These samples were collected between 2003 and 2005. For the evaluation of the efficacy of selective breeding, blood samples were collected from 884 Suffolk sheep in the experimental flock of Shintoku Hokkaido Animal Research Center (HARC). Samples at HARC were collected during 2001–2006. The QIAamp DNA Blood Mini Kit (QIAGEN) and the DNeasy Tissue Kit (QIAGEN) were used for purification of genomic DNA from blood and obex, respectively.

DNA fragments corresponding to the open reading frame of the PrP gene were amplified using PCR with primers sPrP104 and sPrP105 (Table 1). After amplification, the excesses of primers and nucleotides were removed using an S-300HR spin column (GE Healthcare), and the amplified

* CORRESPONDENCE TO: HORIUCHI, M., Laboratory of Prion Diseases, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060–0818, Japan.
e-mail:horiuchi@vetmed.hokudai.ac.jp

Table 1. Nucleotide sequencing of primers and Taqman probes used in this study

Primers for PCR and/or nucleotide sequencing	
sPrP104	5'-CATCATGGTGAAAAGCCACATAGGCAG-3'
sPrP105	5'-ATGAAAACAGGAAGGTTGCCCCTATCC-3'
sPrP109	5'-GGTCAAGGTGGTAGCCACAG-3'
sPrP110	5'-GTCAGTTTCGGTGAAGTTCTC-3'
Primers for SNPs analysis	
356F	5'-CTGCAGCTGGAGCAGTGGTA-3'
450R	5'-GTCCTCATAGTCATTGCCAAAATGTATA-3'
467F	5'-ACATGTACCGTTACCCCAACCA-3'
553R	5'-TGTTGACACAGTCATGCACAAAG-3'
Taqman probes for SNPs analysis[a)]	
136A	5'-FAM-TGCTGGGAAGTGCCA-MGB-3'
136V	5'-VIC-ATGCTGGGAAGTGTCA-MGB-3'
171Q	5'-VIC-CAGTGGATCAGTATAGTAA-MGB-3'
171R	5'-FAM-CAGTGGATCGGTATAGTA-MGB-3

a) Taqman probes possess fluorophore (either FAM or VIC) at 5' and MGB quencher at 3'.

fragments were subjected to direct sequencing with the above primers and additional internal primers, sPrP109 and sPrP110. The sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit, and nucleotide sequences were determined using an ABI-3100 Avant-Genetic Analyzer (Applied Biosystems).

Single nucleotide polymorphisms (SNPs) at codons 136 (GCC for alanine and GTC for valine) and 171 (CAG for glutamine and CGG for arginine) were analyzed by allelic discrimination using Taqman assay. The PCR primers used for SNPs analysis at codon 136 were 356F and 450R, and those for codon 171 were 467F and 553R (Table 1). Taqman probes used for SNPs analysis at codon 136 were 136A and 136V, and those for codon 171 were 171Q and 171R (Table 1). SNPs analysis was performed in a 25 μl reaction mixture consisting of 10 *n*g of genomic DNA, 900 nM of each primer for PCR, 250 nM of each Taqman probe, and 1x Taqman Universal PCR Master Mix (Applied Biosystems). Cycling conditions were one cycle of 2 min at 50°C and 10 min at 95°C, followed by 35 cycles of 15 sec at 92°C and 1 min at 60°C. The PCR reaction and the following allelic determination were carried out with an ABI PRISM 7900 HT using SDS 2.1 software (Applied Biosystems).

If samples showed polymorphisms at both codons 136 and 171, the PCR products amplified with primers sPrP104 and sPrP105 were cloned into a pCRII-TOPO vector (Invitrogen) and then sequenced. The PrP haplotype was determined by the co-incidence of the polymorphisms at codons 136 and 171 in the cloned fragments.

In a previous study, six haplotypes, MARQ, TARQ, MVRQ, MAHQ, MARR, and MARH, based on polymorphic codons at 112, 136, 154, and 171, were identified in Japanese Suffolk sheep [18]. In the present study, nucleotide sequencing of the PrP gene in 98 Suffolk sheep revealed four haplotypes, MARQ, TARQ, MVRQ, and MARR. Since polymorphisms at codons 136 and 171 greatly influence scrapie susceptibility, in the following study we focused on determining polymorphisms at codons 136 and 171 using SNPs analysis. Table 2 shows the frequencies of PrP genotypes in Japanese Suffolk sheep. The frequencies of the scrapie-susceptible AQ/AQ and AQ/VQ genotypes were 48.0%, indicating that nearly half of the Japanese Suffolk sheep are susceptible to the disease. In contrast, only 9.6% of the sheep were homozygous for AR. Nationwide surveillance also revealed that PrP genotype frequencies varied with location; Tochigi and Miyagi prefectures showed higher frequencies of AQ/AQ and AQ/VQ genotypes than Hokkaido ($p<0.05$ in χ^2 test).

Table 3 shows the frequencies of PrP genotypes in meat breeds at 14 private farms in Hokkaido. In the Suffolk sheep, the frequency of the AQ/AQ genotype varied from 30.9% to 100%. In four out of ten farms, more than half of the Suffolk sheep carried the AQ/AQ genotype, indicating that scrapie susceptibility varies with the flocks. At farm D, the percentage of sheep bearing the AR/AR genotype (27.4%) was higher than that at other farms. The breeding records confirmed that a single sire was primarily used for breeding at this farm, suggesting that the genotypes of sires used for breeding influenced the scrapie susceptibility of the flocks. We also investigated the PrP genotypes of Poll Dorset sheep at two farms and those of Southdown sheep and cross-breeds at two other farms (Table 3). The Poll Dorset sheep showed a lower frequency of the AQ/AQ genotype than the Suffolk sheep, and had a high percentage of sheep carrying the AR haplotype (72.2% at farm L and 93% at farm K). Furthermore, the percentage of sheep carrying the VQ haplotype among the Poll Dorset sheep (13.1% at farm

Table 2. Frequencies of PrP genotypes in Suffolk sheep from various parts of Japan

Genotype	Prefectures								Japan[a)]	
	Hokkaido		Aomori		Miyagi		Tochigi			
	n=468	%	n=18	%	n=24	%	n=130	%	n=648	%
AQ/AQ	200	42.7	9	50.0	13	54.2	82	63.1	307	47.4
AQ/VQ	0	0	0	0	2	8.3	2	1.5	4	0.6
AQ/AR	209	44.7	8	44.4	9	37.5	41	31.5	272	42.0
AR/AR	57	12.2	1	5.6	0	0	4	3.1	62	9.6
AR/VQ	2	0.4	0	0	0	0	1	0.8	3	0.5

a)In addition to sheep samples collected from the prefectures indicated in the table (n=640), total number includes 8 additional sheep samples from Niigata (n=7) and Ishikawa (n=1) prefectures.

Table 3. Frequencies of sheep PrP genotypes at private farms in Hokkaido

Farms	Breed	n[a]	Genotype									
			AQ/AQ		AQ/AR		AR/AR		AQ/VQ		AR/VQ	
			n	%	n	%	n	%	n	%	n	%
A	Suffolk	14	8	57.1	5	35.7	1	7.1	0	0	0	0
B	Suffolk	55	17	30.9	30	54.5	8	14.5	0	0	0	0
C	Suffolk	20	10	50.0	9	45.0	1	5.0	0	0	0	0
D	Suffolk	73	24	32.9	29	39.7	20	27.4	0	0	0	0
E	Suffolk	88	37	42.0	39	44.3	12	13.6	0	0	0	0
F	Suffolk	57	22	38.6	26	45.6	8	14.0	0	0	1	1.8
G	Suffolk	32	10	31.3	20	62.5	2	6.3	0	0	0	0
H	Suffolk	12	6	50.0	6	50.0	0	0	0	0	0	0
I	Suffolk	6	6	100	0	0	0	0	0	0	0	0
J	Suffolk	6	2	33.3	1	16.7	3	50.0	0	0	0	0
K	Poll Dorset	61	14	23.0	27	44.3	12	19.7	3	4.9	5	8.2
L	Poll Dorset	29	0	0	11	37.9	13	44.8	2	6.9	3	10.3
M	Southdown	8	4	50.0	3	37.5	1	12.5	0	0	0	0
	PD × SD[b]	76	10	13.2	45	59.2	17	22.4	3	3.9	1	1.3
N	Southdown	12	3	25.0	3	25.0	6	50.0	0	0	0	0
	S × SD[c]	44	13	29.5	19	43.2	11	25.0	1	2.3	0	0

a) Number of sheep analyzed.
b) Poll Dorset crossed with Southdown.
c) Suffolk crossed with Southdown.

Table 4. PrP genotype of sires and ewes used for selective breeding at HARC

	PrP genotype	2001	2002	2003	2004	2005
Sire	AQ/AQ	4	0	0	0	0
	AQ/AR	3	3	5	4	6
	AR/AR	0	0	0	2	3
Ewe	AQ/AQ	–	59	68	56	61
	AQ/AR	–	37	43	53	67
	AR/AR	–	8	11	12	18

L and 17.2% at farm K) was higher than that in the Suffolk sheep. The PrP genotype distributions of the Poll Dorset sheep were similar to those reported in other countries [17].

At HARC, a trial of selective breeding using sires with the AQ/AR and AR/AR genotypes has been conducted since 2002. Table 4 shows the PrP genotype of sires and ewes used for the breeding program. More than 100 ewes were used every year, however, only sires have been selected on the basis of PrP genotype since 2002. Table 5 shows the transition of the frequencies of PrP genotypes in lambs born in each season. There were significant differences in the frequencies of PrP genotypes between lambs born before

Table 5. The transition of the frequencies of PrP genotypes in the experimental flock at HARC

a. Genotype frequency

Genotypes[a]	2002		2003		2004		2005		2006	
	n=186	%	n=220	%	n=214	%	n=220	%	n=154	%
AQ/AQ	97	52.2	86	39.1	60	28.0	44	20.0	37	24.0
AQ/AR	71	38.2	104	47.3	116	54.2	132	60.0	78	50.6
AR/AR	18	9.7	30	13.6	38	17.8	44	20.0	39	25.3

b. Statistical analysis[b]

	2002	2003	2004	2005	2006
vs 2002	–	7.09 (<0.05)*	24.85 (<0.001)*	46.64 (<0.001)*	32.20 (<0.001)*
vs 2003	–	–	6.14 (<0.05)*	19.54 (<0.001)*	13.17 (<0.01)*
vs 2004	–	–	–	3.96 (0.14)	3.21 (0.20)
vs 2005	–	–	–	–	3.25 (0.20)

a) Genotypes of lambs born in each year were determined by SNPs analysis.
b) Differences in the frequencies of PrP genotypes between years were analyzed by χ^2 test. χ^2-values and p-values (in parentheses) are shown. Asterisks indicate significant differences.

(born in 2002) and after selective breeding (born in 2003 and later). In the selective breeding during 2002, which brought lambs early in 2003, we used only three sires with the AQ/AR genotype. However, the frequencies of the PrP genotypes in lambs born during 2003 differed significantly from those in 2002. In 2005, three years after the implementation of selective breeding, the ratio of the AQ/AQ genotype was reduced from 52.2% to 20%, and that of the scrapie-resistant genotypes (AQ/AR and AR/AR) increased from 47.9% to 80%. However, no increase in the ratio of scrapie-resistant genotypes was observed between 2005 and 2006.

The results in this study revealed that the Japanese Suffolk sheep population, the main breed in Japan, is largely comprised of scrapie-susceptible sheep. The frequencies of the scrapie-susceptible genotypes are higher, and those of the resistant genotypes are lower than those reported in European countries [12]. In the UK, where selective breeding was started in accordance with the National Scrapie Plan, more than 90% of Suffolk sheep are reported to have the AR/AR or AQ/AR genotype [10]. Restriction on the use of rams carrying the VQ haplotype is the highest priority in the selective breeding of scrapie-resistant flocks. Although PrP genotype distributions differ with breed, the Suffolk and Poll Dorset sheep used here possessed the VQ haplotype. Selective breeding programs have not been implemented in Japan, however the high percentage of scrapie-susceptible sheep and the presence of the VQ haplotype suggest a requirement for selective breeding to raise the ratio of scrapie-resistant sheep.

Before beginning selective breeding, sires carrying AR/AR in the flock at HARC were rare so that we used sires with the AQ/AR genotype in the first two years of breeding. Although we used sires with the AQ/AR genotype and ewes were used randomly, the breeding program could convert flocks into more scrapie-resistant ones within 2 or 3 years. However, the increase in the ratio of scrapie-resistant sheep appeared to plateau between 2005 and 2006. This suggests that the use of sires with AR/AR and the selection of ewes based on PrP genotype are required to raise the ratio of scrapie-resistant genotypes to a higher level.

It is also important to determine whether the selection of sheep with a specific PrP genotype affects any traits such as meat quality and production. Several studies have reported that some traits, such as litter size, 135 days weight, and daily liveweight gain, could be influenced by PrP genotypes in some breeds [1, 5, 8, 19]. However, it is unlikely that unfavorable associations of scrapie-resistant PrP genotypes with performance parameters, if they really exist, will be a common occurrence in sheep. In fact, no association between PrP genotypes and traits has been observed in the Suffolk sheep produced by the selective breeding at HARC so far (Tokari et al. unpublished results). In Japan, the selective breeding for scrapie-resistance has not been implemented yet; however, because of the small number of sheep populations, it is relatively easy to control sheep PrP genotypes using high-performance sires with scrapie-resistant genotypes. The PrP genotyping carried out in this study will contribute to the selection of sires and ewes used for breeding in the future.

Unusual scrapie cases in sheep with the ARR/ARR and ARQ/ARR genotypes have recently been reported in Germany, the UK, France, and Belgium [6, 7, 10, 13]. This raises questions regarding the increase in sheep populations bearing the ARR haplotype, although the risk of the atypical scrapie to public health remains to be elucidated [3]. The unusual scrapie cases have not been recognized in Japan so far, thus increasing the frequency of scrapie-resistant genotypes is still believed to be effective in reducing and/or eliminating a prevalence of classical scrapie, even though sheep carrying the ARR/ARR genotype are not fully resistant to scrapie [18]. Sheep carrying ARR/ARR genotype were resistant to BSE [3, 4, 20] although not completely [2, 15]. This fact also suggests that selection of the ARR haplotype will contribute to reducing the possibility of human exposure to BSE via sheep. The control of PrP genotype distributions through genotyping, not only for codons 136 and 171, but also for codons 141 and 168 that appear to influence TSE susceptibility especially for atypical scrapie, in addition to continuous surveillance of TSE occurrence, will contribute to the control of TSE in small ruminants.

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