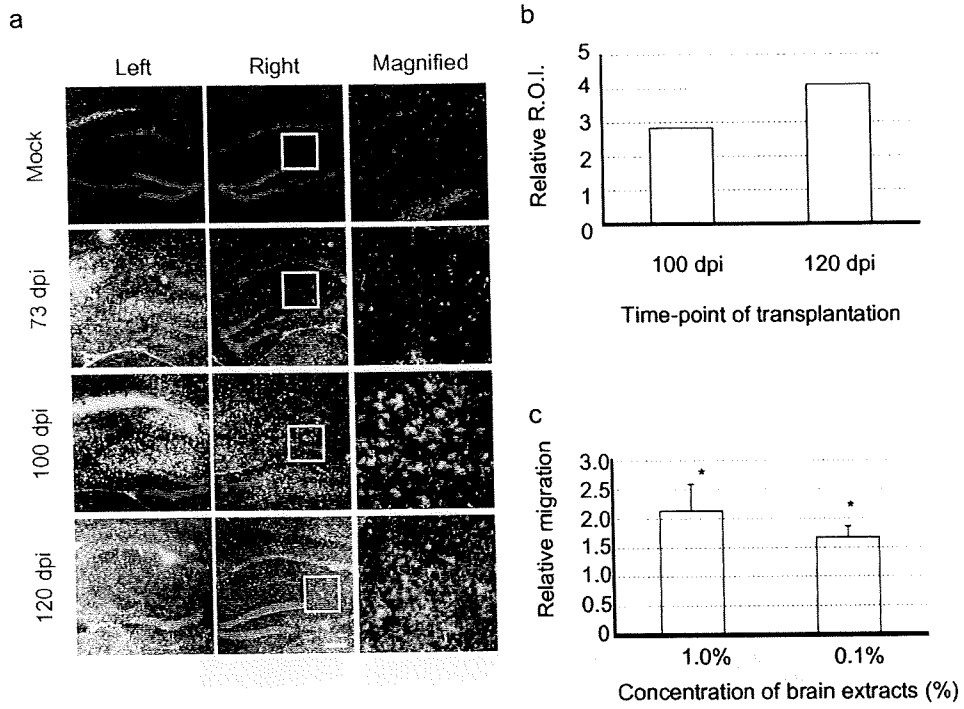

FIG. 2. Migration of hMSCs in response to prion-specific lesions. hMSCs (1×10^5 cells) were transplanted into the left hippocampi of mice infected with strain Chandler at 73, 100, or 120 dpi and into those of mock-infected mice at 120 dpi. One week after transplantation, the migration of hMSCs (green) to the contralateral hippocampus (right) was analyzed. The rightmost panels show magnified images of the regions boxed in the panels immediately to the left. Bar, 200 μm. (b) Quantification of hMSC migration. Areas positive for β-Gal in the contralateral hippocampus (regions of interest [ROI]) were measured using NIH Image J. The graph shows the levels of ROIs for mice transplanted at 100 and 120 dpi relative to that for a mouse transplanted at 73 dpi. (c) Migration of hMSCs to extracts from the brains of prion-infected mice. Insert wells containing an hMSC suspension were placed in the lower chambers, which contained 1.0 or 0.1% brain extract in serum-free DMEM, and were incubated for 24 h. The mean migration of hMSCs to brain extracts from mock-infected mice was arbitrarily set at 1, and the relative migration to brain extracts from mice infected with strain Chandler is indicated. Means and standard deviations from three independent assays (triplicate in each assay) are shown. *, $P < 0.05$.

eral hippocampus. In contrast, migration of hMSCs to the thalamus, where moderate PrP^{Sc} deposition had already occurred, was clearly observed (data not shown). In addition, more hMSCs were detected in the contralateral hippocampus when the transplantation was carried out at later time points (Fig. 2a). To compare the migration of hMSCs quantitatively, the total area of the hMSCs in the contralateral hippocampus (areas positive for β-Gal) was measured using the NIH Image J program. Compared to the migration of hMSCs to the contralateral hippocampus a week after the transplantation at 73 dpi, 2.8 and 4.1 times more hMSCs were detected when the transplantation was done at 100 and 120 dpi, respectively (Fig. 2b). We examined at least two mice from each experimental group and confirmed the consistency of the findings. Since PrP^{Sc} accumulation and astrocytosis in the hippocampi of mice infected with strain Chandler were first detected around 90 dpi and the levels of PrP^{Sc} accumulation and astrocytosis increased gradually thereafter (see Fig. S1 in the supplemental material), the migration of hMSCs to the contralateral hippocampus appeared to correlate with the severity of pathological changes.

Next, we analyzed the migration of hMSCs to brain extracts from prion-infected mice in vitro. Insert wells of a QCM 24-well colorimetric cell migration assay kit containing hMSCs were placed in the lower chambers, which contained 1.0 or 0.1% brain extract from prion- or mock-infected mice, and were incubated for 24 h. Cells that had migrated to the back sides (facing the lower chamber) of the membranes of the insert wells were quantified according to the supplier's instructions. Compared to the migration to brain extracts from mock-infected mice, approximately 2 and 1.5 times more hMSCs had migrated into the lower chambers containing 1.0% and 0.1% brain extracts from prion-infected mice, respectively (Fig. 2c). This suggests that chemoattractive factors that promote the migration of hMSCs are produced by the lesion caused by prion infection.

Migration of hMSCs into the brain after intravenous transplantation. MSCs have been reported to migrate to a site of brain injury even when they are introduced via intravenous injection (37, 38). To test if a similar phenomenon could be observed in prion-infected mice, hMSCs were intravenously inoculated into mice infected with strain Chandler or into mock-infected mice at 120 dpi. In mice infected with strain Chandler, hMSCs were observed in the hippocampus and thalamus even at 2 days after transplantation (data not shown). The cells showed a symmetrical distribution and appeared to increase in number in these tissues by 3 weeks posttransplantation (Fig. 3a; see also Fig. S2 in the supplemental material). In contrast, few MSCs were detected in the brains of mock-

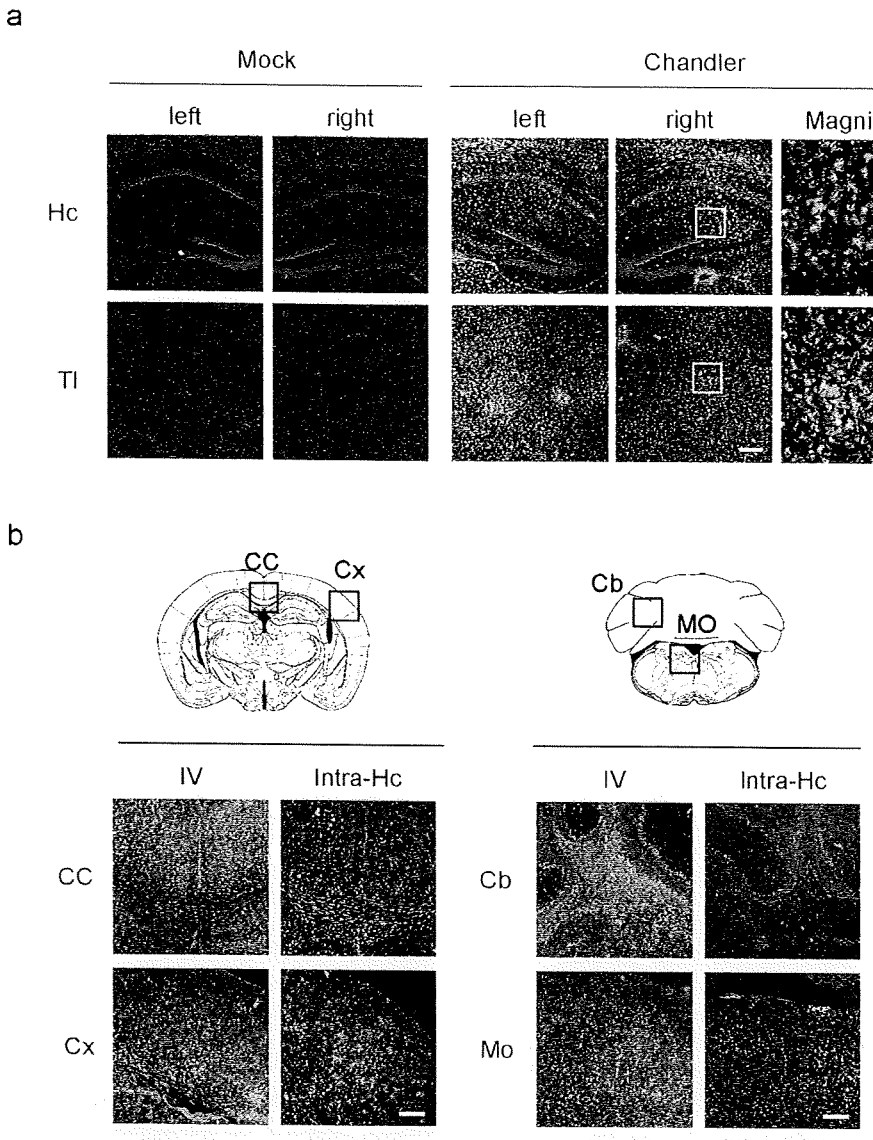

FIG. 3. Migration of hMSCs into the brain after intravenous transplantation. hMSCs (1×10^6 cells) were intravenously injected into mice infected with strain Chandler and into mock-infected mice at 120 dpi. Three weeks after injection, cryosections were prepared and stained with an anti-β-Gal MAb. (a) Presence of hMSCs in the left and right hippocampus (Hc) and thalamus (Tl) 3 weeks postinjection. The rightmost panels show magnified images of the regions boxed in the panels immediately to the left. Bar, 200 μm. (b) Distribution of hMSCs transplanted into the left hippocampus (Intra-Hc) or introduced via intravenous injection (IV). At 120 dpi, the hMSCs were transplanted into the left hippocampi (1×10^5 cells) or injected via the tail veins (1×10^6 cells) of mice infected with strain Chandler. The presence of hMSCs in the corpus callosum (CC), cortex (Cx), cerebellum (Cb), and medulla oblongata (MO) 3 weeks posttransplantation is shown. These brain regions are boxed on the images taken from Paxinos and Franklin and reprinted with permission of the publisher (39). Bars, 200 μm.

infected mice, demonstrating that the hMSCs migrated to the brain lesions caused by prion propagation. At each time point, we examined two mice for each experimental group and confirmed the consistency of the findings. The hMSCs were also well distributed in other brain regions, including the cerebral cortex, cerebellum, and medulla oblongata (Fig. 3b); however, consistent with the results shown in Fig. 3b, they did not migrate well to the hypothalamus (see Fig. S2 in the supplemental material). There was no difference in the area of hMSC distribution following intravenous versus intrahippocampal transplantation except at the corpus callosum. More hMSCs were observed in the corpus callosum after transplantation into the hippocampus than after intravenous injection, suggesting that cells migrate to the contralateral side through the corpus callosum after intrahippocampal transplantation (2).

Effects of transplantation of hMSCs on the survival of prion-infected mice. To examine whether the transplantation of hMSCs can ameliorate prion diseases, hMSCs were transplanted into the left hippocampi of mice infected with strain Chandler at 90 dpi. Figure 4 shows the survival curve for these mice. The intrahippocampal transplantation of hMSCs prolonged the survival of mice infected with strain Chandler

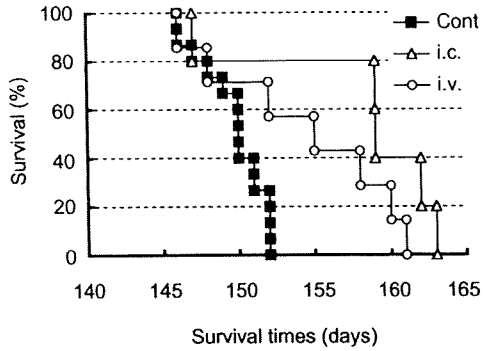

FIG. 4. Prolongation of survival of prion-infected mice by transplantation of hMSCs. For intracerebral (i.c.) transplantation, hMSCs (1×10^5 cells) were transplanted into the left hippocampi of mice infected with strain Chandler at 90 dpi ($n = 5$). For intravenous (i.v.) transplantation, 1×10^6 hMSCs were injected via the tail vein at 120 dpi ($n = 7$). The hMSC-transplanted and nontransplanted control (Cont) ($n = 15$) mice were observed until they reached the terminal stage of the disease. The graph shows survival curves.

(158 ± 6 days; $n = 5$) over that of the nontransplanted control group (150 ± 2 days; $n = 15$). Thus, hMSC transplantation prolonged mean survival by only 8 days, but this difference was statistically significant ($P < 0.01$ by the log rank test). We also transplanted hMSCs via the tail veins of mice infected with strain Chandler at 120 dpi. The transplantation of hMSCs via this peripheral route appeared not to be effective for nearly half of the mice; however, the remaining mice survived beyond the mean survival of the nontransplanted control group. Although the mean survival of hMSC-transplanted mice (154 ± 6 days; $n = 7$) was only a little longer than that of the control group, this difference was also significant ($P < 0.05$ by the log rank test). The fact that survival time was prolonged even when hMSCs were transplanted via a peripheral route after clinical onset (120 dpi) suggests that hMSCs have therapeutic potential for prion diseases. Since both intracerebral and intravenous transplantation of hMSCs prolonged the survival of prion-infected mice, we further analyzed the transplanted hMSCs.

Proliferation of hMSCs after transplantation. To examine the proliferation state of hMSCs that had migrated to lesions, BrdUrd was systemically administered after the transplantation of hMSCs to the left hippocampus. Three weeks posttransplantation, many BrdUrd-labeled nuclei were detected in the contralateral hippocampi and thalami of mice infected with strain Chandler (see Fig. S3 in the supplemental material), where many hMSCs had migrated (Fig. 1a). In contrast, few BrdUrd-labeled nuclei were detected in the contralateral hippocampi and thalami of mock-infected mice (see Fig. S3 in the supplemental material), although a few cells with BrdUrd-labeled nuclei were detected on the transplanted side (data not shown). BrdUrd-labeled cells were also observed in the cerebella and medullae oblongatae of mice infected with strain Chandler (data not shown). We examined two mice for each experimental group and confirmed the similar results. These results suggest that transplanted hMSCs are capable of proliferating in the microenvironment caused by prion propagation.

Expression of trophic factors in hMSCs. It is known that MSCs migrate to a site of injury in the brain and produce various trophic factors (8, 29). To ask if something similar happens in the case of prion disease, we next assayed the production of trophic factors in our model system. hMSCs were transplanted into the left thalamus at 120 dpi, and one mouse in each group was sacrificed and examined for the production of human trophic factors at 2 days and 1 and 3 weeks after transplantation. Immunoreactivities for human BDNF, NT3, and VEGF in the ipsilateral thalami of mice infected with strain Chandler became more intense from 2 days to 3 weeks posttransplantation. In contrast, no obvious increases, but rather decreases, in the signals of these trophic factors were observed for mock-infected mice (Fig. 5). Additionally, the expression of NGF, NT4/5, and CNTF was also upregulated (data not shown). These results suggest that hMSCs produce a variety of trophic factors in response to the neurodegeneration caused by prion infection.

Interestingly, only subpopulations of the hMSCs appeared to be positive for NT3 and BDNF. In addition, parts of the regions positive for these factors did not overlap with β-Gal staining. Since these antibodies are specific to human trophic factors and will not react with the corresponding mouse trophic factors, the presence of NT3 and BDNF in areas negative for β-Gal may represent trophic factors secreted from hMSCs and bound to mouse brain cells.

Differentiation of hMSCs. MSCs are known to differentiate into cells of neuronal and glial lineages in vivo and in vitro (11, 48, 61). We next asked if hMSCs differentiate into neuronal and glial cells in response to the lesions of prion diseases. At 3 weeks after transplantation into the thalamus, hMSCs positive for the neurodifferentiation marker MAP2, GFAP, or CNPase were detected in the brains of mice infected with strain Chandler (Fig. 6), although a relatively small number of hMSCs were positive for each marker. In contrast, no hMSCs positive for MAP2, GFAP, or CNPase were observed in the brains of mock-infected mice (data not shown), suggesting that neuronal and glial differentiation of hMSCs occurs in response to the neurodegeneration caused by prion infection. The GFAP-positive hMSCs were detected in the hippocampus, thalamus, and medulla oblongata. In contrast, MAP2-positive hMSCs were detected primarily in the hippocampus, cortex, and cerebellum, and CNPase-positive hMSCs were detected mainly in the cortex (data not shown).

DISCUSSION

The primary purpose of this study was to evaluate the potential of MSCs for treating prion diseases in a mouse model. For this purpose, the use of mouse MSCs would have been desirable; however, no appropriate method for the isolation of mouse MSCs from bone marrow had been established by the beginning of this study. On the other hand, it was well known that MSCs avoid allogeneic rejection (47). Thus, we adopted hMSCs to the mouse model and showed that hMSCs responded to the neuropathological lesions of prion diseases and may have therapeutic potential. Transplantation of MSCs is known to ameliorate neurological dysfunctions in experimental models (7, 23, 24, 30, 37). In clinical trials in which autologous MSCs are transplanted into patients with multiple system atrophy (28) or amyotrophic lateral sclerosis (33), or into patients who have suffered a stroke (3), there is some evidence of

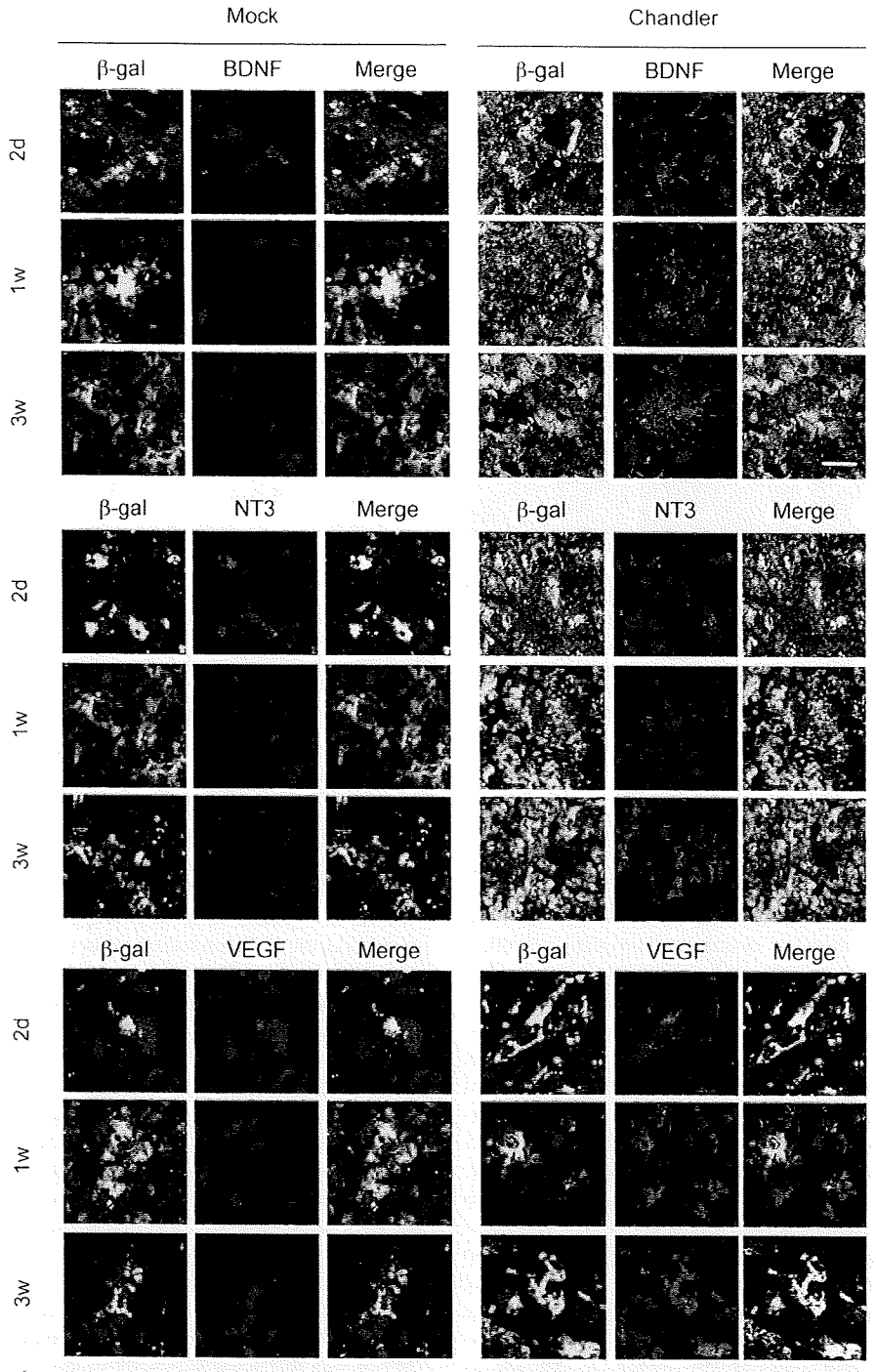

FIG. 5. Expression of trophic factors in hMSCs. hMSCs (1×10^5 cells) were transplanted into the left thalami of mice infected with strain Chandler and into those of mock-infected mice at 120 dpi. Two days (2d), 1 week (1w), and 3 weeks (3w) posttransplantation, cryosections were prepared and doubly stained with an anti-β-Gal MAb, for hMSCs (green), and an antibody against a human trophic factor (BDNF, NT3, or VEGF) (red). Nuclei were counterstained with DAPI (blue). Bar, 20 μm.

a beneficial effect without any adverse effects. How the introduction of MSCs leads to improved outcomes is not yet clear. However, the transplanted MSCs are known to migrate and home to a site of injury. Moreover, in this context, MSCs are expected to restore injured tissues by protecting neural tissues via secretion of various trophic factors (8), promotion of angiogenesis (20), stimulation of the proliferation and differentiation of endogenous neural stem cells (36), integration into tissues by differentiation or cell fusion (1), and modulation of the local immune response (64).

Here we showed that the level of migration of MSCs to the contralateral side of the mouse brain correlates with the de-

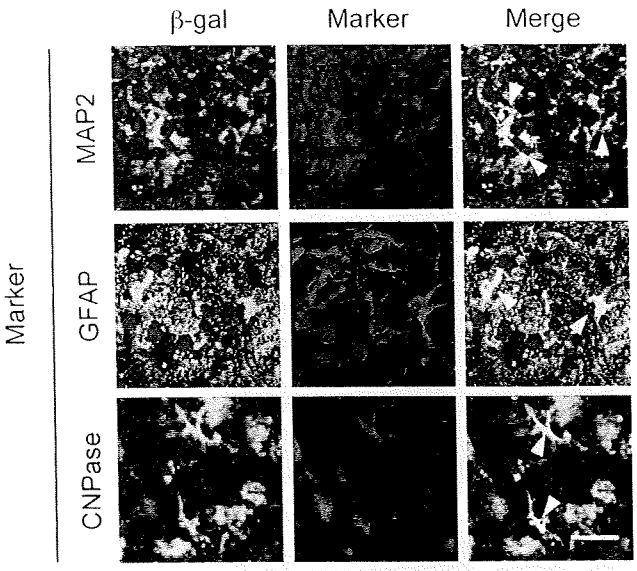

FIG. 6. Differentiation of hMSCs into cells of neuronal and glial lineages. At 120 dpi, hMSCs (1×10^5 cells) were transplanted into the left thalami of mice infected with strain Chandler. Three weeks post-transplantation, cryosections were prepared and doubly stained with an anti-β-Gal MAb, for hMSCs (green), and an antibody against a marker for neurons (MAP2), astrocytes (GFAP), or oligodendrocytes (CNPase) (red). Nuclei were counterstained with DAPI (blue). Arrows indicate hMSCs positive for each marker protein. The brain region for MAP2 and GFAP is the ipsilateral hippocampus, while that for CNPase is the cerebral cortex. Bar, 20 μm.

gree of PrP^{Sc} accumulation and the severity of histopathological changes in the prion-infected brain (Fig. 1d and 2). Although no antigen-specific humoral or cellular immune response is provoked in prion diseases, microglial activation and astrocytosis are prominent features of the diseases. Indeed, the expression of inflammatory cytokines and chemokines, which are likely to be produced in glial cells, is upregulated in the middle to late stage of prion infection (5, 6). In addition, brain extracts from prion-infected mice promoted chemotaxis of hMSCs in vitro. These results suggest that certain factors produced in the brains of prion-infected mice act as chemoattractive factors for hMSCs, although it is not clear whether the effects of mouse factors on human MSCs are as efficient as those on homologous MSCs. Monocyte chemoattractive protein 1 (known as CCL2), interleukin-8 (IL-8), and macrophage inflammatory protein 1α (known as CCL3) have been reported to enhance the migration of MSCs to ischemic brain tissue (57, 58). It was recently reported that an interaction between stromal cell-derived factor 1 (known as CXCL12), produced in ischemic brain lesions, and CXCR4, expressed on MSCs, plays an important role in the migration of MSCs (59). Because the inflammatory response and glial activation are common events in many neurological disorders, it is conceivable that a similar mechanism may account to some extent for the migration of MSCs to brain lesions associated with prion disease. For instance, we have found that the expression of CCL3 is upregulated in the thalami and medullae of prion-infected mice (unpublished observation). Experiments are under way to identify the chemoattractive factors by use of an in vitro chemotaxis assay.

MSCs have been reported to migrate to a site of brain injury even when intravenously injected (37, 38). Consistent with previous reports, our results showed here that hMSCs transplanted via intravenous injection travel to areas of brain lesions in prion-infected mice (Fig. 3). In prion diseases, although impairment of the blood-brain barrier (BBB) was observed in the cerebellum (56), no significant impairment of the BBB was observed in the hippocampus or cerebral cortex at the time of clinical onset or even at a later stage (42, 56). Thus, passive translocation of MSCs to the brain parenchyma through a disrupted BBB seems unlikely. Instead, active transendothelial migration of MSCs, similar to the recruitment of leukocytes and monocytes from the bloodstream to an inflammation site, is expected to be involved in the engraftment of MSCs transplanted via intravenous injection. Vascular cell adhesion molecule 1 and p-selectin expressed on the endothelium are important for the adhesion of MSCs to the endothelium via the β1 integrin VLA-4 (16, 46, 51). Proinflammatory cytokines, such as tumor necrosis factor alpha and IL-1β, upregulate the expression of adhesion molecules in endothelial cells (32). Indeed, tumor necrosis factor alpha and IL-1β are upregulated during the course of prion disease (6, 49), suggesting that these cytokines induce the adherence of MSCs to the endothelium and their subsequent transendothelial migration to the brain lesions. Understanding how the migration of MSCs to brain lesions affected by prion diseases is regulated, and further elucidation of the mechanisms underlying the tropism of MSCs, may provide new insight into the engraftment of MSCs as it relates to the progression and possible treatment of neurodegenerative diseases.

The ability of MSCs to migrate to a site of injury has been given particular attention, because it suggests that these cells can act as a vehicle for gene therapy in addition to aiding in the regeneration of degenerated tissues. Indeed, MSCs expressing genes of therapeutic potential showed a greater positive effect on functional recovery than unmodified MSCs (24, 37, 38). Transgenic expression of anti-PrP antibodies (22), a fusion protein between PrP^C and the Fc portion of immunoglobulin (PrP-Fc) (34), and dominant-negative PrP mutants (40) inhibited prion propagation. In addition, expression of anti-PrP Fab fragments and PrP-Fc in the brain by virus vectors has been reported to antagonize prion propagation in the brain (18, 62). Furthermore, intraventricular infusion of an anti-PrP MAb slowed the formation of neuropathological lesions and prolonged the survival of prion-infected mice even when the MAb was administered at clinical onset (53). However, large macromolecules, such as immunoglobulins, are expected to be delivered to the lesions inefficiently. Indeed, the distribution of MAbs was restricted primarily to the hippocampus and thalamus, even when the MAbs were infused directly into the lateral ventricle (53). Therefore, the observation that hMSCs target and home to brain lesions associated with prion diseases indicates the potential utility of hMSCs as a cellular vehicle for the delivery of therapeutic genes to brain lesions.

We showed here that microenvironments in the brain lesions associated with prion disease stimulate MSCs to produce various trophic factors: BDNF, NGF, VEGF, and others. These trophic factors are reported to have antiapoptotic effects, to promote nerve fiber regeneration, and to induce endogenous cell proliferation and angiogenesis in injured brains (9, 29, 31).

It remains to be elucidated whether the prolonged survival of prion-infected mice by hMSC transplantation can be attributed to the secretion of trophic factors from hMSCs. Although hMSCs alone may have the ameliorative effect to some extent, they could not arrest the disease progression caused by prion propagation. Similarly, it has been shown that antagonizing prion propagation can slow disease progression but cannot ameliorate functional deficits (13, 25, 53). Thus, it seems possible that the combination of MSCs with inhibitors of prion propagation would have a synergistic effect in the treatment of prion diseases.

Replacement of damaged neurons with differentiated MSCs or their fusion with MSCs after MSC transplantation is an attractive possible route to the restoration of neurological functions (11, 54, 60). In this study, we showed that small populations of MSCs were differentiated into cells expressing neuronal, astrocyte, or oligodendrocyte markers. Because only a small portion of transplanted MSCs differentiated into a neuronal and a glial lineage in vivo, it seems unlikely that the prolongation of survival could be attributed directly to differentiation. However, induction of neuronal differentiation in vitro prior to transplantation improves functional outcomes in a rat model of Parkinson's disease and cerebral infarction (12, 35). Therefore, appropriate preconditioning may enhance the effects of *trans*-differentiation on the restoration of degenerated tissues.

To our knowledge, this is the first report showing the therapeutic potential of MSCs for prion diseases. We showed that hMSCs home to the lesions, produce trophic factors, and differentiate into neuronal and glial lineage cells in response to the microenvironment in the lesions. As we are already aware, not only inhibition of prion propagation but also regeneration of damaged nervous tissues is required for recovery from prion diseases. Thus, a combination of genes possessing antiprion effects with MSCs, which can deliver therapeutic genes and have potential for neuroprotection and the regeneration of damaged tissues, may provide an effective treatment for prion diseases.

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Generation of monoclonal antibody that distinguishes PrP^{Sc} from PrP^{C} and neutralizes prion infectivity

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ABSTRACT

To establish PrP^{Sc}-specific mAbs, we immunized *Prnp*$^{-/-}$ mice with PrP^{Sc} purified from prion-infected mice. Using this approach, we obtained mAb 6H10, which reacted with PrP^{Sc} treated with proteinase K, but not with PrP^{Sc} pretreated with more than 3 M GdnHCl. In contrast, reactivity of pan-PrP mAbs increased with increasing concentrations of GdnHCl used for pretreatment of PrP^{Sc}. In histoblot analysis, mAb 6H10 showed a positive reaction on a non-denatured histoblot but reactivity was lower when the histoblot was pretreated by autoclaving. Epitope analysis suggested that the extreme C-terminus of PrP is likely to be part of the epitope for mAb 6H10. MAb 6H10 immunoprecipitated PrP^{Sc} from brains of mice, sheep, and cattle infected with prions. Furthermore, pretreatment of purified PrP^{Sc} with mAb 6H10 reduced the infectious titer more than 1 log. Taken together, these results suggest that mAb 6H10 recognizes a conformational epitope on PrP^{Sc} that is related to prion infectivity.

Introduction

Prion diseases are fatal neurodegenerative diseases which include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE), and Creutzfeldt-Jakob diseases (CJD) in humans. A hallmark of the diseases is the accumulation of a pathogenic, abnormal isoform of prion protein, designated PrP^{Sc}, in the central nervous system of affected animals. PrP^{Sc} is generated from the host-encoded, cellular prion protein, PrP^{C}, by certain post-translational modification including conformational transformation (Prusiner et al., 1998). Although the two isoforms are encoded by the host gene, *PrP*, they differ from each other biochemically and biophysically. For example, PrP^{C} is soluble in non-ionic detergents and sensitive to protease treatment, whereas PrP^{Sc} is insoluble due to its propensity to form aggregates that are partially resistant to protease treatment (Oesch et al., 1985; Meyer et al., 1986). PrP^{C} has a high α-helix but low β-sheet content, whereas PrP^{Sc} has a higher β-sheet content (Caughey et al., 1991; Pan et al., 1993; Safar et al., 1993). However, PrP^{Sc} is comprised of PK-sensitive and PK-resistant PrP^{Sc}, such that PrP^{Sc} cannot be distinguished from PrP^{C} simply using protease treatment (Bessen and Marsh, 1994; Safar et al., 1998, 2005; Silveira et al., 2005).

To date, there have been a number of reports on the production of monoclonal and polyclonal antibodies against PrP molecules. Most of these are pan-PrP antibodies that recognize either linear or discontinuous epitopes on PrP^{C} and react with PrP^{Sc} pretreated with denaturant (Kascsak et al., 1987; Serban et al., 1990; Williamson et al., 1996; Peretz et al., 1997; Kim et al., 2004a). Because of the co-existence of PrP^{C} and PrP^{Sc}, and the propensity of PrP^{Sc} to form aggregates, removal of PrP^{C} by protease treatment and subsequent denaturation are prerequisites for specific detection of PrP^{Sc} by pan-PrP antibodies. However, the ability to analyze the properties of PrP^{Sc} using pan-PrP antibodies is limited, as the biological and biochemical properties of PrP^{Sc} are affected by protease treatment and denaturation.

Molecular probes that specifically react to PrP^{Sc} and distinguish PrP^{Sc} from PrP^{C} can be a powerful tool for analysis of the entity of PrP^{Sc}. For instance, PrP^{C} will be act as a molecular probe for PrP^{Sc}, as PrP^{C} binds to PrP^{Sc} (Horiuchi et al., 1999). A fusion protein comprised of PrP^{C} and the immunoglobulin Fc region and a genetically modified antibody possessing PrP^{C} segments have been demonstrated to bind to PrP^{Sc} possibly via the PrP^{C} segments (Meier et al., 2003; Moroncini et al., 2004). Moreover, plasminogen binds to PrP^{Sc} not to PrP^{C}; however, plasminogen will bind to other serum proteins and thus, selectivity of the binding is obscure (Fischer et al., 2000). In contrast to many reports of pan-PrP mAbs, only a few studies have reported anti-PrP^{Sc} antibodies that specifically discriminate PrP^{Sc} from PrP^{C} (Korth

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et al., 1997; Paramithiotis et al., 2003; Curin Serbec et al., 2004; Jones et al., 2009).

Availability of a panel of PrP^{Sc}-specific antibodies is indispensable for analysis of the biochemical properties of PrP^{Sc}. Thus, in order to obtain PrP^{Sc}-specific antibodies, we immunized PrP-ablated (*Prnp*$^{-/-}$) mice with PrP^{Sc} purified from prion-infected mouse brains. Prion infectivity is thought to be associated with PrP^{Sc} oligomers; thus, we used non-denatured, purified PrP^{Sc} as the immunogen. One of the mAbs, clone 6H10, showed interesting reactivity to PrP molecules; mAb 6H10 reacted with non-denatured PrP^{Sc} but not with recombinant mouse PrP (rMoPrP) or denatured PrP^{Sc} in an enzyme-linked immunosorbent assay. This pattern of reactivity implied that mAb 6H10 recognizes PrP^{Sc} but not PrP^{C}, allowing for detailed characterization of mAb 6H10.

Results

Reactivity of mAb 6H10 to purified PrP^{Sc}

To obtain PrP^{Sc}-specific mAbs, we immunized *Prnp*$^{-/-}$ mice with a purified PrP^{Sc} fraction and hybridoma supernatants were screened with the purified PrP^{Sc} and rMoPrP by enzyme-linked immunosorbent assay (ELISA). We established one mAb, 6H10 (isotype: IgG2b), which reacted with purified PrP^{Sc} but not with rMoPrP or PrP^{Sc} denatured with 6 M GdnHCl. This pattern of reactivity suggests that mAb 6H10 might specifically recognize the PrP^{Sc} conformation and thus, we further analyzed mAb 6H10.

Figure 1 shows reactivity of mAb 6H10 and pan-PrP mAbs to purified PrP^{Sc}. The mAbs 31C6, 44B1, and 72, which were characterized as pan-PrP mAbs, reacted with purified PrP^{Sc} before proteinase K (PK) treatment (0 μg/ml), but reactivity disappeared when PrP^{Sc} was pretreated with 20 μg/ml or higher concentrations of PK (Fig. 1a). It is known that PrP^{Sc} forms sedimentable aggregates, and PrP^{C} or protease-sensitive PrP molecules, which are expected to expose the epitopes for pan-PrP mAbs, are usually co-purified with PrP^{Sc} during purification. Thus, the drastic decline in reactivity of pan-PrP mAbs with increases in PK concentration is probably due to removal of protease-sensitive PrP species from the purified PrP^{Sc} fraction. In contrast to the results of pan-PrP mAbs, mAb 6H10 reacted with PrP^{Sc} treated with up to 320 μg/ml of PK (Fig. 1a).

Most of epitopes for pan-PrP mAbs are buried in aggregates of purified PrP^{Sc}, and become exposed as denaturation reveals cryptic epitopes (Kim et al., 2004a). In contrast, PrP^{Sc}-specific conformational epitope(s), if they exist, would be expected to be destroyed upon denaturation. Consistent with this, reactivity of mAb 6H10 gradually decreased and disappeared when PK-treated PrP^{Sc} was treated with more than 3 M GdnHCl (Fig. 1b). This is different from pan-PrP mAbs, which did not react with PK-treated PrP^{Sc} without denaturation (at 0 M GdnHCl) but reactivity became apparent and increased as the GdnHCl concentration is increased (Fig. 1b). Taken together, the results suggested that mAb 6H10 recognizes a conformational epitope on PrP^{Sc}. Consistent with this, mAb 6H10 did not react with PrP^{Sc} in immunoblot analysis (Fig. 2a). To confirm reactivity of mAb 6H10 to PrP^{Sc}, we performed immunoprecipitation analysis. The mAb 6H10 precipitated PrP^{Sc} into the bead-bound fraction from a suspension of a purified PrP^{Sc} fraction, whereas most of PrP^{Sc} remained in the unbound fraction in the case of mAb 31C6 or a negative control mAb (Fig. 2b). Following immunoprecipitation of PrP^{Sc} by mAb 6H10, the amount of PrP^{Sc} that remained in the corresponding supernatant was decreased.

Immunoprecipitation of PrP^{Sc} from brain homogenates by mAb 6H10

Next we performed immunoprecipitation from brain homogenates. The mAb 31C6 immunoprecipitated bands corresponding to PrP^{C} from brain homogenates of uninfected mouse, whereas mAb

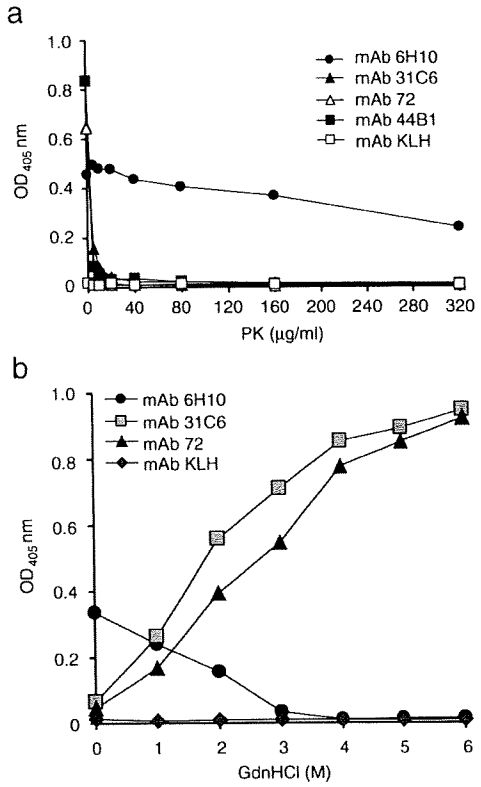

Fig. 1. Reactivity of mAb 6H10 to PrP^{Sc} in ELISA. (a) Reactivity to PK-treated PrP^{Sc}. Wells were coated with 200 ng/well of purified, PK-untreated PrP^{Sc} of the Obihiro strain. After adsorption, wells were treated with the indicated concentrations of PK. After terminating PK activity with Pefabloc, the wells were subjected to the antibody reaction. The anti-PrP mAbs used were 6H10, 31C6, 72, and 44B1. Anti-KLH mAb was used as a negative control mAb. (b) Effect of denaturation of PrP^{Sc}. Purified PrP^{Sc} adsorbed to the wells was digested with 20 μg/ml PK and treated with GdnHCl (0–6 M) at r.t. for 1 h. Then wells were subjected to the antibody reaction.

6H10 did not. PrP precipitated from brain homogenates of mice infected with the Obihiro or Chandler strain by mAb 31C6 disappeared after PK treatment. In contrast, 6–7 kDa smaller PrP bands were detected when fractions immunoprecipitated from brain

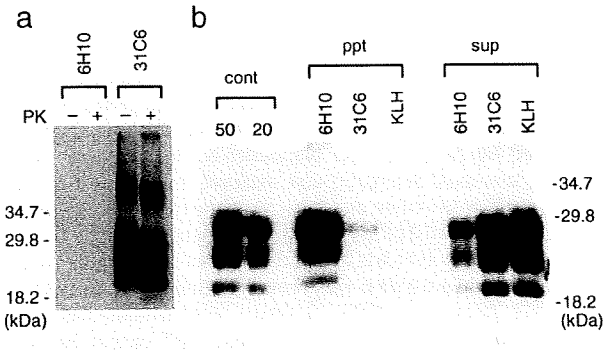

Fig. 2. Reactivity of mAb 6H10 to PrP^{Sc} in Immunoblotting. (a) Reactivity of mAbs 6H10 and 31C6 on an immunoblot. PK-treated (+) and PK-untreated (-) brain homogenates of mice infected with the Obihiro strain were subjected to immunoblotting. (b) Immunoprecipitation. PK-digested purified PrP^{Sc} of the Obihiro strain in PBS containing 1% Triton X-100 was incubated with antibodies as indicated at the top. The antigen–antibody complexes were collected with magnetic beads coated with protein G. PrP^{Sc} in precipitates (ppt) and supernatants (sup) were detected by immunoblotting using HRP-conjugated mAb 31C6. Anti-KLH mAb (KLH) was used as a negative control mAb. PK-digested PrP^{Sc} fractions at 50 and 20 μg brain equivalents were loaded as controls.

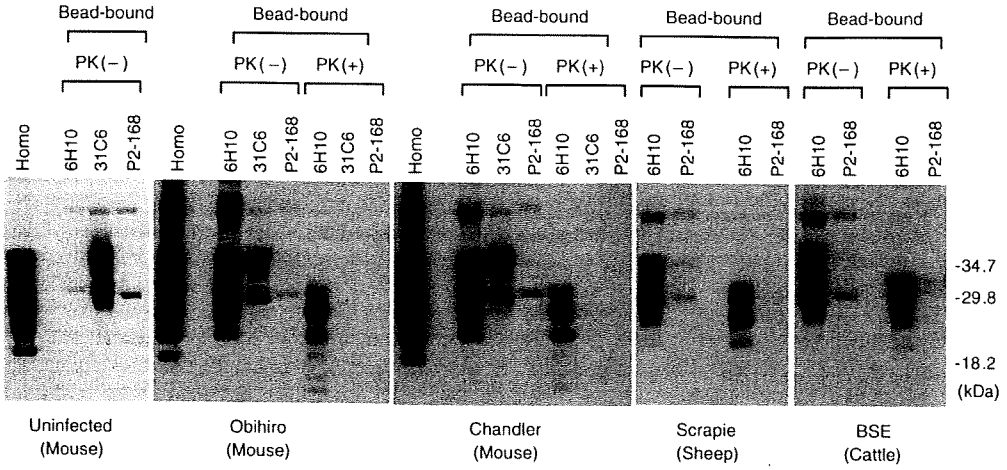

Fig. 3. Immunoprecipitation of PrP^{Sc} by mAb 6H10. Brain homogenates (2.5%) of prion-infected or uninfected animals were incubated with 10 μg of mAbs (6H10, 31C6, P2-168) and then immunoprecipitated with protein G coupled magnetic beads. Bead-bound fractions were treated with PK [PK(+)] or not [PK(-)] and then subjected to immunoblotting. The blots were probed with HRP-conjugated mAb 31C6. MAb P2-168 was used as a negative control mAb. Homo, brain homogenates of uninfected mice or mice infected with prions.

homogenates of the Obihiro or Chandler strain-infected mice with mAb 6H10 were treated with PK (Fig. 3). Thus, at least, some fraction of the PrP immunoprecipitated with mAb 6H10 was resistant to PK treatment. These results suggest that mAb 6H10 selectively recognizes an epitope specific to PrP^{Sc} and thus has a potential to distinguish PrP^{Sc} from PrP^{C}. Moreover, mAb 6H10 exhibited broad host specificity; the mAb reacted with PrP^{Sc} in the brains of scrapie-affected sheep and BSE-affected cattle (Fig. 3). These results suggest that, if not all, at least a certain population of PrP^{Sc} in the brains of prion-infected animals possesses the epitope for mAb 6H10 on its surface.

Recently, Biasini et al. (2008) reported that mAb 15B3, which is purported to be specific to PrP^{Sc} (Korth et al., 1997), also reacted with non-infectious PrP aggregates. Thus we analyzed reactivity of mAb 6H10 to PrP aggregates prepared from rMoPrP89-231 purified from *Escherichia coli* (Fig. 4). The mAb 31C6 immunoprecipitated both non-aggregated and aggregated rMoPrP89-231. In contrast, mAb 6H10 did not immunoprecipitate non-aggregated rMoPrP89-231, but showed weak reaction to aggregated rMoPrP89-231 at the same level as an isotype-matched negative control (mAb P2-168). Thus, the reaction of mAb 6H10 to aggregated rMoPrP89-231 appears to be a non-specific reaction that is possibly caused by a hydrophobic property of aggregated rMoPrP89-231. The same results were obtained when rMoPrP23-231 was used (data not shown). Considering that mAb P2-168 did not immunoprecipitate PrP^{Sc} from brains of prion-infected animals, again, the reactivity of mAb 6H10 to PrP^{Sc} in immunoprecipitation is thought to be a specific reaction between mAb 6H10 and the epitope on PrP^{Sc}.

Reactivity of mAb 6H10 to prion-infected and uninfected brain in histoblot

To further analyze the reactivity of mAb 6H10 to PrP^{Sc}, we performed histoblot analysis (Fig. 5a). Two pan-PrP anitbodies, one of which is antiserum of $Prnp^{-/-}$ mice immunized with rMoPrP (NIAH), and the other is mAb 110, showed positive reaction to histoblot of prion-infected and uninfected mouse brains. However, the positive signals nearly disappeared when the blots were pretreated with PK, indicating that the signals represented protease-sensitive PrP. When the PK-treated histoblots were further processed by autoclaving, which partially denatures PrP^{Sc} and thus exposes cryptic epitopes, these antibodies reacted only with prion-infected mouse brains. In contrast, mAb 6H10 reacted intensely to a histoblot of prion-infected mouse brain pretreated with PK and the intensity was drastically reduced by autoclaving of the histoblot. These results indicated that PrP^{Sc} accumulated in the brain possessed the epitope for mAb 6H10. Unexpectedly, mAb 6H10 showed weak reaction to PK-untreated, uninfected mouse brain sample, although the reaction was not observed on a PK-treated histoblot. To examine whether mAb 6H10 reacts not only with PrP^{Sc} but also PrP^{C} in a histoblot, we used brains of wild-type ($Prnp^{+/+}$) and $Prnp^{-/-}$ mice. The mAb 44B1, classified as pan-PrP mAb, reacted only with the brain of $Prnp^{+/+}$ mice as expected. However, mAb 6H10 reacted weakly with both $Prnp^{+/+}$ and $Prnp^{-/-}$ mice brains (Fig. 5b). Thus, the weak reaction of mAb 6H10 to the brains of uninfected mice may be the result of the presence of a host antigen(s) other than PrP^{C} that shares an epitope similar to that for mAb 6H10.

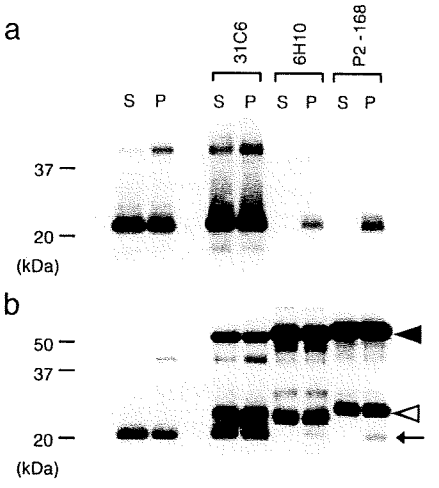

Fig. 4. Reactivity of mAb 6H10 to non-infectious PrP aggregates. (a) Immunoprecipitation of non-aggregated rMoPrP89-231 (S) and aggregated rMoPrP89-231 (P) with mAbs 31C6, 6H10, and P2-168. The mAb P2-168 is an isotype-matched negative control for mAb 6H10. Samples were prepared as described in Materials and Methods, and 10 μl of bead-bound fraction was loaded in each lane. The blot was probed with HRP-conjugated mAb 44B1. (b) After chemiluminescence reaction, the same blot in (a) was washed with PBST and re-probed with HRP-conjugated goat anti-mouse Igs (GE Healthcare) to ensure comparable amount of mAbs were used for each immunoprecipitation. Closed and open arrowhead indicate immunoglobulin heavy and light chain, respectively. Arrow indicates rMoPrP89-231 detected in the re-probing process.

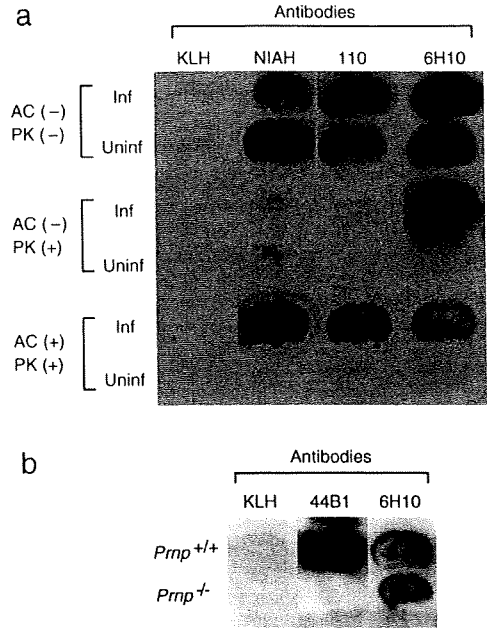

Fig. 5. Histoblot analysis. (a) Cryosections of Obihiro strain-infected (Inf) and uninfected mice brains (Uninf) were blotted onto PVDF membranes. The histoblots on the 1st row were neither treated with autoclave (AC-) nor PK (PK-). The blots on the 2nd row were treated with 50 μg/ml PK (PK+) but not with autoclave (AC-), and those on the 3rd row were treated with PK (PK+) and then autoclaved (AC+). Antibodies: KLH, negative control mAb; NIAH, serum of *Prnp*-/- mouse immunized with rMoPrP; 110, mAb 110; 6H10, mAb 6H10. (b) Histoblots of wild-type ($Prnp^{+/+}$) and $Prnp^{-/-}$ mouse brains were stained with antibodies as indicated.

Neutralization of prion infectivity by mAb 6H10

Next, we examined whether mAb 6H10 can neutralize prion infectivity. Purified PrP^{Sc} from mice infected with the Obihiro strain was pre-incubated with mAbs and then inoculated intracerebrally to mice for a bioassay (Table 1). Pre-incubation of PrP^{Sc} with mAb 6H10 prolonged the mean incubation time by 19 days compared to PrP^{Sc} pretreated with negative control mAbs anti-KLH ($p<0.01$), whereas pre-treatment with mAb 31C6 or 110 appeared to prolong the incubation time a little but the differences were not significant. A prolongation of 19 days was inferred as corresponding to a more than 95% reduction in prion infectivity based on the infectivity–incubation time standard curve obtained from bioassays of serially diluted brain homogenates from the Obihiro strain-infected mice (Table 1). Several pan-PrP mAbs have been reported to inhibit the accumulation of PrP^{Sc} in prion persistently infected neuroblastoma cells when the cells were incubated with those mAbs (Enari et al., 2001; Peretz et al., 2001; Perrier et al., 2004; Kim et al., 2004b). Cells persistently infected with the Obihiro strain were unavailable so far, we used mouse neuroblastoma cells persistently infected with the Chandler strain (I3/I5-9 cells, Kim et al., 2004b) to examine whether mAb 6H10 inhibits PrP^{Sc} formation in cells. Cells were cultured with mAb 6H10 (up to 20 μg/ml) for 4 days; however, mAb 6H10 did not affect the PrP^{Sc} formation in I3/I5-9 (data not shown). Our previous study showed that anti-PrP mAbs strongly reacted with PrP^{C} on the cell surface could inhibit PrP^{Sc} formation in I3/I5-9 cells (Kim et al., 2004b). However, mAb 6H10 did not react with cell surface of I3/I5-9 cells by flowcytometirc analysis (data not shown).

Table 1
Neutralization of prion infectivity by mAb 6H10.

mAb	Numbers of mice		Incubation time to terminal stage [mean±SD (days)]
	Inoculated	PrP^{Sc} positive	
Anti-KLH	5	5	161±4
mAb 31C6	5	5	166±6
mAb 110	5	5	169±7
mAb 6H10	5	5	180±8[a]
Brain homogenate[b]			
10%	10	10	161±6
1%	12	12	164±6
0.1%	12	12	175±6
0.01%	11	11	189±18
0.001%	12	12	212±12
0.0001%	11	11	245±31
0.00001%	6	3	278±52
0.000001%	6	0	>393

[a] $p<0.01$ by ANOVA followed by Dunnett's *post hoc* test.
[b] To obtain an infectivity–incubation time standard curve, 10-fold serial dilutions of brain homogenates of mice infected with the Obihiro strain were bioassayed. The 50% lethal dose (LD_{50}) of the original homogenates (10% brain homogenates) was estimated to be 10^6 LD_{50}/20 μl by the Reed-Muench method. The standard curve for incubation periods (x) shorter than 190 days was fitted by the approximation of $LD_{50}=e^{41.48-0.183x}$. The standard curve for incubation periods (x) longer than 190 days was fitted by the approximation of $LD_{50}=e^{21.07-0.076x}$.

Epitope for mAb 6H10

To investigate the epitope for mAb 6H10, we adopted phage display analysis. Fig. 5a shows consensus amino acid (aa) residues deduced from selected phage clones with mAbs 31C6 or 110, or rabbit polyclonal antibodies (pAb) B103. Three or four aa residues of the phage clones selected with mAbs 31C6 and 110 were identical to the aa residues in regions determined by pepspots analysis (Kim et al., 2004a). Similarly, three or four aa residues of selected phage clones with pAb B103 were identical to the synthetic peptide used as the immunogen. These results suggest that at least three residues are required to form an epitope for antibody. In total, 56 phage clones selected after five rounds of panning with mAb 6H10, and these were subjected to DNA sequencing so that the aa sequences could be deduced. The consensus aa residues of two abundant phage clones are listed in Fig. 6a. The most abundant phage clone ph#121 contained four residues identical to the C-terminus of MoPrP (SPSQAWLYMRHE, underlined residues). The second most abundant phage clone ph#125 also had three residues identical to the C-terminus of MoPrP (TQNWSMSMLLKQ, underlined residues). In contrast, no aa sequence identity to MoPrP was observed in the third most abundant phage clone ph#98 (IPLTGKYLDEQS, 6/56, 11%). Binding of antibodies to selected phage clones was confirmed by captured ELISA (Fig. 6b). The mAb 6H10 reacted with phage clones ph#121 and ph#125, originally selected by mAb 6H10, but not with ph#6, originally selected by mAb 31C6. On the other hand, mAb 31C6 reacted only with phage clone ph#6. Taken together, these data demonstrate the specificity of phage clone selection. Moreover, the results suggest that the C-terminus of MoPrP is involved in the formation of the epitope for mAb 6H10. We also analyzed the epitope for mAb 6H10 using a pepspots membrane; however, mAb 6H10 did not react with any spots (data not shown).

Discussion

Immunization of $Prnp^{-/-}$ mice and screening of hybridomas with non-denatured PrP^{Sc} is one of the ways to establish PrP^{Sc}-specific mAbs. Using this approach, we obtained mAb 6H10, which reacted with non-denatured PrP^{Sc} but not with denatured PrP^{Sc}. It is well known that epitopes for pan-PrP antibodies on PrP^{Sc} become accessible by antibodies after dissociation of PrP^{Sc} aggregates and/or denaturation of PrP^{Sc} (Kascsak et al., 1987; Serban et al., 1990; Williamson et al., 1996). However, the reactivity of mAb 6H10 to PrP^{Sc} decreased with the increases in GdnHCl concentration for the pretreatment of PrP^{Sc} to 3 M, indicating that the properties of mAb

a

Antibody: mAb 31C6

aa sequence of epitope	143-DWEDRYYRE-231	
Deduced aa sequences	DxxxRxxxE	(6/11)
	DWxxR	(2/11)
Possible epitope	DWEDRYYRE	

Antibody: mAb 110

aa sequence of epitope	83-PHGGGWG-89	
Deduced aa sequences	PxGxGW	(6/10)
	PxGxxW	(1/10)
Possible epitope	PHGGGWG	

Antibody: pAb B103

aa sequence of epitope	90-QGGTHGQWNKPSKPKTNMK-109	
Deduced aa sequences	NKxxxP	(7/11)
	HxxxNKxxxP	(2/11)
Possible epitope	QGGTHGQWNKPSKPKTNMK	

Antibody: mAb 6H10

aa sequence of epitope	NA	
Deduced aa sequences	SQAxxxxR	(24/56)
	TQxxxxS	(13/56)
Possible epitope	213-CVTQYQKESQAYYDGRRSS-231	

b

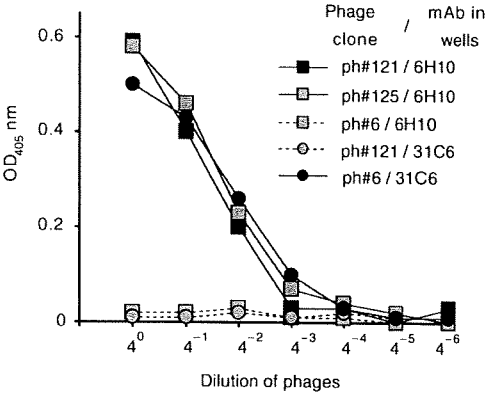

Fig. 6. Epitope analysis by peptide phage display. (a) Identity of aa sequences between selected phage clones and the region to which antibodies are expected to bind. The aa sequence of the epitopes of mAbs 110 and 31C6 were determined by pepspots analysis (Kim et al., 2004a), whereas that of pAb B103 corresponds the synthetic peptide used for immunization (Horiuchi et al., 1995). The deduced aa sequences indicate that consensus aa residues of selected phage clones. Shown are two abundant consensus sequences deduced from selected phage clones, which were obtained after three rounds (for pAb B103, and mAbs 110 and 31C6) or five rounds (for mAb 6H10) of panning. Amino acid residues identical to the expected region are indicated in bold. Numbers in parentheses indicate the total number of phage clones analyzed for each antibody (denominators) and the numbers of phage clones that possess the corresponding consensus aa sequence (numerators). The residues that are expected to constitute the epitope for each antibody are underlined (possible epitope). (b) Binding of antibodies to selected phage clones. Wells coated with mAb 6H10 or 31C6 were incubated with four-fold serial dilutions of the selected phage clones (ph#121, ph#125, or ph#6). Phage–antibody complexes were detected as described in Materials and Methods. Phage clones ph#121 and ph#125, selected by mAb 6H10, express the peptides SPSQAWLYMRHE and TQNWSMSMLLKQ, respectively. Phage ph#6, selected by mAb 31C6, expresses peptide *SDWHTRFHYSMN* (underlining indicates the consensus aa residues for the corresponding epitope).

6H10 differ from those of pan-PrP mAbs. The infectivity of the Obihiro strain, which was used as a source of PrP^{Sc} in this study, was dramatically reduced by the treatment with 3 M GdnHCl (Shindoh et al., 2009), consistent with a decrease in the reactivity of mAb 6H10 to PrP^{Sc} of the Obihiro strain pretreated with 3 M GdnHCl. Furthermore, mAb 6H10 neutralized infectivity of PrP^{Sc} purified from mice infected with the Obihiro strain (Table 1). Thus, mAb 6H10 may recognize a conformational epitope on non-denatured PrP^{Sc} that is related to the oligomerization interface on the PrP^{Sc} molecule. Reduction of the hydrophobic surface on PrP^{Sc} correlated with the dissociation and/or denaturation of PrP^{Sc} by GdnHCl at concentrations between 0 and 3 M (Safar et al., 1994). Circular dichroism analysis also revealed that GdnHCl dissociated PrP^{Sc} aggregates with a midpoint of transition around 2 M (Safar et al., 1993). The half-maximal GdnHCl concentrations, which are required to denature 50% of PrP^{Sc}, have been reported to range from 1.5 to 3 M for most of mouse-adapted scrapie and BSE prions (Legname et al., 2006; Shindoh et al., 2009). In addition, treatment of samples with 2–4 M GdnHCl resulted in decrease of prion infectivity (Caughey et al., 1997; McKenzie et al., 1998). Although these reports used different prion strains, the decrease in reactivity of mAb 6H10 to PrP^{Sc} with an increase in GdnHCl concentration for pretreatment of PrP^{Sc} concurs with structural alteration of PrP^{Sc} or the decrease of infectivity induced by GdnHCl at similar concentrations.

The results of phage display analysis suggested the possibility that the extreme C-terminus of PrP molecule is part of the epitope for mAb 6H10. Among the amino acid residues expected to compose the epitope for mAb 6H10 (aa215-TQxxxxSQAxxxxR-aa228 of MoPrP), the underlined residues are identical to those of sheep and bovine PrP. This may be consistent with the fact that mAb 6H10 could immunoprecipitate PrP^{Sc} from sheep and cattle samples. The mAb 6H10 showed no reaction with denatured PrP^{Sc} by immunoblotting or ELISA (Figs. 1 and 2), or with the corresponding C-terminal peptide by pepspots analysis (data not shown). In contrast, reactivity of mAbs that recognize linear epitopes on the extreme C-terminus increased after pretreatment of PrP^{Sc} with denaturants (Peretz et al., 1997; Kim et al., 2004a). Thus it is unlikely that mAb 6H10 binds linear epitope composed of the C-terminal residues. One mAb, V5B2, which was raised against a synthetic peptide corresponding to the C-terminal residues of human PrP (aa214-CITQYERESQAYY-aa226), is reported to discriminate PrP^{Sc} in brains of CJD patients from PrP^{C} in non-CJD brains possibly by recognizing the oligomerized C-terminal region in PrP^{Sc} oligomers (Curin Serbec et al., 2004; Ulrih et al., 2006). Therefore, the C-terminal regions of two PrP molecules may cooperate to form a conformational epitope; however, the exact properties of the epitope for 6H10 are not yet fully elucidated. Alternatively, in the proposed model of PrP^{Sc} protofibrils, the C-terminal region is thought to be located in close proximity to other regions on the same or different PrP molecule (DeMarco and Daggett, 2004; Govaerts et al., 2004). Cross-linking of recombinant PrP oligomers revealed that an interaction between the N- and C-terminal regions (Kaimann et al., 2008). Thus, the C-terminal region and other regions on the same or different PrP molecule may participate in constituting the epitope for mAb 6H10. The mAb 15B3, which was produced by immunizing recombinant bovine PrP and is purported to be specific to PrP^{Sc}, reacted with three PrP segments, residues 141–147, 161–269, and 213–215 of MoPrP in pepspots analysis (Korth et al., 1997). Involvement of the extreme C-terminus in the epitope for mAb 15B3 is intriguing; however, mAb 6H10 did not react with any PrP peptides in pepspots analysis (data not shown). Thus, the epitope for mAb 6H10 seems to differ from that for mAb 15B3. Recently, mAb 15B3 is reported to react with infectious and non-infectious PrP

aggregates (Biasini et al., 2008), therefore, we analyzed the reactivity of mAb 6H10 to aggregated rMoPrP (Fig. 4). Although a weak non-specific reaction to aggregated rMoPrP was observed, mAb 6H10 did not show any specific reaction to aggregated rMoPrP, suggesting mAb 6H10 is more specific to PrP^{Sc} generated in brains of prion-infected animals.

Some mAbs against either PrP or non-PrP molecules have been reported to immunoprecipitate PrP^{Sc} via an epitope-independent reaction when mAbs were bound to a solid phase such as magnetic beads, as binding of immunoglobulins on the limited area of the solid surface increases the concentration of immunoglobulins in that area and thus a low-affinity interaction between PrP^{Sc} and immunoglobulins on the beads may occur (Morel et al., 2004). We considered that this possibility is unlikely in the case of mAb 6H10 for several reasons. First, bead-free mAb 6H10 reacted with PrP^{Sc} in ELISA and histoblot analysis (Figs. 1 and 5). In addition, a direct interaction of fluorescent dye-labeled mAb 6H10 with non-denatured PrP^{Sc} in brain homogenates of mice infected with prions can be detected in solution by fluorescent correlation spectroscopy (K. S. and M.H., in preparation). Secondly, a Fab' fragment of mAb 6H10 still reacted with native purified PrP^{Sc} in ELISA (data not shown). However, a weak non-specific reaction to aggregated rMoPrP would be expected depending on experimental conditions (Fig. 4), careful experimental design, such as the use of negative control mAb and comparison between prion-infected and mock-infected materials, will be required to ensure specific reactivity of mAb 6H10 to PrP^{Sc}.

The reactivity of mAb 6H10 to PrP^{Sc} in biochemical analysis strongly suggests that mAb 6H10 discriminates PrP^{Sc} from PrP^{C}. However, in histoblot analysis, the mAb showed weak reaction to certain host molecule in *Prnp*$^{-/-}$ mice. This raises two possibilities. First, the mAb may recognize a host molecule other than PrP with an epitope similar to that for mAb 6H10 on PrP^{Sc}. The presence of third abundant phage clone (ph#98) that selected by mAb 6H10, aa sequence of which showed no apparent homology to PrP, may clue the identification of such host molecule. However, no protein or peptide domain was found by homology search using protein blast and tblastn or domain search using Conserved Domain Database. Secondly, the mAb may recognize a host factor tightly associated with PrP^{Sc} in the prion-infected mouse brain, rather than reacting with PrP^{Sc} itself. The latter possibility suggests that the positive reaction to PK-treated PrP^{Sc} in ELISA was due to a reaction to a PK-resistant molecule tightly associated with PrP^{Sc}, which seems unlikely because the PrP^{Sc} fraction used in this study was estimated to be nearly 90% pure after PK treatment (Kim et al., 2004a). However, this possibility cannot be completely ruled out at present, particularly as several macromolecules, including glycosaminoglycans (Snow et al., 1989), ubiquitin (Lowe et al., 1990), apolipoprotein E (Namba et al., 1991) have been shown to co-localize with PrP^{Sc} in brains of prion-infected animals and human. In addition, nucleic acids (Aiken et al., 1990; Sklaviadis et al., 1993) and sphingolipid (Klein et al., 1998) have been co-purified with PrP^{Sc}.

In this study, we showed the possibility that the C-terminal region forms a PrP^{Sc}-specific epitope. Recently, immunization of β-form recombinant human PrP could induce production of mAbs that react with the N-terminal region of PrP^{Sc} (aa91–110; Khalili-Shirazi et al., 2007). The immunization of aggregated synthetic peptide corresponding to aa106–126 of PrP also generated a mAb that discriminates PrP^{Sc} from PrP^{C} (Jones et al., 2009). These results suggest that PrP^{Sc}-specific epitopes are present at various regions on PrP^{Sc}. A fusion protein of PrP and Fc-region of immunoglobulin and PrP-peptide-grafted antibodies have been reported to recognize PrP^{Sc} (Meier et al., 2003; Moroncini et al., 2004, 2006; Solforosi et al., 2007; Lau et al., 2007). In addition, there are nucleotide aptamers that possess higher affinity to PrP^{Sc} than to PrP^{C} (Rhie et al., 2003), and small chemicals such as 9-aminoacridine, streptomycin, luminescent conjugated polymers, polyionic polymers Seprion (Microsens biotechnologies) and others, may also act as PrP^{Sc}-specific probes (Moussa et al., 2006; Phuan et al., 2007; Sigurdson et al., 2007). Despite technical difficulties in manipulating PrP^{Sc} due to its aggregation-prone propensity and heterogeneity, PrP^{Sc}-specific molecular probes are gradually accumulating. As the availability of a panel of anti-PrP mAbs has greatly contributed to characterization of the biochemical properties of PrP, a panel of PrP^{Sc}-specific molecular probes appears to be indispensable tools for analyzing the biochemical and biological properties of native PrP^{Sc} with which prion infectivity is believed to be associated.

Materials and methods

Purification of PrP^{Sc} and production of mAbs

The purification PrP^{Sc} from brains of mice infected with prion Obihiro strain and purity of PrP^{Sc} were reported elsewhere (Kim et al., 2004a). Immunization of PrP^{Sc} and production of mAbs were carried out as described (Kim et al., 2004a).

Antibodies

The following mAbs against mouse PrP molecules were used: 31C6 (IgG1, epitope: aa 143–149), 44B1 (IgG 2a, epitope: aa 155–231), 72 (IgG1, epitope: aa 89–231), and 110 (IgG2b, epitope: aa 59–89) (Kim et al., 2004a). Anti-keyhole limpet hemocyanin (KLH) mAb (IgG2a) and anti-parvovirus mAb P2-168 (IgG2b) (Horiuchi et al., 1997) were used as negative controls. Rabbit antiserum raised against bovine PrP synthetic peptide 103-121 (pAb B103) was also used (Horiuchi et al., 1995). Purification of mAbs was carried out as described elsewhere (Kim et al., 2004a). Conjugation of mAbs with horseradish peroxidase (HRP) was carried out as follows: purified mAbs were digested with pepsin and reduced by 2-mercapthoethanolamine (MEA) to generate Fab' fragments. After removal of MEA with a PD-10 size exclusion column (GE Healthcare), the Fab' fragments were mixed with HRP coupled with the bi-directional cross-linker GMBS (Dojin).

ELISA

Ninety-six well plates (MaxiSorp, Nunc) were coated overnight at 4 °C with either 200 ng purified PrP^{Sc} or 100 ng rMoPrP in 50 μl of 20 mM phosphate buffer (pH 7.0). After adsorption, wells were blocked with 5% fetal bovine serum (FBS) in PBS containing 0.1% Tween 20 (PBST) for 2 h at room temperature (r.t.), and incubated with antibodies diluted with 1% FBS in PBST for 1 h. After washing with PBST, wells were incubated with HRP-conjugated secondary antibodies for 1 h. The antigen–antibody complexes were visualized with 2,2′-azino-bis(3-etht-benzthiazoline-6-sulfonic acid), 0.04% H_2O_2 in 50 mM citrate–phosphate buffer, pH 4.0, and the absorbance at 405 nm was measured with a microplate reader. In some cases, PrP^{Sc} adsorbed to the well was digested with various concentrations of proteinase K (PK) in Tris-buffered saline (TBS; 50 mM Tris–HCl [pH 8.0] and 150 mM NaCl) at 37 °C for 45 min. After terminating PK activity by adding Pefabloc (Roche Diagnostic) to a final concentration at 2 mM, the plates were subjected to the immune reaction.

Immunoblotting

SDS-PAGE and immunoblotting of proteins on an Immobilon-P Transfer Membrane (Millipore) were carried out as described elsewhere (Kim et al., 2004a, 2004b). Membranes were blocked with 5% skim milk in PBST for 1 h and then incubated with HRP-conjugated Fab fragment of anti-PrP mAbs (direct staining) or anti-PrP mAb followed by incubation with HRP-conjugated secondary antibodies (indirect staining). ECL Western blotting detection reagents (GE Healthcare) and X-ray film were used for visualization.

Immunoprecipitation

Protein G-coupled magnetic beads (Dynabeads) were blocked with blocking buffer containing 5% skim milk and 50% Sea Block (Pierce) in PBS. Brain homogenates (2.5%) from prion-infected or uninfected animals were prepared with PBS containing 0.5% I-Block (Applied Biosystem), and were incubated with 10 μg of mAb and protein G magnetic beads for 45 min at 37 °C. The magnetic beads were washed four times with PBS containing 2% Triton X-100 using a magnetic separator. After washing, the beads were suspended with 50 μl of TBS and divided into two tubes. An equal volume of 2 sample buffer (8 M urea, 10% SDS, 8% *b*-mercaptoethanol, 125 mM Tris–HCl [pH 6.8], 6 mM EDTA, 10% glycerol, and 0.04% bromophenol blue) was added to one tube to make samples without PK treatment. The other was treated with 40 μg/ml of PK for 30 min at 37 °C. The reaction with PK was stopped with 2 mM Pefabloc before adding 2 sample buffer.

To examine the reactivity of mAbs to non-infectious PrP aggregates, rMoPrP23-231 or rMoPrP89-231 (Kim et al., 2004a) were diluted to 20 μg/ml with 1 ml of 1% Triton-X 100 in PBS (PBS-Triton, pH 7.2) and kept for 30 min at 20 °C. Then samples were centrifuged at 100,000×*g* for 45 min at 20 °C. The supernatant was recovered and used as non-aggregated rMoPrP, while the resulting precipitate was resuspended with 1 ml of PBS-Triton by sonication and used as aggregated rMoPrP. Protein G-coupled magnetic beads (100 μl) were incubated with 20 μg of mAbs and then blocked with 5% skim milk and 5% N102 blocking reagent (NOF Corporation, Japan) in PBS-Triton for 1 h at r.t. After washing with PBS-Triton once, the beads were mixed with 300 μl of non-aggregated or aggregated rMoPrP for 45 min at r.t. The beads were washed four times with 1 ml of 2% Triton X-100 in PBS and finally washed with PBS. Proteins bound to the magnetic beads were eluted with 100 μl of 1× sample buffer.

Histoblot analysis

Histoblot analysis was carried out as described by Taraboulos et al. (1992). Immobilon-P Transfer Membranes were activated with methanol and then equilibrated with lysis buffer (0.5% Nonidet P-40, 0.5% sodium deoxycholate, 100 mM NaCl, 10 mM EDTA, 10 mM Tris–HCl [pH 7.8]). Mouse brain cryosections (8 μm) were prepared and placed on glass slides. The glass slides carrying the sections were immediately pressed onto membranes on layers of filter paper saturated with lysis buffer for 1 min. The membranes were thoroughly air-dried and stored at -80 °C until use. Before immunostaining, the membranes were rehydrated in PBST for 1 h at r.t. and then immunostaining was carried out as described above for immunoblotting.

Neutralization of prion infectivity

Fifteen micrograms of purified PrP^{Sc} were incubated with 36 μg of mAb in 300 μl of PBS containing 0.1% Zwittergent 3-12 for 2 h at r.t. The mixture (20 μl) containing 1 μg PrP^{Sc} was then inoculated intracerebrally into 4-week-old female slc:ICR mice. For dose–infectivity correlation, 20 μl each of serially diluted brain homogenates from mice infected with the Obihiro strain were inoculated intracerebrally to 4-week-old female slc:ICR mice.

Peptide phage display

The Ph.D.-12™ Phage Display Library Kit (New England Biolabs), a combinatorial library which expresses random 12-mer-peptides at the N-terminus of a minor coat protein of M13 phage, was used according to the supplier's instructions. Polystyrene Petri dishes (60×15 mm) were coated overnight at 4 °C with 1.5 ml of 100 μg/ml antibodies in 0.1 M $NaHCO_3$ (pH 8.6). After blocking with 0.5% FBS in PBS for 1 h, dishes were rinsed six times with PBST and then inoculated with 4×10^{10} of the phage library. After 1 h incubation at r.t., the dishes were washed 10 times with PBS and then bound phage was eluted by addition of 1 ml 0.2 M glycine–HCl (pH 2.2) for 10 min. The eluate was immediately neutralized with 150 μl of 1 M Tris–HCl (pH 9.1). In total, 1 ml of the eluate was used for amplification of the selected phage pool and the amplified phage stock was subsequently used for the next panning.

After three to five rounds of panning, phage DNA encoding the selected random peptide sequences was amplified directly from individual plaques by PCR using primers PD12F (5′-TCAAGCTGTT-TAAGAAATTCACC-3′) and PD12R (5′-TAAAGTTTTGTCGTCTTTCCA-GAC-3′). The PCR products were purified by S-300 HR spin column (GE Healthcare) and used as templates for DNA sequencing. DNA sequences were determined with an automated DNA sequencer (ABI-373A, Applied Biosystems) and using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit.

Binding of antibodies to selected peptides was confirmed by a captured ELISA. Briefly, 96-well plates coated with antibodies (20 ng/well) were incubated with four-fold serial dilutions of the phage stock for 1 h at r.t. After washing with PBST, plates were incubated with HRP-conjugated anti-M13 antibody to detect the phage captured by the antibodies. Phage–antibody complexes were detected as described for ELISA.

Pepspots analysis

Pepspots analysis was carried out as described elsewhere (Kim et al., 2004a).

Statistical analysis

Statistical analysis was done with JMP software (SAS Institute Inc.).

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GABAA receptor subunit β1 is involved in the formation of protease-resistant prion protein in prion-infected neuroblastoma cells

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ABSTRACT

γ-Aminobutyric acid type A (GABAA) receptor β1 (gabrb1), a subunit of GABAA receptors involved in inhibitory effects on neurotransmission, was found to associate with the formation of protease-resistant prion protein in prion-infected neuroblastoma cells. Silencing of gabrb1 gene expression significantly decreased the abnormal prion protein level but paradoxically increased the normal prion protein level. Treatment with a gabrb1-specific inhibitor, salicylidene salicylhydrazide, dose-dependently decreased the abnormal prion protein level, but silencing of other GABAA receptor subunits' gene expression and treatments with the receptor antagonists and agonists did not. Therefore, gabrb1 involvement in abnormal prion protein formation is independent of GABAA receptors.

1. Introduction

Transmissible spongiform encephalopathies or prion diseases are fatal neurodegenerative disorders that include Creuzfeldt–Jakob disease in humans, and bovine spongiform encephalopathy and scrapie in animals. These diseases are characterized by deposition of a partially protease-resistant abnormal isoform of prion protein (PrPres), which is the main component of the pathogen and which is converted from the normal cellular isoform of prion protein (PrPc) in the central nervous system and lymphoreticular system [1]. Cell biology of the biosynthesis and metabolism of PrPc and PrPres has been eagerly investigated in prion-infected cells [2,3] but has not been fully elucidated. Especially, endogenous factors involved in the formation of PrPres or the conformational change from PrPc into PrPres [4,5] remain enigmatic.

Using gene screening by the gene silencing technique with small interfering RNA (siRNA) or short hairpin RNA (shRNA) [6,7], we have sought endogenous factors affecting the metabolism of PrPres in prion-infected neuroblastoma cells. We report here a possible linkage of γ-aminobutyric acid A receptor β1 (gabrb1) with the formation of PrPres. In fact, gabrb1 is a subunit of γ-aminobutyric acid type A (GABAA) receptors responsible for most of the fast inhibitory synaptic transmission in mammalian brain [8]. Belonging to the ligand-gated ion channels, they are formed by the pentameric assembly of homologous subunits. Numerous GABAA receptor subunits have been identified (α1–6, β1–3, γ1–3, δ, π, ε, and θ), all of which are products of separate genes, and most GABAA receptors contain two α subunits, two β subunits and either one γ subunit or one δ subunit [8]. It has been well documented that GABAergic neurons are affected by prion infection [9–15], but it is not clear whether GABAA receptors are involved in PrPres formation. Therefore, to address this query, we performed gene silencing experiments for gabrb1 and other representative GABAA receptor subunits (α5, β3, γ2, and δ) as well as GABAA receptor modulating experiments using the antagonists and agonists in prion-infected cells.

2. Materials and methods

2.1. siRNAs and compounds

Double-stranded siRNAs for GABAA receptor subunits used for this study (Table 1) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Some GABAA receptor antagonists (bicuculline

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methiodide and picrotoxin) and agonists (GABA, muscimol, and isoguvacine hydrochloride) were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). Another agonist, pentobarbital, was purchased as a 5% (approximately 200 mM) solution of its sodium salt, nembutal, from Dainippon Sumitomo Pharma Co. Ltd. (Osaka, Japan). A specific inhibitor of GABAA receptor β1 subunit, salicylidene salicylhydrazide, was obtained from Tocris Bioscience (Missouri, USA).

2.2. shRNA expression vectors

The DNA fragments flanking 5′ *Bam*HI recognition sequence and 3′ *Hind*III recognition sequence, which corresponded to shRNA sequences for GABAA receptor subunits, were produced by annealing pairs of sense and antisense 64mer oligonucleotides, of which sense sequences were designed as follows: 5′-GATCC***GAgGGTGGAGTTCAtAAtAGG***CTTCCTGTCA***CCTGTTGTGAACTCCACCTTC***-TTTTTA-3′ targeting to nucleotides 663–683 of β1 subunit coding sequence; 5′-GATCC***ACCTGGTgTGAtAGAtGTTTG***CTTCCTGTCA***CAAACGTCTGTCATACCAGGT***TTTTTTA-3′ targeting to nucleotides 355–375 of γ2 subunit coding sequence; 5′-GATCC***GAATGGGCTgCTTtACtATCC***CTTCCTGTCA***GGATGGTGAAGTAGCCCATTC***TTTTTTA-3′ targeting to nucleotides 809–829 of γ2 subunit coding sequence; 5′-GATCC***ACATGGAgTACACtATGAtTG***CTTCCTGTCA***CAGTCATGGTGATTTCCATGT***TTTTTTA-3′ targeting to nucleotides 245–265 of δ subunit coding sequence. Bold italic letters and small bold italic letters, respectively denote target sites and mismatch-induced sites. The DNA fragments were ligated into a pBAsi-hH1 (Takara Bio Inc., Shiga, Japan) cut with *Bam*HI and *Hind*III. The ligated vectors were introduced into *Escherichia coli*; then plasmids of interest were harvested and sequenced.

2.3. Mutated gabrb1 expression vector

Mouse gabrb1 gene was cloned from a ready mouse brain cDNA library (Marathon; Takara Bio Inc.) using PCR with KOD-plus DNA polymerase (Toyobo Co. Ltd., Osaka, Japan). A PCR product was inserted into a pcDNA3.1 Myc/His expression vector (Invitrogen Corp.). The ligated vectors were introduced into *E. coli*; then plasmids of interest were harvested and sequenced. Mutations were induced by site-directed mutagenesis using PCR technique with primers of 5′-GATGCATCTGCAGCgcGtGTgGCtCTtGGtATaACaACGGTGCTG-3′ (small italic letters indicate silent mutations induced) and 5′-TGCAGATGCATCATAGTTGATCCA-3′. The PCR products were treated with *Dpn*I for digesting template plasmids and introduced directly into *E. coli*; then plasmids of interest were harvested and sequenced.

2.4. Gene silencing experiments

Mouse neuroblastoma N2a cells infected persistently with RML prion strain (ScN2a) or 22L prion strain (N167) were diluted to 10% or 15% confluence with Opti MEM I (Invitrogen Corp.) including 10% fetal bovine serum (FBS), and 2.4 ml each was seeded onto six-well plates. Transfection was performed on the next day of seeding. TransFectin (3.0 μl/well; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for the transfection of shRNA expression vectors, and siLentFect (3.0 μl/well, Bio-Rad Laboratories, Inc.) was used for transfection of double-stranded siRNAs. The amounts of vectors or siRNAs used in transfection were, respectively, 0.2 μg per well or 20 nM per well. Medium was changed on the day after transfection. Cells were harvested after washing with PBS 3 days after transfection.

Table 1
siRNAs and shRNAs used for this study.

GABAA receptor subunit examined	Nucleotide position of target region*	
	siRNA (catalog no.**)	shRNA
α5	311–335 (MSS201426)	
β1	820–844 (MSS204523)	663–683
β3	873–897 (MSS204527)	
β3 transcript variant 1	62–86 (MSS204528)	
γ2		355–375, 809–829
δ		245–265

* Nucleotide position in the coding sequence of each GABAA receptor subunit gene.
** siRNAs were obtained from Invitrogen Corp. (Carlsbad, CA, USA).

2.5. Rescue experiment

The ScN2a cells were diluted to 15% confluence with Opti MEM I including 10% FBS, and 2 ml each was seeded onto six-well plates. Transfection was performed on the day after seeding. TransFectin (2.0 μl/well) was used for the transfection of both mutated gabrb1 expression vector and double-stranded siRNA. The amounts of the vector and the siRNA used for transfection were, respectively, 0.8 μg per well and 5 nM per well. Medium was changed on the day after transfection. Cells were harvested after washing with PBS 3 days after transfection.

2.6. Immunoblotting

Cells were lysed with lysis buffer (0.5% sodium deoxycholate, 0.5% Nonidet P-40, PBS) after rinsed with PBS, and debris was eliminated by centrifugation at 3000×*g* for 10 min at 4 °C. Protein contents of each sample were measured using a modified Lowry method [16] with Dc protein assay reagent (Bio-Rad Laboratories, Inc.) with bovine serum albumin as a standard. For PrPres detection, cell lysate containing the same protein amount was treated with 10 μg/ml of proteinase K for 30 min at 37 °C, and PrPres was pelleted by centrifugation at 20 000×*g* for 20 min at 4 °C. After denaturation in sample buffer by heating at 95 °C for 10 min, PrP was separated using SDS–PAGE and then transferred onto Immobilon-P membrane (Millipore Corp., Bedford, MA, USA). Subsequently, PrP was detected using a monoclonal antibody SAF83 as a primary antibody, which recognizes residues 126–164 of mouse PrP (1:5000; SPI-Bio, Massy, France), and an alkaline phosphatase-conjugated goat anti-mouse antibody (1:20 000; Promega Corp., Madison, WI, USA) as a secondary antibody. Immunoreactivity was visualized using CDP-Star detection reagent (Amersham, Piscataway, NJ, USA) and was analyzed densitometrically using the ImageJ program (National Institutes of Health, Bethesda, MD, USA). To check the sample integrity, protein levels of GAPDH and β-actin were analyzed in the same samples used for PrPres detection.

2.7. Quantification of mRNA level

Cells were lysed with RNAiso-plus reagent (Takara Bio Inc.). Total RNA was extracted using FastPure RNA (Takara Bio Inc.). Poly A^+ RNA was purified from total RNA using an isolation kit (MicroFast Track MAG micro mRNA; Invitrogen Corp.). In addition, cDNA was synthesized with first strand cDNA synthesis kit (Takara Bio Inc.). The mRNA level was measured by real-time PCR using SYBR Premix Ex Taq II (Takara Bio Inc.) or using TaqMan probe with gene expression assay master mix for gabrb1 (Mm00433461_m1; Applied Biosystems). Fold change of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as an internal control.

2.8. Statistical analyses

Statistical significance was analyzed using one-way analysis of variance followed by the Tukey–Kramer post-hoc test for multiple

sample comparisons, or using a *t*-test for the two sample comparisons. Statistical significance for each analysis was defined as $P < 0.05$.

3. Results

3.1. Effects of gabrb1 gene silencing

Gene silencing of gabrb1 in ScN2a cells by transfection of shRNA expression vector targeting at nucleotides 663–683 of gabrb1 coding sequence demonstrated a significant decrease in both gabrb1 mRNA level (Fig. 1a) and PrPres formation (Fig. 1b). On the other hand, the PrP mRNA level increased significantly (Fig. 1a); upregulation in PrPc protein level was confirmed in uninfected N2a cells as well as ScN2a cells (Fig. 1a and c). Similar results were also observed in the gene silencing experiment using double-stranded siRNA, which was designed to target at nucleotides 820–844 of gabrb1 coding sequence (Fig. 1d–f). Protein levels of GAPDH and β-actin in the cells treated with the gabrb1 shRNA or siRNA were consistent with those of the mock controls (Fig. 1b and e), indicating that the reduction of PrPres level was not attributable to either sample preparation artifacts or cell viability difference. On the other hand, in comparison with ScN2a cells, N167 cells showed a less remarkable decrease in PrPres formation when treated with the gabrb1 siRNA (Supplementary Fig. 1).

Other representative subunits of GABAA receptor, including β3 and one of its transcript variants, as well as α5, γ2, and δ, were subjected to gene silencing experiments in ScN2a cells, but no modification in the PrPres level was observed despite marked reduction in mRNA levels of the target genes (Supplementary Fig. 2). These subunits were chosen from the following facts. Most GABAA receptors contain two α subunits, two β subunits, and either one γ subunit or one δ subunit [8]. The α5 subunit is reportedly upregulated in the brain when prion is inoculated into the mice expressing anchorless PrP [17]. The β3 subunit is the most abundant β subunit observed in ScN2a (data not shown). The γ2 subunit is the most abundant subunit in the brain and is incorporated in most GABAA receptor subtypes [18]. The δ subunit comprises only one member. In addition to the subunits described, the β2 subunit was also examined in this study, but its gene silenc-

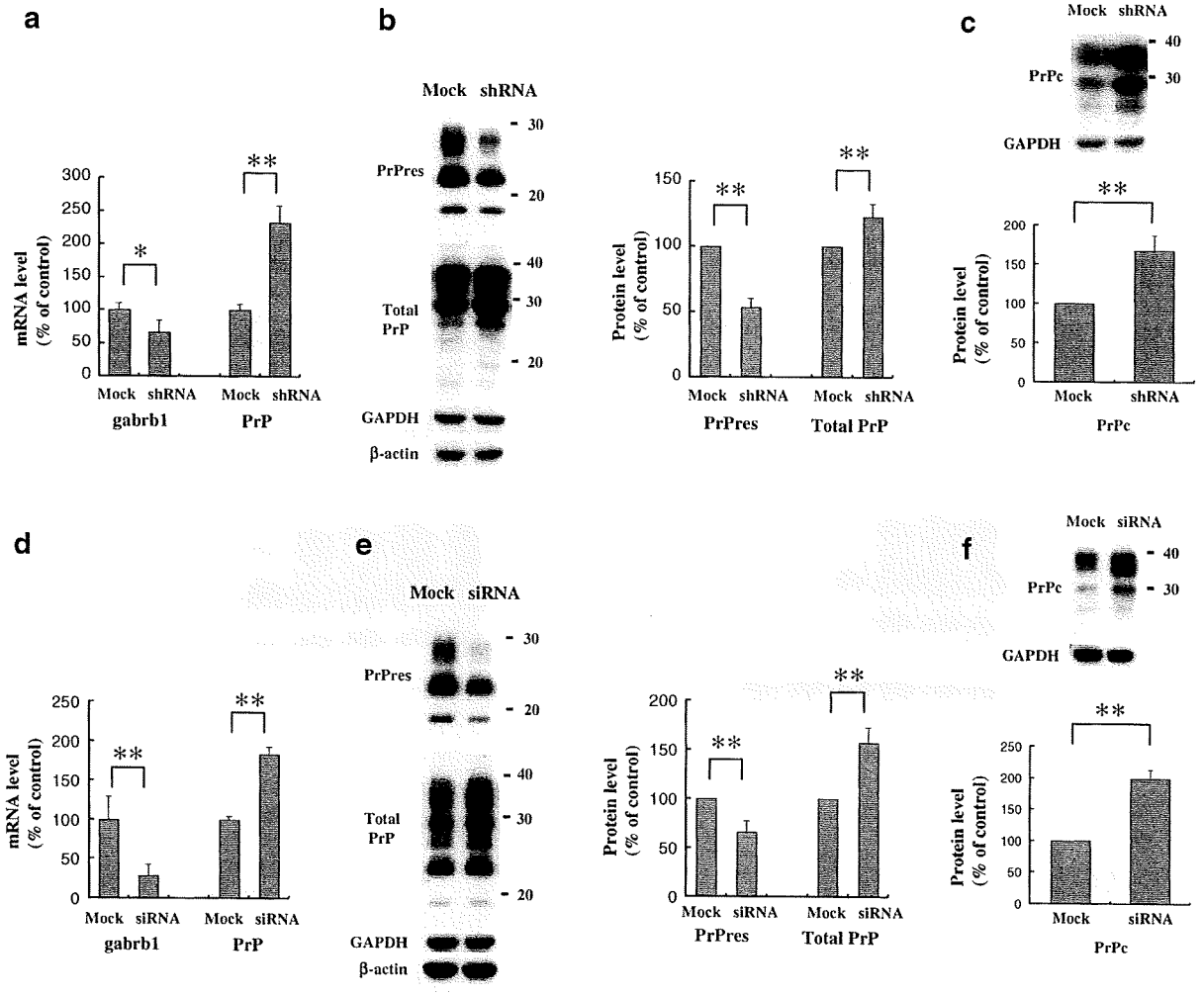

Fig. 1. Effects of gabrb1 gene silencing. Data from ScN2a cells transfected with shRNA expression vector or mock vector are shown: mRNA level of gabrb1 and PrP (a); immunoblot and protein level of PrPres and total PrP (b). Immunoblot and protein level of PrPc from N2a cells transfected with shRNA expression vector or mock vector are shown in (c). Data from ScN2a cells transfected with double-stranded siRNA or transfection reagent only (mock) are shown: immunoblot and protein level of PrPres and total PrP (d); mRNA level of gabrb1 and PrP (e). Immunoblot and protein level of PrPc from N2a cells transfected with double-stranded siRNA or transfection reagent only (mock) are shown in (f). Molecular size markers in the right side of the immunoblots are shown in kilodaltons. Immunoblot data shown here are representative examples; the graphic data shown here are the average and standard deviation from results of independent triplicate experiments ($^{*}P < 0.05$, $^{**}P < 0.01$).

ing effects were not evaluated because of its very low gene expression level in ScN2a cells.

3.2. Rescue of gene silencing effect

To confirm the specificity of gabrb1 gene silencing, a rescue experiment was performed using an expression vector for the mutated gabrb1 gene, which contained silent mutations at the targeting region of the double-stranded siRNA. Introduction of this mutated gabrb1 gene into ScN2a cells did not modify the PrPres level (Fig. 2; lanes 1 and 2). Co-transfection of the mutated gabrb1 expression vector and the double-stranded siRNA into ScN2a cells caused no reduction of the PrPres level, although co-transfection of the mock expression vector and the double-stranded siRNA caused substantial reduction in the PrPres level (Fig. 2; lanes 3 and 4). Consequently, the gabrb1 gene silencing effect on the PrPres formation was rescued by the expression of the silent mutation-containing gabrb1 gene.

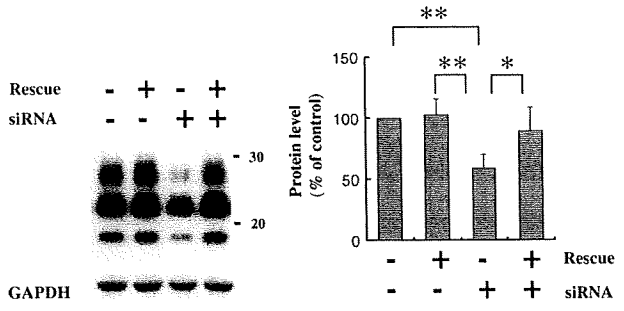

Fig. 2. Rescue of gabrb1 gene silencing. Immunoblot and protein level of PrPres from ScN2a cells transfected with silent mutation-containing gabrb1 expression vector (rescue +) or mock vector (rescue −) in the presence of (siRNA +) or the absence of (siRNA −) the siRNA used in the experiment shown in Fig. 1. Molecular size markers in the right side of the immunoblots are shown in kilodaltons. Immunoblot data shown here are representative examples; the graphic data shown here are the average and standard deviation from results of independent triplicate experiments (*$P < 0.05$, **$P < 0.01$).

3.3. Effects of GABAA receptor-related compounds

Salicylidene salicylhydrazide is a selective inhibitor of β1 subunit-containing GABAA receptors [19]. Treatment of ScN2a cells with this compound inhibited PrPres formation dose-dependently with a 50% inhibition dose of 450 nM (Fig. 3a). However, it did not modify the PrP mRNA level and the PrPc level, as demonstrated in the experiment using N2a cells (Supplementary Fig. 3). Another GABAA receptor antagonist, bicuculline methiodide [8], was also effective in inhibiting the PrPres formation dose-dependently but at almost a thousand times higher dose than that of salicylidene salicylhydrazide (Fig. 3b). No other GABAA receptor-related compound modified the PrPres level in ScN2a cells (Supplementary Fig. 4). The compounds tested in this study included an antagonist, picrotoxin [8], and agonists such as GABA [8], muscimol [8], pentobarbital [8], ethanol [20], and isoguvacine hydrochloride [21].

4. Discussion

Results show that gabrb1 is involved in the PrPres formation in ScN2a cells. The PrPres level was reduced significantly by gabrb1 gene silencing using either shRNA or siRNA, designed to target each different region of the gene. This gabrb1 gene silencing effect was rescued by co-transfection of silent mutation-containing gabrb1 gene, which was designed not to be targeted by the siRNA. Moreover, the results of the experiment with gabrb1-specific inhibitor salicylidene salicylhydrazide were coincident with those of gabrb1 gene silencing experiments. These results indicate that gabrb1 gene silencing effects are not artifacts such as off-targeting. We confirmed the gabrb1 gene silencing effects in the mRNA level but not in the protein level. We attempted unsuccessfully to detect gabrb1 in protein level using several gabrb1-specific antibodies ob-

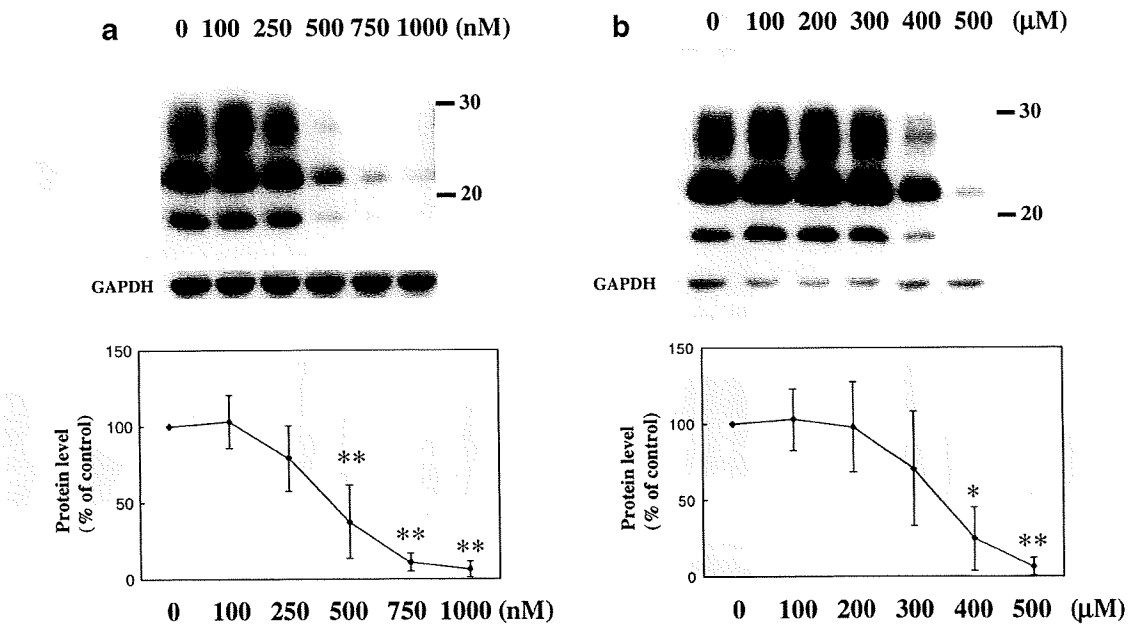

Fig. 3. Effects of gabrb1-specific inhibitor and GABAA receptor antagonist. Immunoblot and protein level of PrPres from ScN2a cells treated with gabrb1-specific inhibitor salicylidene salicylhydrazide (a) or GABAA receptor antagonist bicuculline methiodide (b) are shown. Molecular size markers in the right side of the immunoblots are shown in kilodaltons. Immunoblot data shown here are representative examples; the graphic data shown here are the average and standard deviation from results of independent triplicate experiments (*$P < 0.05$, **$P < 0.01$).

tained from different sources. Failure was mainly attributed to the very low expression level of gabrb1 protein in the cells and to technical difficulties in distinguishing gabrb1 signals from immunoglobulin heavy chain signals on the blot. Successful detection of endogenous gabrb1 protein in immunoblotting from cultured cells has not been reported.

Gene silencing of other GABAA receptor subunits tested in this study did not affect the PrPres formation in ScN2a cells, suggesting that gabrb1 involvement in the PrPres formation is in a manner irrespective of GABAA receptor. Results from treatments of ScN2a cells with an antagonist picrotoxin and agonists such as GABA, muscimol, pentobarbital, ethanol, and isoguvacine hydrochloride were consistent with this hypothesis. No new function of gabrb1 unrelated to GABAA receptors, except gabrb1 homomeric chloride ion channels, has been reported in the relevant literature. These homomeric channels, expressed in Xenopus oocytes or A293 cells, are sensitive to picrotoxin [22,23], but picrotoxin did not affect the PrPres formation in ScN2a cells. Consequently, gabrb1 function in ScN2a cells might differ from the homomeric channels, which remains to be evaluated.

The gabrb1 gene silencing increased the PrP mRNA level and PrPc protein level. It remains unclear how this happened and whether this resulted from upregulation of PrP gene transcription or from increased stability of PrP mRNA. However, it is noteworthy that the formation of PrPres was reduced despite the increased PrPc expression level. Furthermore, it is noteworthy that treatment with gabrb1 inhibitor, salicylidene salicylhydrazide, decreased the PrPres level but did not modify the PrP expression in either the mRNA level or protein level. The discrepancy between the results of gabrb1 gene silencing and those of gabrb1 inhibitor might reflect the difference in the mRNA/protein expression level of gabrb1: a decreased gabrb1 expression level in the gene silencing versus an unmodified gabrb1 expression level but functional inhibition in the inhibitor. However, further study is necessary to elucidate this speculation.

Compared to ScN2a cells (RML prion-infected N2a cells), N167 cells (22L prion-infected N2a cells) showed less remarkable reduction of PrPres formation in gabrb1 gene silencing. This gap of gabrb1 involvement between ScN2a cells and N167 cells might reflect prion strain difference. Gabrb1 might be more influential in the PrPres formation of RML prion strain, than 22L prion strain. However, we could not exclude possibilities that other factors than prion strain might be responsible for the gap observed between the two cells.

Involvement of GABAergic system in the pathogenesis of prion diseases has been reported [9–15]. The GABAergic neurons are degenerated in an early stage of the disease [13,14]. On the other hand, Trifilo and colleagues [17] report upregulation of GABAA receptor subunits in the prion-inoculated mouse brains expressing anchorless PrP. According to their speculation, inhibitory synaptic transmission is over-stimulated while PrPres formation is promoted; then the inhibitory synaptic transmission system collapses from overwork. Taken together with the findings in this study, suppression of gabrb1 function by inhibitors or other means might be useful for both calming overwhelmed GABAergic systems and inhibiting PrPres formation when conducted at an appropriate stage of the disease.

In conclusion, we identified gabrb1 as a new host factor involved in the PrPres formation in ScN2a cells. Although gabrb1 is a subunit of GABAA receptors, our results suggest that gabrb1 acts on the PrPres formation in a GABAA receptor-independent manner. Because previous literature has not revealed any association of gabrb1 with PrPc [24–30], gabrb1 might function through other cellular factors or directly interact with PrPres as observed in the prion-inoculated mouse brains expressing anchorless PrP [17], where direct association of GABAA receptors with PrPres was demonstrated. The mechanism of gabrb1 involvement in the PrPres formation remains to be elucidated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.02.029.

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