

Microglial Cell Line Established from Prion Protein-Overexpressing Mice Is Susceptible to Various Murine Prion Strains[∇]

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Several lines of evidence suggest that microglia have important roles in the pathogenesis of prion diseases. Here, we establish a novel microglial cell line (MG20) from neonatal *tga20* mice that overexpress murine prion protein. After exposure to Chandler scrapie, we observed the replication and accumulation of disease-associated forms of the prion protein in MG20 cells up to the 15th passage. Furthermore, MG20 cells were susceptible to ME7, Obihiro scrapie, and bovine spongiform encephalopathy agents. Thus, MG20 cell lines persistently infected with various murine prion strains provide a useful model for the study of the pathogenesis of prion diseases.

Prion diseases (transmissible spongiform encephalopathies) are fatal, neurodegenerative disorders and include mainly Creutzfeldt-Jakob disease (CJD), bovine spongiform encephalopathy (BSE), scrapie, and chronic wasting disease. The pathological features of prion diseases are brain vacuolation, neuronal death, astrocytosis, and microgliosis (7, 29). The key event in the pathogenesis of prion diseases is the conformational change from the normal host prion protein (PrP^C) into the abnormal, disease-associated form (PrP^{Sc}). PrP^C is rich in α -helical structures and is protease sensitive, whereas PrP^{Sc} is largely composed of β -sheets (9, 22) and is partially protease resistant. PrP^{Sc} is thought to be the main, if not the only, constituent of the agent of prion diseases (24, 25), and the detection of PrP^{Sc} correlates with the presence of infectivity (18, 26).

Several studies have indicated that microglia are related to the pathogenetic events in the loss of neurons in prion diseases (14, 19, 35). Cell culture models have provided valuable tools for investigating the roles of microglia in the pathogenesis of prion diseases (4, 5). The neuronal toxicity of an amyloid, fibril-forming, PrP-derived peptide (PrP106-126) and PrP^{Sc} has been documented in *in vitro* studies (12, 13), and this toxicity is greatly enhanced in the presence of microglia (5, 8), possibly due to the secretion of cytokines and neurotoxic reactive oxygen species from activated microglia (8, 10, 23, 34). A recent study of the rodent CJD model revealed that the presence of infectivity is detected in microglial cells isolated from the brain (3). Therefore, a better understanding of the roles of microglia in neurodegeneration could be obtained from the

physiological and biochemical characterization of prion-infected microglial cells. However, to date, persistent prion replication has been reported in only a limited number of cell lines (1, 20, 27, 28, 33) and none of them were of microglial origin.

It has been demonstrated that a large amount of PrP in cultured cells improves the susceptibility to *in vitro* challenges with various murine prion strains (20). We therefore have generated a microglial cell line from brains of transgenic mice (*tga20*) overexpressing murine PrP (11) and examined whether these cells are susceptible to persistent infection to various prion strains.

Establishment of microglial cell lines from *tga20* and PrP-deficient mice. Primary microglial cells were cultured from the brains of newborn murine PrP overexpressing mice (*tga20*) (11) and PrP-deficient mice (*PrP^{0/0}* mice) (36), as previously described (31). These cells were immortalized by infection with a *c-myc*-containing replication-deficient retroviral vector. Wild-type microglial cells (MG6 cells) were similarly established from C57BL/6 mice (32). The immortalized cell clones derived from *tga20* mice (MG20 cells) and *PrP^{0/0}* mice (MG0 cells) were characterized by immunolabeling with the following cell type-specific antibodies: anti-Mac-1 and F4/80 for microglia, anti-glial fibrillary acidic protein for astrocytes, and anti-microtubule-associated protein 2 for neuronal cells. In a manner similar to that of the wild-type MG6 cells, MG20 and MG0 cells expressed Mac-1 (Fig. 1A) and F4/80, but did not express glial fibrillary acidic protein and microtubule-associated protein 2 (data not shown). MG20 and MG0 cells were mostly rounded or flattened in shape, but occasionally had a spindle or ramified morphology in culture. These morphological characteristics were similar to those of primary microglia (3) and microglial cell lines (21), including MG6 cells (32). In addition, they actively phagocytosed latex beads and produced inflammatory cytokines, such as tumor necrosis factor alpha, interleukin-1 α , and interleukin-6, when stimulated by lipopolysac-

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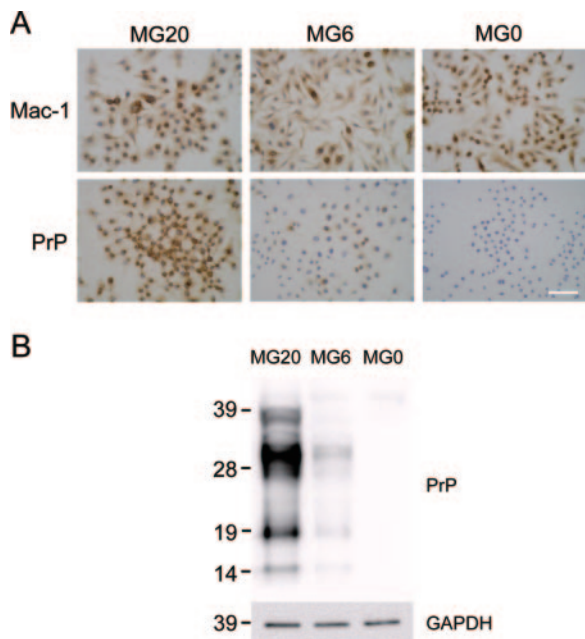


FIG. 1. Immunostaining and PrP expression of microglial cell lines derived from PrP-overexpressing (MG20), C57BL/6 (MG6), and PrP-deficient (MG0) mice. (A) Each cell line was immunolabeled with anti-Mac-1 and PrP antibodies and stained with 3,3-diaminobenzidine substrate as chromogen (brown). Nuclei were lightly counterstained with hematoxylin (blue). Scale bar, 50 μ m. (B) The levels of PrP were compared in microglial cell lines by semiquantitative immunoblotting with MAb T2. Proteins in postnuclear cell extracts prepared without proteinase K treatment were precipitated with acetone prior to electrophoresis. Molecular mass standards (kDa) are indicated on the left. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a control to confirm protein content of cell extracts.

charide (data not shown), indicating that established cell lines possess morphological, physiological, and immunological characteristics of microglia in the mouse brain.

Expression of PrP in microglial cell lines. The levels of PrP expression in MG20, MG6, and MG0 cells were analyzed by immunocytochemistry with monoclonal antibody (MAb) T2 (15). Wild-type MG6 cells showed moderate staining with MAb T2, whereas PrP-overexpressing MG20 cells were more densely stained than MG6 cells (Fig. 1A). No immunoreactivity was found in PrP-deficient MG0 cells. PrP expression levels were further evaluated by immunoblotting. Both MG6 and MG20 but not MG0 cells expressed PrP, which was in agreement with the immunocytochemistry results. MG20 cells expressed a PrP level approximately nine times that of MG6 cells as shown by densitometry. These data indicate that MG20 cells overexpressed PrP at levels similar to that of the brain tissue in *tga20* mice from which they were derived (11). Thus, the MG20 cell line could be a potential candidate for persistent infection with prion strains *in vitro*.

Infection of MG20 cells with murine scrapie prions and screening of PrP^{Sc}-positive subclones. First, we examined the susceptibility of MG20 cells to murine scrapie infection. MG20 cells were incubated with 1% (wt/vol) brain homogenates from terminally ill mice infected with Chandler scrapie (16). PrP^{Sc}-positive subclones were screened by the enzyme-linked immu-

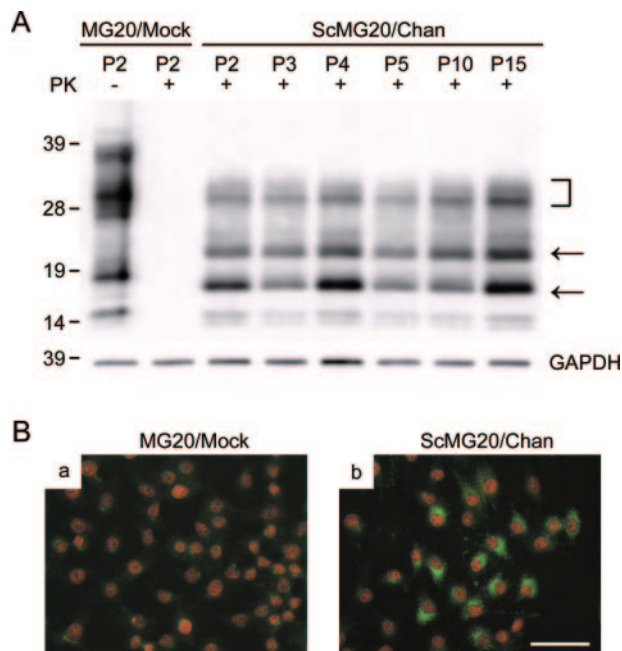


FIG. 2. Immunoblotting and immunofluorescence detection of PrP^{Sc} in MG20 subclone. (A) A mock-infected subclone (MG20/mock) was obtained from MG20 cells exposed to normal brain homogenates. The PrP^{Sc}-positive subclone (ScMG20/Chan) was chosen after screening by ELISA using the Seprion ligand system. MG20/mock cells and ScMG20/Chan cells were passaged every 4 to 5 days. Cell extracts from cultures at the indicated passage number (P2 to P15), either with (+) or without (-) PK treatment, were analyzed by immunoblotting with T2 monoclonal antibody (2.5×10^5 cells equivalent per lane). The arrows and bracket indicate un-, mono-, and diglycosylated PrP^{Sc} molecules, respectively. Molecular mass standards (kDa) are indicated on the left. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a control to confirm the protein content of cell extracts. (B) MG20/mock cells (a) and ScMG20/Chan cells (b) growing on slide glasses were fixed with 4% paraformaldehyde for 30 min and were permeabilized with 0.1% Triton X-100. Following denaturation with 5 M GdnScn in 8.5% polyethylene glycol 8000, cells were immunolabeled with T2 antibody, followed by Alexa Fluor 488-conjugated secondary antibody (green). Nuclei were counterstained with propidium iodide (red). Scale bar, 50 μ m.

nosorbent assay (ELISA) method using the Seprion ligand system (Microsens Biotechnologies, United Kingdom) (17). The proportion of PrP^{Sc}-positive subclones was around 20% (22 out of 103 tested subclones) in MG20 cells. Several subclones with strong signals in the ELISA were transferred into 100-mm petri dishes at the second passage and then passaged at a 1:5 split ratio every 4 to 5 days.

Production of PrP^{Sc} in scrapie-exposed MG20 cell culture. We examined seven representative PrP^{Sc}-positive MG20 subclones by immunoblotting after treatment with proteinase K (PK) at the fifth passage. Mock-infected MG20 subclones showed no accumulation of PrP^{Sc}, whereas all of the infected MG20 subclones had PrP^{Sc} detectable by immunoblotting (data not shown). One of the subclones (termed ScMG20/Chan) with a significantly strong PrP^{Sc} signal was selected, and the production of PrP^{Sc} was examined after continuous passages. The PrP^{Sc} signals were detected for at least up to 15 passages without any loss of signal intensity during passages (Fig. 2A). In contrast to MG20 cells, MG6 cells expressing PrP

levels nine times lower than those of MG20 cells did not support PrP^{Sc} production upon exposure to Chandler and ME7 scrapie (data not shown). This result is consistent with a previous study reporting that the poor efficiency of prion infection in wild-type N2a is improved by increasing the level of PrP (20). It is likely that the overexpression of PrP increases the susceptibility of microglial cells to prion infection.

By using immunofluorescence detection, we next assessed the proportion of cells accumulating with PrP^{Sc}. After fixation with 4% paraformaldehyde, infected and mock-infected MG20 cells were treated with 5 M GdnSCN in 8.5% polyethylene glycol 8000 to enhance the binding of PrP antibodies (33). Around 60% of the ScMG20/Chan cells showed intracellularly bright and punctate guanidine-dependent fluorescent signals, which were assumed to reflect PrP^{Sc} accumulation, whereas these were not seen in mock-infected cells (Fig. 2B). No major morphological alterations were identified between mock-infected MG20 cells and ScMG20/Chan cells.

Infectivity in scrapie-exposed MG20 cell culture. The infectivity in ScMG20/Chan cells at the 12th passage was assayed using *tga20* and CD-1 mice. Freeze-thawed cell lysates were inoculated intracranially into *tga20* mice and CD-1 mice (5×10^5 cells/20 μ l per head). As a positive control, 20 μ l of 10% (wt/vol) mouse brain homogenates were inoculated. All of the ScMG20/Chan cell-infected mice developed typical signs of Chandler scrapie and the times of death were 60 ± 0.8 days (mean \pm standard deviation) for *tga20* mice ($n = 8$) and 144 ± 7.9 days for CD-1 mice ($n = 5$), respectively. These mean incubation periods were similar to those of positive control mice, whose times of death were 59 ± 1.3 days for *tga20* mice ($n = 6$) and 147 ± 4.4 days for CD-1 mice ($n = 7$), respectively. The accumulation of PrP^{Sc} in their brains was confirmed by immunoblotting. Any mice inoculated with mock-infected MG20 cells or normal mouse brain homogenates did not exhibit scrapie symptoms at all. In addition to incubation periods, the lesion profiles and the immunoblot profiles of PK-treated PrP^{Sc} from CD-1 mouse brains infected with ScMG20/Chan cells were similar to those infected with Chandler scrapie mouse brain homogenates (data not shown). These results indicate that the biological characteristics of the prion strain were well maintained in ScMG20/Chan cells.

Biochemical features of PrP^{Sc} propagated in MG20 cell culture. Birkett et al. (6) have reported that the biological characteristics of the scrapie strains were maintained in infected cells, although the biochemical features of PrP^{Sc} (e.g., glycoform profiles and molecular mass) differed from those of PrP^{Sc} propagated in the brains. Therefore, the immunoblot profiles of the PK-treated PrP^{Sc} from ScMG20/Chan cells, ScN2a58 cells (20), and Chandler scrapie-infected CD-1 mouse brains were compared. As shown in Fig. 3A, un- and monoglycosylated PrP^{Sc} derived from ScN2a58 and ScMG20/Chan cells migrated faster, by around 0.5 kDa and 1 kDa, respectively, than did the brain-derived isoform. The difference in molecular masses was further confirmed after the removal of carbohydrates with N-glycosidase F (PNGase F). To examine whether the difference in molecular masses comes from the PK cleavage site heterogeneities of PrP^{Sc}, we analyzed infected or uninfected brain homogenates and cell lysates without PK treatment by immunoblotting after the removal of carbohydrates. Consistent with PK-treated PrP^{Sc}, cell-derived ungly-

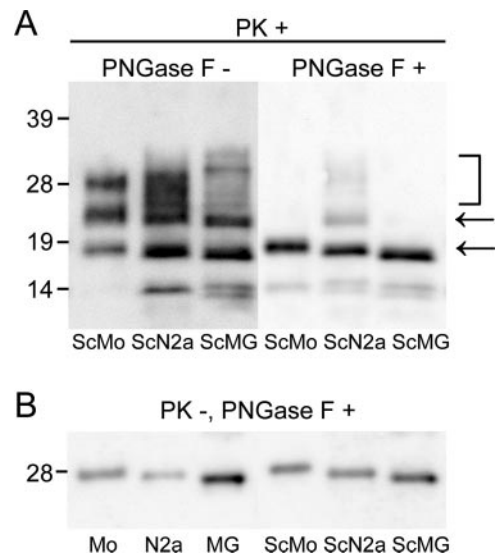


FIG. 3. Comparison of PrP^{Sc} glycosylation patterns and molecular size of unglycosylated PrP^{Sc} derived from scrapie-infected brain and ScN2a58 and ScMG20/Chan cells with (+) or without (-) PK treatment. (A) Immunoblot analysis of PK-treated samples from Chandler scrapie-infected mouse brain (ScMo), ScN2a58 cells (ScN2a), and ScMG20/Chan cells (ScMG) with or without deglycosylation by PNGase F treatment. PrP^{Sc} was demonstrated by immunoblotting with MAb T2. The arrows and brackets indicate un-, mono-, and diglycosylated PrP^{Sc} molecules, respectively. (B) Immunoblot analysis of PK-untreated samples from normal mouse brain (Mo), N2a58 cells (N2a), MG20 cells (MG), Chandler scrapie-infected mouse brain (ScMo), ScN2a58 cells (ScN2a), and ScMG20/Chan cells (ScMG) with deglycosylation by PNGase F. PrP was demonstrated by immunoblotting with MAb 3H2, which recognizes the epitope in N-terminal PrP. Molecular mass standards (kDa) are indicated on the left.

cosylated PrP without PK treatment migrated faster than did the brain-derived isoform (Fig. 3B). These results suggest that the differences in the molecular masses of PrP^C among brain tissues and cell cultures were probably responsible for those of PrP^{Sc}. Our observations are consistent with a recent study on scrapie- and CJD-infected GT1-7 cells, suggesting that heterogeneity in the constitution of glycosylphosphatidylinositol (GPI) moieties is involved in the size differences of unglycosylated PrP between brain tissues and cells (2).

Exposure of MG20 cells to various prion strains. To assess the susceptibility of MG20 cells to other murine prion strains, we exposed MG20 cells to 1% (wt/vol) brain homogenates from terminally ill mice infected with ME7, Obihiro scrapie (30), and BSE agents (15). As in the case of ScMG20/Chan cells, several subclones positive for PrP^{Sc} by ELISA were selected and expanded. The accumulation of PrP^{Sc} in these infected MG20 cells was examined by immunoblotting at the fifth passage. All the subclones infected with each prion strain demonstrated substantial PrP^{Sc} accumulation. (Fig. 4). In BSE-infected MG20 cells, un- and monoglycosylated PrP^{Sc} migrated faster, by around 1 kDa, than did scrapie-infected, cell-derived isoforms, possibly due to the difference in the N-terminal cleavage site after protease treatment (15). This unique characteristic of PrP^{Sc} from the mouse-propagated BSE agent was also preserved in the cell-propagated BSE agent.

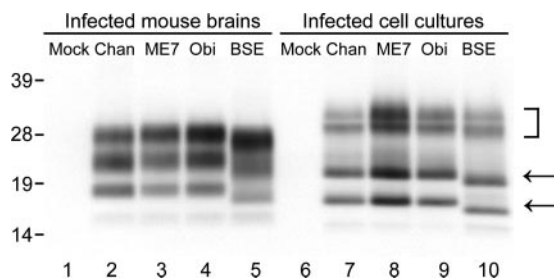


FIG. 4. Immunoblot analysis of PrP^{Sc} derived from mouse brains and MG20 cells infected with various prion strains. Lanes 1 to 5, infected mouse brains; lanes 6 to 10, infected MG20 cells; lanes 1 and 6, mock infected; lanes 2 and 4, Chandler (Chan) infected; lanes 3 and 8, ME7 infected; lanes 4 and 9, Obihiro (Obi) infected; lanes 5 and 10, BSE infected. The arrows and bracket indicate un-, mono-, and diglycosylated PrP^{Sc} molecules, respectively. Molecular mass standards (kDa) are indicated on the left.

In conclusion, we demonstrated that prions could replicate and conserve their biological characteristics in the microglial cell line derived from PrP-overexpressing mice. Although the involvement of microglia in the induction of neuropathogenesis has been considered elsewhere, the precise roles of microglia still remain largely obscure. Therefore, cell culture models using infected microglial cell lines offer a potentially powerful route to investigate the possible roles of microglia in prion diseases.

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