G1-Dependent Prion Protein Expression in Human Glioblastoma Cell Line T98G

Yutaka Kikuchi,* a Tomoshi Kakeya, a Takeshi Yamazaki, b Kaori Takekida, c Naoto Nakamura, d Haruo Matsuda, a Kosuke Takatori, a Akio Tanimura, c Ken-ichi Tanamoto, a and Jun-ichi Sawada e

Abstract

Human glioblastoma cell line T98G produced a cellular form of prion protein (PrP C), and we confirmed expression of PrP mRNA by RT-PCR. Immunoblot analysis of whole cell lysate revealed one major (35 kDa) and two faint bands (31, 25 kDa) that reacted with monoclonal anti-human PrP antibody 3F4. Cells treated with tunicamycin produced only a 25 kDa band, representing a deglycosylated form of PrP. Similarly, peptide: N-glycosidase F treatment of whole cell lysate altered the Asn-linked form to the deglycosylated form. When T98G cells were cultured for a longer period, the amount of PrP C per cell increased on Day 4 to 16 in a time-dependent manner. When the cells were cultured at high cell-density, the cells on Day 4 produced the same amount of PrP C as those cultured on Day 16 of the usual culture. Moreover, in a serum-free medium, cells cultured at a low cell-density produced the same amount of PrP C as those cultured at the high cell-density. These results demonstrate that PrP C production in T98G cells was dependent on the phase of the cell cycle, probably the G1 phase.

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

Materials and Methods

Materials Primer sets for human PrP C (5′GGCAGTGACTATGGAGACCGTTAAC3′ and 5′GGCTTGACCACTATCCAGGTCTA3′; the expected product size 251 bp) 23) were chemically synthesized. Reaction mixture contained 1 μL of each primer (25 pmol), 0.5 μL of Taq polymerase, 5 μL of dNTPs, 0.5 μL of MgCl 2 (15 mM), 5 μL of DNA, and 2 μL of distilled water. Amplification was performed in the presence of 30 cycles of denaturation, annealing, and extension.

Results

Expression of PrP C in the brain. PrP Sc, which is derived from a cellular prion protein (PrP C or PrP sen) that is easily solubilized and degraded by proteinase K, exists as an insoluble aggregate that is resistant to proteinase K digestion. There are some differences in the secondary structure between the two isoforms: PrP C is rich in α-helical structure whereas PrP Sc is rich in β-sheet structure. The conversion mechanism from PrP C to PrP Sc, however, remains unclear.

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

PrP Sc may play a role in neural differentiation, lymphocyte proliferation, or cell adhesion. PrP C does not cause neurodegeneration, but a synthetic PrP peptide (consisting of residues 106–126) is toxic to neurons that express PrP C. In culture, the neurotoxic PrP peptide enhances proliferation of microglia but not astrocytes.

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

The currently available cell lines are relatively low in expression level of PrP C to clarify its function. We examined PrP C expression in several cell lines and found that human glioblastoma cell line T98G highly expresses endogenous PrP Sc. We used the cell line to investigate the mode of PrP C expression, which will contribute to the development of therapies of prion diseases.
acid and stored at −80 °C under N₂ gas until used. Peptide: N-glycosidase F (PNGase F) was purchased from New England Biolabs (Beverly, MA, U.S.A.). Oligo-dT primer and Hybrid-P: PVDF membrane were purchased from Amersham PLC (Buckinghamshire, U.K.). Fetal calf serum (FCS) and [methyl-³H]thymidine (740 GBq/mmol) were purchased from ICN Pharmaceuticals (Costa Mesa, CA, U.S.A.). Anti-human PrP monoclonal antibody 3F4 was purchased from Senetek PLC (Napa, CA, U.S.A.). Anti-human PrP monoclonal antibody 6H4 was purchased from Prionics AG (Zürich, Switzerland). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated rabbit anti-chicken IgG, bovine serum albumin (BSA), 3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS), and tunicamycin were purchased from Sigma (St. Louis, MO, U.S.A.). Affi-gel Protein A agarose was purchased from Bio-Rad (Hercules, CA, U.S.A.). Complete Freund’s adjuvant (FCA) was from Difco (Detroit, MI, U.S.A.), vanadyl ribonucleoside complex (VRC) and SuperScript II reverse transcriptase from Life technologies (Rockville, MD, U.S.A.), KOD DNA polymerase from Toyobo (Osaka, Japan), DNase-I from Takara (Tokyo, Japan).

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

Cell Culture
The human glioblastoma cell line T98G (JCRB9041) was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). T98G cells were cultured at 37 °C in monolayers in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 60 μg kanamycin/ml, and 10 mM Hepes/NaOH. The medium was changed every 4 days. Usually, the cells were replated at 5.0×10⁵ cells per 9-cm dish (55 cm²). For high cell-density cultures, the cells were replated at 8.0×10⁶ cells per 9-cm dish.

RNA Extraction
Extraction of total RNA from the cells was carried out according to the method with slight modification. Briefly, T98G cells (1.5×10⁷ cells) were resuspended in 250 μl of lysis buffer (10 mM Tris–HCl, pH 7.8, 150 mM NaCl, 10 mM VRC). After incubation for 1 min on ice, 28 μl of NP-40 was added and the suspension mixed briefly. Insoluble material was eliminated following centrifugation at 19000×g for 2 min at 4 °C, and then 250 μl of preparation buffer (40 mM Tris–HCl, pH 7.8, 40 mM EDTA, 700 mM NaCl, 2% SDS) was added to the supernatant. RNA was extracted from the supernatant with phenol–chloroform–isoamylalcohol and chloroform–isoamylalcohol.

RT-PCR Analysis
RT-PCR analysis was performed according to the method with slight modification. Briefly, 5 μg of total RNA was treated with DNase-I for 15 min at room temperature. After addition of oligo-dT primer and SuperScript II reverse transcriptase, and 20 μl (2.5 μg total RNA) was incubated at 42 °C for 60 min to synthesize cDNA. Subsequently, 10 μl of cDNA solution was subjected to PCR in a total volume of 50 μl that included 1× KOD buffer, 0.2 mM dNTPs, 1 mM MgCl₂, 1 μl KOD DNA polymerase, and 50 pmol sense and antisense primers. The amplification program was as follows: denaturation at 98 °C for 15 s, annealing at 65 °C for 5 s, and elongation at 74 °C for 40 s for 40 cycles. Final elongation was performed at 74 °C for 1 min. PCR was carried out in a GeneAmp PCR system 2400 (Applied Biosystems; Foster city, CA, U.S.A.). PCR products were electrophoresed in 2% agarose gel and checked by ethidium bromide staining.

Preparation of Whole Cell Lysate
T98G cells were maintained in a 9-cm dish in 10 ml medium. At the indicated times, the cells were washed twice with ice-cold phosphate buffered saline (PBS) and scraped into lysis buffer (1.8×10⁵ cells/μl; 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% NP-40, 10 mM NaF, 1 mM EDTA, 0.5 mM Na₃VO₄, 10 mM tetrasodium pyrophosphate, aprotinin 90 KIU/ml, 20 mM leupeptin, 1 mM phenylmethyl-sulfonyl fluoride). After sonication, insoluble material was eliminated by centrifugation at 14000×g for 15 min at 4 °C to yield whole cell lysate. Protein concentration was determined by the BCA protein assay. To remove Asn-linked oligosaccharides, aliquots of whole cell lysate were treated with PNGase F as follows (Chen et al. 1995): the lysate (50 μg protein) was denatured by boiling for 10 min in 0.5% SDS, 1% 2-mercaptoethanol. After addition of NP-40 to 1%, the lysate was incubated at 37 °C for 2 h with 0.15 IUB unit of PNGase F in 50 mM phosphate buffer, pH 7.5.

Immunoblotting
Usually, 50 μg total protein prepared from approximately 1.7×10⁶ cells was subjected to SDS-gel electrophoresis. Briefly, aliquots of whole cell lysate treated with or without PNGase F were mixed with 2× electrophoresis sample buffer. After boiling for 10 min, the samples were electrophoresed on 12.5% acrylamide gel and the proteins were transferred onto PVDF membranes. The membranes were blocked with 0.5% casein in PBS (casein-PBS) and incubated with anti-prion antibodies in casein-PBS. Immunoreactive bands were visualized using HRP-conjugated anti-IgG and SuperSignal West Femto Maximum Sensitivity Substrate, according to the manufacturer’s instructions. Quantitative analysis of the 25 kDa deglycosylated form of PrPc was performed by computer-assisted densitometry.

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

Human Glioblastoma Cell Line T98G Produced PrPC

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

PrPC Increased in a Time-Dependent Manner

We next examined the correlation between PrPC production and T98G cell growth. The growth curve (Fig. 3A) and phase-contrast microscopic examination (Fig. 5A, photos I and III) revealed that T98G cells were confluent on Days 12 to 16. In contrast microscopic examination (Fig. 5A, photos I and III) revealed that T98G cells were confluent on Days 12 to 16. In culture, the 35, 31, and 25 kDa band intensities increased on Days 4 to 16 (Fig. 3B, left panel). To estimate the amount of immunoreactive PrPC, we digested the T98G cell lysate with PNGase F to produce the deglycosylated form of PrPC. The 25 kDa band which represents the deglycosylated increased in a time-dependent manner (Fig. 3B, right panel). The PrPC content on Day 16 was approximately twice that on Day 4 (Fig. 3C).

Production of Endogenous PrPC was Dependent on Cell-Density

T98G cells cultured for 4 d at low cell-density produced less PrPC than those cultured for 16 d at high cell-density (Fig. 4, lanes 2 and 3). Quantitative analysis of the 25 kDa deglycosylated form of PrPC from Fig. 4 (right panel) reveal that the amount of deglycosylated 25 kDa of Day 4 was almost half that of Day 16 (Table 1). The cells were almost confluent on Day 4 when cultured at a high cell-density (1.7×10^5 cells/cm²), but the amount of deglycosylated 25 kDa they produced was almost the same as that produced by Day 16 cells (Table 1), demonstrating that PrPC production was dependent on cell-density.

T98G Cells in Serum-free Cultures Produced a Large Amount of Endogenous PrPC

T98G cells are arrested in G1 phase under conditions of high cell-density or low serum concentration, so we examined how plating density and/or cell proliferation affect PrPC production. To address the effect of density, we compared two culture conditions with similar average cell-densities but distinct cell proliferation rates: one with medium containing 10% FCS and the other
with serum-free medium. Day 3 T98G cell cultures were incubated for another 5 d in serum-free medium to shift them to the quiescent phase at low cell-density. As shown in Fig. 5, morphology (A) and cell-density (B) of the serum-free cultures (II) were almost the same as those of the log-phase cultures (III) in medium containing 10% FCS for 4 d. Compared with the log-phase cultures (Fig. 5C, III), the serum-free cultures (Fig. 5C, II) showed dramatically reduced [3H]thymidine incorporation, similar to that of the usual Day 16 confluent cultures (Fig. 5C, I). While PrPC production in log-phase cultures at low cell-density (Fig. 5D, III) was lower than in confluent cultures (Fig. 5D, I), it was higher in serum-free cultures even at low cell-density (Fig. 5D, II). Moreover, the deglycosylated 25 kDa bands of the serum-free cultures (Fig. 5E, II) were almost equal to those of the confluent cultures (Fig. 5E, I). Taken together, these results suggest that T98G cells produced PrPC at the G1 phase of cell cycle.

DISCUSSION

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

In the present study, we showed that human glioblastoma cell line T98G constitutively expressed PrP\(^{\ominus}\) mRNA. Quantitative immunoblot analysis of the 25 kDa deglycosylated form of PrP\(^{\ominus}\) shown in B (right panel) were performed by computer-assisted densitometry. The integrated intensity of each bands was percentage of the intensity on Day 16. On the panel is shown the bar of integrated intensity versus incubation time. Bars are means±ranges from two independent experiments.
The mechanism of that cleavage and the physiological role of the C terminal fragment are unknown; T98G cells might provide a useful model for studying the proteolytic cleavage site.

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

The results we present here also demonstrate a correlation between endogenous PrP\(^C\) production and cell growth.

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

Immunoblotting with anti-PrP antibodies is the most common procedures for identifying PrP\(^Sc\) in prion diseases. In this study, we used 3 monoclonal antibodies (3F4, 6H4, and HUC2-13) and 1 polyclonal antibody (HPC2) to detect PrP\(^C\). These are directed to distinct epitopes throughout PrP\(^C\): HUC2-13 reacts with the N terminus, 3F4 and 6H4 react with the mid-region, and HPC2 reacts with the C terminus.
HPC2 showed a different reactivity pattern; it reacted strongly with the deglycosylated form of PrP\(^{C}\) but weakly with the glycosylated form. Other antibodies also react differently with the PrP\(^{C}\) bearing heterogeneous Asn-linked oligosaccharides. We have shown that quantitative analysis of the 25 kDa deglycosylated form of PrP\(^{C}\) treatment with PN-Gase F was helpful in obtaining precise measurements of PrP content. These methods will be useful in comparing the PrP\(^{C}\) production in cultured cells.

Prion diseases are characterized by gliosis, loss of neurons, and formation of amyloid plaques.\(^{5,38,39}\) PrP\(^{C}\) accumulates in white matter and in glia cytoplasm.\(^{38,39}\) In addition, PrP mRNA is expressed not only in neurons but also in glia.\(^{38,39}\) Therefore, more evidence is needed from cell culture models to clarify the role of glia cells in prion disease.

In conclusion, cell cycle arrests in G\(_1\) phase induced endogenous PrP\(^{C}\) production in human glioblastoma cell line T98G. T98G cells should be useful for studying the cellular function and molecular mechanisms of action of PrP\(^{C}\).

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