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Characterization of early transient accumulation of PrP^{Sc} in immune cells

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ABSTRACT

PrP^{Sc} is known to elicit no specific immune response and the immune cells are suspected to support its accumulation. In the present study, we investigated the response of some immune cell types to PrP^{Sc} to characterize an observed early transient accumulation of PrP^{Sc}. After cells were treated with PrP^{Sc}-brain homogenate, PrP^{Sc} was transiently accumulated for the first 8–12 h post-exposure then completely cleared by the 5th day of the experiment. The accumulated PrP^{Sc} was not a *de novo* product of the cell PrP^{C}. Further investigation of this phenomenon revealed some potential factors influencing it. These factors included cholesterol homeostasis, temperature, the degradation power of the cell and the availability of sufficient PrP^{C}. Our *in vitro* results suggest that immune cells, especially macrophages are potential risk factors for the accumulation and intercellular spread of PrP^{Sc} if the complete clearance of PrP^{Sc} were not fulfilled.

1. Introduction

Transmissible spongiform encephalopathies (TSE, prion diseases) are invariably fatal neurodegenerative diseases affecting animals and humans. These diseases develop following conformational changes in cellular prion protein (PrP^{C}) which result in the misfolded isoform of prion protein (PrP^{Sc}) at post-translational modification [1]. Animal TSE infections have arisen through feeding with PrP^{Sc}-contaminated animal food. After oral uptake, PrP^{Sc} first accumulates in gut-associated lymphoid tissues (GALT) such as Peyer's patches in the intestines before neuroinvasion occurs [2,3].

Unlike other infectious diseases, TSEs do not elicit specific immune responses because the infectious agent is composed of a protein with a primary structure identical to a host encoded protein [4,5]. Although comprehensively studied, the roles of mononuclear phagocytes in prion pathogenesis remain a matter of ongoing debate [6]. Several studies suggest that cells of the immune system, including macrophages, support the replication and spread of prions to the central nervous system [7,8]. Peripheral macrophages have demonstrated both accumulation [7,9] and proteolysis of PrP^{Sc}[7,9]. Macrophages in lymphoid follicles have been shown to contain PrP^{Sc} at early stages of TSE infection [10].

In a previous study [8], we investigated the responses of various cell types including immune, neural, intestinal and fibroblast cells to PrP^{Sc} for up to 28 days. Different patterns of response to PrP^{Sc} exposure varied between accumulation or degradation were identified. Some of the cell types showed a characteristic transient propagation of PrP^{Sc} which preceded its clearance. The aim of our study was to characterize the transient PrP^{Sc} accumulation and its significance in disease development. Our *in vitro* results suggest that immune cells, despite their important role as main protective shield against prion disease, may potentially be involved in the accumulation and intercellular spread of PrP^{Sc}, especially if their proteolytic function was impaired before the complete clearance of PrP^{Sc}.

2. Materials and methods

2.1. Cell lines

Two mouse macrophage cell lines (Raw and J774) and primary macrophage cells of mice bone marrow were used in the study. The bone marrow-derived macrophages (BMMs) were prepared from BALB/c mice by the method described previously [11]. Briefly, after culture in L-cell-conditioned medium, BMMs were resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS; PAA Laboratories, GmbH, Haidmenweg, Austria). The cells were cultured in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; WAKO, Osaka, Japan) supplemented with 10% FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

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2.2. Escherichia coli cell lysate and polystyrene microspheres

Escherichia coli (*E. coli*) ATCC11775 of 1×10^9, were incubated in L-broth (0.5 g of yeast extract, 1 g of tryptone and 0.5 g of NaCl) overnight. The cell pellets were collected and resuspended in 1 ml of PBS and boiled for 10 min. The *E. coli* cell lysate and polybead polystyrene microspheres (2.5% Solids-Latex, 1.0 μm; Polysciences Inc., Warrington, USA) were used as stimulants in the Real-Time PCR and Western blot analysis experiments. They were added to cells at a rate of 10 μl/dish.

2.3. Prion strains

Brain homogenates were prepared from mice terminally affected with mouse-adapted scrapie strains, Chandler [12], MHM2/Chandler [13] or Obihiro [14]. The MHM2/Chandler brain samples were kindly provided by Dr. T. Yokoyama (National Institute of Animal Health, Ibaraki, Japan). This strain is transmissible to a transgenic mouse strain which expresses a chimeric PrP derived from mouse and hamster. Infected and normal mouse brains were mechanically homogenized in PBS and diluted to a final concentration of 10% (w/v) in PBS, sonicated and stored at −20 °C until use.

2.4. Infection of macrophage cells with prion

Macrophage cells were cultured in 60-mm culture dishes at the optimum cell numbers to provide 60–70% confluence after overnight incubation. Infected brain homogenates equivalent to 0.5 mg of brain tissue were added to the cell dishes and incubated until harvest at the indicated intervals. The cells and supernatants were collected and centrifuged at 1000*g* for 5 min. Pellets were then stored at −20 °C until PK treatment.

2.5. Cholesterol depletion

To evaluate the influence of cholesterol metabolism on PrP^{Sc} infected-cells, U18666A (Sigma, St. Louis, MO, USA) was used as an inhibitor of cholesterol synthesis and intracellular transport [15]. Raw cells were treated with 5 μg of U18666A/dish and kept overnight before the addition of 0.5 mg of Chandler brain homogenate. After 2 h, cells were rinsed twice and incubated with U18666A at 37 °C until harvest.

2.6. Proteinase K treatment

Cells were lysed with 300 μl of lysis buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate and 5 mM EDTA, pH 8.0) on ice for 30 min. The lysate was centrifuged at 500*g* for 5 min and the supernatant was recovered into a 2-ml tube. PK (Roche Diagnostics) was added to each sample at a concentration of 20 μg ml^{-1} and incubated for 20 min at 37 °C. Proteolysis was terminated by the addition of 1 mM Pefabloc (Roche Diagnostics). The samples were then incubated with 0.3% sodium phosphotungstate at 37 °C for 30 min instead of ultracentrifugation as described previously [16]. PK-treated samples were centrifuged at 20,000*g* for 45 min and the resulting pellets were dissolved in sample buffer, boiled for 10 min and stored at −20 °C until loading.

2.7. Western blot analysis

Proteins were separated using 12% SDS–polyacrylamide gel electrophoresis. The gel were then transferred to Immobilon P (Millipore, Billerica, MA, USA), blocked with 5% non-fat milk in 0.1% Tween-20 Tris-buffered saline, pH 7.5, probed with anti-prion monoclonal antibody (mAb) 31C6 [12] or 3F4 [17] at 1:4000 followed by incubation with a peroxidase-conjugated anti-mouse antibody (GE Healthcare, Buckinghamshire, UK). Immunodetection was visualized using an enhanced chemiluminescence kit (ECL; GE Healthcare, UK) and exposure to X-ray film. Analysis of ECL images was performed using the public domain Image-J program (developed at the National Institutes of Health, Bethesda, MD, USA) according to the manufacturer's instructions.

2.8. Proteasome assay

The proteasome activity in the infected and uninfected cells was measured using a 20S Proteasome Assay Kit (Cayman Chemical Co., MI, USA) according to the manufacturer's protocol. Briefly, triplicates of 5×10^4 Raw cells per well in 100 μl of culture medium were seeded in a 96-well plate and incubated overnight. The next day, cells were treated with 100, 30 or 0 μg Chandler-infected brain homogenate and incubated at 37 °C or 24 °C for 12 h. The plates were then centrifuged at 500*g* for 5 min. The culture medium was aspirated and 200 μl/well of 20S Proteasome Assay Buffer were added. The plate was centrifuged again at 500*g* for 5 min and the supernatants were aspirated and 100 μl/well of the 20S Proteasome Lysis Buffer were added. The plate was incubated with gentle shaking for 30 min and centrifuged at 1000*g* for 10 min. An amount of 90 μl of the supernatant was transferred from each well to the corresponding well in a black plate and mixed with 10 μl of assay buffer or 20S inhibitor solution. A 100 μl of the positive control solution followed by 10 μl of the substrate solution were added to the corresponding wells in the black plate. The plate was incubated at 37 °C for 1 h. Fluorescent intensity of each well was measured (excitation 360 nm, emission 480 nm) using Wallac 1420 ARVOsx-1 Fluoroscan (Perkin Elmer Life Sciences, Tokyo, Japan). Differences between proteasome activity in infected and normal cells were analyzed using Student's *t*-test and were considered statistically significant at $p < 0.05$.

2.9. RNA extraction and Quantitative Real-Time PCR

Total RNA was extracted from treated and untreated cell pellets using the Qiagen RNeasy Kit protocol (Qiagen, Tokyo, Japan). RNA was quantified using a NanoVue spectrophotometer (GE Healthcare, Tokyo, Japan) and cDNA was then synthesized using the SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen) according to the manufacturer's protocol. Quantitative Real-Time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. PCR was carried out in 48-well plates on cDNA equivalent to 0.5 μg of total RNA. Thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C followed with 45 cycles at 95 °C for 3 s and 60 °C for 30 s. Data were collected using the StepOne analytical thermal cycler (Applied Biosystems). The used primers for prion protein gene (*PRNP*) were mPrion-F: ACGACTGCGTCAATATCACCAT and mPrion-R: GGTACTGGGTGACGCACATCT. Primers for the internal standard control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were AmGAPDH-F: TGCACCACCAACTGCTTAG and AmGAPDH-R: GGATGCAGGGATGATGTTC.

3. Results

3.1. Transient accumulation of PrP^{Sc} in macrophages and its origin

The early cellular responses to 0.5 mg of Chandler-infected brain homogenate were investigated in PK-treated and untreated Raw cells. Both cell groups were harvested at 0, 4, 8, 12, 24, 48, 72 and 120 h after PrP^{Sc} exposure and assayed by Western blot analysis. An incremental accumulation of PrP^{Sc} was observed with

maximum levels of PrP at 4 h post-exposure in the group assayed without PK treatment (Fig. 1A) and at 12 h in the PK-treated group. To investigate whether these results were specific to the Raw cell line or to the Chandler strain, we conducted similar experiments using J774 and BMMs macrophages and PrP^{Sc} strains of Obihiro and MHM2/Chandler. Almost identical biphasic response patterns to Chandler PrP^{Sc} were also observed with J774 and BMMs and with the MHM2/Chandler strain of PrP^{Sc}. However, when the Obihiro strain was used, the peak of PrP^{Sc} was recorded at 24 h post-exposure and the clearance rate was slower (data not shown).

The accumulated PrP^{Sc} was further investigated to elucidate whether it was a *de novo* product of the macrophage cells or if it originated from the PrP^{Sc} inoculum of the brain homogenate. A similar experiment was conducted using MHM2/Chandler strain which can easily be distinguished by 3F4 mAb. Western blot analysis revealed correlation between the 2 curves of PrP^{Sc} (Fig. 1B). This result shows that the accumulated PrP^{Sc} is not *de novo* produced by the cells.

3.2. Characterization of PrP^{Sc} degradation in macrophages at variable incubation temperatures

As way to make unfavorable conditions for normal cell physiology, the effect of low temperature on PrP^{Sc} cellular accumulation and degradation in Raw cells was investigated. After infection with 0.5 mg of Chandler brain homogenate, cells were incubated at 37 °C or 24 °C for 5 days. Western blot analysis revealed differences in the PrP^{Sc} accumulation curves between the two groups. The cells incubated at 37 °C gradually cleared PrP^{Sc} after 24 h, while those incubated at 24 °C showed no degradation of PrP^{Sc} up to the 5th day post-exposure (Fig. 2A). Interestingly, when the cells were moved from the 24 °C to 37 °C after the 5th day, the normal cell physiology was restored and PrP^{Sc} was completely cleared by the 9th day (data not shown). These data show that the ability of macrophages to degrade PrP^{Sc} was impaired at the unfavorable conditions such as low temperature.

To confirm this observation, a main cellular degradation system, 20S proteasome, was assayed in infected and normal Raw cells at different incubation temperatures. Results revealed that proteasome activity was significantly decreased in both infected and normal cells that were incubated at 24 °C compared to those incubated at 37 °C (Fig. 2B).

3.3. Effect of additional PrP^{C} on the accumulation of PrP^{Sc} in Raw cells

To investigate if additional PrP^{C} from an external source may affect the pattern of PrP^{Sc} accumulation and clearance, Raw cells were treated with PrP^{Sc} brain homogenates with or without additional amounts of normal brain homogenate as a source of PrP^{C}. Treated cells with PrP^{Sc}-brain were incubated for 2 h, washed, treated with 2 mg/dish of normal brain homogenate and incubated at 37 °C for 5 days. Western blot results showed that PrP^{Sc} remained for longer time in the cells that were treated with additional normal brain compared to the complete disappearance after 3 days in the untreated cells (Fig. 3A).

To further characterize the accumulation of PrP^{Sc} in macrophages, the effect of the cholesterol synthesis and intracellular transport inhibitor, U18666A, on the uptake and accumulation of PrP^{Sc} in macrophages was investigated. Raw cells were pre-incubated with U18666A compound, exposed to PrP^{Sc} for 2 h and washed. The Western blot analysis showed a significant decrease in both the endocytosed and accumulated PrP^{Sc} levels in the cells treated with U18666A (Fig. 3B).

3.4. Characterization of the specificity of early prion accumulation

Prion accumulation in macrophages was further investigated to elucidate whether it is specific to Raw cells and to PrP^{Sc} agent. An identical experiment was conducted using J774 and BMMs cell

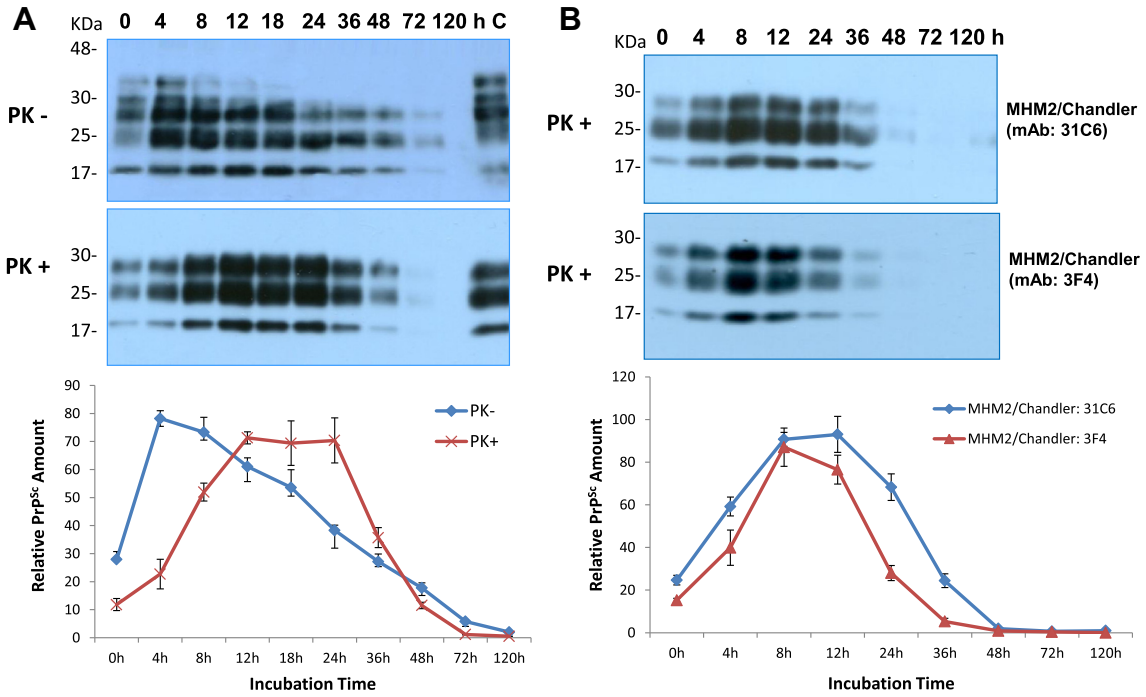

Fig. 1. Kinetics of early accumulation of PrP^{Sc} in Raw cell line. (A) Western blot and graphical representation show PrP^{Sc} levels in the infected cells during 5 days of incubation. Chandler-infected mouse brain homogenate was used and cells were harvested at the indicated intervals. The mAb 31C6 was used for detection of the PrP^{Sc}. (B) Western blot and graphical representation shows PrP^{Sc} levels in Raw cells infected with MHM2/Chandler during 5 days of incubation. The 31C6 or 3F4 mAbs were used for detection of the PrP^{Sc}. The relative amounts of PrP^{Sc} were quantified using the Image-J software. Error bars represents the SD values obtained from 3 observations of 3 independent experiments.

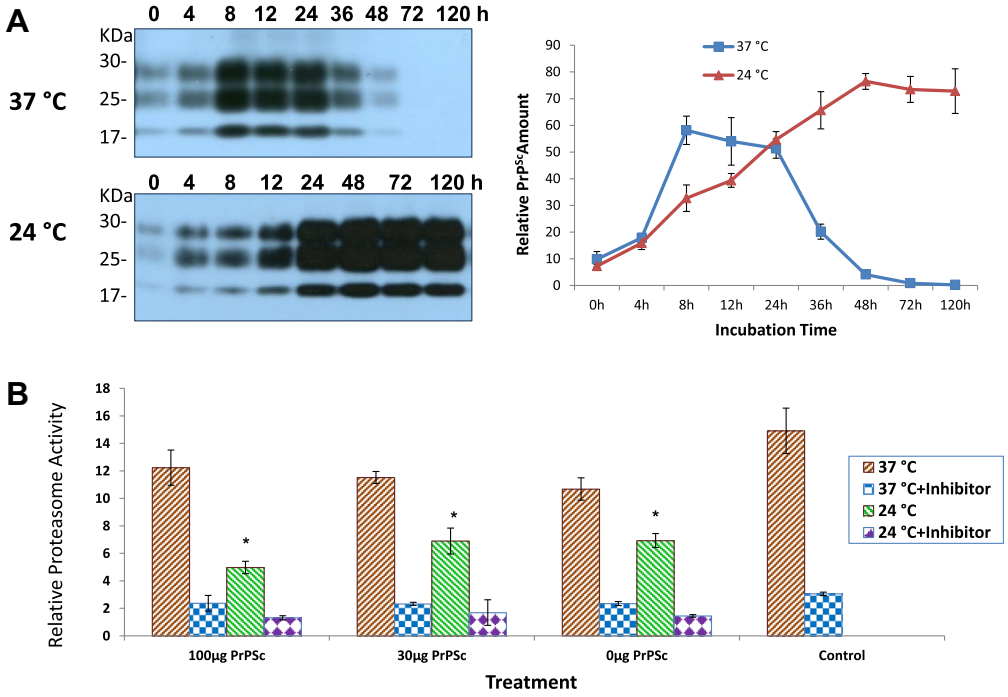

Fig. 2. Kinetics of PrP^{Sc} accumulation and proteasome activity levels in Raw cell line at different incubation temperatures. (A) Western blot results and graphical representation show PrP^{Sc} levels in infected cells incubated at 37 °C or 24 °C. The 31C6 mAb was used for detection of PrP^{Sc}. The ECL-developed images were analyzed using Image-J software and the relative amounts of PrP^{Sc} are expressed in pixels. (B) Graphical representation of proteasome activity levels in Raw cells infected with 100, 30 or 0 µg of PrP^{Sc}-brain and incubated at 37 °C or 24 °C. A significant difference was observed between 37 °C and 24 °C ($p < 0.05$). Error bars represents the SD values obtained from 3 observations of 3 independent experiments.

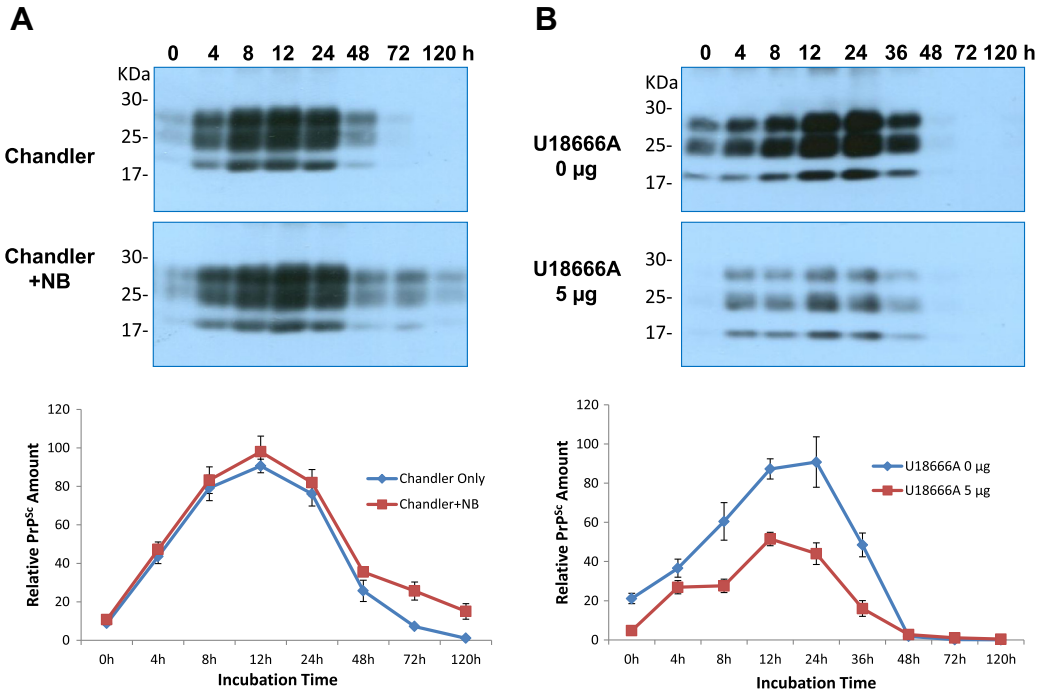

Fig. 3. Kinetics of PrP^{Sc} accumulation in Raw cells treated with normal brain homogenate or U18666A compound. (A) Western blot results and graphical representation show PrP^{Sc} levels in the infected cells treated with 2 mg additional normal brain homogenate. (B) Western blot results and graphical representation showing PrP^{Sc} levels in the infected cells treated with 5 µg U18666A compound. PrP^{Sc} was detected using mAb 31C6 and images were analyzed using the Image-J software. Error bars represents the SD values obtained from 3 observations of 3 independent experiments.

cultures. Furthermore, cell cultures were treated with normal brain homogenate, *E. coli* cell lysate or microspheres. Western blot results revealed that PrP^{Sc} accumulation occurred only in the cells treated with PrP^{Sc}-brain homogenates (Fig. 4A). However, a transient non-specific accumulation of PrP^{C} was observed in the cells treated with normal brain (Fig. 4A).

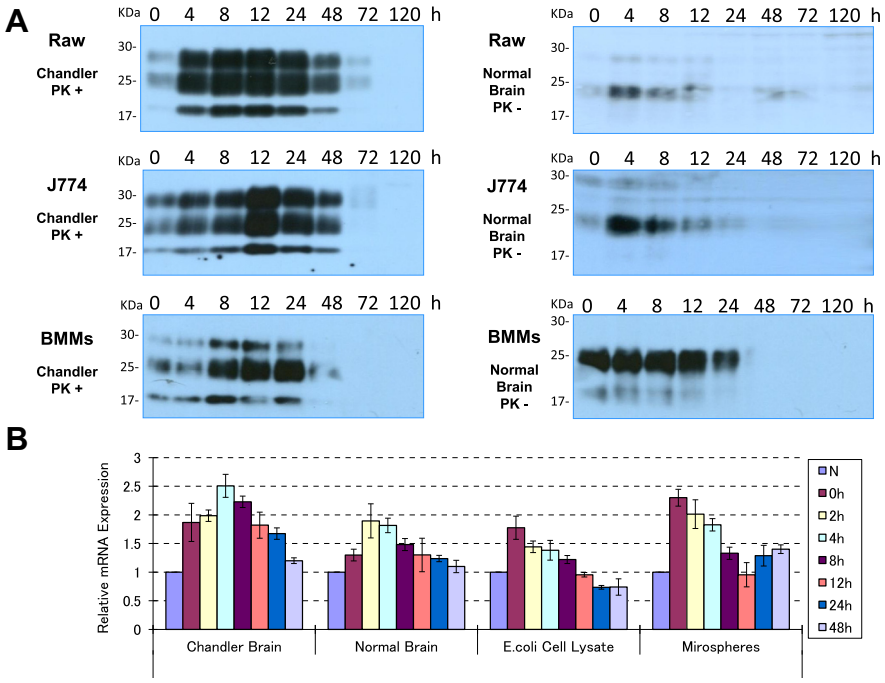

Fig. 4. Kinetics of accumulation of PrP^{C}, PrP^{Sc} and mRNA expression of *PRNP* in macrophage cells exposed to different antigens. (A) Representative Western blot results showing PrP^{Sc} and PrP^{C} levels in Raw, J774 cells and BMMs treated with Chandler-PrP^{Sc} or normal brain homogenate. PrP^{Sc} was detected using mAb 31C6. (B) Graphical representation of the relative *PRNP* mRNA expression levels determined using Real-Time PCR in Raw cells treated with different antigens. Error bars represent the SD values obtained from 3 Real-Time PCR readings of each sample.

3.5. mRNA expression of prion-encoding gene, PRNP, in macrophages

To characterize the potential relationship between *PRNP* activation and the observed accumulation of PrP^{Sc} in macrophages, *PRNP* mRNA expression during exposure to PrP^{Sc} was investigated in Raw cells by Real-Time PCR. A gradual up-regulation of *PRNP* gene, up to 2.5 times the normal level, was observed at 4 h of exposure (Fig. 4B). However, these minute changes at the mRNA expression level may not be enough to affect the protein levels of PrP.

The specificity of the increased of mRNA expression in Raw cells to PrP^{Sc} exposure was further investigated by analysis of mRNA expression in other cell types and other antigens. Raw, J774 and BMMs cells were treated with PrP^{Sc}-brain, normal brain, *E. coli* cell lysate and microspheres. A non-specific variable *PRNP* up-regulation in Raw cells was observed with all of the treatment agents (Fig. 4B). Similarly, variable up-regulation of *PRNP* was also observed in J774 cells and BMMs with each of the treatment agents (data not shown). These results suggest that the mRNA expression of the PrP gene was not a specific response to PrP^{Sc} exposure but may be a normal response that occurs in immune cells exposed to various kinds of antigens.

4. Discussion

We have previously observed three variable forms of cellular response to PrP^{Sc}[8]. In some cell types, degradation was preceded by a transient PrP^{Sc} accumulation around the 5th day of exposure. In this study, we further investigated a similar characteristic phenomenon of a prominent and transient PrP^{Sc} accumulation in three macrophage cell types. The source of the propagated PrP^{Sc}, the factors influencing the conversion of PrP^{C} into PrP^{Sc} and its clearance in these cells were investigated.

In the present study, we showed that the accumulated PrP^{Sc} was derived from the inoculated brain homogenate and was not a *de novo* product of the PrP^{C} in cells. In regard to the factors influencing this transient accumulation of PrP^{Sc}, the effect of unfavorable physiology conditions for the cells were investigated by incubating cells at lower temperature than appropriate. Results showed that such conditions affected the cells metabolic activity including the degradation power and led to unsuccessful clearance of PrP^{Sc}. Moreover, higher PrP^{Sc} accumulation level than that of 37 °C was observed. When the conditions around cells are unfavorable, cellular biophysiological mechanisms may be directed to enhance the PrP^{C}–PrP^{Sc} conversion process and inhibit its clearance resulting in propagation of PrP^{Sc}. This continuous PrP^{Sc} propagation may lead to its transfer to neighboring cells, which could potentially be neural cells. The effect of that unfavorable condition on the cellular degradation systems was proved by a significant decrease in proteasome activity in infected and non-infected cells incubated at 24 °C. This suggests that the accumulation of PrP^{Sc} is enhanced when degradation systems are impaired. Results showed also that PrP^{Sc} itself impairs the cellular 20S proteasome system even at optimum temperatures, confirming other previous studies [18,19]. Another factor that was found to enhance PrP^{Sc} accumulation in cells was the availability of PrP^{C} supply where the exposed cells to additional PrP^{C} maintained PrP^{Sc} for a longer time. This data indicates that PrP^{C} availability may be important to support the PrP^{C}–PrP^{Sc} conversion process in the cells. However, the saturation of phagocytic capacity of the cells could not support the PrP^{Sc} propagation rate to be at higher rate than the degradation rate. These results come in concomitance with other studies that have shown the importance of PrP^{C} in disease permissibility and progression [20,21]. Here, we also demonstrate that the cellular PrP^{Sc} content is dependent on the original amount that could be endocytosed into cells through lipid rafts and further cycling with PrP^{C} around the cell membrane. This was also demonstrated when the U18666A compound was added to cell cultures and proved to perturb lipid raft formation and block PrP^{C} transport to the plasma membrane [22]. This may be attributed to the compromised cellular phagocytic activity as a result of impaired cholesterol synthesis and PrP^{C} trafficking. The effect of this agent on the inhibition of PrP^{Sc} was also supported by other studies [15,23].

Investigating the correlation between the accumulation of PrP^{Sc} in macrophages and mRNA expression of *PRNP* during exposure to PrP^{Sc} revealed some up-regulations of this gene. However, these up-regulation rates may not be effective at the protein levels. Moreover, in some of the obtained results, there was no direct correlation between the mRNA and protein data. Thus, the *PRNP* gene up-regulation may express a nonspecific response of immune cells to variable antigens. This is understandable as many studies have shown that there is not always agreement between mRNA and protein levels as many parameters may influence mRNA and protein correlation [24,25].

Studies in mice and mouse-derived cell cultures have shown that several cell types, especially immune cells, may harbor the agent, enhance its expression and transfer it to other cells [7,8,26]. We have focused in this study on the potential role of immune cells, especially macrophages, in prion disease. If that complete clearance of PrP^{Sc} were not fulfilled because of extracellular factors or cell death, these cells may act as reservoirs for PrP^{Sc} particles until the opportunity arises to transfer to other cells. Our data provides insights into the relationship between PrP^{Sc} propagation in cells and the factors of PrP^{C} availability, cholesterol homeostasis, temperature and the condition of cellular degradation systems. This study suggests that further investigation of macrophages may be significant in controlling prion diseases.

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