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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 $1 \times 10^9$, 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 $5 \times 10^4$ 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