Effect of PrP105-132 on the secretion of interleukin-6 and interleukin-8 from microglial cells *in vitro*

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Abstract. In the present study, the effect of prion protein (PrP) on the secretion of interleukin-6 (IL-6) and IL-8 from microglial cells *in vitro* and its possible underlying pathway were investigating by establishing a cell model for prion disease. Rat neuroglial cells were cultured *in vitro*, and were treated with 80 μM PrP peptides 105-132 (PrP105-132) only, PrP+MG132 or PrP+cyclosporin A (CsA). After 48 h, the IL-6 and IL-8 levels in the supernatant fluid of the treated cells were detected using enzyme-linked immunosorbent assay. In addition, the expression levels of nuclear factor-κB (NF-κB) and nuclear factor of activated T cells (NFAT) were evaluated using reverse transcription-polymerase chain reaction. The results indicated that the microglial cells were activated by treatment with PrP peptides. Cell bodies were augmented and appeared to have round, rod and amoeba-like shapes. In addition, the protuberances were shortened and eventually disappeared. Furthermore, the mRNA expression levels of NF-κB and NFAT in microglial cells increased, as well as the IL-6 and IL-8 levels in the supernatant fluid after treatment with PrP. However, the mRNA expression levels of NF-κB, and the IL-6 and IL-8 levels decreased after these cells were treated with MG132, a specific inhibitor of NF-κB. The mRNA expression of NFAT decreased after these cells were treated with CsA, a specific inhibitor of NFAT; however, the IL-6 level decreased, while no significant difference was observed in the IL-8 level. In conclusion, PrP-treated microglial cells secreted IL-6 and IL-8, and the secretion of IL-6 was associated with the activation of NF-κB and NFAT pathways. In addition, the secretion of IL-8 was mainly dependent on the NF-κB pathway.

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Introduction

Prion disease, also known as transmissible spongiform encephalopathy (TSE), is a lethal neurodegenerative disease that affects both humans and livestock, and is characterized by amyloidosis of the brain tissue (1). Abnormal prion protein (PrP) deposition is the main component of amyloidosis, and large quantities of glial cells have been observed around PrP deposition sites (2), which results in progressive neuronal degeneration and neuronal vacuolation (3). Human prion diseases include Kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI). The most prevalent human prion disease is CJD. It is reported that 85-90% of CJD cases occur sporadically and affect 1-1.5 people per million annually (4). Glial cells are important in providing support, nutrition, protection and repair for the survival and vital movement of neurons. These cells present various immunocompetencies and constitute the initial protection of the central nervous system (CNS) against the invasion of pathogens. Cytokines are the key regulators of innate and adaptive immunity (5). Among CNS infectious diseases, tissue-infiltrating immunocytes, CNS-associated macrophages, microglial cells and astrocytes are the sources of cytokines in CNS-specific inflammation (6). Microglial cells are the main source of pivotal proinflammatory factors and immune regulatory cytokines *in vivo* and *in vitro* (7). In addition, the levels of the proinflammatory cytokine interleukin (IL)-6 and the chemokine IL-8 have been demonstrated to significantly increase in the cerebrospinal fluid of patients with Creutzfeldt-Jakob disease (CJD) (8,9).

The mechanisms of neuronal loss during prion disease are not fully understood. Previous studies have shown that glial activation precedes neuronal loss (10), and that cytokines secreted by activated microglia are important in neurodegeneration and neuronal loss (11). In our previous study, IL-8 was secreted from microglial cells treated with PrP *in vitro* (12). In the present study, microglial cells were treated with PrP to investigate the source and possible pathways of IL-6 and IL-8 in prion disease.

Materials and methods

Ethics statement. The present study was performed in strict accordance with the recommendations of the Guide for

the Care and Use of Laboratory Animals of the National Institutes of Health. Furthermore, the protocols were approved by the Institutional Animal Care and Use Committee of Inner Mongolia Medical University (Hohhot, China; permit no. YKD2013163). All surgical procedures were performed under sodium pentobarbital anesthesia (1% sodium pentobarbital, 40 mg/kg, intraperitoneal injection; Shanghai Westang Bio-Tech Co., Ltd., Shanghai, China) with all efforts made to minimize animal suffering.

Materials and animals. A total of 10 healthy newborn (1-day-old) Wistar rats (weight, 5.3±0.3 g) were acquired from the Experimental Animal Center of Jilin University (Jilin, China). ELISA kits for the determination of IL-6 and IL-8 levels were purchased from Shanghai Westang Bio-Tech Co., Ltd. (cat. nos. F01310 and F15880). TRIzol reagent was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA; cat. no. 15596-026). One Step RNA polymerase chain reaction (PCR) kit and DL2000 DNA marker were purchased from Takara Bio, Inc. (Otsu, Japan; cat. nos. RR024A and 3427A). In addition, the primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc., Shanghai, China), while PrP105-132 peptides, MG132 and cyclosporin A (CsA) were obtained from Shanghai Bootech Bioscience & Technology Co., Ltd. (Shanghai, China), EMD Millipore (Billerica, MA, USA; cat. no. 474790) and Huadong Medicine Co., Ltd. (Hangzhou, China; cat. no. 59865-13-3), respectively. Anti-CD68 antibody was purchased from Thermo Fisher Scientific, Inc. (cat. no. MS-397-R7). Streptavidin-peroxidase (SP) immunohistochemistry kit and DAB color developing reagent kit were purchased from Fuzhou Maxim Bioscience & Technology Co., Ltd. (Fuzhou, China; cat. nos. KIT-9710 and DAB-0031).

Treatment of PrP peptides. PrP105-132 (KTNLKHVAG AAAAGAVVGGLGGYMLGSA) was synthesized using the solid-phase method (13). Small quantities of peptides (0.5 mg) were transferred to an Eppendorf tube, and then dissolved using diluted acetic acid. Subsequently, the peptides were further diluted by adding distilled water (1:1), and the pH was neutralized using diluted acetic acid.

Nerve glial cell culture. The crania of 10 newborn Wistar rats were opened under sterile conditions. The brain tissues containing cortex and medulla were dissected and placed in a dish with D-Hanks solution (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Blood was removed by repeated washing with D-Hanks solution. Next, the meninges and blood vessels on the surface of the brain tissue were also removed, and the brain tissue was washed once or twice with D-Hanks solution. The brain tissue was then cut into cubes of ~1-3 mm^3, and 40 times volume of trypsin (Sigma-Aldrich) was added according to the tissue mass. The mixture was repeatedly pipetted at 37˚C for 5-10 min until it became cloudy. Subsequently, the digestion was terminated by adding complete medium, consisting of high-glucose DMEM/F12 (1:1; Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). The single-cell suspension was transferred into a sterile Eppendorf tube, and the cells were centrifuged at 111.8 x g, 4˚C for 10 min. The supernatant was discarded, and the cells were resuspended by adding complete medium. Cells were then seeded into six 50-ml cell culture flasks at a density of 1.0-1.2x10^5 cells/ml and cultured in an incubator at 37˚C until further use.

Separation, purification and passage of microglial cells. Microglial cells were separated from the nerve glial cell solution, purified and passaged as described previously (14). Briefly, the complete culture medium in the culture of glial cells (1.0-1.2x10^5 cells/ml per 50-ml cell culture flask) was replaced on days 3 and 12 of culture. A solution of trypsin-ethylenediaminetetraacetic acid (EDTA) was prepared by mixing 0.25% trypsin and 0.02% EDTA at a ratio of 1:1, and was further diluted using D-Hanks solution at a ratio of 3:1. At 24 h after refreshment of the medium for the second time, the cells were treated with a diluted trypsin-EDTA solution at 37˚C for 40 min. The microglial cells were separated from the adherent astrocytes by shaking the flask, and these cells were transferred into a new cell culture flask. After 24 h, the medium was replaced. Finally, these cells were passaged using trypsin at a confluence of 100%, and purified microglial cells were obtained.

Identification of cultured cells. The obtained microglial cells were seeded onto a coverslip and washed with 0.9% saline three times for 5 min. Next, these cells were fixed by 4% paraformaldehyde for 40 min, and further washed with 0.01 M phosphate-buffered solution (pH 7.3) three times for 5 min. The coverslip was then stored at 4˚C. Immunocytochemical identification of the microglial cells was performed by staining with anti-CD68 antibody (1:50) at 4˚C for 24 h and the SP immunohistochemistry kit, following the manufacturers' protocols. Five fields of high magnification were selected randomly, and the number of CD68-positive microglial cells in these fields was counted under an optical microscope.

PrP105-132 treatment of microglial cells and sample collection. The cultured microglial cells were divided into four groups as follows: i) Control, ii) PrP, iii) PrP+MG132 and iv) PrP+CsA groups. In these groups, MG132 served as a specific inhibitor of nuclear factor (NF)-κB, and CsA as a specific inhibitor of nuclear factor of activated T cells (NFAT). Six repeated wells were established for each group, and 1x10^6 cells were seeded into each well. The microglial cells were cultured with complete medium only in the control group. In the PrP group, the cells were treated with 80 μM PrP105-132. In the PrP+MG132 and PrP+CsA groups, the cells were treated with 80 μM PrP105-132 for 24 h, followed by treatment with 3 μmol/l MG132 and 1.0 μg/ml CsA, respectively. After 48 h of culture, the cells were centrifuged at 1,000 x g, 4˚C for 10 min. The supernatant and cells were collected and stored at 20˚C until further use to detect the IL-6 and IL-8 protein levels or the NF-κB and NFAT mRNA expression.

Detection of IL-6 and IL-8 levels. The levels of IL-6 and IL-8 in the various groups were detected using the aforementioned ELISA kits in accordance with the kit manufacturer's instructions.

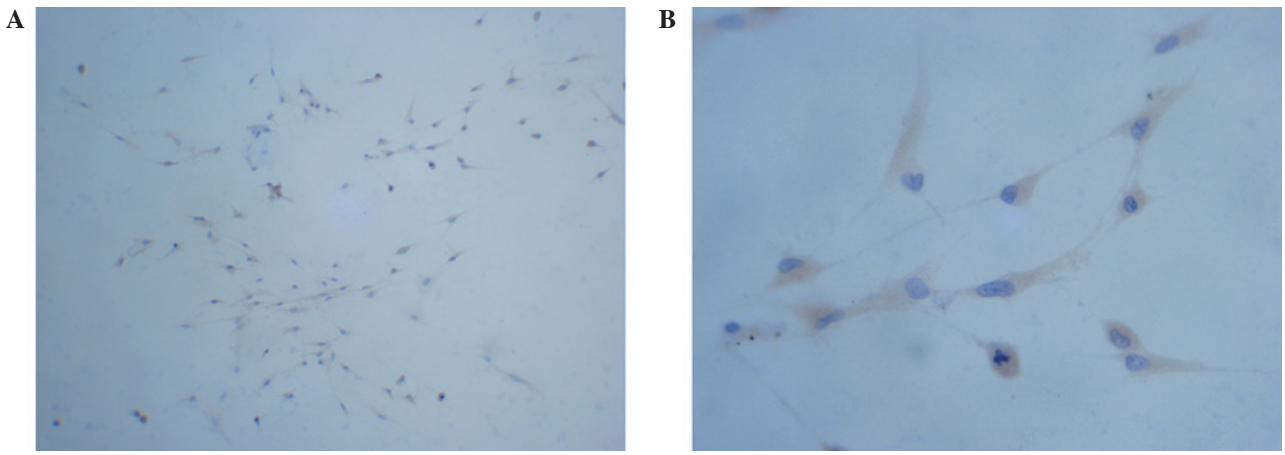

Figure 1. CD68 staining of microglial cells at a magnification of (A) x200 and (B) x400.

Detection of NF-κB and NFAT at the mRNA level. The mRNA expression of NF-κB and NFAT was detected using reverse transcription (RT)-PCR. Briefly, total RNA was extracted from the treated microglial cell samples using an RNA extraction kit. The target gene in the RNA was then amplified by RT-PCR using the One Step RNA PCR kit. The PCR primers for NF-κB (accession no. NM002502), NFAT (accession no. NM001107425) and β-actin (accession no. BC063166) were designed using Primer Premier 6.0 (Premier Biosoft, Palo Alto, CA, USA) according to their accession number. The primer sequences were as follows: β-actin (147 bp), 5'-GTCAGGTCATCACTATCGGCAAT-3' (sense) and 5'-AGAGGTCTTTACGGATGTCAACGT-3' (antisense); NF-κB (378 bp), 5'-ATGCGTTTCCGTTACAAG TGCGAGG-3' (sense) and 5'-GACCGCATTCAAGTCATA GTCCCCG-3' (antisense); NFAT (365 bp), 5'-GACCTGGAA TCGCCCAAGTCCCTGT-3' (sense) and 5'-GTTACTTAC CCCCACGGCTGAGGAG-3' (antisense). PCR was conducted using the CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), with a reaction mixture consisting of 2.5 μl 10X One Step RNA PCR Buffer, 5 μl $MgCl_2$ (25 mM), 5 μl dNTP Mixture (10 mM), 0.5 μl AMV-Optimized Taq (5 U/μl), 1 μl forward and reverse primer (100 pM), 2 μl cDNA (5 ng/μl) and 10.5 μl dH_2O. The PCR cycling conditions were as follows: 95˚C for 15 min, followed by 30 cycles of 94˚C for 30 sec and 55˚C for 30 sec, and a final extension step at 72˚C for 1.5 min. Following PCR assay, the products were analyzed using 1.5% TAE (40 mmol/l Tris-HAc, 1 mmol/l EDTA) agarose gel electrophoresis.and the densities of the electrophoretic bands were analyzed using a gel imaging analysis system (BOT-860SR; Beijing Zhongyiboteng Technology Co., Ltd., Beijing, China). The relative expression of each gene was obtained using the following formula: Relative expression of gene = (density of the gene band) / (density of β-actin band).

Statistical analysis. Results are expressed as mean ± standard deviation. One-way analysis of variance was used to compare groups. P<0.05 was considered to indicate a statistically significant difference. The statistical analysis was conducted using SPSS 13.0 software (IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Observation of cultured microglial cells in vitro. First-generation microglial cells were obtained, which floated in the medium, and presented rotundity and strong refractivity. The cells were passaged and further adhered to the culture dish, presenting numerous short and bent protuberances after 5-10 days of culture. The cell bodies of the PrP105-132-treated microglial cells were enlarged and presented round, rod and amoeba-like morphologies. In addition, the protuberances were shortened and eventually disappeared following treatment with PrP. However, no significant morphological changes were observed after these cells were treated with MG132 or CsA (data not shown).

Purity of cultured microglial cells. The microglial cells were detected by immunohistochemistry using a CD68 monoclonal antibody. As shown in Fig. 1, large quantities of round cells were confirmed by the observation of strong positive staining, and some cells exhibited short protuberances. The percentage of cells that were positively stained (microglial cells) was >95% (Fig. 1).

IL-6 and IL-8 levels. ELISA kits were used to determine the protein levels of IL-6 and IL-6 in the various groups. The levels of IL-6 and IL-8 in the supernatant of the PrP group were significantly higher compared with those in the control group (P<0.001; Tables I and II). Furthermore, IL-6 and IL-8 levels in the PrP+MG132 group were markedly lower compared with those in the PrP group (P<0.001). However, the IL-6 protein level was markedly decreased in the PrP+CsA group compared with the PrP group (P=0.024; Table I), while that of IL-8 did not show a significant reduction (P=0.180; Table II). In addition, the IL-6 and IL-8 levels in the PrP+MG132 group were significantly lower than those in the PrP+CsA group (P<0.001; Tables I and II).

mRNA expression levels of NF-κB and NFAT. As shown in Table III and Fig. 2, the mRNA expression of NF-κB in the PrP group was significantly higher compared with that in the control group (P<0.001). However, the mRNA expression of NF-κB was significantly decreased after these cells were

Table I. Secretion of IL-6 from microglial cells *in vitro*.

Group	No. of wells	Maximum value (pg/ml)	Minimum value (pg/ml)	Concentration (mean ± SD)
Control	6	73.56	65.25	68.09±3.04
PrP	6	169.48	142.13	157.79±9.69[a]
PrP+MG132	6	71.30	68.71	70.13±1.04[b]
PrP+CsA	6	145.97	134.52	138.55±3.99[b,c]

[a]P<0.01 vs. control group; [b]P<0.01 vs. PrP group; [c]P<0.01 vs. PrP+MG132 group. The level of IL 6 in the supernatant of the PrP group was significantly higher than that in the control group (P<0.001). The level of IL 6 in the PrP+MG132 group was significantly lower than that in the PrP group (P<0.001) and the PrP+CsA group (P<0.001). The level of IL 6 in the PrP+CsA group was decreased compared with that in the PrP group (P=0.024). IL, interleukin; SD, standard deviation; PrP, prion protein; CsA, cyclosporin A.

Table II. Secretion of IL-8 from microglial cells *in vitro*.

Group	No. of wells	Maximum value (pg/ml)	Minimum value (pg/ml)	Concentration (mean ± SD)
Control	6	21.15	17.26	19.44±1.40
PrP	6	39.66	35.05	37.94±1.69[a]
PrP+MG132	6	30.49	22.99	27.07±2.74[b]
PrP+CsA	6	40.65	32.77	35.78±3.22[c]

[a]P<0.01 vs. control group; [b]P<0.01 vs. PrP group; [c]P<0.01 vs. PrP+MG132 group. The level of IL 8 in the supernatant of the PrP group was significantly higher compared with that in the control group (P<0.001). The level of IL 8 in the PrP+MG132 group was significantly lower than that in the PrP group (P<0.001) and the PrP+CsA group (P<0.001). No significant difference was observed between the PrP+CsA and PrP groups (P=0.180). IL, interleukin; SD, standard deviation; PrP, prion protein; CsA, cyclosporin A.

Table III. Expression of NF-κB and NFAT at the mRNA level.

Group	NF-κB expression (mean ± SD)	NFAT expression (mean ± SD)
Control	0.6323±0.0414	0.6476±0.0168
PrP	1.0221±0.0184[a]	0.9727±0.0122[a]
PrP+MG132	0.8334±0.0232[b]	1.0058±0.0308
PrP+CsA	0.9549±0.0365	0.7934±0.0461[b]

[a]P<0.01 vs. control group and [b]P<0.01 vs. PrP group. Expression levels were determined according to the gray scale of the target against that of β-actin. mRNA expression of NF-κB in the PrP group was significantly higher compared with that in the control group (P<0.001), and significantly reduced in the PrP+MG132 group compared with the PrP group (P<0.001). mRNA expression of NFAT in the PrP group was significantly higher than that in the control group (P<0.001), and significantly lower in the PrP+CsA group than the PrP group (P<0.001). NF-κB, nuclear factor κB; NFAT, nuclear factor of activated T cells; SD, standard deviation; PrP, prion protein; CsA, cyclosporin A.

treated with MG132 (P<0.001). Furthermore, the mRNA expression of NFAT in the PrP group was significantly higher compared with that in the control group (P<0.001). However, the mRNA expression of NFAT significantly decreased following the treatment of these cells with CsA (P<0.001).

Discussion

Prion disease, also known as TSE, is a lethal neurodegenerative illness that affects human beings and livestock. Previous evidence indicates that prion disease is mainly caused by the transformation of normal to abnormal PrP (PrP^{C} to PrP^{Sc}) (15). PrP^{C} and PrP^{Sc} present the same amino acid sequence but have different spatial configurations. In the brain tissue of scrapie-infected mice, the activation of microglial cells, the inflammatory mediator IL-1β, and the prostaglandins E_2 (PGE_2) and $PGF_{2\alpha}$ were demonstrated to be associated with the accumulation of PrP in the brain (16). In addition, the distribution of activated microglial cells was consistent with the distributions of PrP^{Sc} and dead neurons (17,18). These observations indicate that the activation of microglial cells serves an important role in the neuropathological changes of PrP-mediated scrapie infection (19). In the present study, microglial cells were activated by treatment with PrP, as demonstrated by the cell morphological changes. Furthermore, the possible sources and pathways of IL-6 and IL-8 in prion disease were explored.

The PrP105-132 peptide (KTNLKHVAGAAAAGAVVG GLGGYMLGSA) is the transmembrane region of PrP^{C},

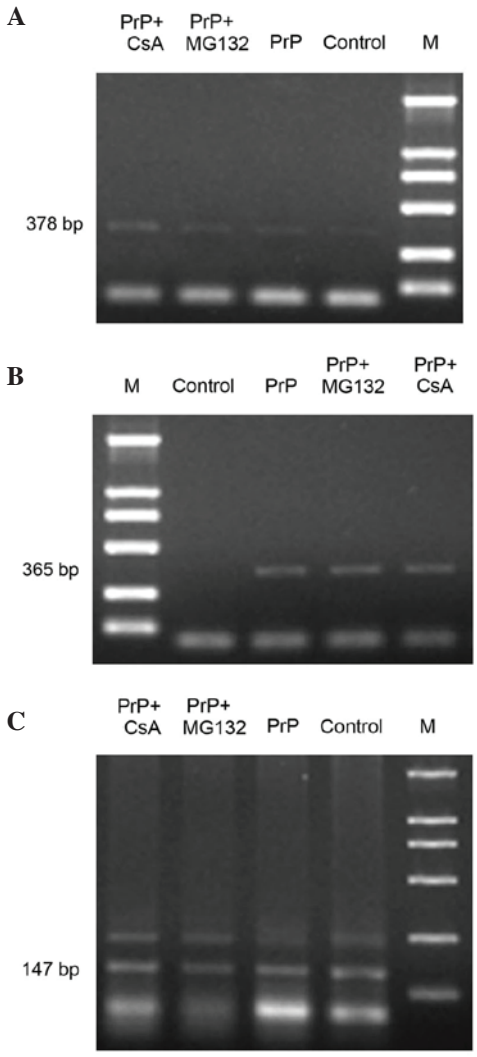

Figure 2. mRNA expression levels of (A) NF-κB, (B) NFAT and (C) β-actin, in the various treatment groups, as determined by reverse transcription-polymerase chain reaction and agarose gel electrophoresis. NF-κB, nuclear factor κB; NFAT, nuclear factor of activated T cells PrP, prion protein; CsA, cyclosporin A; M, marker.

and it is a key position that mediates the transformation of PrP^{C} to PrP^{Sc} (20). In addition, the PrP105-132 peptide is the common structure of all abnormal PrP isoforms and exhibits different secondary structures under different conditions, including ion strength, pH value and solute composition (21). Furthermore, PrP105-132 shares certain common characteristics with the entire PrP^{Sc} structure, and it is able to form amyloid fibrils with proteinase K resistance (21). In the present study, the cell bodies of PrP105-132-treated microglial cells were enlarged, with round, rod and amoeba-like morphologies. The protuberances on these cells were shortened and eventually disappeared following treatment with PrP. In addition, the results indicated increased secretion of IL-6 and IL-8 following treatment with PrP105-132. Thus, the role of PrP105-132 in the activation of microglial cells was further clarified.

The activation of microglial cells is a neuropathological characteristic of prion disease, and previous histological analyses have indicated that the activation of microglial cells in the CNS is associated with the accumulation of abnormal PrP in prion disease (22). In addition, PrP induced an inflammatory reaction mediated by microglial cells, leading to a deficiency of neurons (23). Therefore, the synthesis and participation of various cytokines are required for an inflammatory reaction mediated by microglial cells. It has previously been demonstrated that PrP promotes the expression of cyclooxygenase-2 and the synthesis of IL-1β and PGE_2 in microglial cells (23,24). In the present study, IL-6 and IL-8 levels in the supernatant increased following the treatment of microglial cells with PrP, confirming that microglial cells are one of the sources of IL-6 and IL-8 in prion disease.

The expression of NF-κB has been reported to be elevated in brain microglial cells of CJD patients, and the 20S proteasome was observed on the cell membranes of neurons and glial cells of pathologically changed brain tissue (25). These previous findings indicate that the proteasome system is involved in the pathogenesis of prion disease. In addition, the mRNA expression of NF-κB increased after microglial cells were treated with PrP; simultaneously, the IL-6 and IL-8 levels increased in the supernatant. By contrast, the mRNA expression of NF-κB and the IL-6 and IL-8 levels in the supernatant fluid decreased compared with that in the PrP group after cells were treated with MG132, a specific inhibitor of NF-κB. Therefore, the association between the activation of NF-κB and the secretion of IL-6 and IL-8 in microglial cells was further confirmed.

However, the mechanism of NF-κB activation induced by PrP remains largely unknown. The possible mechanisms are hypothesized as follows: i) PrP-activated protein kinases and protein phosphatases act directly on microglial cells, and IκB, an NF-κB inhibitory protein, is further degraded by the proteasome; subsequently, the activated NF-κB is released. ii) PrP activates microglial cells, and these activated cells release IL-1 and tumor necrosis factor (TNF)-α (16,26), which then promote the degradation of IκB, activating NF-κB. iii) Activated NF-κB promotes the expression of IL-1, TNF-α and IL-6, and these cytokines reversely activate NF-κB, resulting in a positive feedback loop (27).

In the present study, the mRNA expression of NFAT, as well as the IL-6 and IL-8 levels in the supernatant, increased after microglial cells were treated with PrP *in vitro*. This result suggests that PrP can activate NFAT in microglial cells. After these cells were treated with CsA, the mRNA expression of NFAT decreased, and the IL-6 level rather than the IL-8 level in the supernatant decreased. These observations indicate that the secretion of IL-6, but not that of IL-8, may be promoted through the NFAT pathway in microglial cells.

In conclusion, PrP treated microglial cells secreted IL 6 and IL 8, and the secretion of IL 6 was associated with the activation of NF-κB and NFAT pathways. In addition, the secretion of IL 8 was mainly dependent on the NF-κB pathway. These results will provide an experimental basis for further studies on the pathogenesis of prion disease.

Acknowledgements

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