ELSEVIER

Journal of Neuroimmunology 199 (2008) 10–17

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

Aspirin inhibits cytotoxicity of prion peptide PrP106-126 to neuronal cells associated with microglia activation *in vitro*

Lifeng Yang, Xiangmei Zhou, Jianmin Yang, Xiaomin Yin, Lifang Han, Deming Zhao *

National Animal Transmissible Spongiform Encephalopathy Laboratory, College of Veterinary Medicine, China Agricultural University, Beijing 100094, China

Received 22 December 2007; received in revised form 27 March 2008; accepted 16 April 2008

Abstract

The synthetic peptide consisting of amino acid residues 106–126 of the human prion protein PrP106-126 has been demonstrated to generate fibrils, which damage neurons either directly by interacting with components of the cell surface to trigger cell apoptosis signaling or indirectly by activating microglia to produce inflammatory mediators. In our study, rat microglia cells were treated with PrP106-126 or scramble PrP106-126 (Scr PrP). Activated nuclear factor κB (NF-κB) was determined using immunofluorescence staining and the expression of TNF-α and IL-1β mRNA was measured by quantitative RT-PCR. Inhibitory activity of aspirin on neurotoxicity of PrP106-126 associated with microglia activation was determined using an apoptosis detection kit. Treatment of microglia with 25 μM PrP106-126, but not Scr PrP, resulted in activation and translocation of NF-κB, which peaked after 20 min of treatment. The activation of NF-κB was followed by increased mRNA expression of TNF-α and IL-1β peaking at about 20 h. In the presence of microglia, aspirin significantly inhibited neuro-2a cell death induced by PrP106-126. The number of neuro-2a cells in apoptosis and necrosis with 5 mM aspirin was about 3-fold lower than the cell culture without aspirin ($P<0.05$). These data suggest that increased production of cytokines by microglia cells in prion disease is probably regulated by NF-κB translocation and may contribute to neurotoxicity of prions, and neurotoxicity of PrP106-126 may be inhibited by aspirin.

Keywords: PrP106-126; Microglia; Neuron; NF-κB; Cytokines; Aspirin

1. Introduction

Transmissible spongiform encephalopathy (TSE) is a fatal and infectious neurodegenerative disease caused by prions, and can occur in humans as Kuru, Creutzfeldt–Jakob disease (CJD), fatal familial insomnia (FFI) and Gerstamann Straussler Sheinker syndrome (GSS), in cattle as bovine spongiform encephalopathy, and in sheep and goats as scrapie (Prusiner, 1998). According to the "protein-only" hypothesis, the mechanism of pathogenicity is thought to be the conversion of the cellular isoform of prion protein (PrP^{C}) to the pathogenic isoform (PrP^{Sc}), which has a high content of β-sheet secondary structure, forms amyloid fibrils, and is partially resistant to proteolysis (Konturek et al., 2005; Safar et al., 1993). The main characteristics of TSE are apoptosis of neurons, vacuolation of neuropil and recruitment of microglia in brain.

A synthetic prion fragment, PrP106-126, consisting of amino acid residues 106–126 of the human prion protein and residing near the N-terminal of the protease resistant region of PrP^{Sc}, which possesses many properties of the entire PrP^{Sc} (Forloni et al., 1993). It has been used for studying the pathogenesis of PrP^{Sc} and was demonstrated to accumulate fibrils which damage neurons either directly by interacting with components of the cell surface to trigger cell apoptosis signaling or indirectly by activating microglia to produce inflammatory mediators (Marella and Chabry, 2004).

In experimental prion disease models, microglia activation is found in brain areas with accumulation of PrP^{Sc} before signs of neurodegeneration become apparent (Veerhuis et al., 2005). Microglia cells are usually quiescent, but they are very sensitive to changes in the surrounding environment and readily become activated in response to infection or injury (Streit, 2002). Microglia play an important role in neuron degeneration (Brown et al., 1996; Dheen et al., 2007). Activated microglia provide an initial protective role favoring neuronal survival and axonal regeneration. However, sustained secretion of inflammatory

* Corresponding author.
E-mail address: zhaodm@cau.edu.cn (D. Zhao).

0165-5728/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2008.04.028

mediators by microglia can be harmful and lead to damage of neighboring cells (Ciesielski-Treska et al., 2004). Indeed, neuron vacuolation was frequently detected in the vicinity of activated microglia (Muhleisen et al., 1995), and neuron degeneration is exacerbated by co-culture with activated microglia (Brown et al., 1996). Whether microglia play a major role in the progression of neuronal injury induced by PrP106-126 has not been studied.

Neuron degeneration is exacerbated by co-culture with activated microglia (Brown et al., 1996). TNF-α and IL-1β are two main cytokines released by activated microglia (Liu and Hong, 2003). The transcription factor NF-κB is a regulator of the expression of these proinflammatory cytokines, and NF-κB binding sites in the promoter region of their genes serve as inducible transcriptional regulatory elements (Baeuerle and Henkel, 1994; Bales et al., 1998; Kim et al., 1999; van Loo et al., 2006). Little is known about the effects of cytokines which are produced by microglia exposed to abnormal PrP. However, mechanisms through which microglia mediate neuronal cell injury have not been defined precisely. The current study assesses the response of microglia to PrP106-126 stimulation by examining NF-κB activation and translocation and the expression of proinflammatory cytokines, TNF-α and IL-1β, and inhibitory activity of aspirin on neurotoxicity of PrP106-126 associated with microglia activation.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (D-MEM/F-12), and Fetal Bovine Serum (FBS) was from GIBCO (USA). Opti-MEM was from OMEGA (USA). Murine anti-NF-κB p65 (activated subunit of NF-κB) antibodies and anti-CD11b antibodies were purchased from Santa Cruz Biotechnology, Inc (USA). SYBR green was from MJ Research (Waltham, USA). The Taq Dye-Deoxy Terminator kit was obtained from PE-Applied Biosystems (Foster City, USA). The apoptosis detection kit with annexin V-FITC and propidium iodide was purchased from Beyotime Institute of Biotechnology (China). Reagents for RT-PCR were obtained from OMEGA unless specified otherwise. Trizol kit was from Invitrogen (USA). MLV reverse transcriptase and DNA wizard cleanup kit were from Promega (USA).

The PrP106-126 peptide (KTNMKHMAGAAAAGAV VGGLG), scramble PrP106-126 peptide (MEVGWYRSPFSRV VHLYRNGK; Scr PrP) and primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services (China). The purity of prion peptides was >95% according to the synthesizer's data. The peptide solution (500 μM) in PBS (pH 7.4) was aged for 2–3 days at 37 °C or room temperature before use to increase fibrillogenicity of the peptide (Ciesielski-Treska et al., 2004).

2.2. Isolation and culture of microglia cells

Primary mixed glia cells were isolated from brain of 1- to 2-days Sprague–Dawley rats according to Garcao et al. (2006). After sterilization with 75% ethanol for 3–5 min, the brain was dissected and the meninges were carefully removed under a stereomicroscope. Cerebral cortexes were collected and the tissue was dissociated by pipetting up and down for approximately 10 times, and then digested with trypsin (0.125%) for 15–20 min at 37 °C, which was terminated with DMEM containing serum. The digested tissue was repeatedly pipetted to obtain the maximum number of single cells (Bonifati and Kishore, 2007; Ciesielski-Treska et al., 2004). Then the cells were passed through a 200 μm mesh to obtain a single-cell suspension. After centrifugation at 100 g for 5 min and the cellular pellet was resuspended in DMEM containing 10% FCS, penicillin and streptomycin, and then plated in a 35 mm plastic tissue culture flask (Yao et al., 1992). The mixed glia cells were cultured for about 6–7 days. The cells were suspended by agitation for 12 h on a rotary shaker (180 rpm) at 37 °C and transferred to another flask. After incubation for 2 h at 37 °C, microglia that selectively adhered to the plastic flask were washed with Ca^{2+}/Mg^{2+}-free PBS and grown in a serum-free medium before treatment with PrP106-126 (see below). The purity of the microglia cells was approximately 90% determined using anti-CD-11b antibodies.

2.3. Neuro-2a cell culture

The murine neuroblastoma cell line neuro-2a was obtained from Shanghai Institute for Biological Sciences. Neuro-2a cells were grown in Opti-MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin as described previously (Higuchi et al., 2002).

2.4. Treatment of microglia and neuro-2a cells with PrP106-126

2.4.1. Neurotoxicity of PrP106-126

Neuro-2a cells were incubated with 25 μM PrP106-126 or Scr PrP for up to 72 h for the determination of neurotoxicity of these peptides.

2.4.2. Microglia activation by PrP106-126

For the determination of NF-κB activation and translocation, microglia cells were grown for 2 days before treatment with 25 μM PrP106-126, 25 μM SCR PrP or 100 ng/ml LPS (as positive control) for up to 2 h. The cells were processed for immunofluorescence and Hoechst 33258 staining described below. For the measurement of cytokine mRNA expression by

Table 1
Primers used for real-time quantitative PCR

Genes	Primer sequences	PCR fragment size (bp)	Locus
TNF-α	5′CTGAACTTCGGGGTGAT 3′ 5′TGCTTGGTGGTTTGCTA 3′	153 bp	NM_012675
IL-1β	5′GTTCCTGACTTGTTTGA 3′ 5′AAGGTGGACATCTTTGA 3′	185 bp	NM_031512
β-actin	5′AACACCTCAAACCACTC 3′ 5′ATCTTCCGCCTTAATAC 3′	131bp	AA875570

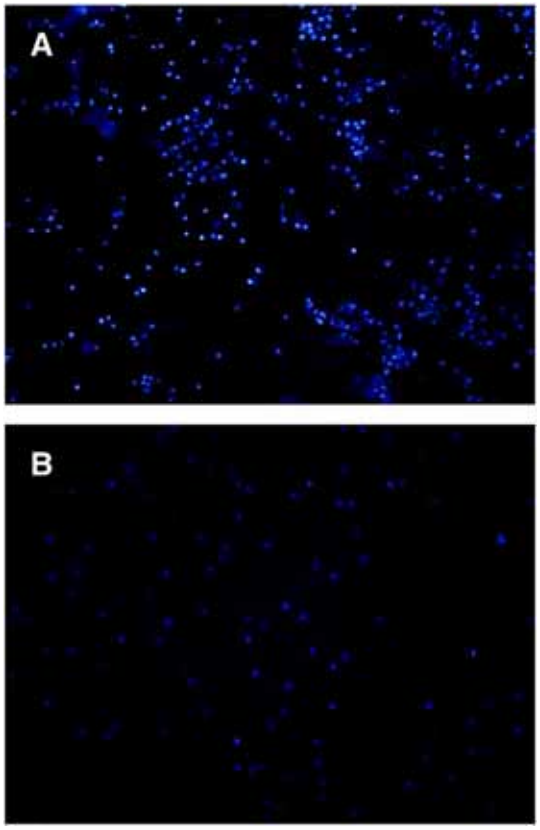

Fig. 1. Neura-2a cells incubated with 25 μM PrP106-126 (A) or scramble PrP106-126 peptide (B) for 48 h and stained with Hoechst 33258 stain. PrP106-126, but not the scramble peptide, induced apoptosis of Neuro-2a cells.

RT-PCR (see below), microglia cells were similarly treated for up to 54 h.

2.4.3. Effects of aspirin on neurotoxicity of PrP106-126 associated with microglia activation

The effect of aspirin on microglia-mediated neurotoxicity of PrP106-126 was studied in co-culture of microglia and neuro-2a cells. Neuro-2a (5×10^5) and microglia cells (5×10^5) were cultured for 24 h before incubation with 25 μM PrP106-126 and aspirin (0, 5 or 10 mM) for 20 h. Apoptotic and necrotic neuro-2a cells were determined by flow cytometry. Briefly, the culture medium was collected, and the treated cells were digested with 0.05% pancreatic enzyme for 3 to 5 min. The digested cells were washed with the collected culture medium, and then suspended in PBS. Approximately $5–10\times10^4$ cells (a mixture of microglia and neuro-2a cells) were incubated with the apoptosis detection kit according to the manufacturer's instructions before analysis by flow cytometry. A negative control culture of neuro-2a cells were not incubated with PrP106-126 or aspirin, one neuro-2a cell culture was treated PrP106-126 without microglia, and one positive control (neuro-2a and microglia) was treated with 100 ng/ml LPS. Control cells were similarly processed. Cells undergoing early stage apoptosis are stained with annexin V-FITC only and cells at late stage apoptosis or necrotic cells are stained with both annexin V-FITC and propidium iodide.

2.4.4. NF-κB activation and translocation assay

Peptide- or LPS-treated microglia cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton

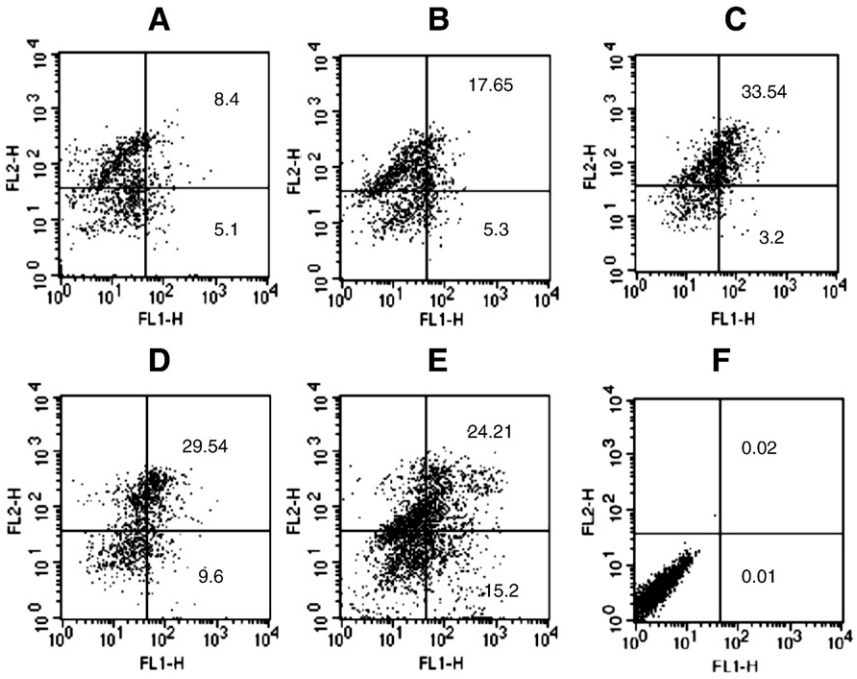

Fig. 2. Flow cytometry of neuro-2a cells co-cultured with microglia, 25 μM PrP106-126 and aspirin. The cells were incubated for 20 h before double staining with annexin V-FITC and propidium iodide. Dotted plot showed annexin V-FITC in *x*-axis and propidium iodide in *y*-axis. A) Neuro-2a + microglia + PrP106-126 + 5 mM aspirin; B) Neuro-2a + microglia + PrP106-126 + 10 mM aspirin; C) Neuro-2a + microglia + PrP106-126; D) Neuro-2a + microglia + 100 ng/ml LPS (positive control); E) Neuro-2a + PrP106-126 (no microglia); F) Negative control (no PrP106-126 or microglia).

X-100 for 10 min at room temperature, and incubated for 30 min with 5% goat serum to block nonspecific binding for 1 h at room temperature. After treatment with blocking solution, the cells were incubated with anti-NF-κB p65 antibody for 1 h at 37 °C or overnight at 4 °C. After several rinses in PBST, the cells were incubated with FITC-conjugated goat anti-mouse IgG as secondary antibody in the dark for 1 h at room temperature. Finally, nuclei were stained with the Hoechst 33258 stain for 5 min and washed for 20 min with PBST before examination under fluorescent microscope.

2.5. Real-time quantitative reverse transcriptase PCR (RT-PCR)

Total cellular RNA was purified from peptide- or LPS-treated microglia cells by the Trizol kit. In brief, a single extraction with an acid guanidinium thiocyanate/phenol/chloroform mixture was performed. Whole RNA was reverse transcribed in cDNA using oligo(dT)12–18 primers and MLV reverse transcriptase. Quantitative RT-PCR reactions were performed using SYBR green fluorescent reagent. Each PCR was carried out in 20 μL reaction volume containing 10 μL DyNAmo™ SYBR Green qPCR mix, 2.0 μL specific primer pairs at 2.5 μM each for IL-1β, TNF-α and β-actin (Table 1) and 2 μL cDNA. PCR amplification conditions were as follows: 30 s denaturation at 94 °C, 30 s hybridation at 49 °C, 40 s elongation at 72 °C. This was followed by 35 cycles of 30 s at 94 °C for DNA denaturation; 30 s at 49 °C for TNF-α and β-actin and 48 °C for IL-1β for primer annealing; 40 s at 72 °C for primer extension; and 1 s at an elevated temperature of 82 °C for fluorescent data acquisition. A final extension at 72 °C for 8 min concluded the PCR and the reaction was then subjected to a melting protocol from 65 to 95 °C with a 0.2 °C/s increment and 1 s holding at each increment to examine the specificity of the amplified products. Five microliters of each PCR product were electrophoresed on 1% agarose gel and visualized by ethidium bromide staining. The representative PCR products were gel-purified with a DNA wizard clean up kit and sequenced using the Taq Dye-Deoxy Terminator kit.

For the quantitation of IL-1β and TNF-α gene expression, the amplified cDNA fragments of IL-1β, TNF-α and β-actin genes were cloned and the standard curves of the cycle threshold (Ct) values for each gene were obtained from serial dilutions of the purified recombinant plasmids. For each sample, the amount of IL-1β, TNF-α and β-actin mRNA was determined from the respective standard curves. All samples were analyzed in triplicate.

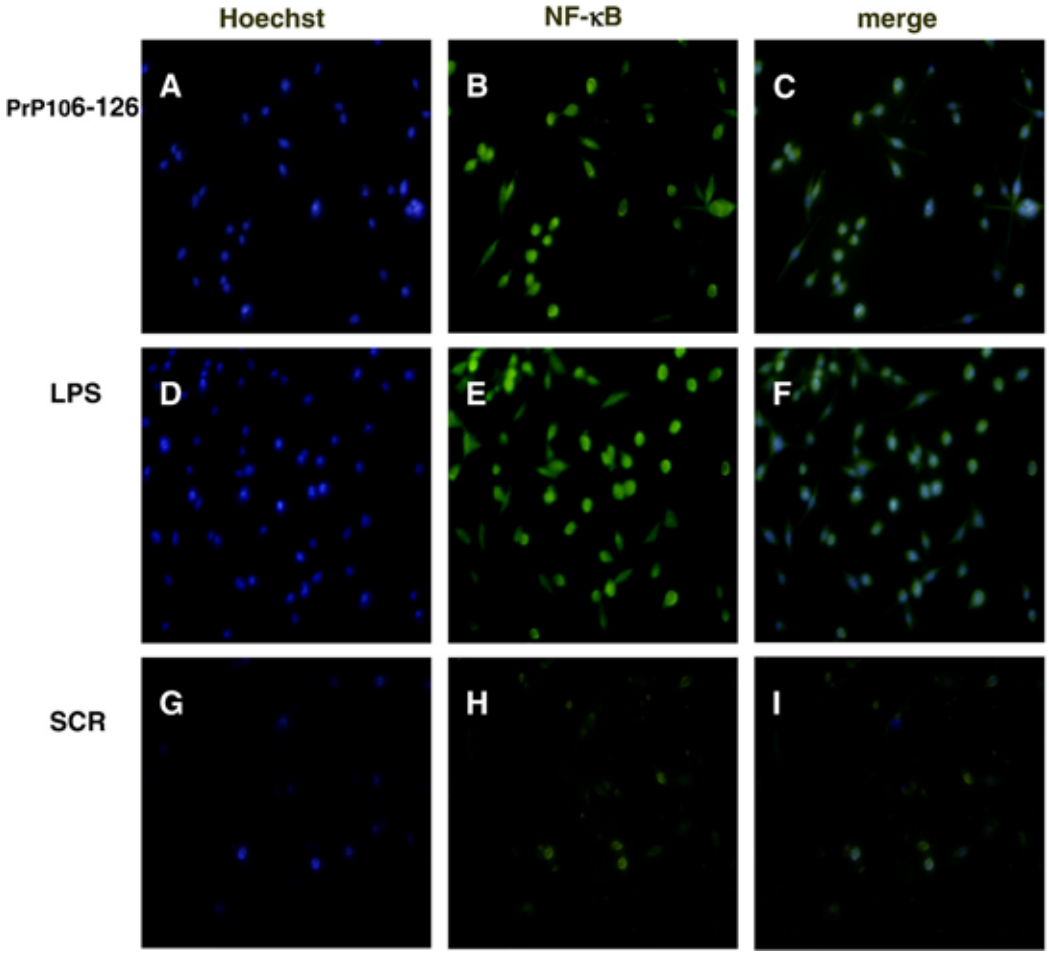

Fig. 3. PrP106-126 induced NF-κB activation and translocation in microglia. Microglia were treated with 25 μM PrP106-126 (A, B, C), 100ng/ml LPS (D, E, F) or 25 μM Scr PrP106-126 (G, H, I) for 20 min, and stained with Hoechst 33258 (A, D, G) or incubated with murine anti-NF-κB p65 antibody and FITC-conjugated goat anti-mouse IgG (B, E, H). Nuclei stained with Hoechst appear blue. Activated NF-κB appears green. Co-localized areas (nuclei) appear light blue (C, F, I) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

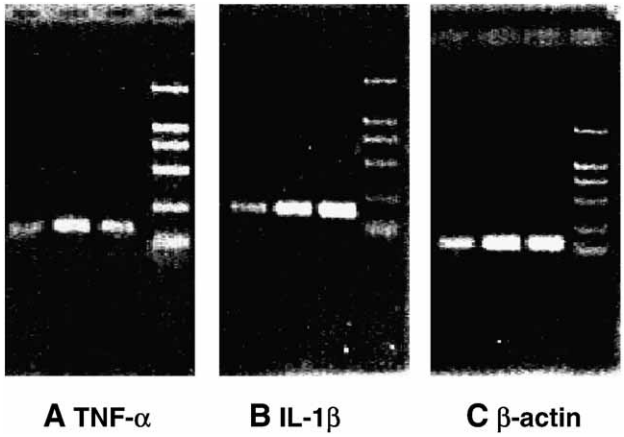

Fig. 4. Electrophoresis of RT-PCR-amplified TNF-α (153 bp), IL-1β (185 bp), and β-actin (131 bp) genes on 1% agarose gel and stained with ethidium bromide. Lane 1: untreated control microglia; Lane 2: microglia treated with PrP106-126 (25 μM) for 20 h; Lane 3: microglia treated with LPS (100 ng/ml) for 20 h. Lane 4: molecular weight markers (DL2000).

2.6. Statistical analysis

The distribution of all data sets was tested by the Kolmogorov–Smirnov normality test and then one-way analysis of variance and *T*-tests were performed. An independent sample *T*-test (SPSS for Windows, version 13.0) was performed to analyze differences in cytokine mRNA expression between control and treatment groups. Differences with $P<0.05$ were considered to be significant.

3. Results

3.1. Effects of aspirin on neurotoxicity of PrP106-126 in the presence of microglia

To determine whether the synthetic PrP106-126 peptide is neurotoxic, neuro-2a cells were treated with 25 μM of PrP106-126 or Scr PrP106-126 for 12, 24, 48 and 72 h. Hoechst 33258 staining of the treated cells showed that PrP106-126 induced cell death after treatment for ≥24 h (Fig. 1A). Neuro-2a cell survival was unaffected by the Scr PrP106-126 (Fig. 1B). These results demonstrated that the PrP106-126 peptide was neurotoxic. Flow cytometric analysis of neruo-2a cells after double staining with annexin V-FITC and propidium iodide showed that neurotoxicity of PrP106-126 was enhanced in co-incubation with microglia. A large number of neruo-2a cells (33.5%) were in late stage apoptosis or necrosis after co-culture with microglia in the presence of PrP106-126 (Fig. 2C), similar to the culture (29.5%) treated with LPS (Fig. 2D). The number of cells in later stage apoptosis or necrosis in the culture without microglia was 24.2% (Fig. 2E). No apoptotic or necrotic cells were detected in the culture without the peptide and microglia (Fig. 2F).

Aspirin significantly inhibited cell death induced by PrP106-126 (Fig. 2A, B). The number of neuro-2a cells in apoptosis and necrosis in the presence of 5 mM aspirin was about 3-fold lower than the culture without aspirin ($P<0.05$). However, increasing the aspirin concentration to 10 mM did not enhance the inhibitory activity; the number of apoptotic and necrotic neuro-2a cells treated with 10 mM aspirin was about 2-fold higher than that treated with 5 mM aspirin.

3.2. NF-κB activation by PrP106-126 in microglia cells

We investigated whether PrP106-126 stimulates the activation and translocation of transcription factors in microglia cells using the NF-κB activation and translocation assay. As shown in Fig. 3, PrP106-126 treatment induced NF-κB activation and nuclear translocation in microglia, indicated by binding to anti-NF-κB P65 antibodies and subsequently to FITC-conjugated goat anti-mouse IgG. NF-κB activation and translocation peaked after 20 min of incubation with PrP106-126. Similar activation and translocation of NF-κB were seen in LPS-treated culture, but not in the microglia treated with the Scr PrP.

3.3. Induction of mRNA expression of cytokines by PrP106-126 in microglia

To investigate the role of NF-κB activation in relation to cytokine gene transcription, inflammatory cytokine mRNA expression was determined by quantitative RT-PCR. Microglia

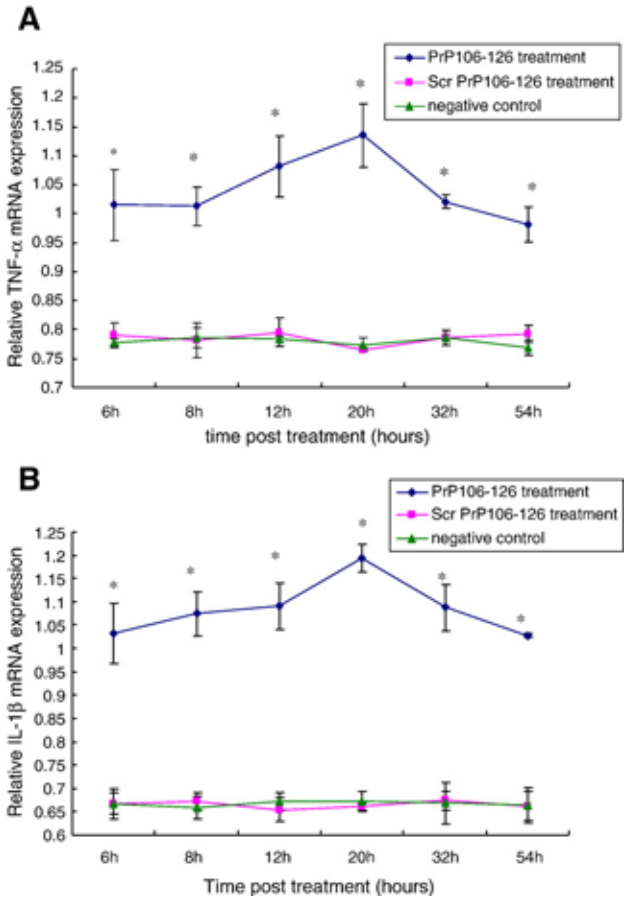

Fig. 5. The mRNA expression change of TNF-α (A) and IL-1β (B) in different time of treatment with PrP106-126 and Scr PrP106-126. Cultured microglia cells were treated with PrP106-126 (25 μM), and scrambled PrP (25 μM) for different periods ranging from 6, 8, 12, 20, 32, and 54 h, as indicated in the figure legends. Untreatment Untreated cells as negative control. *$P<0.05$ vs. negative control.

cells were treated with 25 μM PrP106-126 or Scr PrP for 6, 8, 12, 20, 32, and 54 h. IL-1β, TNF-α and β-actin genes were amplified and confirmed by gel electrophoresis and gene sequencing (Fig. 4). PrP peptide treatment up-regulated the expression of both inflammatory cytokines, IL-1β and TNF-α in a time-dependent manner (Fig. 5). PrP106-126 induced a significant increase in mRNA levels of TNF-α and IL-1β from 6 h, increasing steadily to a peak at 20 h before the expression declined slightly but it remained significantly higher than that of the negative control and Scr PrP-treated cells. TNF-α and IL-1β mRNA levels in PrP106-126-treated microglia were significantly higher than the negative control and Scr PrP106-126-treated microglia at all time points ($P<0.05$). The Scr PrP did not increase TNF-α and IL-1β mRNA expression.

4. Discussion

Microglia are normally quiescent, but when activated, they undergo proliferation, chemotaxis, and morphological alteration and produce numerous cytokines involved in inflammatory and immunomodulatory responses (Ghoshal et al., 2007). Several cytokines produced by activated microglia can damage neurons (Iacobelli et al., 1999; Peyrin et al., 1999), and activated microglia are thought to play a pivotal role in neurodegenerative diseases, such as Alzheimer's disease and Creutzfeldt–Jakob disease (Brown et al., 1996; Fabrizi et al., 2001; Iacobelli et al., 1999). It was reported that in mouse models of prion disease the onset of microglial activation coincided with the earliest changes in neuronal morphology, many weeks before neuronal loss and subsequent pathological symptoms of the disease (Dash and Moore, 1995; Giese et al., 1998; Shepard et al., 2001). Our study showed activation and translocation of NF-κB and up-regulation of mRNA expression of inflammatory cytokines, TNF-α and IL-1β in rat microglia by the fibrillogenic prion peptide fragment PrP106-126 *in vitro*, indicating microglia activation by the pathogenic peptide fragment. NF-κB activation and translocation peaked after 20 min of incubation with PrP106-126, while increased mRNA expression of the two cytokines was detected from 6 h to 54 h. The activation of NF-κB before the increase in cytokine gene expression suggests that the cytokine expression in rat microglia is probably regulated by activated NF-κB, consistent with the postulation that NF-κB is the regulating factor of TNF-α expression (Fabrizi et al., 2001).

Rao et al. (1995) have reported that the combination of TNF-α and IL-1β, but not either cytokine alone, induced degeneration of cortical neuron. Our data showed that the mRNA expression of TNF-α and IL-1β was up-regulated almost simultaneously, and these two cytokines may synergistically induce death of neuronal cells. Furthermore, our data showed a steady and long lasting increase in TNF-α and IL-1β mRNA expression, which remained significantly higher than in untreated control culture during the entire incubation period (54 h). Cytokine concentrations in the microglia culture incubated with PrP106-126 were not measured in our study. Given the significant and persistent increase in TNF-α and IL-1β gene expression, the concentrations of these cytokines in the PrP106-126-treated migroglia culture were most likely increased. Increased TNF-α levels were observed in human microglia culture treated with PrP106-126 *in vitro* (Fabrizi et al., 2001). It will be of interest to investigate whether other cytokines are also produced by microglia cells exposed to PrP106-126 and whether they contribute to neuron degeneration and death.

It has been reported that prion susceptibility varied between neuro-2a cell clones and between subclones (Uryu et al., 2007). We showed that PrP106-126, but not the scramble peptide, induced apoptosis/necrosis of neuro-2a cells *in vitro*, suggesting the neuro-2a cells used in our study were susceptible to prion infection. NF-κB is known to play a critical role in the regulation of cell survival genes and to coordinate the expression of proinflammatory enzymes and cytokines, such as iNOS, COX-2, IL-1β and TNF-α (Ashikawa et al., 2002; Schmedtje et al., 1997). Treatment of cells with proinflammatory cytokines such as TNF-α and IL-1β,or with bacterial products such as LPS, leads to the activation of a specific IκB kinase (IKK) complex that phosphorylates IκB and thereby tags it for ubiquitination and degradation by the proteasome. The degradation of IκB allows NF-κB to translocate into the nucleus where it can act as a transcription factor (Ghosh et al., 1998; Karin and Ben-Neriah, 2000). Studies demonstrate that the anti-inflammatory properties of aspirin are mediated in part by their specific inhibition of IKK-b, which the mechanism of inhibition is due to binding of these agents to IKK-b to reduce ATP binding, thereby preventing activation by NF-κB of genes involved in the pathogenesis of the inflammatory response (Yin et al., 1998). Our studies showed that aspirin inhibited neuronal toxicity of PrP106-126 in co-cultures of neuro-2a and microglia cells. There was a 3-fold decrease in neuronal cell death in PrP106-126-treated neuro-2a and microglia cultures with 5 mM aspirin compared to the cultures without aspirin. The protective effect of aspirin was decreased at higher concentrations, and concentrations greater than 10 mM was cytotoxic (data not shown). Studies have shown that aspirin blocks NF-κB activation in rat neuronal cultures (Forloni et al., 1993; Grilli et al., 1996). The inhibitory activity of aspirin against PrP106-126 induced neuro-2a cell apoptosis/necrosis co-cultured with rat microglia cells observed in our study was probably mediated by blockade of NF-κB activation in microglia. Inhibition of NF-κB activation would decrease the production of inflammatory cytokines by microglia, and thus reduction of cell apoptosis. It is understood that the inflammatory cytokine expression profile of microglia from CJD-affected brain are different from that of healthy brain (Baker and Manuelidis, 2003). Additional investigations will be necessary to understand how PrP^{Sc} aggregates may influence the expression levels of microglial and neuronal chemokines. It is significant to study the mechanisms of between activated microglia and neurotoxic of neuron cells by PrP106-126.

PrP106-126 induces microglia proliferation (Brown et al., 1996; Forloni et al., 1993). Because of this proliferative effect of the prion peptide on microglia, total cell numbers after PrP106-126 treatment would be higher in neuro-2a and microglia mixed cell cultures treated with PrP106-126 than those without

PrP106-126 treatment. Therefore, no comparison was made between mixed cell cultures co-incubated with PrP106-126 and that without PrP106-126. Co-culture of the neuronal cells with microglia enhanced the neurotoxicity of PrP106-126 probably due to increased cytokine release from activated microglia. It is likely that widespread neuronal degeneration also induces microglial activation, which in turn may further contribute to neurotoxicity.

No effective therapy is currently available for prion protein diseases. It is imperative to investigate inflammation in neurodegenerative progression and ultimately find signal transduction pathways that lead to the deleterious in the central nervous system as therapeutic standpoint. In a mouse model of spinal cord injury, inhibition of NF-κB in astroglial cells reduced inflammation and promoted functional recovery (van Loo et al., 2006). Our study suggest that suppression of microglia activation by inhibiting the NF-κB activation pathway may alleviate the progress of inflammation and neuronal degeneration in prion diseases. It is significant that the neuron degeneration mechanism were elucidated in transmissible spongiform encephalopathy.

Acknowledgments

This work was supported by the China 973 Program (Project no. 2005CB523000), National Key Technologies R&D Program, China (Project no. 2006BAD06A13) and the Natural Science Foundation of China (Project no. 30500371).

References

Ashikawa, K., Majumdar, S., Banerjee, S., Bharti, A.C., Shishodia, S., Aggarwal, B.B., 2002. Piceatannol inhibits TNF-induced NF-kappaB activation and NF-kappaB-mediated gene expression through suppression of IkappaBalpha kinase and p65 phosphorylation. J. Immunol. 169, 6490–6497.

Baeuerle, P.A., Henkel, T., 1994. Function and activation of NF-kappa B in the immune system. Annu. Rev. Immunol. 12, 141–179.

Baker, C.A., Manuelidis, L., 2003. Unique inflammatory RNA profiles of microglia in Creutzfeldt–Jakob disease. Proc. Natl. Acad. Sci. U. S. A. 100, 675–679.

Bales, K.R., Du, Y., Dodel, R.C., Yan, G.M., Hamilton-Byrd, E., Paul, S.M., 1998. The NF-kappaB/Rel family of proteins mediates Abeta-induced neurotoxicity and glial activation. Brain Res. Mol. Brain Res. 57, 63–72.

Bonifati, D.M., Kishore, U., 2007. Role of complement in neurodegeneration and neuroinflammation. Mol. Immunol. 44, 999–1010.

Brown, D.R., Schmidt, B., Kretzschmar, H.A., 1996. Role of microglia and host prion protein in neurotoxicity of a prion protein fragment. Nature 380, 345–347.

Ciesielski-Treska, J., Grant, N.J., Ulrich, G., Corrotte, M., Bailly, Y., Haeberle, A.M., Chasserot-Golaz, S., Bader, M.F., 2004. Fibrillar prion peptide (106–126) and scrapie prion protein hamper phagocytosis in microglia. Glia 46, 101–115.

Dash, P.K., Moore, A.N., 1995. Enhanced processing of APP induced by IL-1 beta can be reduced by indomethacin and nordihydroguaiaretic acid. Biochem. Biophys. Res. Commun. 208, 542–548.

Dheen, S.T., Kaur, C., Ling, E.A., 2007. Microglial activation and its implications in the brain diseases. Curr. Med. Chem. 14, 1189–1197.

Fabrizi, C., Silei, V., Menegazzi, M., Salmona, M., Bugiani, O., Tagliavini, F., Suzuki, H., Lauro, G.M., 2001. The stimulation of inducible nitric-oxide synthase by the prion protein fragment 106–126 in human microglia is tumor necrosis factor-alpha-dependent and involves p38 mitogen-activated protein kinase. J. Biol. Chem. 276, 25692–25696.

Forloni, G., Angeretti, N., Chiesa, R., Monzani, E., Salmona, M., Bugiani, O., Tagliavini, F., 1993. Neurotoxicity of a prion protein fragment. Nature 362, 543–546.

Garcao, P., Oliveira, C.R., Agostinho, P., 2006. Comparative study of microglia activation induced by amyloid-beta and prion peptides: role in neurodegeneration. J. Neurosci. Res. 84, 182–193.

Ghosh, S., May, M.J., Kopp, E.B., 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16, 225–260.

Ghoshal, A., Das, S., Ghosh, S., Mishra, M.K., Sharma, V., Koli, P., Sen, E., Basu, A., 2007. Proinflammatory mediators released by activated microglia induces neuronal death in Japanese encephalitis. Glia 55, 483–496.

Giese, A., Brown, D.R., Groschup, M.H., Feldmann, C., Haist, I., Kretzschmar, H.A., 1998. Role of microglia in neuronal cell death in prion disease. Brain Pathol. 8, 449–457.

Grilli, M., Pizzi, M., Memo, M., Spano, P., 1996. Neuroprotection by aspirin and sodium salicylate through blockade of NF-kappaB activation. Science 274, 1383–1385.

Higuchi, Y., Otsu, K., Nishida, K., Hirotani, S., Nakayama, H., Yamaguchi, O., Matsumura, Y., Ueno, H., Tada, M., Hori, M., 2002. Involvement of reactive oxygen species-mediated NF-kappa B activation in TNF-alpha-induced cardiomyocyte hypertrophy. J. Mol. Cell. Cardiol. 34, 233–240.

Iacobelli, M., Rohwer, F., Shanahan, P., Quiroz, J.A., McGuire, K.L., 1999. IL-2-mediated cell cycle progression and inhibition of apoptosis does not require NF-kappa B or activating protein-1 activation in primary human T cells. J. Immunol. 162, 3308–3315.

Karin, M., Ben-Neriah, Y., 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu. Rev. Immunol. 18, 621–663.

Kim, J.I., Ju, W.K., Choi, J.H., Choi, E., Carp, R.I., Wisniewski, H.M., Kim, Y.S., 1999. Expression of cytokine genes and increased nuclear factor-kappa B activity in the brains of scrapie-infected mice. Brain Res. Mol. Brain Res. 73, 17–27.

Konturek, P.C., Bazela, K., Kukharskyy, V., Bauer, M., Hahn, E.G., Schuppan, D., 2005. Helicobacter pylori upregulates prion protein expression in gastric mucosa: a possible link to prion disease. World J. Gastroenterol. 11, 7651–7656.

Liu, B., Hong, J.S., 2003. Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. J. Pharmacol. Exp. Ther. 304, 1–7.

Marella, M., Chabry, J., 2004. Neurons and astrocytes respond to prion infection by inducing microglia recruitment. J. Neurosci. 24, 620–627.

Muhleisen, H., Gehrmann, J., Meyermann, R., 1995. Reactive microglia in Creutzfeldt–Jakob disease. Neuropathol. Appl. Neurobiol. 21, 505–517.

Peyrin, J.M., Lasmezas, C.I., Haik, S., Tagliavini, F., Salmona, M., Williams, A., Richie, D., Deslys, J.P., Dormont, D., 1999. Microglial cells respond to amyloidogenic PrP peptide by the production of inflammatory cytokines. Neuroreport 10, 723–729.

Prusiner, S.B., 1998. Prions. Proc. Natl. Acad. Sci. U. S. A. 95, 13363–13383.

Rao, P., Hsu, K.C., Chao, M.V., 1995. Upregulation of NF-kappa B-dependent gene expression mediated by the p75 tumor necrosis factor receptor. J. Interferon Cytokine Res. 15, 171–177.

Safar, J., Roller, P.P., Gajdusek, D.C., Gibbs Jr., C.J., 1993. Conformational transitions, dissociation, and unfolding of scrapie amyloid (prion) protein. J. Biol. Chem. 268, 20276–20284.

Schmedtje Jr., J.F., Ji, Y.S., Liu, W.L., DuBois, R.N., Runge, M.S., 1997. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. J. Biol. Chem. 272, 601–608.

Shepard, L.W., Yang, M., Xie, P., Browning, D.D., Voyno-Yasenetskaya, T., Kozasa, T., Ye, R.D., 2001. Constitutive activation of NF-kappa B and secretion of interleukin-8 induced by the G protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus involve G alpha(13) and RhoA. J. Biol. Chem. 276, 45979–45987.

Streit, W.J., 2002. Microglia as neuroprotective, immunocompetent cells of the CNS. Glia 40, 133–139.

Uryu, M., Karino, A., Kamihara, Y., Horiuchi, M., 2007. Characterization of prion susceptibility in Neuro2a mouse neuroblastoma cell subclones. Microbiol. Immunol. 51, 661–669.

van Loo, G., De Lorenzi, R., Schmidt, H., Huth, M., Mildner, A., Schmidt-Supprian, M., Lassmann, H., Prinz, M.R., Pasparakis, M., 2006. Inhibition

of transcription factor NF-kappaB in the central nervous system ameliorates autoimmune encephalomyelitis in mice. Nat. Immunol. 7, 954–961.
Veerhuis, R., Boshuizen, R.S., Familian, A., 2005. Amyloid associated proteins in Alzheimer's and prion disease. Curr. Drug Targets, CNS Neurol. Disord. 4, 235–248.
Yao, J., Keri, J.E., Taffs, R.E., Colton, C.A., 1992. Characterization of interleukin-1 production by microglia in culture. Brain. Res. 591, 88–93.
Yin, M.J., Yamamoto, Y., Gaynor, R.B., 1998. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature 396, 77–80.