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In vitro effect of prion peptide PrP 106–126 on mouse macrophages: Possible role of macrophages in transport and proliferation for prion protein

X.M. Zhou, G.X. Xu, D.M. Zhao*

Animal Transmissible Spongiform Encephalopathies Laboratory, College of Veterinary Medicine, China Agricultural University, Beijing 100094, PR China

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Abstract

While there is a growing consensus on the understanding that the immune system plays an important role in facilitating the spread of prion infections from the periphery to the central nervous system, little is known about the key players in the first steps of the infection and about the sites of the disease development. Owing to their subepithelial location and their migratory capacity, macrophages could be early targets for prion transportation or propagation during the later stages of disease. In order to investigate the role of macrophages, we studied *in vitro* the effect of exposing primary peritoneal macrophages to a synthetic peptide homologous to residues 106–126 of the human prion protein (PrP), PrP 106–126. As shown by MTT assay, macrophage viability treated with less than 50 μM PrP 106–126 for 72 h was not inhibited but slightly stimulated at 10 and 25 μM, while there was significant decrease when exposed to 100 μM PrP 106–126 for 72 h. The expressions of PrP at mRNA and protein level were up-regulated following treatment with PrP 106–126 for 72 h. Cytokine TNF-α production were elevated by the PrP peptide in a time-dependent manner, which demonstrated a proinflammatory response linked to the presence and progression of prion disease took place in macrophages. These findings suggested that macrophages may play roles in the transportation and replication of the infectious agent.
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Keywords: Prion peptide 106–126; Primary peritoneal macrophages; TNF-α

1. Introduction

The transmissible spongiform encephalopathies (TSEs) or prion diseases are fatal neurodegenerative diseases that affect humans and both domestic and free-ranging animals. They are mostly due to oral infection with the infectious agent, which is thought to be a misfolded form (scrapie associated PrP (PrP^{Sc})) of a normal host protein (cellular isoform of PrP (PrP^{C})). Replication of PrP^{Sc} depends critically on the normal prion protein PrP^{C} in affected tissues such as the central nervous system (CNS) and lymphoid tissues [1]. However, how and when PrP^{Sc} are acquired in the course of natural infection is not known. After experimental intragastric or oral exposure of rodents with scrapie, infectivity and PrP^{Sc} accumulate first in Peyer's pathches, gut-associated lymphoid tissues (GALT) and ganglia of the enteric nervous system [2,3] long before their detection in the CNS. Early accumulation of PrP^{Sc} in GALT, including mesenteric nodes, has also been described in sheep with scrapie [4–6].

Soon after peripheral infection, follicular dendritic cells (FDCs) in the germinal centers of lymphoid organs or tissues are reported to be sites of PrP^{Sc} accumulation [7–9]. In mouse scrapie models, mature FDCs are critical for scrapie replication and PrP^{Sc} accumulation in lymphoid tissues, and in their absence neuroinvasion following peripheral challenge is significantly impaired [10–12].

The transport mechanisms by which TSE agents reach the germinal centers from the gut lumen are not known.

Abbreviations: TSEs: transmissible spongiform encephalopathies; PrP: prion protein; PrP^{Sc}: scrapie associated PrP; PrP^{C}: cellular isoform of PrP; CNS: central nervous system; GALT: gut-associated lymphoid tissues; FDCs: follicular dendritic cells; DCs: dendritic cells; NK-1R: neurokinin-1 receptor; CT: threshold cycle

*Corresponding author. Tel.: +86 10 62732980; fax: +86 10 62732975.
E-mail address: zhaodm@cau.edu.cn (D.M. Zhao).

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Microfold (M) cells dependent or M cells independent routes were suggested for transport of TSE agents across the intestinal epithelium. Once across the intestinal epithelium, current data suggest that the TSE agent might be acquired by migratory dendritic cells (DCs) and macrophages [13]. Macrophages of the lymphoid follicles have been shown to contain PrP^{Sc} at early stage of TSE infection and at terminal disease [3,14,15]. Accumulation was found in tingible body macrophages, a subset which seems to be specialized in phagocytic activity in the germinal centers [14]. Tingible-body macrophages within the B-cell follicles also acquire TSE agents [16]. Cells resembling macrophages were found to contain PrP^{Sc} in FDC-deficient mice infected with scrapie [11,17]. Thus, macrophages could provide the alternative sites of prion accumulation and replication in the absence of functional FDCs. Although the precise involvement of macrophages in TSE pathogenesis is uncertain, these data do not exclude the possibility that macrophages might have additional roles in pathogenesis, such as TSE agent transportation or propagation during the later stages of disease.

Many studies have been carried out using a peptide corresponding to residues 106–126 of the human PrP sequence (PrP 106–126). *In vitro*, it maintains most of the characteristics of PrP^{Sc}, including a strong tendency to aggregate into a β-sheet structure forming amyloid fibrils and a partial resistance to proteolysis. It has been reported that this peptide induced neuronal apoptotic death in primary cultures of various brain origins [18–22], as well as *in vivo* apoptotic-mediated mouse retinal cell death and altered electrical activity of the retina [23].

In the present report, we examined the effect of PrP 106–126 on peritoneal macrophages *in vitro*. To address this question, we administrated PrP 106–126 into primary peritoneal macrophages. Cell viability was demonstrated by means of MTT test. Prion protein (PrP) gene mRNA expression was detected by real-time PCR. The level of protein expression was confirmed by immunocytochemical technique. In order to understand whether there happened a proinflammatory response in macrophages after exposure to the prion peptide, induction of inflammatory cytokine production by PrP 106–126 was measured by ELISA.

2. Materials and methods

2.1. Primary culture of peritoneal macrophages

Adult female BALB/c mice at 11 weeks of age were supplied by the Center of Laboratorial Animals in Chinese Academy of Science, Beijing, China. The procedure for harvesting of resident peritoneal cells was carried out according to David and Gordon [24]. Resident peritoneal macrophages were purified by adhesion. The resident peritoneal cells were plated in macrophage culture medium at 3×10^5 cells per well in a 24-well tissue culture plate and incubated for 60 min at 37 °C. The nonadherent cells were removed by washing five times in 500 μl warm PBS with a gentle swirling action. After purification, the adherent cells should consist of a population of cells, more than 90% of which should be macrophages. Cells were cultured in 5% CO_2, at 37 °C.

2.2. Prion protion peptide

PrP peptides PrP 106–126 with sequence of KTNMKH-MAGAAAAGAVVGGLG was synthesized by Sangon Bio-Tech. The peptides were dissolved in deionized water at a concentration of 5 mM and stored at −20 °C as the stock solutions.

2.3. Peptide treatment

Peptide aggregation was performed with a 24 h period of agitation (300 vibration/min at room temperature) at 2 mM in PBS followed by a 24 h period of agitation at final concentration (ranging between 10 and 100 μM) in culture medium (RPMI1640 with 10% HI FCS containing 50IU of penicillin, 50 μg streptomycin, and 2 mM glutamine per milliliter). Then, microphages in primary culture were treated with the aggregated peptide PrP 106–126 in above medium. A scrambed prion peptide (scrPrP) was used as a negative control. At least three wells were used in each group of experimental conditions.

2.4. Cell survival assay

Survival of the cultured macrophages treated with different concentrations of PrP 106–126 for 72 h was determined using MTT assay, respectively. The assay was operated according to the instruction of a commercial MTT kit (MTT Cell Proliferation and Cytotoxicity Assay Kit, Beyotime, China). The absorbance was measured at 570 nm on a microplate reader (Bio-Rad 550, USA).

2.5. RNA extraction and detection of the expression of PrP and Neurokinin-1 receptor by real-time RT-PCR

After being treated with 25 μM PrP 106–126 for 72 h, the culture medium was discarded, and RNA-*Solv* reagent (Omega Bio- tek, Lilburn, GA) was added. The total RNA of macrophages was extracted according to the manufacturer's instruction. The RNA extracts were treated with RNase-free DNase I to remove DNA, and quantified on a spectrophotometer (BioPhotometerw Eppendorf; Germany) then stored at −80 °C.

The RNA from each sample was reverse transcribed with oligo dT to cDNA using Reverse Transcription System (Promega, Madison, USA). The expression level of PrP and NK-1R mRNA were quantified by real-time amplification of PrP and NK-1R gene, and endogenous housekeeping gene β-actin as control from the above prepared cDNA. The real-time PCR was carried out using DNA Engine OpticonTM 2 continuous fluorescence detection

system and Power SYBR Green PCR Master Mix (2 ×) kit (Applied Biosystems, Foster, CA, USA). Each PCR reaction contained 500 nM of each primer (Table 1), 2 μl cDNA, 10 μl Power SYBR Green PCR Master Mix (2 ×) in a final volume of 20 μl. After an initial denaturation for 10 min at 95 °C, the PCR reaction were subjected to 40 cycles of amplification consisting of denaturation at 95 °C for 15 s, followed with annealing step at 60 °C for 30 s and extension at 72 °C for 30 s. A final elongation step at 72 °C for 10 min concluded the PCR. To check the specificity of the amplified products, melting curve analysis was performed immediately following the completion of the PCR. Data were collected using PCR baseline deduction mode. After adjusting baseline cycles and calculating threshold value, the sample threshold cycle (CT) was obtained. All data were analyzed by software SPSS (Statistical Package for the Social Sciences, version 10.0 for Windows; SPSS Inc., Chicago, IL). An independent sample T-test was used to analyze differences in mRNA expression between different groups. Differences with $p < 0.05$ were considered to be statistically significant.

2.6. Immunocytochemical detection of PrP following PrP 106–126 treatment

After being treated with peptides for 72 h, cells were washed with 0.01 M PBS. Cells were then fixed in paraformaldehyde for 20 min. After three washes with PBS, cells were incubated with normal rabbit serum for 1 h at room temperature to block nonspecific binding. Then cells were incubated with monoclonal antibody 6H4 against PrP at 37 °C for 1 h. Cells were rinsed in PBS and incubated for 1 h with an anti-mouse FITC-conjugated antibody (1:2000, Sigma) at room temperature. Coverslips were put into culture wells and analyzed under an inverted fluorescence microscope (Olympus IX71, Japan). Negative controls including both the untreated cells and cells treated with scrPrP were fixed and stained in the same way. Three wells were used in each group of experimental conditions.

2.7. ELISA for TNF-α

Enzymed-linked immunosorbent assay (ELISA) kit (RnD, USA) for TNF-α was used. The assay was performed as instructed in the protocol provided by the manufacturer. In brief, 100 μl of supernatants was added to antibody-coated wells and incubated for 90 min at 37 °C. The plate was washed with the provided buffer solution and incubated with 100 μl of biotinylated antibody reagent for 1 h at 37 °C. The plate was washed again, treated with 100 μl of prepared streptavidin-horseradish peroxidase (HRP) solution, and incubated for 30 min at 37 °C. After an additional wash, 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate solution was added to each well, and color was allowed to develop at 37 °C for 10–15 min. The reaction was stopped by the addition of 100 μl of stop solution to each well. The plate was read on a microplate reader (Bio-Rad 550, USA).

3. Results

3.1. Effect of PrP 106–126 on peritoneal macrophages viability

To determine different concentration of PrP 106–126 on the primary peritoneal macrophages viability, cells proliferation and cytotoxicity were examined by MTT Assay after treatment with PrP 106–126 for 72 h. As demonstrated in Fig. 1, macrophages viability was significantly inhibited by PrP 106–126 at concentration of 100 μM ($P < 0.05$) compared with either other concentration treatment or scrable prion peptide (data not shown). Cells viability decreased to 88% after exposure to 100 μM PrP 106–126 for 72 h, while maintained to 99% after exposure to 50 μM PrP 106–126. It seemed that macrophages viability was slightly stimulated when they were treated with PrP 106–126 at 10 and 25 μM.

3.2. Effect of PrP 106–126 on mRNA expression of PrP gene and NK-1R gene

It was shown that the relative quantities of PrP transcripts in macrophages following treatment with 25 μM PrP 106–126 for 72 h was higher than the controls and ScrPrP treatment (Table 2), while NK-1R transcripts was lower than those. Although there was no significant difference between cells treated with PrP 106–126 and cells treated with ScrPrP or untreated cells, there appeared that PrP 106–126 up-regulated the genes expression of PrP after 3 days of treatment.

Table 1
Primers used for real-time RT-PCR

Genes	Primer sequences (5′–3′)	Nucleotide positions	PCR product size (bp)	Genbank accession no.
PrP	ggaccgctactaccgagaa tggtgactgtatgctgcttg	436–569	134	NM 011170
NK-1R	gaaatccacccgatacct gaccttcctctggctcat	991–1100	110	NM 009313
β-Actin	gtgggaatgggtcagaag ggtacttcagggtcaggata	214–288	75	NM 007393

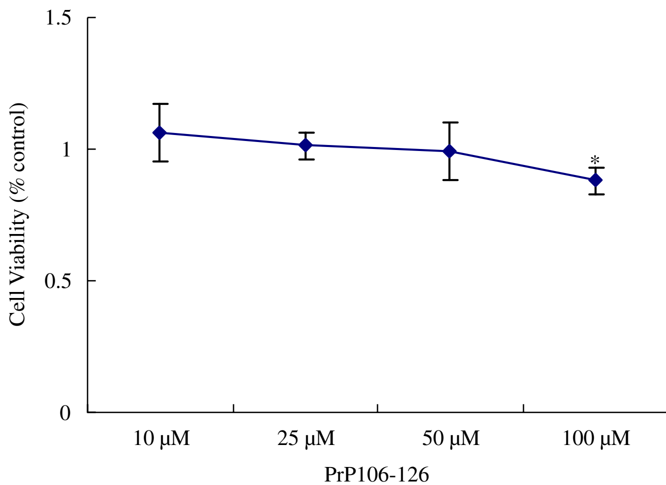

Fig. 1. Effect of different concentration of PrP 106–126 (from 10 to 100 μM) and scrPrP (25 μM) on viability of the cultured macrophages after 72 h treatment. Cells were kept in culture for 3 days and then treated with or without PrP 106–126 for 72 h. Cell viability was assessed by MTT. PrP 106–126 (100 μM) can significantly inhibited activity of macrophage after 72 h ($p<0.05$), while 50 μM or less than 50 μM PrP 106–126 cannot inhibit the viabilities of the macrophages after 72 h treatment. Data are expressed as mean percentages of the untreated controls ± SEM of triplicate samples measured in quadruplicate. *$P<0.05$ versus respective control values.

Table 2
Effect of PrP 106–126 on mRNA expression of PrP gene and NK-1R gene at 72 h

Gene	Group	Replicates	The relative CT levels[a]
PrP			
	Control	5	1.01 ± 0.03
	ScrPrP	5	1.02 ± 0.02
	PrP 106–126	5	0.97 ± 0.01
NK-1R			
	Control	5	0.87 ± 0.02
	ScrPrP	5	0.88 ± 0.03
	PrP 106–126	5	0.91 ± 0.04

[a]Mean ± SD.

3.3. Effect of PrP 106–126 on PrP expression in macrophages culture

Activation of PrP expression in macrophages culture was assessed by immunocytochemistry using monoclonal antibody 6H4 and FITC-conjugated anti-mouse antibody. PrP appeared in green. A basal level of PrP immunoreactivity was found in control cells (Fig. 2A). A 72 h exposure of primary peritoneal macrophages to 25 μM PrP 106–126 resulted in up-regulation of PrP expression (Fig. 2B). PrP immunoreactivity showed a distinct increase in macrophages culture, positive cells appeared greater than those untreated or treated with scrPrP (Fig. 2C). Ratios of prion positive macrophages to total number of macrophages under three scopes in each group were counted. Exposure to PrP 106–126 induced 62% macrophages expressed PrP, while they just reached 33% in untreated cells and 40% in cells treated with ScrPrP.

3.4. Induction of TNF-α production by PrP 106–126 in primary peritoneal macrophages

In order to further determine whether PrP 106–126 induced primary peritoneal macrophages to produce differential expression of inflammatory cytokine, TNF-α expression in treated and untreated cells or scrPrP-treated cells were demonstrated. We observed that macrophages treated with 25 μM PrP 106–126 showed elevated expression of TNF-α in a time-dependent manner as demonstrated by ELISA assays (Fig. 3). Cells treated with PrP 106–126 for 24 h expressed TNF-α at a 7-fold higher lever compared with untreated cells ($P<0.01$), but decayed as nearly same level as untreated cells by 72 h. The scrambled PrP peptide had no such stimulatory effect on TNF-α expression.

4. Discussion

The results obtained from this study showed that being exposed to PrP 106–126 less than 50 μM, macrophages viability was not inhibited. Macrophage viability was

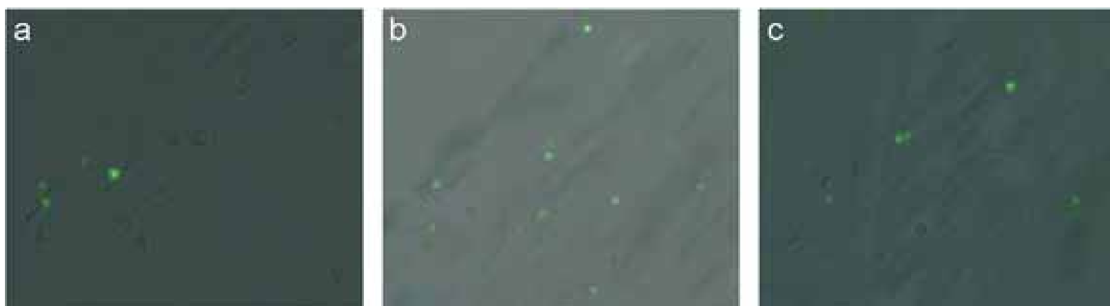

Fig. 2. Immunocytochemical detection of prion protein on primary peritoneal macrophages after 72 h of PrP 106–126 treatment: (A) negative control; (B) PrP 106–126 treatment (final concentration, 25 μM); (C) scrPrP (25 μM) treatment; prion protein in macrophages culture was detected with monoclonal antibody 6H4 and then immunostained with FITC-conjugated anti-mouse antibody. Prion protein appeared in slight green. Photos were taken at normal light and fluorescence at same scope, and were superposed. Exposure to PrP 106–126 induced activation of PrP expression.

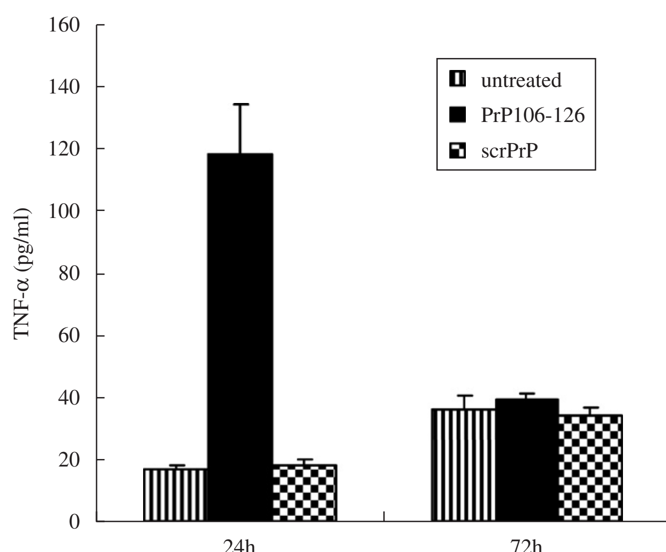

Fig. 3. Effect of PrP 106–126 on TNF-α protein production in peritoneal macrophages. Macrophages were incubated with PrP 106–126 (25 μM) for 24 and 72 h, respectively. TNF-α protein in the supernatants were then assayed using enzyme-linked immunosorbent assay (ELISA). The data shown are presented as the mean ± SD of quadruple cultures (**$P<0.01$).

significantly inhibited when treated with 100 μM PrP 106–126. This result suggests that high concentration PrP 106–126 was toxic to macrophages and caused some of them death. Macrophages were widely used as a model to study microglial activation by the amyloidogenic fragments of PrP [25]. Some studies showed that the poorly degraded aggregates PrP was capable of promoting macrophage survival [26]. Subsequent activation of the more numerous microglia and macrophages may contribute further to the pathological processes in prion disease or play an important role in the clearance of the neurotoxic agents.

The present study examined the effect of PrP 106–126 on endogenous PrP at both the transcript and the protein level. The level of PrP gene and protein expression was up-regulated in the cultured macrophages, which was consistent with previous observations [13]. However, while the protein expression became evident after the treatment for 3 days, effect of PrP 106–126 on the gene expression was not significant until 3 days after the treatment. The observation confirms the conclusions of previous reports that the effect of the PrP 106–126 depends on the cellular expression of PrP^{C} [27,28]. PrP 106–126 may promote the aggregation of cellular PrP and induced the synthesis of transmembrane PrP as demonstrated on neuron cells [29].

Kaneider et al. [30] reported that a specific G protein-coupled receptor (GPCR) neurokinin receptor 1 was identified as an important coreceptor with which PrP 106–126 interacts on DCs. Other *in vitro* studies have demonstrated GPCR formyl peptide receptor-like 1 (FPRL1) was identified as being necessary and sufficient for PrP 106–126-induced cell activation [31]. In this study, however, the expression of neurokinin receptor 1 at mRNA level has been shown to be down-regulated on primary peritoneal macrophages. Further studies were currently underway to identify the receptor complex and signaling pathway used by PrP 106–126 in macrophages.

In other studies indicate that nuclear factor κB (NF-κB) and mitogen-activated protein kinase-dependent pathways and proinflammatory cytokines they are capable of activating have been previously linked to the presence and progression of prion disease [32,33]. Our results showed that cytokine TNF-α expression was elevated in activated macrophages by PrP 106–126. The induction of cytokine TNF-α expression by PrP 106–126 activated macrophages is consistent with other reports in neurological disorders including TSE, Parkinson's disease and Alzheimer's disease. Up-regulation of TNF-α mRNA expression has been detected in the brains of mice experimentally infected with Creutzfeldt-Jacob disease or scrapie [34,35]. In addition, TNF-α mRNA expression was found to be elevated in human astrocytes and microglial cells when exposed *in vitro* to PrP 106–126 [33]. In human fetal microglial cells, it was estimated that PrP 106–126 stimulated the induction of TNF-α through the mitogen-activated protein kinase signaling pathway [33].

How macrophages might affect TSE agent accumulation is not know. In the absence of the TNFR1-signalling pathway, the RML scrapie isolate can accumulate to high levels within lymph-node macrophages [17]. In sheep, macrophages were thought to acquire PrP^{Sc} from FDCs by phagocytosis and proteolytically removed the N terminus [15]. Although macrophages are not capable of activate naïve T cells and not the most potent antigen-presenting cells compared with DCs, the capacity of macrophages to migrate to secondary lymphoid organs is not different to DCs. Macrophages may also have role in carrying prions from sites of exposure to germinal centers and then sites of neuroinvasion.

In summary, this study provides *in vitro* evidence of the role of mucosal-associated macrophages in the initial replication or transportation of TSE agents. PrP 106–126 at moderate concentration could sustain macrophages survival. Both PrP gene expression and protein expression were up-regulated in macrophage cultures following PrP 106–126 treatment. TNF-α production in macrophage cultures was elevated after PrP 106–126 treatment. The results may suggest that PrP expression was necessary for the effect of PrP 106–126 on macrophages. A proinflammatory response linked to the presence and progression of prion disease happened. Macrophages may play an additional role in the transportation or replacation of the infectious agent.

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