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Influence of PrP 106—126 on expression of laminin and fibronectin in astrocyte

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Astrogliosis is a hallmark of prion disease, but the metabolic alterations of astrocytes remain poorly documented. A synthetic peptide corresponding to amino acid 106—126 of the human prion protein (PrP) has been shown to be toxic to neurons. In this study, the effects of PrP 106—126 on astrocytes were investigated *in vitro*. The proliferation of astrocytes was significantly (P < 0.05) increased when grown in media conditioned with PrP 106—126 (80 μmol/L) from microglia. The expression of laminin (LN) and fibronectin (FN) was examined at both mRNA and protein levels. The results showed that exposure of astrocytes to PrP 106—126 enhanced the expression of LN and FN. The increase of FN in astrocyte cultures required cytokines previously released by activated microglia. This study reveals the expression of LN and FN affected by PrP106—126.

Prion peptide 106—126 (PrP 106—126), astrocyte, microglia, laminin, fibronectin

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are a group of fatal neurodegenerative diseases in humans and a variety of mammals[1]. The accumulation of disease-specific prion protein (PrP^{Sc}), a pathological isoform of a host-encoded prion protein (PrP), is believed to be the major mechanism of the disease pathogenesis. However, whether the accumulation of PrP^{Sc} gives rise to the profound neurodegeneration of mammalian tissues remains enigmatic.

Gliosis is a hallmark of prion disease that occurs early in the progression of the diseases[1−4]. A number of proteins, including glial fibrillary acid protein (GFAP), IL-1R, IL-6R and cell cycle proteins, have been found to be associated with the metabolic changes of gliosis[5−7]. Astrocytes are known to be accumulated in areas where neurons have been damaged, while laminin (LN) and fibronectin (FN), extracellular matrix proteins produced by astrocytes, can enhance neurite outgrowth and neuronal survival[8,9]. Despite a wealth of information on the expression of these molecules in astrocyte cultures[10,11], the expression level of LN and FN remains unknown.

Many studies have been carried out to investigate the neurotoxicity of PrP^{Sc} *in vitro* by using a peptide corresponding to residues 106—126 of the human PrP sequence (PrP 106—126)[12−17]. This peptide, defined as P106 through this text, mimics most of the characteristics of PrP^{Sc} including two prominent features: a strong tendency to aggregate into a β-sheet structure thus forming amyloid fibrils and a partial resistance to proteolysis[18]. This peptide is considered a valuable model to study the mechanisms of neuronal damage caused by PrP^{Sc}, especially its toxic effects on neurons[19−21].

A recent study has shown that the astrocyte proliferation and up-regulation of GFAP expression caused by

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P106 are dependent on co-culture with microglia[15]. In our research, the direct and indirect effects of P106 on astrocyte cultures were investigated under four treatments: with P106; conditioned medium from microglia with or without P106; and with scrambled P106 (a control). All media were added to astrocyte cultures for three days. The proliferation of astrocytes and the expression of LN and FN at mRNA and protein levels were measured.

1 Materials and methods

1.1 Reagents

Monoclonal anti-fibronectin and monoclonal anti-laminin were obtained from Sata (USA). Dulbecco's minimal essential medium, DMEM (Gibco, USA) was used throughout and the MTT kit (MTT Cell Proliferation and Cytotoxicity Assay kit) was from Beyotime, China.

1.2 Peptide synthesis

Peptides used in these experiments consisted of human prion protein peptide 106－126 with the sequence KTNMKHMAGAAAAGAVVGGLG (PrP 106－126), Mr = 1912.3 and PrP 106－126 scrambled control (Scr), which consisted of the same amino acids as PrP 106－126 but in a random order LVGAHAGKMGANTAKAGAMVG, Mr = 1912.3. Peptides were synthesized by Omega Bio-Tech (USA). The peptides were dissolved in deionized water at a concentration of 5 mmol/L and stored at −20℃.

1.3 Glial subtype isolation

Mixed glial cultures were prepared from dissociated cerebral cortices of newborn rats. Briefly, 4－5 cortices were digested in 0.05% trypsin (Sigma, USA) and plated in a 75 cm^2 culture flask in DMEM supplemented with 10% fetal calf serum (Sigma, USA) and 1% of an antibiotic solution, penicillin and streptomycin (Gibco, USA). Cultures were maintained at 37℃ with 5% CO_2 for 14 d or until they became confluent.

Astrocytes and microglia were isolated and characterized as described previously[15,19,22]. Briefly, some mixed glia cultures were treated with 5 mmol/L L-leucine methylester (Sigma), which was applied to cultures 24 h after plating. After 2 h, the medium was removed and fresh medium was supplied.

1.4 Conditioned medium preparation from microglia and the treatments of astrocyte

The medium was prepared as described above and added to purified microglia. A portion of the same stock medium was further treated with 80 μmol/L P106 and an aliquot was then added to microglia cells. An additional batch of medium was treated with Scr. After 48 h of microglia cell growth, the medium was centrifuged to remove debris and the supernatant was taken as the conditioned medium. The conditioned medium, defined as either MiCM or MiCM-P106 in this test, was used throughout.

Astrocyte cells were grown on all four treatment media for 3 d under standard conditions and then tested for cell proliferation and protein expression.

1.5 Cell survival assay

Survival and growth of the primary astrocyte cells were determined using the MTT assay according to the instructions of a commercial MTT package. The absorbance was measured at 570 nm on a microplate reader (Bio-Rad 550, USA).

1.6 Western blot analysis of LN and FN

For protein expression analysis, astrocyte cultures were scraped, centrifuged, resuspended and lysed in a lysis buffer as previously described[23]. Equal amount of cellular protein was loaded into each well of a polyacrylamide-SDS gel. After electrophoresis, proteins were transferred to nitrocellulose. The membranes were blocked with milk and were incubated with specific primary and secondary antibodies. Proteins were detected by enhanced chemiluminescence, and band images were recorded by exposure to Kodak film. Band intensity was quantified by thin layer scanner (AlphaImager™ 2200, USA) relative to the band intensity of β-actin, which was used as an internal control.

1.7 Total RNA isolation and RT-PCR

Total RNA from astrocytes was extracted with RNeasy kit (Qiagen,) and reverse transcription reaction was carried out using the Reverse Transcription System (Promega) according to the manufacture's instruction. The primers for β-actin, FN and LN were designed based on the published gene sequences: β-actin, 5′-TGCTGTCCCTGTATGCCTCTG-3′ and 5′-TTAATGTCACGCACGATTTCC-3′; LN, 5′-GGCTTCTATGACCTGAGTGC-3′ and 5′-GCCCAAGATTGGCTTCCTC-3′; FN,

5′-CCGTGGAGTAGTTGGTTAGT-3′ and 5′-TCAGG-GCTTGAGTAGGTCA-3′. Products were electrophoretically separated and visualized by ethidium bromide staining. The fluorescent intensity of each band was measured with AlphaImager™2200, and the expression of a specific mRNA was determined relative to the expression of β-actin, which was used as an internal control.

1.8 Statistical analysis

Data were analyzed by SPSS software (Statistical Package for the Social Sciences, version 10.0 for Windows; SPSS Inc., Chicago, IL, USA). An independent sample t-test was applied to analyze differences between groups.

2 Results

2.1 Astrocyte proliferation induced with P106 in the presence of MiCM

Relatively pure cultures of astrocytes and microglia isolated according to Brown et al.[19] were used in this study. By this method astrocyte and microglia cultures could be purified close to 100% (Figure 1).

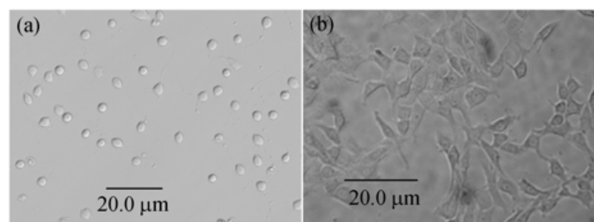

Figure 1 Morphology and purity of the primarily cultured microglia (a) and astrocytes (b).

To determine the effects of P106 on the viability of the primary astrocytes, the proliferation of cells was examined by MTT Assay. The numbers of astrocytes cultured on a medium with formerly grown microglia in the presence of P106 (MiCM-P106) significantly increased 22% ($P < 0.05$), compared to the growth on the Scr medium (Figure 2). The same treatment also resulted in a significant increase (19%, $P < 0.05$), in astrocytes growth compared to MiCM. The astrocytes growths were not affected by MiCM alone or P106 alone. However, MiCM-P106 induced astrocytic proliferation due to the released cytokines of active microglia caused by P106[23]. The results from this study and Hafiz et al.[6] both show that P106-induced proliferation of astrocyte is dependent on the factors.

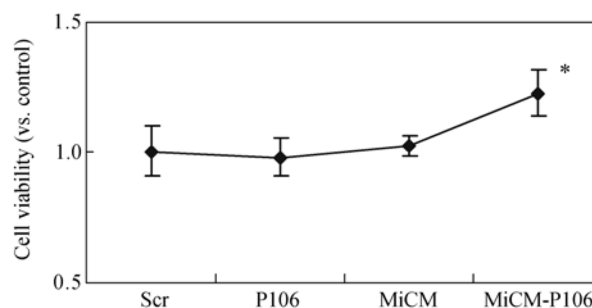

Figure 2 Effect on viability of the primary astrocyte cultures after treatment for 72 h. Cell viability was assessed by MTT. P106 treated microglia conditioned medium (MiCM-P106) significantly promoted the activity of primary astrocyte cultures ($P < 0.05$) compared with the control (Scr). However, P106 or microglia conditioned medium without treatment with P106 (MiCM) had no significant effects on the proliferation of astrocyte cultures. Data are expressed as mean percentages of the untreated controls ± SEM of triplicate samples measured in triplicate. *, $P < 0.05$ versus respective control values.

2.2 Effects of P106 on expression of LN and FN proteins

In addition, the expression of LN and FN at both mRNA and protein levels in astrocytes grown under the four treatments were compared using RT-PCR and Western blot analysis.

In cells grown on the P106 medium, expression of LN mRNA and LN protein increased 1.55 and 1.7 fold significantly ($P < 0.01$), respectively (Figures 3 and 4). In cells grown on the MiCM-P106 medium, expression of LN mRNA and LN protein increased 1.4 and 1.53 fold significantly ($P < 0.01$), respectively (Figures 3 and 4). These data reveal that P106 alone up-regulated LN expression at both mRNA and protein levels in astrocyte cultures. This suggests that up-regulation of LN expression is independent of microglia or the cytokines of microglia growth since there was no treatment effect of MiCM-P106 compared to P106.

Expression of FN mRNA and FN protein was also increased up to 1.6 and 1.5 folds ($P < 0.01$), respectively, but only in cells treated with MiCM-P106 (Figures 3 and 4). Treatment of P106 alone or MiCM alone did not change the levels of FN, suggesting that the increase of FN expression is dependent on the interacting factors of microglia and P106.

3 Discussion

This study shows that astrocyte proliferation is significantly increased after exposure to a medium which formerly grew microglia in the presence of P106. These

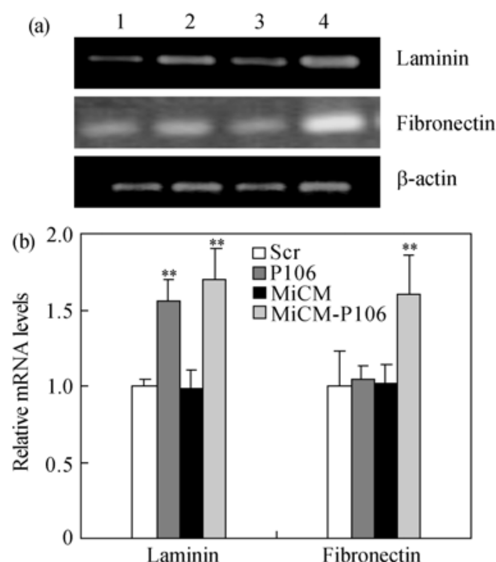

Figure 3 The effect of P106 on the mRNA expression levels of laminin and fibronectin in cells by RT-PCR. The fluorescent intensity of each band was measured with AlphaImager™2200, and the transcription levels of these mRNA were determined relative to that obtained for β-actin. (a) The mRNA expression levels. 1, Scr 80 μmol/L (control); 2, P106 80 μmol/L; 3, MiCM; 4, MiCM-P106. (b) The differences of mRNA expression levels in 4 groups. Data were compared to Scr 80 μmol/L, which was given a relative value of 1. Data are expressed as mean percentages of the controls ± SEM of triplicate samples measured in triplicate. **, $P < 0.01$ versus respective control values.

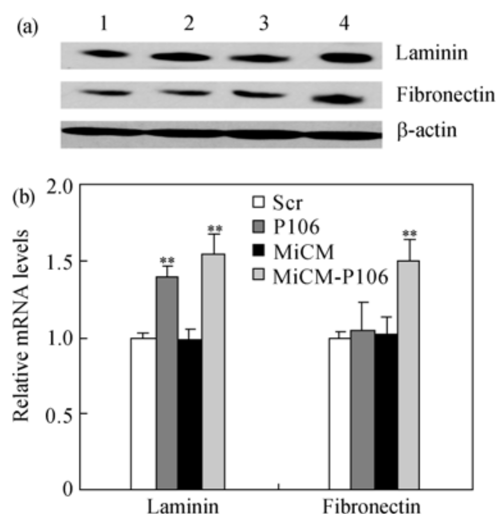

Figure 4 The effects of P106 on the protein expression levels of laminin and fibronectin in astrocyte cultures by Western blot. The fluorescent intensity of each band was measured with AlphaImager™2200, and the levels of these proteins were determined relative to that obtained for β-actin. (a) The protein expression levels. 1, Scr 80 μmol/L (control); 2, P106 80 μmol/L; 3, MiCM; 4, MiCM-P106. (b) The differences of protein expression levels in 4 groups. Data were compared to Scr 80 μmol/L, which was given a relative value of 1. Data are expressed as mean percentages of the controls ± SEM of triplicate samples measured in triplicate. **, $P < 0.01$ versus respective control values.

data agree with the results reported by Brown et al.[19], who also observed that the effect of P106 on astrocyte proliferation was dependent on the presence of microglia. The microglia may be directly activated by P106 which results in the release of various reactive oxygen species and cytokines into the medium[19,23,24]. These cytokines, directly react with the astrocytes and promote cell proliferation[15].

Metabolic changes in astrocytes may be the cellular responses to P106. Our results show that P106 alone can enhance the expression of LN, but an enhancement of FN in astrocyte cultures requires the factors released by activated microglia in addition to P106.

LN, an 800-kD heterotrimeric glycoprotein consisting of two short (α, β)and one long (γ) polypeptide chains, has been shown to mediate neuronal differentiation through its interaction with integrins which has been characterized by neurite formation and extension[25]. It is reported that prion protein (PrP^C) binds LN[26] and interacts with the 37-kD/67-kD LN receptor, which may participate in the internalization of 20%—50% of the membrane-bound cellular PrP^C[27]. The character that both PrP^C and LN interact with the same LN receptor domain site results in the fact that LN competes with PrP^C for the receptor binding site. It is well known that PrP^C levels in astrocytes are enhanced by P106[28,29]. This suggests that the up-regulation of LN is a characteristic competition response to PrP^C. The relationship between LN and PrP^C also explains the difference in the inducement of expression after addition of P106 and MiCM-P106. Since both LN and FN are extracellular matrix proteins produced by astrocytes, the up-regulation of LN by P106 is independent of the active microglia. The expression of LN has no direct relationship with the proliferation of astrocytes, as our results show that P106 does increase the expression of LN but does not increase the proliferation of astrocytes. However, our results that MiCM-P106 enhanced the expression of FN and the astrocyte proliferation suggests that FN may be involved in supporting cell division[9]. These findings are consistent with the results by Mahler et al.[10] who found that the astrocyte proliferation by basic fibroblast growth factor (bFGF) and transforming growth factor (TGF) are associated with the increase of FN.

In conclusion, this is the first report of increased expression of LN and FN in astrocytes under exposure to

P106 *in vitro*. The increase of FN in astrocytes was found to be dependent on the activation of microglia by P106. Further investigation of the LN and FN expression in response to P106 *in vivo* will be necessary.

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