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Studies on the Mechanism of Antagonistic Effect of Human Immunoglobulin on the Toxicity of Amyloid ß-Proteins-Treated Murine Nerve Cells

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Abstract: The antagonistic effects of human immunoglobulin on the toxicity of amyloid ß-proteins (Aβ)-treated nerve cells and the mechanism of such effects. The brain mantle cells of embryonic Kunming mice were primarily cultured through the treatments of 20μM Aβ and human immunoglobulin with concentrations of 5, 10, and15mM, respectively. The cell survival rates were analyzed with the application of MTT method and PI-Hoechst stain method, and the cleavage of P35 and activity of GSK-3β were detected by Western blotting. The results showed that human immunoglobulin effectively antagonized the toxicity of Aβ-treatment, improved the survival rates of such cells by 22%, prevented the cleavage of P35 to P25 and lowered the activity of GSK-3β. Therefore, it can be concluded that immunoglobulin exerts certain antagonistic effects on Aβ-treated nerve cells, and provide protection to brain nerve cells. The antagonistic mechanism is considered to prevent the cleavage of P35 to P25 and lower the activity of GSK-3β, thus inhibiting the collapse of cytoskeletons of nerve cells.

Key words: Human immunoglobulin, amyloid β-protein, brain nerve cells, antagonism.

INTRODUCTION

The neurodegenerative diseases of central nerve system, such as Alzheimer disease (AD), and Parkinson's syndrome have increased tremendously in the advanced countries. Till now, no effective medical treatment is available. One of the characteristics of AD is that large amounts of amyloid β-proteins (Aβ) are deposited in the intercellular gaps of nerve cells which interfere with the functions of these cells and disrupt the signal transductions in nervous system, leading to cell death (Gotz *et al.*, 2001). Another apparent symptom of AD occurs when the nerve cells contain large quantities of neurofibrillar tangles, which basically consist of collapsed cytoskeletal microtubules because of over-phosphorylation of tau protein (Peng and Wang, 2004). It was shown in several laboratories that Aβ represents a common pathway for induction of AD (Mason *et al.*, 1996). If a certain kind of substance can disrupt Aβ creation among nerve cells or can eliminate the formed Aβ, such substance will then inhibit AD occurrence or alleviate AD symptoms. It was reported in Daily Newspaper of Science and Technology issued in April 2005 that US researchers have successfully inhibited AD by immunoglobulin (Ig), which provides a kind of protein created by antigen-activated B lymphocytes.

It has been reported from different laboratories that Ig played protective role in brain nerve cells (An *et al.*, 2005; Arumugam *et al.*, 2007; Hu *et al.*, 2000; Xu *et al.*, 2003). Zhu *et al.* (2004) studied Ig changes in AD patients from various altitudes. Mark *et al.* (1998) reported that the neurodegenerative diseases such as AD were associated with decreased immune functions. In order to further clarify the functions of Ig during the process of AD, human immunoglobulin (HIg) and Aβ were collectively applied to treat the nerve cells cultivated *in vitro*. Through morphological observation, MTT activity detection, and fluorescent double-stain method, the antagonistic effects of HIg on the nervous toxicity of Aβ was testified. The mechanism of such effects was analyzed through western blotting so as to provide a novel idea and theoretical reference for the treatment of neurodegenerative diseases in central nervous system.

MATERIALS AND METHODS

Materials

Kunming mice and newborn bovine serum were purchased from Zhengzhou Institute of

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Experimental Animals and Hangzhou Sijiqing Biological Engineering Material Co. Ltd., respectively. MEM medium, anti-GSK-3β antibody, anti-P35 (C-19) antibody and horseradish peroxidase labeled goat anti-mouse polyclonal antibody were all ordered from Santa Company, and MTT, Aβ (25-35), PI and Hoechst-33342 were ordered from Sigma Company. Human Ig preparations (whose main gradient is IgG) for intravenous use were purchased from Tangshan Huakai Biological Technology Co. Ltd.

Primary culture of the cerebral cortex cells of embryonic mice

Five embryonic mice were extracted from a two-week-old pregnant Kunming mouse and then were immersed into D-Hanks solution. The brains of these mice were taken out through dissection and the finally-acquired hemispheres of mice brains were shredded. The shredded brains were treated in water bath at 37°C for 5-min digestion with the addition of 0.25% trypsin. The brain cells were slightly blown and dispersed with the help of droppers. The dispersed cells were centrifuged for 3 min at a speed of 800r/min. The obtained suspended cells were cultivated in MEM medium with 10% FBS, and were filtered using membranes with pore size of 10μm. The filtered cells were cultured in culture bottles after being diluted by MEM. After a 5%CO_2 incubator-cultivation at 37°C for 4h, the cells were further cultivated into NM-2 medium (which consists of 70% serum-containing MEM and 30% NB).

Determination of antagonistic effects of human immunoglobulin (HIg) on Aβ

Treatment of brain cells with Aβ

After being primarily cultured for 6d, the cerebral cortex cells of embryonic mice were collected, rinsed twice with serum-free medium, diluted with NM-2 medium, and evenly distributed in six Petri dishes, a cover-slip was added to each dish, and the cells were incubated at 37°C for 24 h in 5%CO_2 incubators.

The remaining brain cells in the culture bottle were evenly distributed in five 24-well plates. To one culture plate was added Aβ (25^{th} and 35^{th} amino acids) with a final concentration of 20μM/well. The second culture plate served as control with no Aβ added. Then the cells were incubated at 37°C in 5% CO_2 incubator for 24h, before they were observed and photographed. Each treatment was replicated 24 times.

Co-treatments with Aβ and HIg

Blood immunoglobulin preparation (with a mass concentration of 5%) extracted from healthy people was diluted to 5mM, 10mM and 15mM. and the diluted solutions were added into 3 above remaining culture plates containing cerebral cortex cells of embryonic mice, and incubated for 14h. Aβ with a final concentration of 20μM was then added in the culture cells for one hour at room temperature and then incubated in 5% CO_2 incubator for 24h at 37°C. The control group was also treated the same way.

Analysis of survival rates of cells using MTT method

According to a documented method (An *et al.*, 2005), the above Aβ-treated cerebral cortex cells were washed twice with buffer solutions. Then, 450μl buffer solutions and 50μl of 0.5% 3-(4,5-Dimethylthiazol-2-yl) -2,5- diphenyltetrazolium bromide (MTT) were added into every culture hole. After the addition of MTT to a final concentration of 0.5g/L/hole, the cells were cultivated at 37°C for 1h and part of the solution in every hole was sucked. After the addition of 150μL DMSO, the values of absorbance at 495nm were determined by Microplate Reader. The determined results were calculated to relative survival rates of cells.

The final results was recorded as relative survival rates of cells (%), and the survival rate of control group (CK) was set as 100%.

All the data were analyzed using Excel.

Analysis of the survival rates of stained cells using propidium iodide (PI)-Hoechst

Six cover slips containing cultured cerebral cortex nerve cells of embryonic mice were divided into two groups. One group of these slips was treated with 20μM Aβ and the other group of cells was treated by combined treatments with 20μM Aβ followed by treatment with 5, 10 and 15mM HIg, respectively. After 24h treatments, PI and Hoechst

were added on these slips with final concentrations of 2μM and 0.2 mM, respectively. The cells were incubated at 37°C for 15min. Then the treated cells were taken out, rinsed once using PBS, fixed with paraformaldehyde, and section-sealed by 50% glycerin for ultraviolet-excited observation and photograph.

Determination of antagonistic effect of HIg on Aβ

Determination of P35 cleavage using western blotting

The cells treated with 20μM Aβ, 20μM Aβ + 10mM HIg and control cells were collected, rinsed twice with PBS and then cleaved with 100μL SDS sample buffer. The cleaved cells were boiled for 10min at 100°C, and then centrifuged at 12000rpm (13314×g) for 10min. The supernatants were run on 10% polyacrylamide gel and then transferred to nitrocellulose membranes. The cell samples on the membranes were rinsed by TBS for 10min and soaked in TBS containing 3% skimmed milk powder at 37°C for 2h. The anti-P^{35} antibodies (C-19) of mice were used as primary antibodies which were incubated at 37°C for 1h and rinsed four times for 5min with TBST. The horseradish peroxidase labeled goat anti-mouse IgGs were added secondary antibodies, which were incubated at 37°C for 30min and rinsed four times, 5min each, with TBST. Then, the substrates were added for coloration.

Determination of GSK-3β activity using western blotting

The cells treated with 20μM Aβ, and those treated with 20μM Aβ and 10mM Hig were collected according, lysed, electrophoresed and western blotted as described above. The anti-GSK-3β mouse antibodies were applied as primary antibodies, the horseradish peroxidase labeled goat anti-mouse IgGs were applied as secondary antibodies, and the activity of GSK-3β was determined.

RESULTS

The toxicity of Aβ on nerve cells

After treatment with 20μM Aβ, the cerebral cortex nerve cells of embryonic mice were cultured for 24h. Figure 1 shows the comparison of Aß-treated cells with the untreated cells, Aβ-treated cells exhibited more apparent shrinkages, which indicates that damages were caused in cultured nerve cells by 20μM Aβ treatment.

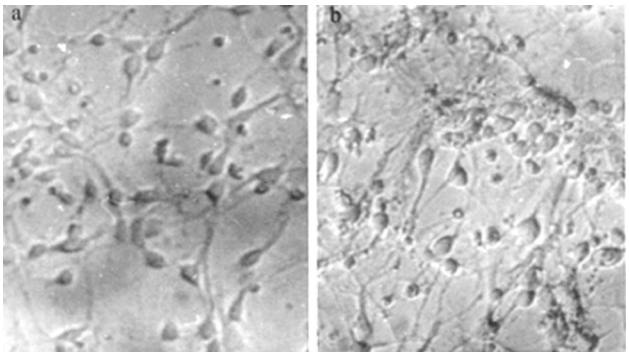

Fig. 1. The primary culture of the cerebral cortex nerve cells in embryonic mice. A, untreated cells; B, Aβ-treated cells (treated for 24h).

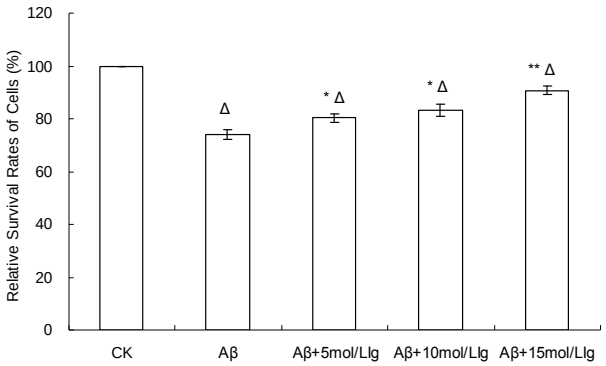

Fig. 2. The relative survival rates of nerve cells as determined by MTT method. Note Δ means significant difference at 0.01 levels between treatments and CK, while ** means significant difference at 0.01 levels between Ig treatments and CK, and * means significant difference at 0.05 levels between Ig treatments and CK.

Survival rates of nerve cells

Figure 2 shows results of MTT detection which represent relative survival rates. It can be seen that the relative survival rates of the nerve cells treated with 20μM Aβ were 30% lower than that of control group (with extremely significant differences ($P<0.01$)), indicating that Aβ exerted

harmful effects on nerve cells, which led to the death of neurons. Meanwhile, comparing with Aβ-treated cells, the HIg and Aβ collectively-treated cells manifested improved relative survival rates, and the extent of such improvements was proportional to the concentration of HIg. The survival rates of cells improved more significantly under the additions of 5mM and 10mM than under mere Aβ-treatments ($P<0.05$), and the survival rates of cells improved more extremely significantly under the additions of 15mM than under mere Aβ-treatments ($P<0.01$), suggesting that Hig can exert antagonistic effects on Aβ and certain protective effects on nerve cells, and there exists a positive correlation between such effects and HIg concentration.

PI-Hoechst stain method

Propidium iodide (PI) is a kind of dye for DNA staining, which marks dead cells and generates red fluorescent light under the excitation of ultraviolet lights. Hoechst 33342 is another DNA-staining dye, which can pass through the plasma membranes of the live cells and thus can be used for staining live cells. Under the excitation of ultraviolet lights, Hoechst 33342 generates blue white fluorescent light. The Aβ and 10mM HIg co-treated cells were double-stained by PI-Hoechst. The experiments were repeated 3 times with consistent results (Fig. 3). It can be seen that after Aß+10 mMHIg treatment the number of dead cells was more than the live cells, showing that Aβ led to massive death of nerve cells. In Figure 3B which shows Aß-treatment, there are more living cells compared to dead cells. This shows that Aβ and 10mM HIg co-treatment improved the survival rates of nerve cells and HIg exerted an inhibitive effect on the toxicity of brain nerve cells, which was in accordance with the experimental results obtained from MTT method.

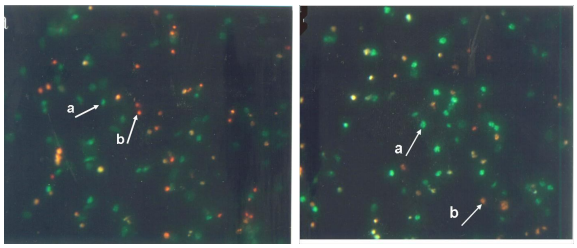

Fig. 3. PI-Hoechst33342 staining of murine nerve cells treated with Aβ+10mMHIg (A) and Aβ (B). Arrow a points to a living cell stained in green, and arrow `b' refers to a dead cell stained in orange.

P35 cleavage

Cyclin-dependent protein kinase 5 (CDK5) and its upstream protein P35 are essential for the normal growth of nerve cells (Xu *et al.*, 2003). In the cerebral neurons of such neurodegenerative diseases in central nerve system as Alzheimer disease (AD), P35 are decomposed to P25 by protease, which results in the over-activation and abnormal distribution of CDK5, thus bringing about the collapse of cytoskeleton and inducing the death of cortex cells (Arumugam *et al.*, 2007; Hu *et al.*, 2000). The cleavage of P35 to P25 stems from Aβ toxicity, and can be transmitted into neurons through a series of signal transduction pathways. The results of western blotting (Fig. 4) of P35 cleavage showed that two bands namely, P35 and P25 (obvious), were formed in Aβ-treated cells, while two bands were created in Aβ and HIg co-treated cells and P25 band significantly weakened, indicating that the cleavage of P35 to P25 was induced by Aβ but inhibited by Ig and HIg holds the antagonistic effects on Aβ-caused cell damages.

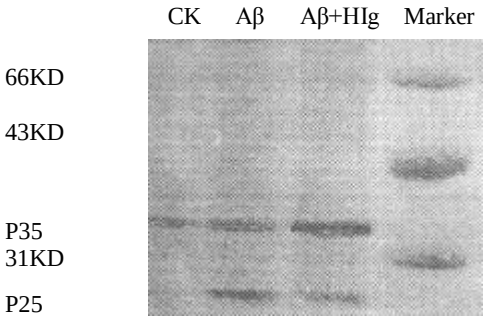

Fig. 4. P35 cleavage in Aß-treated Aß-HIg-treated murine nerve cells. The results of Western blotting showed that HIg inhibited the cleavage of P35 to P25, and CK refers to cells with no Aβ and HIg addition.

GSK-3β activity

Figure 5 shows GSK-3βs in Aβ and HIg co-treated cells held higher mobility and shallower

colorations than those of GSK-3βs in Aβ-treated cells, indicating that HIgs inhibited the phosphorylation of GSK-3βs, thus lowering their activities.

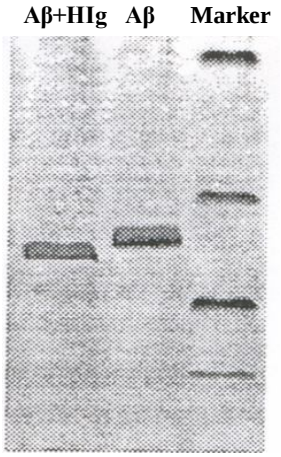

Fig. 5. GSK-3β activity in Aß and Aß+HIg treated murine nerve cells. Here Aβ-treated cells were taken as control (CK) for Aβ and HIg co-treated cells.

DISCUSSION

Among the mechanisms of many neurodegenerative diseases, Aβ deposition provides a major factor in causing the damage or death of nerve cells (Zhu *et al.*, 2004). Currently, the seeking of Aβ's antagonists has become one of the most effective approaches for the prevention and treatment of neurological diseases. Currently, Kunming mice represent the most widely applied experimental animal in China. Kunming mice used in this study was a kind of Swiss mice introduced from India, and such mice hold a genotype close to that of human being, strong resistance to diseases, and good adaptability, which make them suitable for pharmacological, toxicological and immunological studies. The toxicity of Aβ was tested through the addition of certain amounts of Aβ into the cultures of embryonic Kunming mice cerebral cortex cells. According to the collective treatments of HIg and Aβ to cultured cells, it was found that HIgs exerted certain antagonistic effects on Aβ-caused toxicity among nerve cells. In the presence of HIgs, the toxicity of Aβ in the *in vitro* cultured cerebral cortex nerve cells of embryonic Kunming mice lowered and the survival rates of cells improved by 22%. The double-stain fluorescent method also demonstrated that HIgs held antagonistic effects on Aβ-caused toxicity among nerve cells

Through the exploration of the mechanism of HIgs' antagonistic effects on Aβ-caused toxicity among nerve cells, it was found that HIg inhibited the cleavage of P35 to P25, prevented the over-activation of CDK5, and lowered the over-phosphorylation of tau proteins in the cytoskeletons of cerebral cells, thus avoiding the damages in cerebral nerve cells. Through the western blotting experiments for GSK-3β activity determination, it was found that HIg lowered the activity of GSK-3β. However, GSK-3β is an upstream protease of protein tau which induces cytoskeletons of neurons (Mark *et al.*, 1998), and the over-activation of GSK-3β can induce the over-phosphorylation of tau, thus enabling the formation of tangles due to the disorder in the neuron cytoskeletons. Therefore, it can be concluded that GSK-3β may act as a downstream protein of CDK5 in the signaling pathways for AD occurrence, and the association of HIg with Aβ inhibits the cleavage of P35 to P25 and disrupts the activities of both CDK5 and GSK-3β, thus decreasing the occurrence of neurofibrillar tangles and the death rates of nerve cells and improving the survival rates of cells.

The mechanism of Aβ's damage to nerve cells is rather complicated. The direct toxicity of Aβ begins with its combination with cellular membrane receptors, among which, PAGE receptor represents one of the super families of protein molecules locating on cellular surfaces. The combination of Aβ and PAGE induces the formation of a kind of reactive oxygen intermediate (ROI) on the membranes of nerve cells (Zheng, 2006). ROI brings about abnormal pathways for signal transduction in nerve cells and causes cell death. Among the pathways with abnormal signal transductions of nerve cells, the cleavage of P35 to P25 is of vital importance for the "fatal" changes in cells. The cellular distribution of P35 differs from that of P25. P35 is primarily located on cellular membranes while P25 is more frequently distributed in cytoplasm. The formation of P25 leads to the further over-phosphorylation of tau proteins and the bankruptcy of cytoskeletons.

Since Alzheimer disease (AD) was first discovered by German psychiatrist and neuropathologist Alois Alzheimer in 1906, the exploration for the medical treatments of this disease has been continuously going on for over a century (Green *et al.*, 2000). Studies on the causes of AD have always been the hot issues in neuronal cell biology. A variety of hypotheses have been proposed for explaining the causes of AD, such as cholinergic hypothesis, amyloid β-proteins hypothesis, and microtubule-associated protein tau hypothesis etc. (Terry and Bueeafusco, 2003; Li *et al.*, 2007; Hardy and Allsop, 1991). Cholinergic hypothesis has become a deadwood due to the inefficient clinic effects of its agent. Albeit the vaccines of amyloid β-proteins (Aβ) have been in the early stage of pilot clinic tests, unfortunately, they have little assistance in the improvement of the recognition abilities among AD patients (Jack *et al.*, 2004; Schenk *et al.*, 1999). The microtubule-associated protein tau hypothesis holds that the phosphorylated tau proteins can provide the function of maintaining the stability of microtubule skeletons, whilst the over-phosphorylated tau proteins can associate with other tau protein fibers through pairing. Such excess associations can finally lead to the formation of neurofibrillar tangles and further cause the disintegration of microtubule skeleton systems in nerve cells, which not only affects normal signal transduction among nerve cells, but certainly causes the final death of nerve cells.

In 2009, the mechanism for the occurrence of AD has witnessed new advances. It was found that a kind of mad-cow disease-related cellular prion protein (PrPc) can serve as a receptor for Aβ oligomers and mediate Aβ-oligomer-caused prominent disorders. Therefore, it was proposed that the interference of PrPc functions can be seen as a potential approach for AD treatment (Hong, 2003). It was also found that the N-terminus of APP (the precursor of Aβ), namely N-APP can associate with a kind of nerve cell receptor called death receptor 6 (DR6, also named TNFRSF21), which expresses at high levels in relatively significant AD-affected brain regions (Guan, 2003). Therefore, N-APP/DR6 signal transduction pathways may play important parts in the occurrence and progression of AD.

For over a century, human beings have made painstaking explorations for understanding the mechanism of AD. However, this issue is still less known to us with so many unresolved problems left till now, such as how Aβ hypothesis and tau hypothesis interrelate with each other. This research preliminarily demonstrated the relationship between Aβ hypothesis and tau hypothesis, while the signal transduction pathways between both hypotheses still need to be further studied and discussed in future.

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