Journal of Zhejiang University SCIENCE
ISSN 1009-3095
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

JZUS

Investigation on apoptosis of neuronal cells induced by Amyloid beta-Protein*

LUO Ben-yan (罗本燕)[†1], XU Zeng-bin (徐增斌)[2],
CHEN Zhi (陈 智)[3], CHEN Feng (陈 峰)[3], TANG Min (唐 敏)[1]

([1]*Department of Neurology;* [2]*Department of Radiology;* [3]*Institute of Infectious Diseases,*
First Affiliated Hospital, Medical College, Zhejiang University, Hangzhou 310003, China)

[†]E-mail: Lobenyan@263.net

Received Feb. 25, 2004; revision accepted May 19, 2004

Abstract: Objective: To construct a PC12 cell strain with neuronal differentiation, and observe the apoptosis and proliferation activity effects induced these cells by Amyloid beta-Protein (Aβ-43). Methods: 1) PC12 cells in logarithmic growth phase were subcultured for 24 h. After the culture fluid was changed, the cells were treated with Rat-β-NGF and cultured for 9 days. 2) Neuronal differentiation of PC12 cells in logarithmic growth phase were divided into four groups: control group (0), experimental group (1), experimental group (2) and experimental group (3). The concentrations of Aβ in the four groups were 0 μmol/L, 1.25 μmol/L, 2.5 μmol/L and 5 μmol/L, respectively. The cells were harvested at 24, 48 and 72 h later and stained with AnnexinV-FITC/PI after centrifugation and washing. Then flow cytometry was conducted to examine the apoptosis percentage. 3) NGF-induced PC12 cells were selected and Aβ with different concentrations was added. The final concentrations of Aβ were 0 μmol/L, 1.25 μmol/L, 2.5 μmol/L and 5 μmol/L, respectively. After the cells were incubated in an atmosphere of 5% CO_2 at 37 °C in an incubator for 72 h, the OD values were examined. Results: 1) Neuronal differentiated PC12 cell lines were successfully established. 2) Flow cytometric examination indicated that Aβ (1.25, 2.5, and 5.0 μmol/L) could effectively induce apoptosis of neuronal-differented cells at the 24 h, 48 h and 72 h time points. 3) Aβ (0−5.00 μmol/L) had no obvious effect on proliferation or restraining of the neuronal differentiation of the PC12 cells after a 72 h interacting process. Conclusion: This investigation revealed successful neuronal differentiation of the PC12 cell strain. The induction of apoptosis of the neurocytes by various concentrations of Aβ was observed and the influence of Aβ on induced proliferation of PC12 cells by Rat-β-NGF was revealed. This study may provide basis for future research on the molecular cure of AD and interdiction of AD evolution.

Key words: Alzheimer's Disease, Amyloid beta-Protein, Nneurocytes, Apoptosis
Document code: A **CLC number:** R743

INTRODUCTION

Senile dementia of the Alzheimer type called in short Alzheimer's Disease (AD), is the most common type of senile dementia. The pathological characters of AD are neuron loss, accumulation of senile lipofuscin pigment (SP) caused by the extracellular deposition of Amyloid beta-Protein (Aβ) and neurofibrillary tangles (NFT) caused by accumulation of excessively intracellular phosphorylated tau protein. It was found that the level of Aβ, which is the key component of the senile plaque, is evidently related with the stage of AD. One basic toxicity function of Aβ is the apoptosis of neurons. Thus, apoptosis is an important pathology

* Project (No.2003B054) supported by the Public Health Bureau of Zhejiang Province, China

mechanism of AD, and intervention of the apoptosis is a possible new method for curing AD. This study aims at establishing a neurocyte model and observe the effect of Aβ on the apoptosis of the neurocytes in order to provide a basis for future research on AD.

MATERIALS AND METHODS

Materials

1. Cell line: Pheochromocytoma cell strain PC12 cells (rat), were bought from the Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Science, Chinese Academy of Sciences.

2. Cell culture reagents and materials

(1) DMEM high sugar culture medium: Gibco;

(2) Fresh fetal calf serum: Hyclone;

(3) 0.25% trypsin: 0.25 g trypsin was dissolved in 100 ml PBS and sterilized by filtration;

(4) Phosphate buffer saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 were dissolved in 800 ml water. The pH was adjusted to 7.4 with hydrochloric acid (HCl); water was added until the volume reached 1000 ml and then the solution was sterilized for 20 min at high pressure (1.034×10^5 Pa). The solution was stored at room temperature;

(5) Rat-β-NGF, bought from American R&D Co. Ltd;

(6) 24-well and 96-well cell culture plates, one-off cell culture flask;

3. Annexin V-FITC Apoptosis Detection Kit I, bought from American BD Co. Ltd;

4. MTT: (3-[4.5-Dimethylthiazol-2-yl]-2.5-diph-enyltetrazollium bromide). Imported and loaded;

5. DMSO (Dimethyl sulphoxide), bought from American Sigma Company.

Experimental methods

1. Construction of apoptosis model of nerve metrocyte

Establishment of neuronal differentiation PC12 cell strain

(1) PC12 cell culture

Pheochromocytoma cells were seeded in DMEM containing 10% fetal calf serum (FCS) (with 100 μg/ml penicillin, 100 μg/ml streptomycin, 100 L-Glut (2 mmol/L), pH 7.4) and incubated in an atmosphere of 5% CO_2 at 37 °C in an incubator. The culture medium was changed every two days. The cells were subcultured when the cell monolayer reached 80% convergence.

(2) Rat-β-NGF treatment

After PC12 cells in logarithmic growth phase were subcultured for 24 h, the culture medium was changed, and the cells were divided into control group and experimental group. After a corresponding volume of saline was added to the control group, Rat-β-NGF with final concentration of 10 ng/ml was added to the experimental group and the culture medium was changed every 2 days. The cells were continuously incubated for 9 days and photographed using an inverted microscope.

2. Preparation of apoptosis model of nerve metrocyte

Neuronally differentiation PC12 cells in logarithmic growth phase were treated by common digestion, centrifugation and washing, then harvested for counting. Finally, the concentration of cells was adjusted to 1×10^5 ml^{-1}. The above cells suspension was seeded into 12-well cell culture plate, 2 ml per well. The cells were used in the following experiments after complete plastic-adhesion in about 4–6 h. The cells were divided into four groups: control group (0), experimental group (1), experimental group (2) and experimental group (3). The concentrations of Aβ in the four groups were 0 μmol/L, 1.25 μmol/L, 2.5 μmol/L, 5 μmol/L, respectively and there were 3 wells per group. The cell culture plates were incubated in an atmosphere of 5% CO_2 at 37 °C in incubator. At 24, 48 and 72 h, the corresponding plates were taken out. The cells were collected, centrifuged, washed and stained with AnnexinV-FITC/PI. Then flow cytometry was conducted to examine the apoptosis percentage.

Examination of the cell apoptosis was implemented strictly according to the specifications in the kit.

(1) Culture medium was extracted. And the

cells were washed twice with cool PBS.

(2) Cells were resuspended using 1×binding buffer and the cell concentration was adjusted to 1×10^6 ml^{-1}.

(3) 100 μl cell suspension (about 1×10^5 cells) was taken and added into 5 ml centrifuge tube.

(4) 5 μl Annexin V-FITC and 10 μl PI were added into the centrifuge tube.

(5) The cells were incubated in the dark at room temperature for 15 min after gentle blending.

(6) 400 μl 1×binding buffer was added to resuspend the cells.

(7) Flow cytometry was conducted within 1 h.

Flow cytoMeter (Beckman Cailter EPICS XL) Light source was 488 nm argon ion laser.

FITC emits green fluorescence and PI emits red fluorescence under laser. Ten thousand cells were collected for every sample. The data were analyzed on computer with related software.

3. The effect of Aβ on the proliferation activity of the Rat-β-NGF-induced PC12 cells (Examined by MTT)

(1) The NGF-induced PC12 cells were digested with digestive juice and then collected by centrifugation. The cell concentration was adjusted to 1×10^5 ml^{-1} with culture medium.

(2) 100 μl/well cell suspension was added into 96-well plate (at cell concentration of 1×10^4 $well^{-1}$). After complete plastic-adhesion in about 4–6 h, Aβ with different concentrations was added. The final concentrations of Aβ were 0 μmol/L, 1.25 μmol/L, 2.5 μmol/L, 5 μmol/L, 6 wells for every concentration. The cells were cultured in incubator in an atmosphere of 5% CO_2 at 37 °C for 72 h.

(3) At 4 h time point before completion of the culture (i.e. 68 h), 5 mg/ml of MTT was added into each well and the culture was continued for 4 h.

(4) When an obvious bluish dark crystal color could be observed under microscope, the plates were centrifuged for 5 min at 1000 rpm.

(5) The supernatant was extracted, 150 μl of DMSO was added into every well after slight drying of the precipitates and the plates were oscillated gently for 10 min to dissolve the precipitates.

(6) After complete dissolution, the OD values were examined using a Humareader (German) EIA reader. The experimental wavelength and reference wavelength were 570 nm and 655 nm, respectively.

4. Statistical analysis

SPSS10.0 edition software was used for statistical analysis of the data.

RESULTS

Results of the establishment of the apoptosis model of nerve metrocyte

Normally seeded and cultured PC12 cells showed different morphologies under inverted microscope. The bodies of most cells developed round or polygonal forms and the third dimension and refraction of the bodies were strong. While some of the bodies were dim, dense blank grains could be seen in the cytoplasm and there was no tuber growth of the cells (Fig.1, 40×10). NGF-induced group: on the second day, the original round or polygonal morphology of the cell bodies had changed to neuron-like morphology with tuber growth. The sizes of the bodies differed and the third dimension was strong. The tuber ramifications of some cells were quite complex. The blank grains in the cytoplasm were obvious and nucleolei were evident. The length of the tuber was about 1 cm (Fig.2, 40×10) on the fourth day. The cell morphology on the fifth day was almost similar to that on the fourth day. While the proportion of the cells with strong refraction increased, the length of the cell tuber increased furthermore and the tuber ramification increased evidently. On the seventh day, the cell body differentiated into a polygon or tapered morphology. The length of the tuber was about 2.0–2.5 cm. The cells had differentiated into neurocytes. The stretches of the nerve tuber could form the joints between tuber and tuber or between tuber and cell body (Fig.3, 40×10). While the morphology of the cells of the control group (normally cultured group) was not different from the original morphology, the cells still had round or polygonal morphologies. No obvious tuber growth was observed. The only change was that cell density increased with the increase of culture time.

Results of the establishment of apoptosis model of Aβ-induced neuronal differentiation PC12 cells

The results of the apoptosis cell examined by flow cytomtry are shown in Table 1 and Fig.4.

Multifactor analysis of variance: There were differences among different treating time (P<0.05) cells and among different concentration (P<0.05) cells, among the three different concentration cells and the control group cells (P<0.05). The neuronal

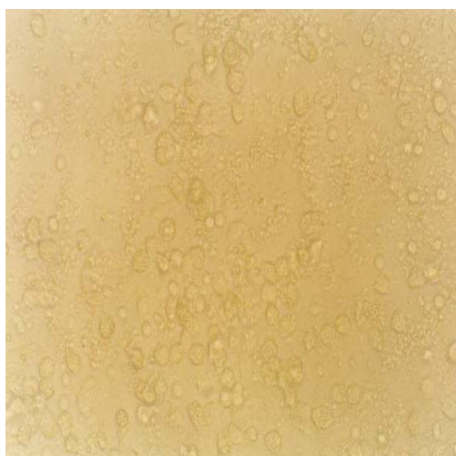

Fig.1 PC12 cells in the control group (40×10)

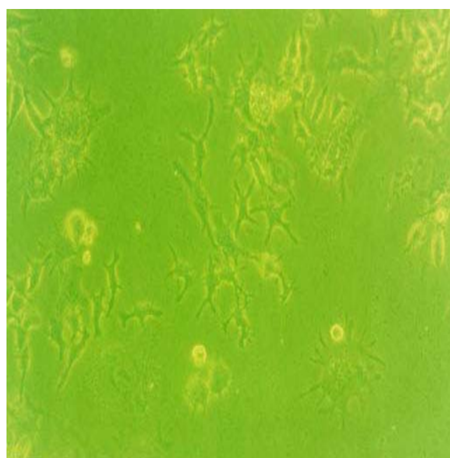

Fig.2 NGF-induced PC12 cells differentiation on the fourth day (40×10)

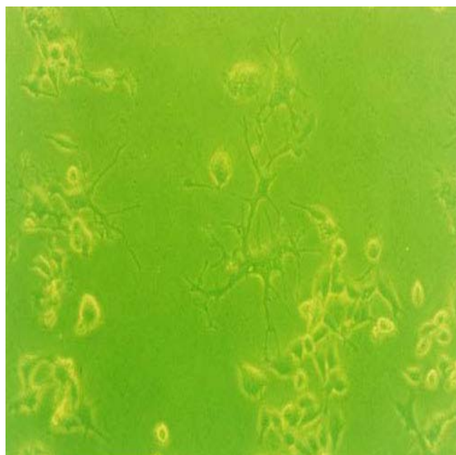

Fig.3 NGF-induced PC12 cells differentiation on the seventh day (40×10)

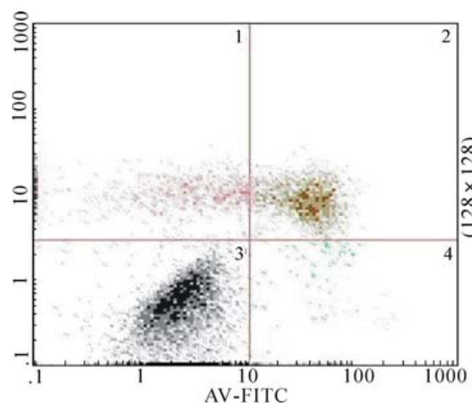

Fig.4 The influence of Aβ concentrations on cell apoptosis

Quadrant 1: physically damaged cells; Quadrant 2: apoptosis cell in the early stage; Quadrant 3: Normal live cells; Quadrant 4: apoptosis cell in the early stage

Table 1 Results of the cell apoptosis of the Aβ-induced neuronal differentiation PC12 cells

	N	Aβ (μmol/L)			
		0	1.25	2.5	5.0
24 h	3	1.12±0.15	20.4±2.78	24.5±1.66	28.3±1.30
48 h	3	1.24±0.21	26.1±1.82	32.8±3.51	36.9±2.40
72 h	3	1.78±0.19	6.06±0.87	7.13±0.61	4.96±0.47

F=188.406, P<0.001

differentiation PC12 cells could successfully be forced to apoptosis after interaction with Aβ-amyloid (1.25, 2.5, 5.0 μmol/L) for 24 h, 48 h and 72 h).

Effect of Aβ on the proliferation activity of the Rat-β-NGF-induced PC12 cells (Examined by MTT shown in Table 2)

Aβ (0~5 μmol/L) had no obvious effect on the proliferation/inhibition of the neuronal differentiation PC12 cells after 72 h interacting process.

Univariable analysis showed that there existed differences between the Aβ (5 μmol/L) treated and Aβ (0, 1.25, 2.5 μmol/L) treated cells. There was no difference among other groups.

DISCUSSION

The "Aβ toxicity theory" holds that the formation, metabolism and toxicity of β-amyloid play a key role in the occurrence and development of Alzheimer's disease (AD). Aβ is a polypeptide composed of 39~43 amino acids (Glenner and Wong, 1984), and is formed from an abnormally metabolized amyloid precursor protein (APP). The content of Aβ in the AD patient's brain is evidently higher than that in the normal brain. Through the interaction with caspase-3 and caspase-8, Aβ can induce the apoptosis of neurons and be cytotoxic. The toxic effect of Aβ on the cultured neurons was as follows: In the earlier period, the cells changed at different speed and the cell membranes were intact. The basic morphological characters, such as nucleus pycnosis, cell shrinkage and apoptotic bodies, could be observed (Loo *et al*., 1993). These changes can also be observed through scanning electron microscopy and transmission electron microscopy. In addition, ladder-like distribution of oligonucleotides in the DNA extracted from nerve cells, treated with Aβ for 24 h, could be found as a characteristic biochemical change in the neuron apoptosis induced by Aβ (Watt *et al*., 1994).

Taking into account the pathological character of AD and based on the "Aβ toxicity theory", this study adopted the cell culture and flow cytometry technique to observe the influence of different amount of Aβ on cell apoptosis. The cells used in this research were Aβ-induced neuronal-differentiation PC12 cells. This study was also aimed at constructing an Aβ-induced nerve metrocyte apoptosis model.

PC12 cells came from a rat adrenal medullary pheochromocytoma cell line and had differentiation potential. By treatment with NGF, PC12 cells can be induced to proliferate and differentiate into sympathetic neurons. This differentiation makes them closer to neuron in the aspects of morphology, physiology, biochemistry and function and provides a neurocyte model for studying diseases such as degenerative diseases of nerve system (Shafer and Atchison, 1991).

NGF plays an important role in the growth, development, differentiation, nutrition and maintaining the functions of neurons. In the presence of NGF (50 ng/ml), PC12 cells can be induced to produce some early changes: (1) Change of the cell morphology, such as cell smoothing, the formation of cytoplasm appendix and a rumpled cell membrane; (2) Promote the transport of amino acids and phosphorylation of some proteins; (3) Change of the ion pump on the cell membrane (Greene and Tischler, 1976). With prolongation of induction time (after about 48 h), the cells revealed long time induction characters: (1) The cells stopped proliferating; (2) The cells showed tuber outgrowth; (3) Cell membrane electric potential (specific character of the neurocyte) appeared. These indicated that di-

Table 2 The effect of Aβ (0~5 μmol/L) on the proliferation/inhibition of the neuronal differentiation PC12 cells after 72 h process

Group	Normal control	Aβ (μmol/L)			
		0	1.25	2.5	5
N	5	5	5	5	5
A	1.39±0.08	1.33±0.03	1.37±0.08	1.33±0.10	1.19±0.10

F=5.583, P=0.002

fferentiated PC12 cells could be used as neurocyte model in neurobiology. However, most researches directly use the PC12 cell tumor (for example, dopamine-induced PC12 cell apoptosis can be used as Parkinson's disease model) instead of neuronal differentiated PC12 cells. The possible causative reason is that the induction period of the differentiation of the cells is too long and that the induction process is troublesome.

The qualitative determination of apoptosis depends on morphological observation and DNA electrophoresis, while the quantitative determination relies on the flow cytometric examination. In 1992, Fadok *et al*.(1992) reported that phosphoryl serine (PS) at the inside of the cell membrane transferred to the outside of the cell in the early stage of apoptosis. Vermes *et al*.(1995) used Annexin V, which had high affinity to the PS, to examine apoptotic cells. Because the PS in the dead cell is also exposed to the outside, which makes Annexin V in combination with positive PI, a dead cell staining positive DNA dye, has to be used to distinguish the dead cells. The exposure of PS precedes the DNA rupture, so it is more effective for detecting early apoptosis by this method. Moreover, Annexin V/PI method not only can detect the early stage of apoptosis, but also can distinguish the apoptotic cells from the dead cells. This method has relatively high sensitivity and specificity and the samples do not have to be fixed. So this method is the preferred method nowadays for flow cytometry to quantitatively examine apoptosis.

In vitro study showed that Aβ proteins has "nutrition" effect for the cells at extremely low concentration while at high concentration, they can stimulate apoptosis of many cultured cells (Cotman, 1998).

Some other researches revealed that the Aβ treated PC12 cells behaved as morphological putrescence. So it is necessary to examine the effect of Aβ on the proliferation activity of the Rat-β-NGF-induced PC12 cells by MTT method.

MTT is tetrazolium salt and reduction of the MTT by the mitochondrial dehydrogenase of viable cells causes formation of a blue formazan product which will be deposited in the cells. The amount of formazan produced is proportional to the number of living cells only. This research has successfully established a neuronal differentiation PC12 cell strain. The induction of apoptosis by different concentrations of Aβ in neurocytes closely observed and the influence of β-AP on the proliferation activity of at-β-NGF-induced PC12 cells was examined. The results provide a basis for future studies on the molecular level of AD.

ACKNOWLEDGEMENT

We appreciate Mr. YAO Hangping (Institute of Infectious Institute, 1st Affiliated Hospital, Medical College of Zhejiang University) for all his work on the cell experiments.

References

Cotman, C.W., 1998. Apoptosis decision cascades and neuronal degeneration in Alzheimer's disease. *Neurobiol Aging*, **19**(1 Suppl):S29-32.

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L., Henson, P.M., 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol*., **148**(7):2207-2216.

Glenner, G.G., Wong, C.W., 1984. Alzheimer's disease: initial report of the purification and characterization of a noved cerebrovasclular amyloid protein. *Biochem Biophys Res Commu.*, **120**(3):885-890.

Greene, L.A., Tischler, A.S., 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *PANS*, **73**(7):2424-2428.

Loo, D.T., Copani, A., Pike, C.J., Whittemore, E.R., Walencewicz, A.J., Cotman, C.W., 1993. Apoptosis is induced by β-amyloid in culture central nervous system neurons. *Proc Natl Acad Sci*, **90**(17):7951-7955.

Shafer, T.J., Atchison, W.D., 1991. Transmitter, ion channel and receptor properties of pheochromocytoma (PC12): Model for neurotoxicological studies. *Neurotoxicology*, **12**(3):473-492.

Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C., 1995. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods*, **184**(1):39-51.

Watt, J.A., Pike, C.J., Walencewicz-Wasserman, A.J., Cotman, C.W., 1994. Ultrastructral analysis of β-amyloid-induced apoptosis in cultured hippocampal neurons. *Brain Res*, **661**(1-2):147-156.