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Cell Research (2004); 14(6):467-472
http://www.cell-research.com

Effect of amyloid peptides on serum withdrawal-induced cell differentiation and cell viability

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ABSTRACT

Abnormal deposition of amyloid-β(Aβ) peptides and formation of neuritic plaques are recognized as pathological processes in Alzheimer's disease (AD) brain. By using amyloid precursor protein (APP) transfected cells, this study aims to investigate the effect of overproduction of Aβ on cell differentiation and cell viability. It was shown that after serum withdrawal, untransfected cell (N2a/Wt) and vector transfected cells (N2a/vector) extended long and branched cell processes, whereas no neurites was induced in wild type APP (N2a/APP695) and Swedish mutant APP (N2a/APPswe) transfected N2a cells. After differentiation by serum withdrawal, the localization of APP/Aβ and neurofilament was extended to neurites, whereas those of APP-transfected cells were still restricted within the cell body. Levels of both APP and Aβ were significantly higher in N2a/APP695 and N2a/APPswe than in N2a/Wt, as determined by Western blot and Sandwich ELISA, respectively. To further investigate the effect of Aβ on the inhibition of cell differentiation, we added exogenously the similar level or about 10-times of the Aβ level produced by N2a/APP695 and N2a/APPswe to the culture medium and co-cultured with N2a/Wt for 12 h, and we found that the inhibition of serum withdrawal-induced differentiation observed in N2a/APP695 and N2a/APPswe could not be reproduced by exogenous administration of Aβ into N2a/Wt. We also observed that neither endogenous production nor exogenous addition of $A\beta_{1-40}$ or $A\beta_{1-42}$, even to hundreds fold of the physiological concentration, affected obviously the cell viability. These results suggest that the overproduction of Aβ could not arrest cell differentiation induced by serum deprivation and that, at least to a certain degree and in a limited time period, is not toxic to cell viability.

Keywords: Alzheimer's disease, amyloid β, cell differentiation.

INTRODUCTION

Alzheimer's disease (AD) is a complex disorder that impairs cognitive and memory functions in the elder population. Aggregated amyloid peptide (Aβ) is the core component of brain senile plaques, a defining feature of Alzheimer's disease. Aβ is composed of 39-43 amino acid residues produced from a large precursor, amyloid precursor protein (APP), and plays a pivotal role in the dysfunction and death of neurons in AD. Among different subtypes of Aβ, $A\beta_{1-40}$ is the most predominant form accounting for more than 90% of the total Aβ, whereas amyloid $A\beta_{1-42}$ is the most toxic form.

APP is a type I integral membrane protein which is processed to generate several intracellular and secreted fragments. Among 10 identified isoforms of APP, APP695, which is composed of 695 amino acid residues, was mainly expressed in human brain. Genetic evidence [1] and the characterization of neurotoxicity of Aβ lead to numerous studies on the cell biology of APP and formation of Aβ [2]. However, the functions of APP and Aβ still remain poorly understood until now. Although it is reported that APP promotes neural differentiation and neurite outgrowth [3], there was no information about whether and how APP or Aβ functions in regulating neurite growth. To investigate the role of APP/Aβ in neurite outgrowth and cell viability, mouse neuroblastoma N2a cells, which are capable of differentiating into neuron-like cells [4], were stably transfected with wild type or Swedish mutated APP695 and used in the study.

MATERIALS AND METHODS

Preparation of β-amyloid peptide

β-amyloid peptides $A\beta_{1-40}$ and $A\beta_{1-42}$ were purchased from Bachem (PA, USA) and prepared as previously described [5]. The peptides were dissolved in double-distilled deionized water at a concentration

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of 1 mM. The stock solutions were 'aged' by incubation at room temperature for 24 h, followed by one freeze-thaw cycle. Aliquots were stored at -70 ºC.

Cell culture

Untransfected mouse neuroblastoma N2a cell line (N2a/Wt), N2a cell lines transfected with empty vector pCB6 (N2a/vector), wild-type APP (N2a/APP695) and Swedish mutant form of APP (N2a/APPswe) were kindly gifted by Dr. Hua Xi XU (Rockefeller University, NY, USA) [6]. The construction of plasmids was described previously [7] and plasmid pAPPswe harbors the Swedish FAD-specific amino acid substitutions (K595N and M596L). N2a cell lines were grown in 50 % Dulbecco's modified Eagle's medium (DMEM), 50 % OPTI-MEM plus 5 % fetal bovine serum (GibcoBRL, Grand Island, NY, USA) in the presence (stably transfected cells) or absence (N2a/Wt) of 200 mg/L G418 (GibcoBRL, Grand Island, NY, USA).

Cell differentiation

Cell differentiation was induced by serum withdrawal for 12 h. To detect the effect of exogenous Aβ on cell differentiation, N2a/Wt was co-cultured with 5 nM (almost the highest Aβ level detected in the medium of transfected cells) or 50 nM $A\beta_{1-40}$ or $A\beta_{1-42}$ (about 10-fold of the highest Aβ level detected in the medium of transfected cells) for 12 h together with serum withdrawal simultaneously. The Aβ concentrations used here were also higher than the physiological concentration of Aβ in blood and cerebrospinal fluid, in which Aβ normally presents in the range of 10-500 pM [8].

Double staining immunofluorescence

Cells were seeded at a concentration of 5×10^4 cells/well in 24-well plates. After differentiated by serum withdrawal for 12 h, cells were fixed for 10 min at room temperature with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) and permeabilized for 5 min at room temperature with 0.3% Triton X-100 in PBS. Cells were further incubated with SMI33 (Sternberger Monoclonal Inc., Lutherville, MD, USA), a monoclonal antibody for neurofilament, for 2 h at 37ºC, and then incubated for 1 h at 37 ºC with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Santa Cruz Biotechnology, CA, USA). After washing, cells were probed with monoclonal antibody 6E10 (CalBiochem, San Diego, CA, USA), which recognize residues 1-17 of Aβ, for 2 h at 37 ºC, and then incubated for 1 h at 37 ºC with Texas Red-conjugated secondary antibodies (Santa Cruz Biotechnology, CA, USA). The cells were visualized by florescence microscopy (Olympus, Japan).

Western blot

Cells were lysed with RIPA buffer containing 50 mM Tris-HCl, pH 7.2, 150 mM sodium chloride, 1% NP-40, 12 mM sodium deoxycholate, 3 mM sodium dodecyl sulfate, 4 mM sodium azide, 0.57 mM phenylmethysulfonyl fluoride and 10 mg/L protease inhibitors (leupeptin, aprotinin and pepstatin). Protein concentrations were determined with BCA Protein Assay Reagent (Pierce, Rockford, USA). Proteins were separated by 10 % SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). To determine the expression of APP, the blots were probed with the monoclonal antibody anti-APP (GibcoBRL, Grand Island, NY, USA) recognizing the N-terminus of APP, then incubated with ALP-labeled antibody to mouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and developed with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT). α-tubulin was also detected as a control by monoclonal antibody DM1A (Sigma, St Louis, MO, USA).

ELISA

Sandwich enzyme linked immunosorbent assay (ELISA) was performed to measure the level of Aβ in culture medium and in cell lysates. In brief, $A\beta_{40}$ and $A\beta_{42}$ were captured with G2-10 (Aβeta, Germany), a monoclonal antibody specific for $A\beta_{40}$, and G2-11 (Aβeta, Germany), a monoclonal antibody specific for $A\beta_{42}$, respectively. The presence of Aβ was then detected specifically by antibody biotin-WO2 (Aβeta, Germany), and further developed with HRP-NeutrAvidin (Aβeta, Germany). HRP activity was assayed by color development using 3, 3', 5, 5'-tetramethylbenzidine (TMB) microwell peroxidase system (Kirkegaard Perry Laboratories, Maryland, USA). The results were expressed as refered to dilutions of standard synthetic control peptides (Sigma, St Louis, MO, USA).

Cell viability assay

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra- zolium bromide (MTT) assay. Cells were plated at a concentration of 5×10^3 cells/well in 96-well plates with 100 µl medium and grown for 24 h. Cells were rinsed with serum-free media and cultured in the presence or absence of 50 nM $A\beta_{1-40}$ or $A\beta_{1-42}$ for 48 h, then incubated with 0.5 mg/ml MTT in PBS for 4 h at 37 ºC. After an additional 12 h of incubation with 100 µl of dimethyl sulfoxide at 37 ºC, absorbance values at 570 nm were determined with a microtitre plate reader (TECAN, Austria). Experiments were repeated independently for 4~5 times.

Statistics

The results were presented as means ± SEM. Statistical significance was determined by ANOVA followed by post-hoc test. *P* values less than 0.05 were considered as significance.

RESULTS

Both APP and Aβ were elevated in APP transfected cell lines

We determined the expression and metabolism of APP. As shown in Fig. 1, APP was present in N2a/APP695 and N2a/APPswe but deprived in N2a/Wt and N2a/vector cells. In addition, the expression levels of APP in N2a/APP695 and N2a/APPswe were comparable. $A\beta_{1-40}$ and $A\beta_{1-42}$ in culture medium and in cell lysates were assayed using ELISA. We found that $A\beta_{1-40}$ both in culture medium and in cell lysates were significantly higher in APP transfected cells, especially in N2a/APPswe (Tab. 1). However, $A\beta_{1-42}$ could only be detected in medium but was absent in cell lysate of the transfected cells, as well as in either fractions of N2a/Wt and N2a/vector (Tab. 1). The generation of $A\beta_{1-40}$ and $A\beta_{1-42}$ in N2a/APPswe were 1~2 folds higher than those of N2a/APP695 though similar levels of APP were detected in these two cell lines.

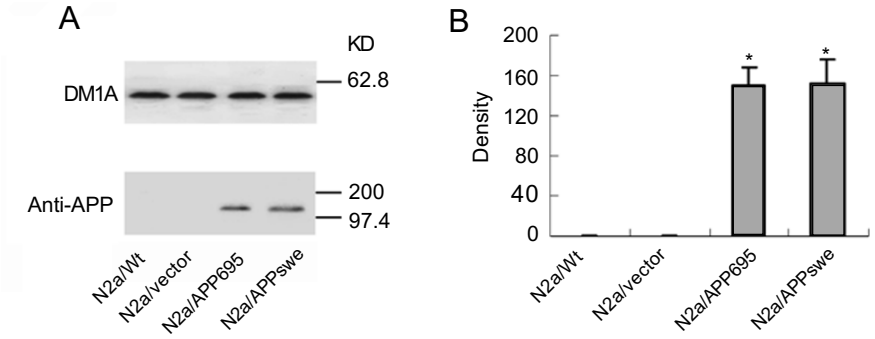

Fig. 1 The expression of APP in N2a/wt, N2a/vector, N2a/APP695 and N2a/APPswe. (**A**) APP expression was detected by Western blot. DM1A was used to confirm the equal loading of each lane. (**B**) Statistical analysis of APP expression detected in (**A**). *presents $P < 0.01$, compared to N2a/Wt.

Tab. 1 Different levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ detected in medium and cell lysate (n = 3).

Cell lines	$A\beta_{1-40}$ (μg/L)		$A\beta_{1-42}$ (ng/L)
	Medium	Cell lysate	Medium
N2a/wt	7.06 ± 2.08	5.50 ± 0.86	
N2a/vector	6.94 ± 1.05	5.59 ± 1.12	
N2a/APP695	11.64 ± 0.75*	9.24 ± 0.32**	10.01 ± 1.21**
N2a/APPswe	16.68 ± 0.76**#	17.59 ± 0.97**##	18.21 ± 4.45**#

$A\beta_{1-40}$ was detected in both the medium and the cell lysates in four cell lines. $A\beta_{1-42}$ could only be detected in the medium of *APP*-transfected cells. *$P < 0.05$, **$P < 0.01$, compared with N2a/Wt. #$P < 0.05$, ##$P < 0.01$, N2a/APPswe *vs* N2a/APP695.

Transfection of *APP* inhibits cell differentiation induced by serum withdrawal

No cell processes or only very short ones were shown both in untransfected and transfected cells cultured in 5 % fetal bovine serum supplemented medium (Fig. 2, higher pannel). After serum withdrawal for 12 h, the majority of the N2a/Wt and N2a/vector extended long and branched neurites, whereas N2a/APP695 and N2a/APPswe displayed similar morphology to that of undifferentiated cells (Fig. 2, lower pannel). The result suggested that APP or Aβ might play a role in inhibiting cell differentiation in N2a/APP695 and N2a/APPswe cells. Immunostaining of APP, Aβ and neurofilament, a specific marker for neuron, was employed to further demonstrate the distribution of APP and its relationship with the outgrowth of cells. It was found that the localization of APP/Aβ and neurofilament in all of the undifferentiated N2a cells was mostly restricted in the cell body (data not shown), suggesting that transfection of *APP* gene did not change the localization of APP/Aβ. After differentiation induced by serum withdrawal, APP, Aβ and neurofilament in N2a/Wt and N2a/vector were found to be co-localized in both of the cell body and processes (Fig. 3); whereas those in N2a/APP695 and N2a/APPswe were still restricted within the cell body (Fig. 3), further confirming that the overexpression of APP or Aβ could inhibit cell differentiation in N2a/APP695 and N2a/APPswe cells.

Inhibition of serum withdrawal-induced cell differentiation could not be reproduced by exogenous addition of Aβ

To further elucidate the involvement of Aβ overproduction in the above-mentioned inhibition of cell differentiation, 5 nM (data not shown), 50 nM $A\beta_{1-40}$ or $A\beta_{1-42}$ (about 10-times of the expression level generated by the transfected cells) were added into the medium of N2a/Wt at the time of serum withdrawal, respectively. It was found that N2a/Wt still extended long neurites and displayed similar morphology to that of untreated control cells following exposure to $A\beta_{1-40}$ or $A\beta_{1-42}$ (Fig. 4), indicating that neither $A\beta_{1-40}$ nor $A\beta_{1-42}$, when added exogenously, affected neurite outgrowth at the concentration used.

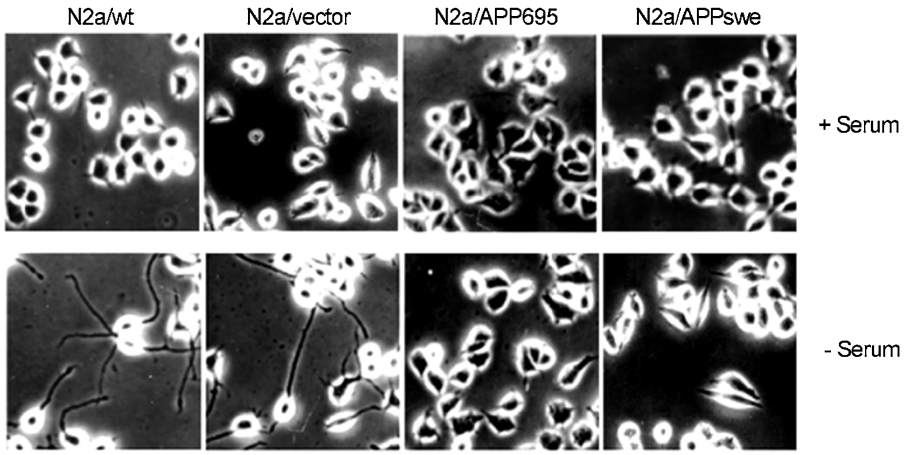

Fig. 2 Phase contrast microscopy showing inhibition of serum withdrawal induced differentiation. N2a/wt, N2a/vector, N2a/APP695 and N2a/APPswe were grown in medium containing 5% fetal bovine serum, then the cells were induced to differentiate by serum withdrawal for 12 h.

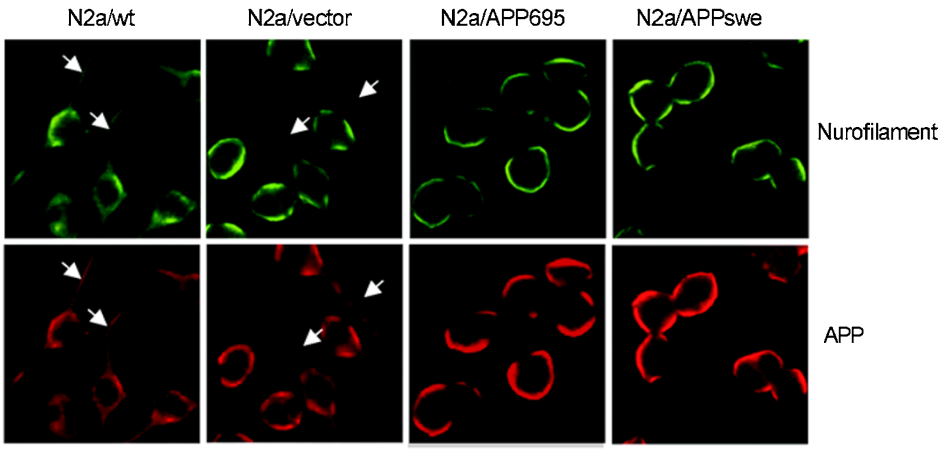

Fig. 3 Immunostaining of neurofilament and APP in differentiated N2a/wt, N2a/vector, N2a/APP695 and N2a/APPswe cells.

Both endogenous production and exogenous addition of Aβ do not affect cell viability

To evaluate the toxicity of endogenously overproduced or exogenously added Aβ, cell viability was measured by MTT assay. No significant difference of the OD_{570} values in N2a/APP695, N2a/APPswe, N2a/vector and N2a/Wt (data not shown) were observed, although Aβ levels in these cell lines varied significantly (see Tab. 1), suggesting that the endogenous overproduction of Aβ did not affect the cell viability. Moreover, the OD_{570} value in N2a/Wt (1.12 ± 0.18) was not altered even after the cells were incubated with either $Aβ_{1-40}$ (1.09 ± 0.29) or $Aβ_{1-42}$ (1.10 ± 0.22) at 50 nM for 48 h.

DISCUSSION

Formation of senile plaques is one of the two major neuropathological features in AD brain. And Aβ, derived from the cleavage of APP, is the major insoluble component of the senile plaques. The presence of dystrophic neurites in the vicinity of senile plaques proposed the possible role of Aβ on neurite growth. The high density of APP in growth cones [9] and increasing expression during neurite outgrowth and synaptogenesis [10] suggest that APP might play a role in neurite outgrowth. However, the involvement of Aβ in this process is not well understood. In the present study, *APP* transfected cell lines N2a/APP695 and N2a/APPswe were resistant to serum-with-

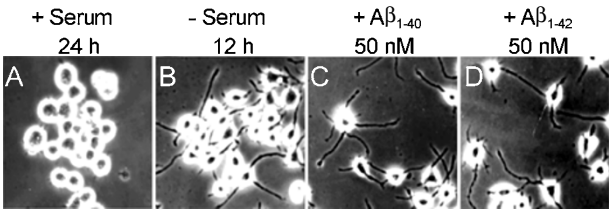

Fig. 4 Effect of exogenous Aβ on the phenotype of differentiated N2a/wt detected by phase contrast micoscopy. (**A**) N2a/wt cells grown in medium containing 5% fetal bovine serum for 24 h. (**B**) Cells were induced to differentiate by serum withdrawal for 12 h. (**C**) 50 nM $A\beta_{1-40}$ and (**D**) $A\beta_{1-42}$ were added to the cultures, respectively.

drawal-induced differentiation, while N2a/vector showed the same morphological feature as N2a/Wt, supporting the involvement of overexpression of APP in inhibiting cell differentiation. It was also observed that the transfection of APP gene did not alter the distribution of APP/Aβ, together with the co-localization of APP/Aβ and neurofilament, demonstrating the inhibitory effect of elevated APP/Aβ on serum deprivation-induced differentiation.

Given the fact that both APP and Aβ in N2a/APP695 and N2a/APPswe cells were significantly higher than that of the N2a/Wt and N2a/vector cells, N2a/Wt cells were co-cultured with exogenous Aβ to elucidate whether the overexpression of APP or Aβ was responsible for the inhibition of cell differentiation. Using the same concentration (5 nM) of Aβ generated in transfected cells or using a 10-times higher (50 nM) of the Aβ concentration, we did not observe any obvious inhibitory effect of the exogenous Aβ on neurite outgrowth. The above findings suggest that the overexpression of Aβ had no effect on inhibiting the serum withdrawal-induced cell differentiation. Another possibility lies in that the exogenous Aβ peptide may work in different way with the endogenous one. For example, we have observed that the administration of exogenous Aβ, up to 50 nM, which is a 100 to 5000-times of the normal endogenous Aβ concentration in blood or cerebrospinal fluid [8], showed no toxic effect on cell viability as detected by MTT assay. In addition, it should be noted that, in the previous studies, the concentration of Aβ used was usually hundreds or thousands times of the physiological concentration (in the level of micromole) [11-14], or different Aβ fragments were employed [11,13], or the cells were directly grew on the slices of AD tissue [15]. The inconsistency between the present study and the previous ones might be partially due to the difference of Aβ concentration.

In addition to Aβ, APP was also elevated in the system, suggesting the effect of APP on inhibiting neurite outgrowth in N2a cell. Generally, APP is a marker for cell differentiation, however, previous studies also demonstrated that the overexpression of APP could inhibite neurite outgrowth in N2a cell [16]. Its membrane location and its structural similarities with cysteine-rich growth factors suggest that APP might be functioning as a cell surface receptor or growth factor [17], and such proteins, such as G0 protein [18], Fe65 [19], and low density lipoprotein receptor-related protein [20], have been reported as its ligands. However, APP is cleaved by different secretases to produce various fragments, whose functions remain largely unknown, except for Aβ. Therefore, no conclusion should be made to the effect of APP on inhibiting cell differentiation untill further studies on these fragments have been done. Furthermore, N2a is a neuroblastoma cell line which has limitation in studying neural differentiation.

Although APP was not detectable by Western blot in N2a/wt and N2a/vector, the expression and processing of endogenous APP in the cell lines were proved by the presence of Aβ in the culture media (see Tab. 1). In addition, the majority of Aβ produced from APP at physiological condition is $A\beta_{1-40}$, and only about 10% is $A\beta_{1-42/43}$. It was demonstrated in N2a cells that the production of $A\beta_{1-40}$ was much higher than that of $A\beta_{1-42}$, which was beyond the sensitivity of the method to be detected in N2a/Wt and N2a/vector. In accordance with previous studies that Swedish mutant APP could enhance the production of Aβ [1], more $A\beta_{1-40}$ and $A\beta_{1-42}$ were detected in N2a/APPswe cells. However, the expression of APP in was found to be comparable to that in N2a/APP695 cells.

Taken together, we have found in the present study that extracellular Aβ could not affect serum withdrawl-induced cell differentiation and cell viability. However, intracellular overproduction and accumulation of Aβ may be harmful to the cells. Therefore, further study on the role of intracellular Aβ is needed.

ACKNOWLEGEMENT

We thank Dr. Cheng Xin GONG from New York State Institute for Basic research for critical reading of the manuscript. This work was supported by grants from the National Natural Science Foundation of China (No. 30100057, 30170221, 30328007, 39925012), and Science and Technology Committee of China (No. G1999054007).

Received, Jan 5, 2004
Revised, Sep 7, 2004
Accepted, Sep 15, 2004

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