Changes in Morphology of Neuroblastoma Cells Treated With All-*trans* Retinoic Acid Combined With Transfer of the C-Terminal Region of the Amyloid Precursor Protein

Saho Honda, Fumio Itoh,* Mitsuru Yoshimoto, Yuji Hinoda, and Kohzoh Imai

First Department of Internal Medicine, Sapporo Medical University, Sapporo, Japan

Alzheimer disease is a progressive neurodegenerative disorder that is characterized by a loss of cognitive and memory functions. Amyloid fibrils deposited in neuritic plaque is mainly β -amyloid protein ($A\beta$) that is derived from amyloid precursor protein (APP). The secreted form of APP, which is corresponded to N-terminal portion of APP, shows neurotrophic activities. On the other hand, $A\beta$ and cytoplasmic domains of APP are thought to be neurotoxic. In order to investigate the effect of C-terminal fragment of APP covering $A\beta$ and the cytoplasmic domain upon cell growth and differentiation, we established a stably transfected cell line producing the C-terminal 100 amino acid pep-

tide of APP. The transfected clones stained positively with anti- $A\beta$ monoclonal antibody, TB-1. The growth rate of the transfected cells was not significantly different from that of mock-transfected cells or native NB39 cells. After treatment with all-*trans* retinoic acid (ATRA), mock-transfected cells extended neurite processes and showed neuronal-like differentiation, while a transfected clone overexpressing C-terminal fragment did not present neuronal-like morphology. These results suggest that ATRA-induced neurite extension may be suppressed by overexpression of the C-terminal fragment of APP. J. Clin. Lab. Anal. 12:172–178, 1998. © 1998 Wiley-Liss, Inc.

Key words: Alzheimer disease; β -protein; all-*trans* retinoic acid (ATRA); differentiation; transfection; monoclonal antibody

INTRODUCTION

Alzheimer disease (AD) is a neurodegenerative disorder that is clinically characterized by a progressive loss of cognitive and memory functions. β -amyloid protein ($A\beta$)(1) is a major constituent of amyloid plaque (neuritic plaque)(2) which is a pathological hallmark of AD. This 39- to 43 amino acid peptide is proteolytically produced from a high molecular weight precursor protein called amyloid precursor protein (APP)(3). APP has features of a transmembrane glycoprotein, and $A\beta$ corresponds to the membrane-spanning region and a part of the extracellular domain. Point mutations of the APP gene on chromosome 21 have been demonstrated in some cases of familial AD, and these mutations have been found in a cluster around the $A\beta$ sequence. A double mutation at the N-terminus of the β sequence was found in the case of a Swedish family (4). Another mutation was found in the middle of the $A\beta$ sequence (5) or at the C-terminus of that sequence (6). Because these mutations result in overproduction of $A\beta$ (7)(8) or increased proportion of $A\beta_{1-42}$ in the $A\beta$ species (6), the abnormal metabolism of APP to $A\beta$ is believed to be a critical process in the pathogenesis of AD (9).

The N-terminal portion of APP can be secreted to the extracellular space by proteolysis in the $A\beta$ region (10). The

secreted form of APP (sAPP) shows neurotrophic activities and is thought to be involved in cell growth (11), neurite extension (12), and synaptogenesis (13). In contrast to sAPP, the $A\beta$ and cytoplasmic domains of APP are thought to be neurotoxic (14)(15), but their physiological roles are not precisely known. $A\beta$ is not a pathological product, but a physiological product found in the cerebrospinal fluid (16). The cytoplasmic domain of APP has a sequence which is capable of coupling to G-protein (17). These observations suggest that $A\beta$ and the cytoplasmic domains of APP may have physiological or pathological roles in the nervous system.

In this study, we clarified the effect of $A\beta$ and cytoplasmic domain on cell growth and differentiation by first establishing a stably transfected cell line producing C-terminal 100 amino acid peptide of APP containing $A\beta$ and the cytoplasmic domain. We then investigated the growth rate of transfected cells and observed key characteristics of the transfected cells.

*Correspondence to: Fumio Itoh, First Department of Internal Medicine, Sapporo Medical University, South-1, West-16, Chuo-ku, Sapporo 060, Japan.

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MATERIALS AND METHODS

Preparation of Complementary DNA (cDNA)

Total RNA was extracted from normal human brain tissue by the acid guanidinium thiocyanate-phenol-chloroform method (18). Randomly primed cDNA was prepared from 1 µg of total RNA by M-MLV reverse transcriptase (Perkin Elmer Cetus, Norwalk, CT) and amplified by PCR (19) using APP specific oligonucleotides (5'-CGGATCCGCTGAA-GTGAAGATGGATG-3' as a sense primer, 5'-GGATCCGAT-GCACTAGTTTGATACA-3' as an antisense primer). These primers contain a Bam HI site at the 5'-terminal of the nucleotide. PCR was performed for 30 cycles (denaturing 94°C for 1 min, annealing 55°C for 1 min, and elongation 72°C for 1 min). The PCR product was purified and determined by DNA sequence analysis to be identical to the sequence corresponding to the C-terminal 100 amino acids and the 3'-untranslated region of the APP cDNA (20)(Fig.1).

Vector Construction

cDNA obtained by PCR was digested with Bam HI and subcloned into the Bam HI site of the expression vector pcDNAI/Neo (Invitrogen, San Diego, CA) to create pAPPc100. This construct served to overexpress the 100 amino acid peptide containing Aβ and the cytoplasmic domain of APP under the control of a cytomegalovirus promoter. Since we did not add a signal sequence to the 5'-end of cDNA, 596-Met becomes an initiation codon and the 100 amino acid peptide (from 596-Met to 695-) is produced (Fig.1).

Cell Culture and Transfection

The human neuroblastoma cell line, NB39, was cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO₂ at 37°C.

The vector pAPPc100 or vector cDNA (alone as a control) were each introduced into the cells by coprecipitation with calcium phosphate (21). Briefly, exponentially growing NB39 cells were trypsinized and plated into 90-mm plate at a concentration of 5×10^5 cells per plate. Then 20 µg of plasmid DNA was mixed with 0.5 ml of 0.25 M CaCl₂, 0.5 ml of 50 mM BES (N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid)-280 mM NaCl-1.5 mM Na₂HPO₄ (BES-buffered saline) was added, and the mixture was incubated for 15 mins at room temperature. This solution (1ml) was added to the plated cells, the mixture swirled gently and incubated for 12h at 37°C under 3% CO₂. The medium was removed, and the cells were washed with PBS, and incubated with fresh medium for 36h at 37°C under 5% CO₂. After the transfected cells were grown and selected with 1 mg/ml of G418 for 6 weeks, 4 stably transfected cell lines were isolated. The most viable clone was used in this study and designated NB-c100. The stably transfected clone with vector plasmid alone was designated NB-c.

Immunocytochemistry

Immunocytochemistry of the cells was performed for the detection of the pAPPc100 product. We used monoclonal antibody TB-1 (IgG₁) (22) that was generated from a BALB/c mouse that had been immunized with a synthetic peptide of

Fig. 1. The RT-PCR system amplifying the C-terminal region of APP and construction of a recombinant pAPPc100 vector. Sense primer and antisense primer were used to amplify the open reading frame corresponding to the C-terminal 100 amino acid fragment and a part of 3'-noncoding

region of human APP cDNA. A BamHI cleavage site is introduced to both terminals of the amplified cDNA. The underline position (.....) corresponds to N-terminal sequence of Aβ. In this system, 596Met(*) is the start site of the sequence.

the first 24 amino acids of A β in our laboratory. It was shown that senile plaque and amyloid angiopathy were clearly stained with TB-1. Analysis with short synthetic peptides revealed that TB-1 recognizes the initial 7 amino acids of A β . The methods used for antigen preparation, immunization, cell fusion, cloning, and characterizations of the antibody have been previously described in detail (22). The cells were cultured on a chamber slide (Nunc, Naperville, IL) for immunostainings of cultured cells. The cells were fixed with 10% acetone and reacted with TB-1 or nonimmune mouse IgG overnight at 4°C after blocking endogenous peroxidase by treatment with 0.3% H₂O₂ and nonspecific binding with 10% normal rabbit serum. After washing, cells were incubated with biotinylated rabbit antimouse IgG (DAKO, Denmark) for 30 mins and then with avidin-biotin-peroxidase. The slides were developed with 0.03% 3,3-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl buffer (pH 7.6) containing 0.006% H₂O₂. Counterstaining was performed with hematoxylin.

Growth Rate

The growth rate of the cells was determined simply by cell counting. Cells were plated into 12-well plates at a concentration of 1×10^5 cells/well. They were then removed by trypsinization and counted with a hemocytometer at 12h, 24h, 36h, and 48h after plating.

Neuronal Differentiation

All-*trans* retinoic acid (ATRA) has been shown to induce neuronal differentiation of neuroblastoma cell lines (23). The transfected cells were plated in 10mm diameter tissue culture dishes. The medium was changed 1-2 days later and ATRA was added to a final concentration of 10 μ M. After 48h, the morphological changes of the cells were observed.

RESULTS

Isolation of Stably Transfected NB39 Cells Overproducing the C-Terminal Region of APP

We isolated a 600bp DNA fragment from the RNA of normal human brain tissue by RT-PCR (Fig. 1). This product was purified, subcloned, and determined to be identical to the cDNA corresponding to the nucleotide sequences of A β , the cytoplasmic domain, and the 3'-untranslated region of APP. The fragment was subcloned into the expression vector pAPPc100, which contains the cytomegalovirus promoter/enhancer for expression and a neomycin resistance gene allowing G418 selection. This construct was introduced into NB39 cells by coprecipitation with calcium phosphate. After 6 weeks of selection with 1mg/ml of G418, the resistant clones were isolated. The most viable clone was designated as NB-c100 and used in this study. The clone transfected with a vector plasmid alone was designated NB-c. RT-PCR analysis

revealed that NB-c100 expressed the C-terminal fragment of APP from the vector pAPPc100. To assess the synthesis of product from pAPPc100, immunostaining was performed with the anti-A β monoclonal antibody, TB-1. As shown in Fig. 2, TB-1 reacted with NB-c100 cells but not with NB-c cells or NB-39 cells. This result indicates a substantial expression of protein products from the transgene in NB-c100 and suggests that any expression level of native APP in host cells can be ignored.

Growth Rate Analysis

The secreted form of APP was reported to be involved in cell growth. Saitoh et al. (11) disclosed a reduced growth rate of fibroblasts expressing decreased level of APP mRNA by overexpression of antisense nucleotides of APP. In order to observe the effect of the C-terminal fragment of APP on cell growth, we compared the respective growth rates of NB39, NB-c100, and NB-c cells. To this end, cells were plated into wells at a concentration of 1×10^5 cells/well and cell counts were made of 12h, 24h, 36h, and 48h after plating and shown in Fig. 3. There were no detectable differences in the rate of cell growth, and overproduction of the C-terminal fragment did not affect the growth rate of NB39 cells.

Neuronal Differentiation of Transfected Cells

NB39 is a cell line derived from neuroblastoma cells. Neuroblastoma cells are composed of primitive cells derived from neural crest and are known to have the capacity to express multiple phenotypes, including those with neuronal, neurilemmal, and melanocytic characteristics. Retinoic acids are known to have potent effects on cell proliferation and differentiation. Human neuroblastoma cell lines are extremely sensitive to retinoic acid-induced growth inhibition and neuronal-like differentiation (24) which is commonly characterized by neurite extension. We observed morphological changes of transfected NB-c cells treated by ATRA which, after 48h, showed obvious extensions of neurites in a large number of cells. However, NB-c100 cells revealed an oval shape and no obvious neurite outgrowth (Fig. 4). These results suggest that overexpression of the C-terminal fragment of APP may reduce the ATRA-induced extension of neurite processes.

DISCUSSION

APP is a transmembrane protein with a receptor-like structure consisting of an N-terminal extracellular region, an A β -containing membrane-spanning region and a cytoplasmic region (3). The A β region is cleaved by a hypothetical protease called α -secretase to release the extracellular domain of APP, sAPP (10), and to generate a membrane-bound 10kDa C-terminal fragment. It is believed that other hypothetical enzymes, designated β - and γ -secretase, cleave APP to generate A β (25).

Recent reports indicated that sAPP may play an important role in the regulation of neurite outgrowth and synaptogenesis

Fig. 2. Expression of the C-terminal fragment of APP in transfected NB39 neuroblastoma cells. Immunostaining was performed by using anti-A β monoclonal antibody, TB-1 as the first antibody. NB-c100 cells (**A**) were clearly stained with TB-1, while NB-c cells (**B**) were not stained.

in developing nervous systems. In contrast to neurotrophic activities of sAPP, the C-terminal fragment of APP, comprising A β and the cytoplasmic domain was shown to have toxic effects on the nervous tissues. Fraser et al. (26) reported that aggregated A β peptides made adjacent neurites to swell and interfered with their extensions. Kammesheidt et al. (14) found aggregated A β immunoreactivity in the cell body and neuropil. He also reported abnormal aggregation of the C-terminal fragment of APP in the cytoplasm and abnormal appearing neurites in the hippocampus of transgenic mice expressing C-terminal fragment of APP under the control of the brain dystrophin promoter. Fukuchi et al. (15) injected P19 cells overproducing the C-terminal fragment of APP into mouse brains, and then observed a remarkable shrinkage character-

ized by neuronal loss and deposits of A β immunoreactivity. In order to investigate the effect of the C-terminal fragment on neuronal differentiation, the pAPPc100 expression vector was introduced into the neuroblastoma cell line, NB39. We established cell lines which overproduce the C-terminal 100 amino acid fragment of APP. After those transfected cells were treated by ATRA, we observed morphological changes of transfected cells and mock-transfected cells. NB-c, showing an obvious extension of neurites, however, we did not observe neurite outgrowth of NB-c100 cells, which were transfected with pAPPc100. These observations suggest that overproduction of the C-terminal 100 amino acid fragment of APP has an inhibitory effect on ATRA-induced neuronal differentiation of NB39 cells.

Fig. 3. Growth rate of NB39, NB-c100, and NB-c cells at 12h, 24h, 36h, and 48h after plating. Significant differences among the growth rate of these cells were not observed.

A region that is responsible for neurite outgrowth has been found in the N-terminal extracellular region, in which the sequence of five amino acids, RERMS (APP 328-332), appears to be critical (27). It was suggested that the secreted form of APP may bind to a cell surface receptor and that the domain containing the RERMS sequence may be required for binding (27). In order to gain an insight into the mechanisms that cause reduced extension of neurites on cells overproducing the C-terminal fragment, another mechanism should be considered. In fact, two possible explanations for this pleasure are the effect of A β , and that of the cytoplasmic region of APP. A β exhibits neurotoxic properties and causes selective neuronal cell death of cultured neuronal cells, while its activity is dependent on the concentration of A β (28). In this regard, it was shown that pathological changes, similar to AD, were induced by injection of A β into rat brains (29). Proposed mechanisms of A β toxicity include ion channel formation (30), kinase activation (31), impairment of mitochondrial succinate dehydrogenase (32), and aggregates formation by interacting with other substances such as apolipoprotein E (33). However, the growth rate of our transfected cells was nearly identical to that of untreated NB39 cells, and A β did not exert any obvious cytotoxic effect in this experimental system. Therefore, it is difficult to conclude that the cytotoxic effect of A β suppressed the neuronal differentiation of transfected cells.

The cytoplasmic region of APP has not been sufficiently investigated, although some notable observations were reported. Thus it was shown that the cytoplasmic region of APP can form a complex with G(o), a GTP-binding protein (17). This finding suggests that APP may have a G(o)-linked receptor function and could play a role in the signal transduction system. This G-protein is known to be involved in signal

transduction as a transducer which is located between a cell surface receptor and a second messenger, such as adenylate cyclase, cGMP phosphodiesterase, or phospholipase C. It was also reported that the expression of G(o) was significantly increased during NGF-induced differentiation of PC12 cells and that the amount of G(o), especially G α_{o1} , correlated with neurite outgrowth (34). As their report demonstrated, G(o) may be involved in neurite outgrowth in neuronal cells. Although it is presently unknown which second messenger enzymes may be activated or inactivated by coupling of APP and G(o), it is highly probable that overexpressed C-terminal fragments may affect neurite outgrowth by interacting with G(o).

Recently, Yamatsuji et al. (35) reported that transfection of mutant APP695, converting valine at position 642 to isoleucine, phenylalanine, or glycine, could induce nucleosomal DNA fragmentation in neuronal cells. These investigators also observed that mutant APP, lacking the 20 cytoplasmic residues (657-His to 676-Lys), could not induce DNA fragmentation. Since this phenomenon was inhibited by pertussis toxin, an inhibitor of G(i) and G(o) protein, it strongly suggests that G(o) mediates mutant APP-induced DNA fragmentation. The cytoplasmic domain of APP may be involved in the pathogenesis of AD by coupling with G(o). Further studies will clarify the signal transduction mechanisms involved in coupling of APP and G(o).

Here we demonstrated the reduction of ATRA-induced neurite extension by the C-terminal fragment of APP. It will be interesting to elucidate the roles of A β and the cytoplasmic domain of APP in physiological or pathological conditions. Although the significance of the cytoplasmic domain is not yet fully understood, the hypothesis that APP can form a complex with G protein is attractive when considering the

Fig. 4. Morphological changes of transfected NB39 cells after treatment with ATRA. ATRA was added to a final concentration of 10 μ M and after 48h NB-c100 cells (A) appeared to be oval shaped and did not show neurite extension, while NB-c cells (B) formed long processes and exhibited neuronal cell-like morphology.

relationship between APP and neuronal cell differentiation. This information should contribute to our understanding of the pathogenesis of AD and may be helpful in developing novel therapeutic strategies.

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