Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 204-209

NEURAL DIFFERENTIATION INCREASES EXPRESSION OF ALZHEIMER AMYLOID PROTEIN PRECURSOR GENE IN MURINE EMBRYONAL CARCINOMA CELLS

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Received July 9, 1990

SUMMARY: Neural differentiation of the embryonal carcinoma P19 cell line markedly increased the abundance of mRNA encoding Alzheimer amyloid β/A4-protein precursor (APP). In P19 cells treated with retinoic acid, the abundance of mRNA encoding APP695, which lacks the protease inhibitor domain, reached a maximum on days 2-4 and decreased thereafter, whereas the abundances of mRNAs encoding APP751 and APP770, both possessing the protease inhibitor domain, slowly increased to reach higher levels than APP695 mRNA at later stages of neural differentiation. The induction of APP695 mRNA was consistent with the appearance of neurons in the P19 cultures. A high abundance of APP695 mRNA was also detected in mouse brain at a stage of the period of neuroblast formation. Thus, neural differentiation of P19 cells may present a suitable model for studying the regulation of APP gene expression during early differentiation of brain cells in vivo.

A major hallmark of Alzheimer's disease is the deposition of amyloid fibrils in the brain. A principal component of amyloid fibrils is β/A4 protein (1,2), which is derived from a membrane-bound glycoprotein precursor (amyloid protein precursor, APP)(3). Three major species of APP mRNA (i.e., APP695, APP751, and APP770) are generated from the single gene by alternative RNA splicing (4,5,6). Little is known, however, about the expression of three species of APP mRNA during early periods of neural differentiation.

Embryonal carcinoma (EC) is a well-studied cell model for cellular commitment, differentiation, and development of mammalian systems. P19 EC cell line can differentiate into neurons and astrocytes by cell aggregation in the presence of retinoic acid (RA) (7). P19 cells committed to neural differentiation may represent the early development of brain cells in vivo (8). Therefore, we have used P19 EC cells as a cell culture model for studying the regulation of APP gene expression during early ontogeny of brain cells.

MATERIALS AND METHODS

Cell cultures: P19 EC cells were provided by Drs. M. McBurney and Dr. H. Hamada. F9 EC cells were donated by the Japanese Cancer Research Resources Bank. These EC cells were cultured and induced to

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Abbreviations: EC, embryonal carcinoma; APP, Amyloid β/A4-protein precursor; RA, retinoic acid; NF-L, neurofilament-L; GFAP, glial fibrillarly acidic protein; DMSO, dimethyl sulfoxide.

0006-291X/90 $1.50

differentiate as described (8). To treat the cells with RA, P19 cells in culture were normally aggregated by plating them at a density of 1 x 10^5 cells/ml into bacterial-grade petri dishes in the presence of 0.5 μM all-trans retinoic acid (RA) (Sigma Chemical Co.). After 4 days of incubation, the aggregates were collected by centrifugation, trypsinized, and plated onto tissue culture-grade surfaces at a density of 3-6 X 10^6 cells/ml. The cells were cultured in MEM Alpha (Sigma Chemical Co.) supplemented with antibiotics and 10 % fetal calf serum under a humidified atmosphere of 95% air/5% CO_2 at 37°C. The culture was maintained without subculturing except for P19 cultures of days 23 and 31 in Fig.3.

Northern blot analysis: Total cellular RNA was extracted from P19 cells, F9 cells and mouse tissues by the differential ethanol precipitation method (9) with modifications (10). Amounts of RNA were quantified by ultraviolet absorption at 260 nm. Yields of total RNA from P19 and F9 cells were 120-290 μg/10 cm dish. Total RNA (5 μg per lane) was electrophoresed on a 6% formaldehyde/1.2% agarose gel containing 0.5 μg/ml ethidium bromide , and blotted onto a nylon membrane (Hybond-N, Amersham). The equality of amounts of blotted RNA on the membrane was closely checked by observing the fluorescent intensities. RNA blots were fixed on the membrane by UV irradiation and hybridized with [^{32}P]labeled probes of rat APP cDNA (11), neurofilament-L (NF-L) (12), glial fibrillary acidic protein (GFAP) (13) (both provided by Dr. N.J. Cowan of New York University), β-actin (a gift from Dr. B.M. Paterson of National Cancer Institute, USA), or synthetic oligonucleotides described below. Hybridization and washing were performed as reported previously (14,15). Densities of autoradiograms were measured with a scanning densitometer (Shimadzu model CS-9000).

Detection of APP mRNAs with synthetic oligonucleotide probes: Oligonucleotides (40mer) complementary to the junctional sequences were synthesized: HK; 5'-GCTGGCTGCTGTCGTGGGAACTCGGACCA CCTCCTCCACG-3' (for APP695 mRNA, Ref.16), IK; 5'-TGCTGGCTGCTGTCGTGGGAAACACGC TGCCACACACCGC-3' (for APP751 mRNA, Ref. 16), IJ; 5'-TCTTGAGTAAACTTTGGGTTGACACGCT GCCACACACCGC-3' (for APP770 mRNA, Ref.16), and KK probe (for total APP mRNA); 5'-TTCCCACGACAGCAGCCAGCACCCCCGACGCCGTCGACAA-3'. In order to gain a high sensitivity for assay, each oligonucleotide was labeled by terminal transferase in the reaction mixture where a molar ratio of [^{32}P] CTP/oligonucleotide was adjusted to add 5-6 molecules of [^{32}P] CTP per each 3' end. Specific activities of the probes were about 2 x 10^9 dpm per μg. RNA blots on the membrane were hybridized with the labeled oligonucleotides (about 2 X 10^6 dpm/ml for all oligonucleotides). After incubating for 16 hr at 42°C in a hybridization buffer containing 50% formamide (14,15), the membrane was washed in 0.2 x SSC + 0.1% SDS at 60°C for 60 min. A separate membrane was used for each oligonucleotide probe.

RESULTS

Fig.1 shows a marked increase of APP mRNA in P19 cells after RA treatment; the increase in APP mRNA in RA-treated P19 cultures on day 12 was about 30 times the undifferentiated control (UN). NF-L mRNA, a marker of neurons, was unexpectedly detected in the undifferentiated stem cells and reached a maximum on

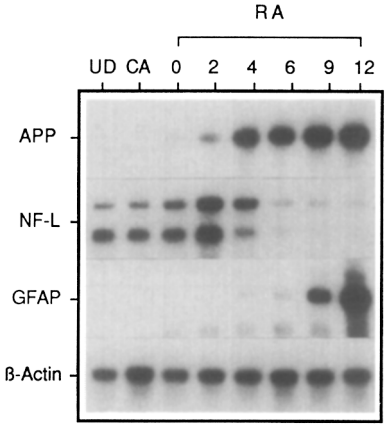

Fig.1. Alteration of abundances of mRNAs encoding APP, NF-L, GFAP, and β-actin. RA-treated P19 cells were harvested after cultivating for the periods indicated on top (0-12 days). Each mRNA species in total RNA was analyzed by Northern analysis. UD: Undifferentiated stem cells CA: Cells treated with aggregation in the absence of RA. The sizes (in kilobases) of hybridizing signals are: APP, 3.5; NF-L, 2.6 and 4.0; GFAP, 3.0; β-actin, 2.0.

day 2 and decreased thereafter, whereas GFAP mRNA, a marker of astrocytes, increased after a lag period of 6 days following RA treatment. The chronology of altered abundances of NF-L and GFAP mRNAs was consistent with that of morphological observations of neurons and astrocytes, respectively (not shown).

We then examined the alteration of three species of APP mRNA during neural differentiation of RA-treated P19 cultures. Using RNA samples from tissues, we first examined the validity of differential analysis of APP mRNA species with oligonucleotide probes (Fig.2A). A low amount of APP695 mRNA was found in germ cells (GC) in mouse testis. In the cerebral cortex, three species of APP mRNA were detected, and APP695 mRNA was the most abundant (APP695> >APP751>APP770), whereas the level of APP751 mRNA was the highest in the kidney (APP751>APP770>APP695). On the other hand, APP mRNAs were hardly detected in the liver. The tissue distribution of APP mRNAs is consistent with those reported previously (16), indicating the validity of differential analysis using oligonucleotide probes. All species of APP mRNA were markedly increased during neural differentiation of RA-treated P19 cultures (Fig.2B RA). The level of APP695 mRNA reached a maximum on day 4 and decreased thereafter, whereas the amounts of APP751 and APP770 mRNA gradually increased. On the other hand, dimethyl sulfoxide-treated P19 cells, which differentiated into cardiac muscle cells (8) exhibited little or no increase in APP695 mRNA, but slightly increased APP751 and APP 770

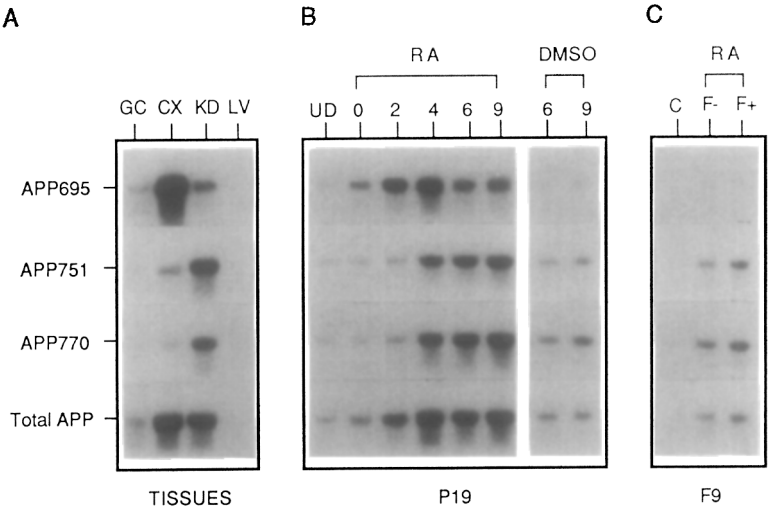

Fig.2. Abundances of three species of APP mRNAs in mouse tissues, P19 EC cells, and F9 EC cells. Abundances of mRNAs encoding APP695, APP751, APP770, and total APP were differentially determined by Northern analysis with synthetic oligonucleotide probes of HK,IK, IJ, and KK, respectively. A: APP mRNAs in tissues from a 55-day-old mouse. GC; testicular germ cells, CX; brain cortex, KD; kidney, LV; liver. B: P19 EC cells treated with RA (RA) or with 1 % dimethyl sulfoxide (DMSO). UD; undifferentiated stem cells. The numbers on top are the durations (days) in culture after the differentiation treatments. C: F9 EC cells after 4-day treatment with RA (0.1 μM) in the absence (F-) or the presence (F+) of forskolin (10μM). C; control (undifferentiated F9 cells). RNA samples of panels A, B, and C were on the same membrane. Autoradiograms were exposed for 16 hr except for total APP mRNA for 5 hr.

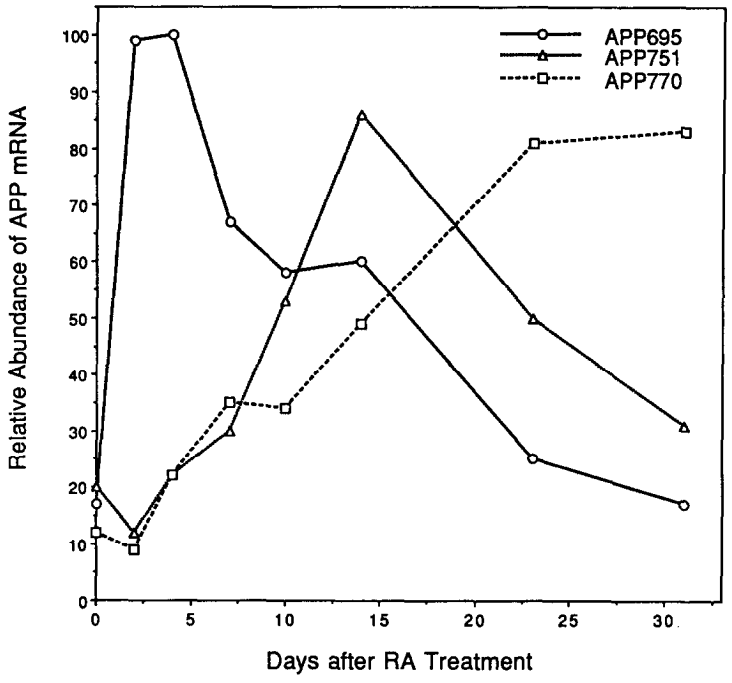

Fig.3. Time course of altered abundances of three species of APP mRNA in P19 cells during neural differentiation. RA-treated P19 cells were harvested after cultivating the cells for the durations indicated. Abundances of mRNAs encoding APP695 (○), APP751 (△), and APP770 (□) were analyzed by Northern analysis with the oligonucleotide probes, and the relative amounts were quantified with a densitometer.

mRNAs (Fig.2B DMSO). When F9 EC cells were treated with RA and forskolin (an adenylate cyclase activator) to induce differentiation into extraembryonic endoderm (8), APP770 and APP 751 mRNAs were moderately increased, whereas little or no increase of APP695 mRNA was noted (Fig.2C). Neither myocardial differentiation of P19 EC cells nor endodermal differentiation of F9 EC cells induced morphological differentiation into neurons (not shown). These results indicate that the induction of APP695 mRNA is consistent with the generation of neurons in P19 cells during neural differentiation.

We further examined the time course, up to day 31, of the changes of APP mRNAs during neural differentiation (Fig.3). The level of APP695 mRNA in RA-treated P19 cultures reached a peak on days 2-4, and then declined to the same level as day 0 after 31 days. On the other hand, the abundance of APP751 mRNA reached a maximal level on day 14, whereas the abundance of APP770 mRNA gradually increased to the highest level among the three APP mRNAs on day 31 (APP770 > APP751 > APP695). These results suggest that each species of APP mRNA is preferentially expressed in distinct type(s) of P19 derivatives which are formed during the course of neural differentiation.

We then examined whether APP695 mRNA is expressed at early stages of neural development in mouse brain (Fig.4). Both cerebral cortex and brain stem on embryonal day 16 (E16)(i.e., a period of neuroblast formation)

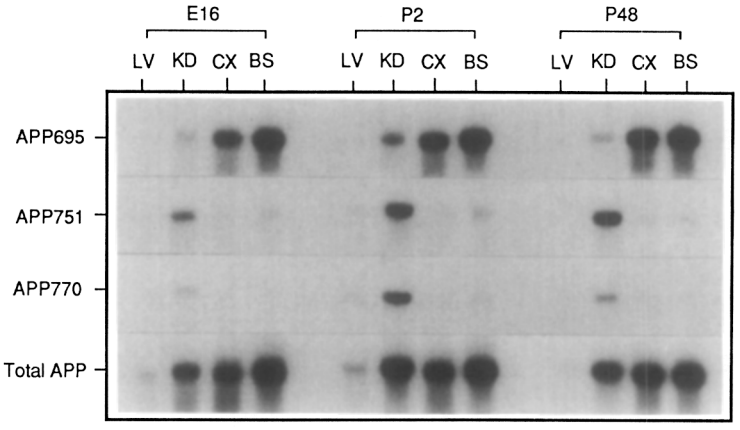

Fig.4. Abundances of three species of APP mRNA in developing mouse brains. Total cellular RNA was prepared from the liver (LV), kidney (KD), cerebral cortex (CX), and brain stem (BS) of mice at various stages of development. Abundances of three species of APP mRNA were determined by Northern analysis using oligonucleotide probes. E16: embryonal day 16, P2: postnatal day 2, P48: postnatal day 48. Yields of total RNA (μg/mg tissue) were: [LV] (E16) 8.6, (P2) 9.2, (P48) 8.1; [KD] (E16) 5.9, (P2) 6.7, (P48) 2.8; [CX] (E16) 3.9, (P2) 3.1, (P48) 1.6; [BS] (E16) 3.1, (P2) 2.4, (P48) 1.3. Autoradiograms of the four APP mRNAs were exposed for 16 hr.

contained a high abundance of APP695 mRNA; the levels were similar to those of postnatal days 2 (P2)(i.e., a period of glioblast formation) and 48 (P48)(i.e., a period of completed brain cytogenesis). At all the stages examined, the relative amounts of APP695 mRNA were consistently more than 90% of the total APP mRNA in the cortex and brain stem. On the other hand, APP751 mRNA was the major species in the kidney (APP751 > APP770 > APP695) at the periods examined. These observations indicate that APP695 mRNA is expressed at early stages of neuronal development in vivo.

DISCUSSION

The present study has demonstrated that APP gene expression is positively regulated by cellular differentiation, especially by neural differentiation; undifferentiated cells such as germ cells and EC stem cells contained low abundances of APP mRNA (see Fig.2), and cellular differentiation markedly increased the abundance of APP mRNA. This raises the possibility that tissue specific factor(s) enhancing APP gene expression is induced by neural differentiation. Since neural differentiation of P19 EC cell line is readily achieved by RA treatment, this system may be of great advantage for identifying the molecular mechanism underlying neural differentiation-associated regulation of APP gene expression.

The brain pathology of Down's syndrome (i.e., trisomy of chromosome 21, where the APP gene is located) is known to closely resemble that of Alzheimer's disease. In fetal brains of Down's syndrome, APP gene expression is increased due to the gene dosage effect (5), and amyloid deposition in the Down's syndrome brain occurs much earlier than that in normal aging subjects (17). These findings suggest that the persistent

overexpression of the APP gene during ontogeny is relevant to the pathogenesis of Down's syndrome. Thus, further studies on regulatory mechanisms of APP gene expression during the development of brain cells may provide an insight into the pathogenesis of Alzheimer's disease and related disorders, and possibly lead to the development of prophylactic strategies.

ACKNOWLEDGMENTS

We are grateful to Drs. M. McBurney (University of Ottawa) and H. Hamada (University of Tokyo) for generous provision of P19 cells, to Dr. D. Allsop for critical review of the manuscript, and to Mr. K. Kato for photographic assistance. This work was supported by Grants-in-aid for General Scientific Research and Special Project Research from the Ministry of Education, Science and Culture of Japan . Also supported by grants from Uehara Memorial Foundation, Shimabara Foundation, and Yamanouchi Foundation.

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