Vol. 182, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1992 Pages 165-173

OVEREXPRESSION OF AMYLOID PRECURSOR PROTEIN ALTERS ITS NORMAL PROCESSING AND IS ASSOCIATED WITH NEUROTOXICITY

Ken-ichiro Fukuchi,[1]* Kouzin Kamino,[1] Samir S. Deeb,[2] Annette C. Smith,[1] Thao Dang,[1] and George M. Martin[1]

[1]Department of Pathology SM-30 and [2]Department of Medicine, Division of Medical Genetics, University of Washington, Seattle, WA 98195

Received November 5, 1991

SUMMARY: The recent discovery that point mutations in the β/A4 amyloid precursor protein may be the cause of certain forms of familial Alzheimer's disease provides strong support for the view that a thorough understanding of the metabolism of this protein may elucidate the pathogenesis of most forms of the disease and thus serve as a basis for rational prevention and therapy. Here we show that overexpression of a portion of the amyloid precursor protein molecule produces at least four distinct fragments of the COOH-terminus of amyloid precursor protein, suggesting altered proteolysis of amyloid precursor protein, and that such overexpression is associated with cytotoxicity. The degree of toxicity in the P19 cell culture model (differentiating mouse embryonal carcinoma cells) is shown to be related to the two larger novel COOH-terminal protein fragments (16 and 14 kilodalton), as well as to levels of expression of these two fragments. The toxicity is manifested in several differentiated cell lineages, including neuronal cells.

The β/A4 amyloid polypeptide accumulates in the aging brains of a variety of mammalian species with contrasting maximum life-span potentials (1). Thus, the rates of its deposition appear to be coupled to intrinsic biological aging rather than merely to chronological time. Large quantities of this material are found within the cerebral blood vessels and neuritic plaques of patients with dementia of the Alzheimer's type, a disorder which exhibits an exponential increase in age-specific incidence in human subjects beginning at about age 60. Subjects born with an extra copy of chromosome 21, which harbors the structural gene for the β/A4 amyloid precursor protein (APP), develop this pathology prematurely, as do certain individuals who carry point mutations in the APP gene (2-4). Mutation at the affected codon is thought to impair translational regulation of the synthesis of APP, possibly resulting in overexpression of the protein (5).

*To whom requests for reprints and correspondence should be addressed.

ABBREVIATIONS: APP, amyloid precursor protein; Ara C, cytosine arabinoside; bp, base pairs; CMV, cytomegalovirus; kilodalton, kDa; RA, retinoic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

0006-291X/92 $1.50

Current evidence indicates that the normal processing of APP cannot result in the β/A4 peptide (6,7). It is possible, however, that an abnormal, minor mode of processing of the precursor protein might be responsible for amyloidosis and for the associated neurotoxicity (which may or may not be directly attributable to the β/A4 aggregates). If so, overexpression of the precursor could be responsible for accelerated rates of deposition of other potentially neurotoxic peptides, β/A4 amyloid, and Alzheimer's disease. To test this hypothesis, we performed overexpression experiments using a very strong promoter and enhancer and a mouse embryonic carcinoma cell line that can be differentiated into neuronal cells by the addition of retinoic acid (RA) (8).

MATERIALS AND METHODS

Construction of the pCA-SβC expression vector: The expression vector, pCA-SβC, was constructed to overexpress a part of APP (signal sequence + 42 amino acid β-amyloid sequence + COOH-terminal sequence of APP) under control of a cytomegalovirus (CMV) enhancer and a chick β-actin promoter. Using the LMG2 human brain stem λgt11 cDNA library (ATCC37432), a partial cDNA of the human APP, extending from base pairs (bp) 901-2851 (9) was isolated and cloned into pGEM -1 plasmid. The EcoRI/ClaI fragment (bp 1796-2473) (9) of the cDNA was subcloned into pBluescript KS^+ (Stratagene) at the same restriction enzyme sites. In a separate reaction, the signal peptide coding sequence for APP and the sequence for the first amino acids of β-amyloid protein (BamHI site + bp -4-51 + bp 1789-1794 + EcoRI site) was chemically synthesized. This fragment was ligated to the EcoRI/ClaI fragment (bp 1796-2473) of APP in the pBluescript KS^+ (Stratagene) using BamHI and EcoRI sites to create the pKS^+-SβC plasmid. This plasmid was digested with BamHI and SalI and the 0.8-kb fragment coding for signal + β amyloid + COOH-terminus of APP was isolated. The SalI site of the 0.8-kb fragment was ligated onto one of the XhoI sites of the expression vector, pCAGGS (10,11), which was previously digested with XhoI. The other ends were ligated after blunt end formation with the Klenow fill-in reaction to create pCA-SβC (Fig. 1).

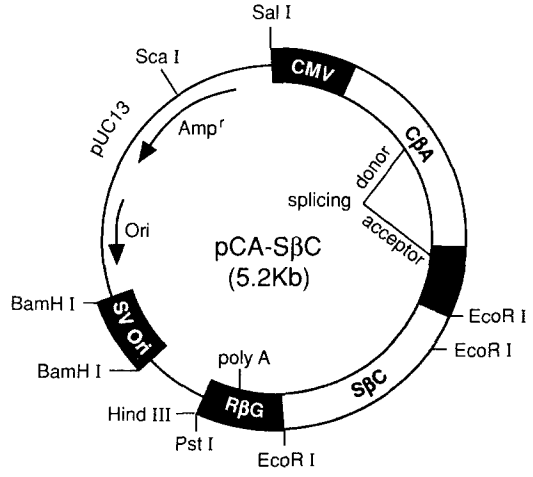


Fig. 1. *The pCA-SβC expression vector.* The expression vector, pCA-SβC, was constructed to overexpress a part of APP (signal sequence + 42 amino acid β-amyloid sequence + COOH-terminal sequence of APP) under control of a CMV enhancer and a chick β-actin promoter. CMV: Cytomegalovirus enhancer; CβA: chicken β-actin promoter and intron; SβC: signal sequence + 42 amino acid β-amyloid sequence + COOH-terminal sequence of APP; RβG: Rabbit β-globin sequence providing splicing acceptor and polyadenylation signal; SVOri: SV40 virus replication origin.

Cell culture: P19 (an embryonal carcinoma cell line) and COS (an African green monkey cell line) were were cultured in Dulbecco's modified Eagle's medium with 4.5 mg/ml D-glucose (GIBCO Laboratories) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. P19 cells were maintained and differentiated according to the methods described by Rudnicki and McBurney (8) with slight modification (12). To eliminate the proliferating non-neuronal cells, the medium was replaced with fresh medium containing 20 μM Ara C (Sigma) one day after plating the cells to tissue culture dishes.

Transient transfection: Five μg of pCA-SβC vector was transfected to the cells using liposome (Lipofectin , BRL) according to the manufacturer's protocol. The cells were lysed with extraction buffer after incubation for 72 hours.

Stable transformation: P19 cells were cotransfected with the pCA-SβC vector and the neomycin-resistant gene at a molar ratio of 10:1 using liposome (Lipofectin , BRL) according to the manufacturer's protocol. Fourteen clones were isolated by selection with 0.8 mg/ml of G418.

Southern blot analysis: Ten μg of DNA was digested with 3 times excess of a restriction enzyme (BamHI) and was separated by electrophoresis on 0.9% agarose gel. The ^{32}P-labeled, 1-kb EcoRI fragment of human APP cDNA (bp 901-2851) (9) was used as a probe.

Northern blot analysis: The total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (13) and 20 μg of the RNA on each lane was electrophoresed through a 1% agarose-formaldehyde gel (14). Both human (bp 901-2851) (9) and mouse (bp 9-1609) (15) APP cDNAs were radiolabeled using a random primed labeling kit (Boehringer Mannheim) with (α-^{32}P) dCTP to a specific activity of 2-4 x 10^8 cpm/μg and were used as probes at the same time.

Western blot analysis: Cells were washed with ice-cold phosphate-buffered saline solution twice, lysed with 2x Laemmli buffer [62.5 mM Tris pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue], and boiled for 5 min. After shearing the samples with a 26-gauge needle, protein concentration was determined by a protein assay (Bio-Rad). Twenty-five μg of the soluble fraction from COS cells and 100 μg from the P19 cell lines were applied to 12.5% or 14% SDS-PAGE (polyacrylamide gel electrophoresis) and electrotransferred to polyvinylidine difluoride membranes. Two different antisera, anti-R37 (16) and anti-C_1 (17) were used to detect COOH-terminal fragments of APP. Anti-R37 and anti-C_1 were raised against synthetic peptides of amino acid 681-695 of APP-695 and amino acid 676-695 of APP-695, respectively. The membranes were immunostained with the antisera using an avidin/biotin peroxidase system according to the manufacturer's protocol (Vector).

RESULTS AND DISCUSSION

COS cells were transfected by the construct and transiently expressed proteins were subjected to a immunoblot analysis (Fig. 2). We observed at least 4 fragments with different sizes of 11-16 kilodalton (kDa) in the transfected COS cells, but no fragments with corresponding sizes were seen on the blot in control COS cells using an antiserum (anti-R37) raised against synthetic COOH-terminal peptides, suggesting that these fragments were proteolytically produced from expressed protein from the introduced vector. Much larger fragments (indicated by arrows in Fig. 2) also were observed only in transfected COS cells, suggesting that the proteins produced from the vector bind each other to form aggregates. In the overexpression experiment using expression vectors for APP, or parts of APP, other investigators observed only 1 COOH-terminal fragment with the molecular weight of 11-16 kDa (8,18,19), 2 fragments (16 and 20 kDa) (20), or a high

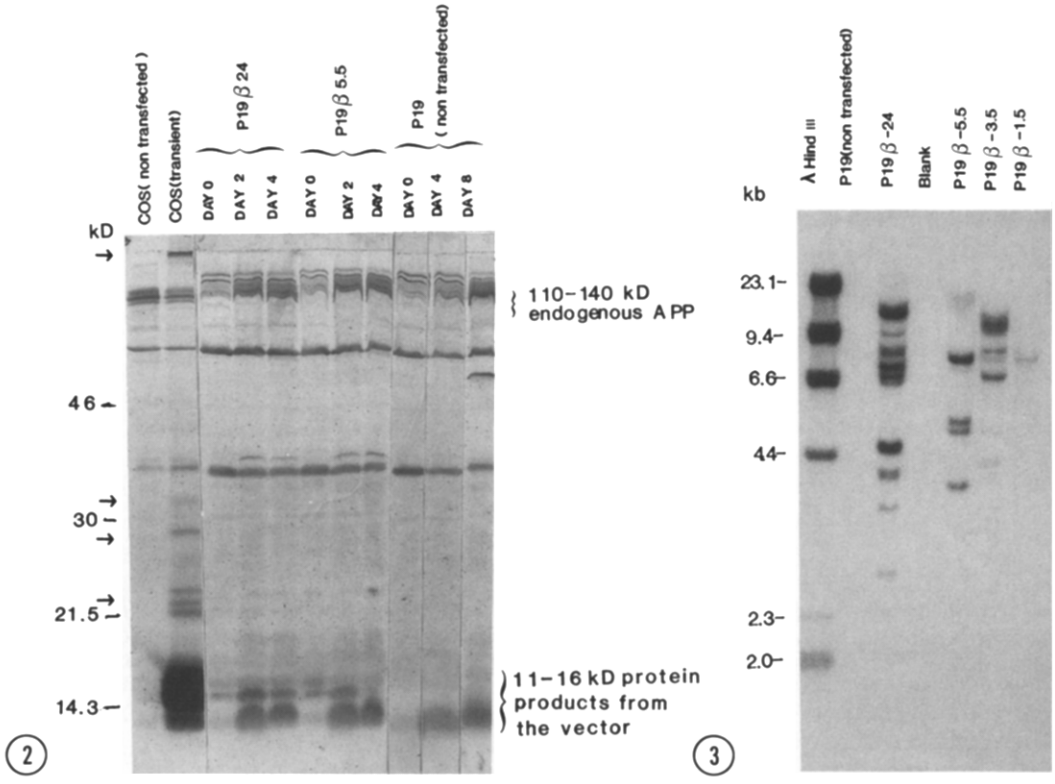


Fig. 2. *Western blot analysis of COS cells (transient) and P19 cells (stable) transfected with the pCA-Sβ C vector.* Twenty-five μg of the protein from COS cells and 100 μg from P19 cells were applied to 14% SDS-PAGE (polyacrylamide gel electrophoresis) and electrotransferred to polyvinylidine difluoride membranes. The membranes were immunostained with the antiserum, anti-R37, using an avidin/biotin peroxidase system according to the manufacturer's protocol (Vector). The arrows indicate aggregates of COOH-terminal fragments expressed from the vector.

Fig. 3. *Southern blot analysis of P19 stably transformed cell lines.* P19 cells were cotransfected with pCA-Sβ C vector and the neomycin-resistant gene and stably transformed cell lines were isolated by selection with 0.8 mg/ml of G418. Ten μg of DNA from each cell line was digested with BamHI and was separated by electrophoresis on 0.9% agarose gel. The radiolabeled 1-kb EcoRI fragment of human APP cDNA (bp 901-2851 of APP-695) was used as a probe. Under the washing condition used, the probe does not hybridize with a mouse APP gene.

molecular aggregated mass (>110 kDa) (21) on immunoblots using antisera against the COOH-terminus of APP. On the other hand, when a part of APP is overexpressed in cultured cells under the very strong promoter, we observed at least 4 different fragments and these aggregates, suggesting that overexpression of APP brings about aberrant proteolysis, including amyloidogenic protein. In the very recent report of Golde *et al.* (22), a set of at least five 8-12 kDa COOH-terminal fragments was also observed using human embryonic kidney (293) cells in which full-length cDNA for APP-695 was overexpressed and the two larger fragments were labeled with antibodies raised against the first 17 amino acids of the β/A4 amyloid protein, corresponding to our observations.

To examine neurotoxicity of the proteins produced from the vector, P19 cells (an embryonic carcinoma cell line) were cotransfected with the expression vector and a

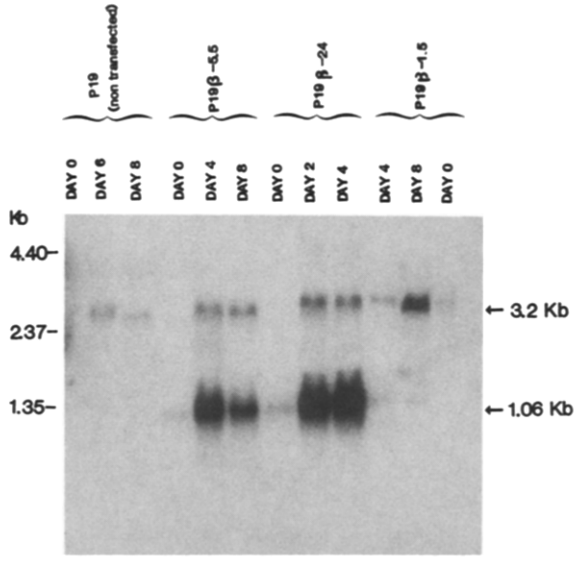


Fig. 4. *Northern blot analysis of stably transformed P19 cell lines.* Twenty μg of the total RNA from each cell line at each differentiation day was electrophoresed through a 1% agarose-formaldehyde gel followed by capillary transfer to nylon membrane. The blot was hybridized with both the radiolabeled human cDNA (bp 901-2851 of APP-695) and mouse (bp 9-1609 of APP-770).

neomycin gene (molar ratio 10:1) and 9 neo^r clones were isolated. Integration and approximate numbers of the vector in the clones were studied by Southern blot analysis (Fig. 3). Different restriction enzyme patterns among these clones suggested that each clone was independent and that multiple copies of the vector were integrated in the distinct sites of the genomes of the cell lines, except for P19 β1.5. These clones were treated with RA (for neuronal differentiation); proliferating non-neuronal cells were eliminated by treatment with cytosine arabinoside (Ara C). Levels of expression in the clones from the vector were studied by Northern blot (Fig. 4) and Western blot analyses (Figs. 2 and 5). Previously we showed that the induction of APP coincided with neurite outgrowth during the neuronal differentiation of the P19 cells (12). Levels of expression from the integrated vectors also increased after treatment with RA. Substantial induction of mRNAs and proteins from the vector was seen in the β5.5, β24 and β3.5 (data not shown) cell lines (Fig. 4). On the other hand, only a small increase of mRNA was observed in β1.5 (Fig. 4) and β15 (data not shown). The levels of expression of mRNA from the vectors were consistent with the levels of expressed proteins (Figs. 2, 4, and 5), except for β5.5 at day 4 of differentiation. As seen in COS cells, at least 4 COOH-fragments with different sizes were also detected in stably transformed P19 cell lines, using antisera, anti-R37 (Fig. 2) and anti-C_1 (Fig. 5), but with differing levels of expression and combinations of fragments.

The larger 2 fragments (16 and 14 kDa) were observed only in transformed cell lines (Fig. 2) and increased during neural differentiation except for the P19 β5.5 cell line. On the other hand, the smaller 2 fragments (13 and 11 kDa) were observed in all cell lines,

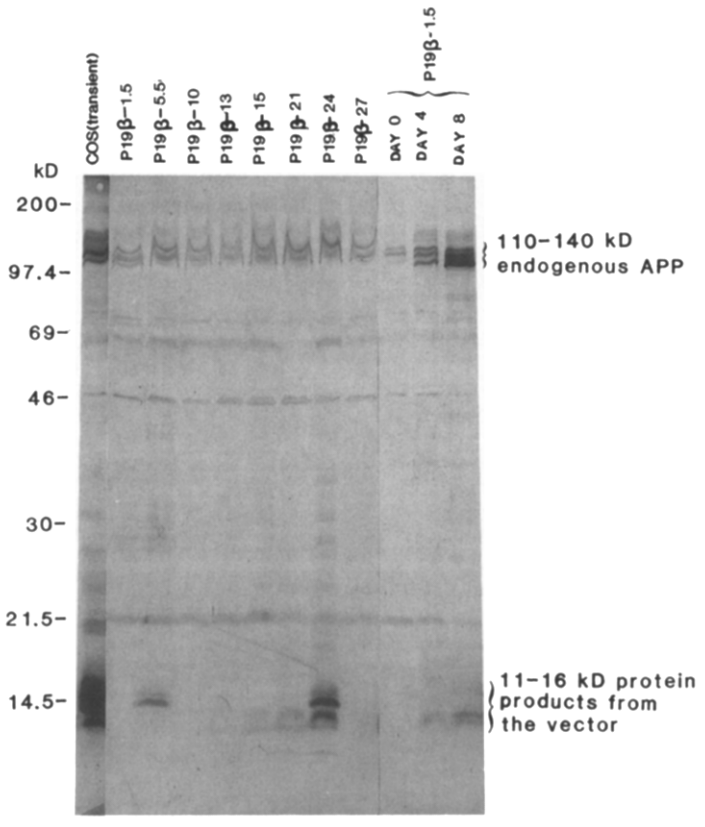
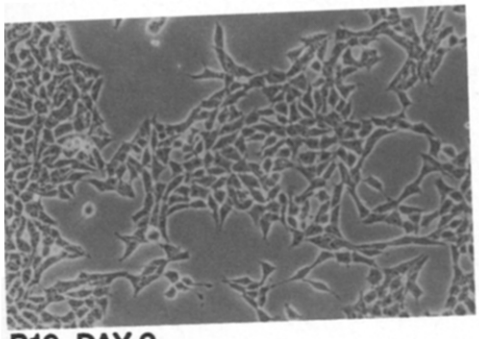


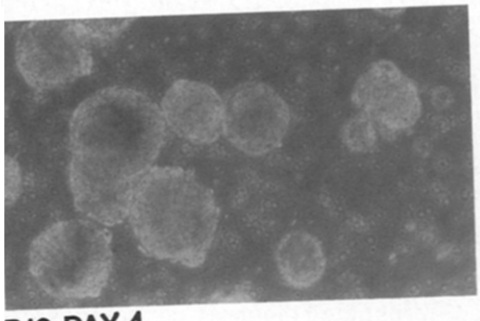
Fig. 5. *Western blot analysis of stable transformants of P19 cells.* An antiserum, anti-C_L (1:50 dilution) (17), raised against synthetic peptide of amino acids 676-695 of APP-695, was used. On each lane, 100 μg of the soluble fraction from the P19 cell line was applied to 12.5% SDS-PAGE.

including nontransfected P19 cells after treatment with RA and increased during neural differentiation (Figs. 2 and 5). The transformed cell lines (β1.5, β3.5, β21, and β24), in which the levels of expression of both mRNA and the larger 2 fragments increased during differentiation, degenerated to various extents. The degree of degeneration appeared to be related to the degree of expression of the larger fragments. P19 β24, which produced the larger 2 fragments in greatest abundance, exhibited the most dramatic evidence of toxicity. Almost all P19 β24 cells which were treated with RA died out within 4 days before treatment with Ara C (Fig. 6). The P19 β3.5 cell line also died out within 5-6 days.

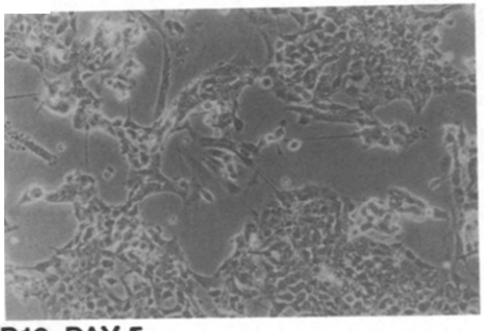
Fig. 6. *Phenotypic changes of stable transformants of P19 cells after treatment with RA.* (**A**) Morphological changes during neuronal differentiation of parental P19 cells. DAY 0: undifferentiated P19 cells. DAY 4: cell aggregates 4 days after treatment with RA. DAY 5: neuronal processes appear on some cells. DAY 10: networks of neuronal processes and aggregates of neurons at the centers of the networks. (**B**) Degeneration of P19 β24 cell line. DAY 0: P19 β24 cell, the cells are not treated with RA and are morphologically indistinguishable from nontransfected P19 cells at day 0. DAY 4: Four days after treatment with RA, degenerating cells were seen in small jagged aggregates of cells. DAY 5: Very small numbers of cells with many vacuoles are seen to be attached to the bottom of the dish. (**C**) Terminally differentiated neurons from P19 β5.5. DAY 10: The cells are very similar to neurons derived from nontransfected P19 cells.



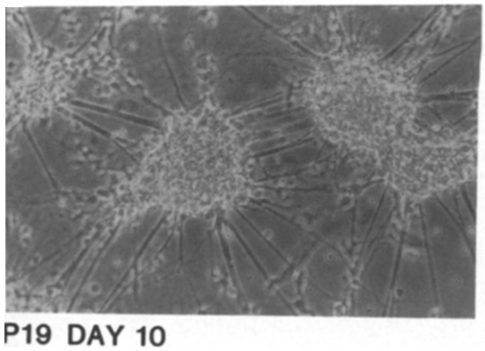
P19 DAY 0



P19 DAY 4

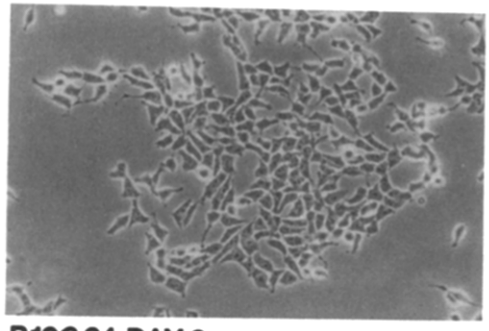


P19 DAY 5

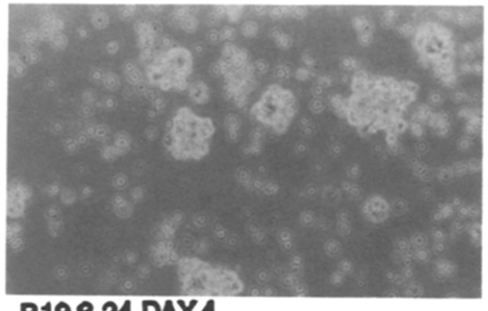


P19 DAY 10

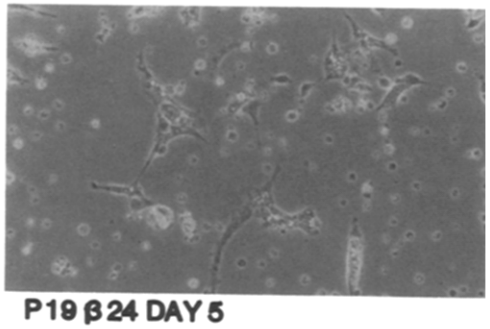
B



P19β 24 DAY 0

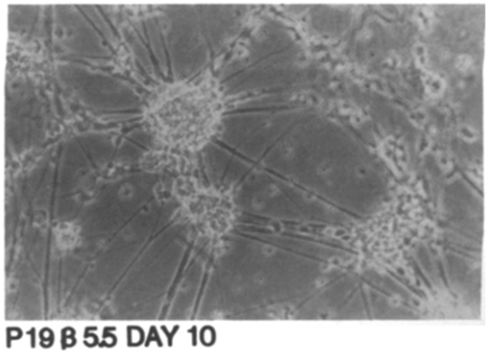


P19β 24 DAY 4



P19β 24 DAY 5

C



P19β 5.5 DAY 10

P19 β1.5 and β15 began to degenerate at days 9-14 (data not shown). In our laboratory, neurons derived from nontransfected P19 cells can be cultured for 2-3 weeks without degeneration.

On the other hand, β5.5 showed an exceptional phenotype and unusual expression of the integrated vector. Changes of morphology of β5.5 during differentiation was very similar to those of nontransfected P19 cells, and β5.5 survived more than 14 days. In β5.5, the larger 2 fragments were observed for the first 3 days of differentiation and, thereafter, were barely detected (Fig. 2). The levels of expression of mRNA from the vector in β5.5 remarkably increased at day 4 of its differentiation and decreased thereafter (Fig. 4). The unusual expression pattern of the integrated vector in β5.5 and the discrepancy between the expression levels of the mRNA and those of the two larger fragments at day 4 of β5.5 may be explainable by changes of transcriptional and translational regulation and proteolysis of APP during the neural differentiation. The degree of degeneration in any of these transformed cell lines was consistent with the degree of expression of the 2 larger fragments.

The nature of the effects of the β/A4 amyloid protein on neuronal cells are disputed. Neurotoxic effects of fragments expressed in stably transformed PC12 rat pheochromocytoma cells have been reported (21). On the other hand, neurotrophic effects of the synthesized amyloid peptides were also reported (23,24). It was also shown that the amyloid peptides were neurotrophic to undifferentiated hippocampal neurons at low concentrations and were neurotoxic to mature neurons at high concentrations, using a variety of portions of synthesized amyloid peptides (25). We did not observe any obvious neurotrophic effects of the expressed proteins to P19 cells at the early stage of differentiation; instead, we observed neurotoxic effects of the protein to neurons at the early stages of differentiation. We did not observe neurotoxic or neurotrophic effects of conditioned media from β24 and β3.5 (data not shown).

Since P19 cells have been shown to differentiate not only to neurons but also to glial and fibroblast-like cells (8), and since these non-neuronal cells also degenerate before treatment with Ara C (Fig. 6), the fragments produced may have toxicity for a number of somatic cell types.

The results described in this paper suggest that neurotoxic fragments can be produced by altered cleavage of APP. In future work, it will be of interest to determine the precise altered cleavage sites of APP by amino acid sequencing of the COOH-terminal fragments found in the transformed cell lines and to create adult brain mouse chimeras by neurotransplantation of these transformed cell lines.

ACKNOWLEDGMENTS: We thank J. Miyazaki for providing the pCAGGS vector, D. Selkoe for the anti-C_1 antiserum, T. Ishii for the anti-R37 antiserum, M. McBurney for providing P19 and J. Garr for preparing the manuscript. This work was supported by grants AG05136, AG00057, and AG01751 from the National Institutes of Health (GMM) and by grant II RG-90-098 from the Alzheimer's Disease and Related Disorders Associations, Inc. (KF).

REFERENCES

1. Selkoe, D.J., Bell, D.S., Podlisny, M.B., Price, D.L., and Cork, L.C. (1987) Science 235, 873-877.
2. Goate, A., Chartier-Harlin, M.-C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C., Pericak-Vance, M., Roses, A., Williamson, R., Rossor, M., Owen, M., and Hardy, J. (1991) Nature 349, 704-706.
3. Naruse, S., Igarashi, S., Kobayashi, H., Aoki, K., Inuzuka, T., Kaneko, K., Shimizu, T., Iihara, K., Kojima, T., Miyatake, T., and Tsuji, S. (1991) Lancet 337, 978-979.
4. Murrell, J., Farlow, M., Ghetti, B., and Bensom, M.D. (1991) Science 254, 97-99.
5. Tanzi, R.E. and Hyman, B.T. (1991) Nature 350, 564.
6. Sisodia, S.S., Koo, E.H., Beyreuther, K., Unterbeck, A., and Price, D.L. Science 248, 492-495.
7. Esch, F.S., Keim, P.S., Beattie, E.C., Blacher, R.W., Culwell, A.R., Oltersdorf, T., McClure, D., and Ward, P.J. (1990) Science 248, 1122-1124.
8. Rudnicki, M.A., and McBurney, M.W. (1987) In Teratocarcinoma and Embryonic Stem Cells: A Practical Approach (E.J. Robertson, Ed.), pp. 19-50. IRL Press, Washington, DC.
9. Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grezeschik, K.H., Multhaup, G., Beyreuther, K., and Müller-Hill, B. (1987) Nature 325, 733-736.
10. Miyazaki, J.-i., Takaki, S., Araki, K., Tashiro, F., Tominaga, A., Takatsu, K., and Yamamura, K.-i. (1989) Gene 79, 269-277.
11. Takaki, S., Tominaga, A., Hitoshi, Y., Mita, S., Sonoda, E., Yamaguchi, N., and Takatsu, K. (1990) EMBO J. 9, 4367-4374.
12. Fukuchi, K., Deeb, S.S., Kamino, K., Ogburn, C.E., Snow, A.D., Sekiguchi, R.T., Wight, T.N., Piussan, H., and Martin, G.M. (1991) J. Neurochem, in press.
13. Chomczynski P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
14. Lehrach, H., Diamond, D., Wozney, J.M., and Boedtker, H. (1977) Biochemistry 48, 4743-4751.
15. Yamada, T., Sasaki, H., Furuya, H., Miyata, T., Goto, I., and Sakaki, Y. (1987) Biochem. Biophys. Res. Commun. 149, 665-671.
16. Ishii, T., Kametani, F., Haga, S., and Sato., M. (1989) Neuropathol. Appl. Neurobiol. 15, 135-147.
17. Selkoe, D.J., Podlisny, M.B., Joachim, C.L., Vickers, E.A., Lee, G., Fritz, L., and Oltersdorf, T. (1988) Proc. Natl. Acad. Sci. USA 85, 7341-7345.
18. Maruyama, K., Terakado, K., Usami, M., and Yoshikawa, K. (1990) Nature 347, 566-569.
19. Ramakrishna, N., Saikumar, P., Potempska, A., Wisniewski, H.M., and Miller, D.L. (1991) Biochem. Biophys. Res. Commun. 174, 983-989.
20. Wolf, D., Quon, D., Wang, Y., and Cordell, B. (1990) EMBO J. 9, 2079-2084.
21. Yankner, B.A., Dawes, L.R., Fisher, S., Villa-Komaroff, L., Oster-Granite, M.L., and Neve, R.L. (1989) Science 245, 417-420.
22. Golde, T.E., Estus, S., Younkin, L., Usiak, M., and Younkin, S.G. (1991) Soc. Neurosci. Abstr. 17 (Part 2), 1293.
23. Whitson, J.S., Selkoe, D.J., and Cotman, C.W. (1989) Science 243, 1488-1490.
24. Whitson, J.S., Glabe, C.G., Shintani, E., Abcar, A., and Cotman, C.W. (1990) Neurosci. Lett. 110, 319-324.
25. Yankner, B.A., Duffy, L.K., and Kirschner, D.A. (1990) Science 250, 279-282.