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Increased gene expression of Alzheimer disease β-amyloid precursor protein in senescent cultured fibroblasts

(aging)

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ABSTRACT **The pathological hallmark of Alzheimer disease is the accumulation of neurofibrillary tangles and neuritic plaques in the brains of patients. Plaque cores contain a 4- to 5-kDa amyloid β-protein fragment which is also found in the cerebral blood vessels of affected individuals. Since amyloid deposition in the brain increases with age even in normal people, we sought to establish whether the disease state bears a direct relationship with normal aging processes. As a model for biological aging, the process of cellular senescence *in vitro* was used. mRNA levels of β-amyloid precursor protein associated with Alzheimer disease were compared in human fibroblasts in culture at early passage and when the same fibroblasts were grown to senescence after more than 52 population doublings. A dramatic increase in mRNA was observed in senescent IMR-90 fibroblasts compared with early-passage cells. Hybridization of mRNA from senescent and early proliferating fibroblasts with oligonucleotide probes specific for the three alternatively spliced transcripts of the gene gave similar results, indicating an increase during senescence of all three forms. A similar, though more modest, increase in message levels was also observed in early-passage fibroblasts made quiescent by serum deprivation; with repletion of serum, however, the expression returned to previous low levels. ELISAs were performed on cell extracts from senescent, early proliferating, and quiescent fibroblasts, and quiescent fibroblasts repleted with serum for over 48 hr, using polyclonal antibodies to a synthetic peptide of the β-amyloid precursor. The results confirmed that the differences in mRNA expression were partially reflected at the protein level. Regulated expression of β-amyloid precursor protein may be an important determinant of growth and metabolic responses to serum and growth factors under physiological as well as pathological conditions.**

A major constituent of the cerebrovascular deposits in the brains of patients with Alzheimer disease (AD) is the β-amyloid protein (1), a self-aggregating protein that consists of 39 or 40 amino acids. Cloning and cDNA sequencing have indicated that the amyloid protein is encoded as part of a larger precursor by a gene located on chromosome 21 (2–4). This gene gives rise to at least three alternatively spliced mRNAs (4–6). Their corresponding proteins (henceforth to be called APP for amyloid precursor protein) consist of 695 (APP 695), 751 (APP 751), and 770 (APP 770) amino acids. The longer forms have inserts whose sequences are similar to a Kunitz-type serine-protease inhibitor.

The predicted amino acid sequence of APP suggests a large extracellular domain, a single membrane-spanning region, and a small cytoplasmic tail (2). *In situ* hybridization studies indicate that within the brain it is expressed predominantly in neurons (7, 8); it is also expressed in a variety of other tissues (4).

Since amyloid deposition in the brain increases with age even in normal people (9), we sought to establish whether the disease state bears a direct relationship to normal aging processes. As a model for biological aging, the process of cellular senescence *in vitro* was used. When fibroblasts are passaged in culture over many generations, they eventually reach a stage where they remain viable but are permanently unable to replicate (10). Such fibroblasts are considered "senescent." Fibroblasts taken from young individuals, moreover, have a greater total doubling capacity compared to fibroblasts derived from older individuals (11). Thus cellular senescence is taken to represent some part of the multifactorial changes which underlie aging.

In this paper we present data that show a dramatic increase in amyloid mRNA and a corresponding more modest increase in protein in nondividing senescent cultured fibroblasts compared with early-passage proliferating fibroblasts. Furthermore, this increase can be reversibly induced, to a lesser extent, in fibroblasts made quiescent by serum deprivation.

MATERIALS AND METHODS

Materials. With the exception of the following, all chemicals were purchased from Sigma. [^{32}P]dCTP (1000 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham; cell-culture supplies, from Irvine Scientific; Sepharose 4B, from Pharmacia; Immulon II plates, from Dynatech; and peroxidase-conjugated goat anti-rabbit IgG, from Bio-Rad.

Cell Culture. Human foreskin fibroblasts from a newborn were passaged in 750-cm^3 roller bottles in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin/streptomycin (5% CO_2 atmosphere at 37°C). Cells were harvested at population doubling (pd) 10–12 and then at pd 40–42 (18 months later) by trypsinization. A second fibroblast line, IMR-90, was obtained from the American Type Culture Collection at passages 10 and 40. The younger cells were passaged to pd 14 and the older cells were passaged to pd 55–60 (at which time they were senescent).

An objective measure of senescence was obtained by serial [^{3}H]thymidine incorporation experiments (12). When doubling times became greater than 4 weeks, the cells had a flattened spread-out morphology and incorporated approximately 10% of the [^{3}H]thymidine incorporated by fibroblasts in early proliferation. Serum-deprived quiescent fibroblasts similarly incorporated lower amounts of [^{3}H]thymidine.

Fibroblasts were made quiescent by changing the fetal bovine serum concentration from 10% to 0.5% for greater

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Abbreviations: APP, amyloid precursor protein; AD, Alzheimer disease; SAP, sphingolipid activator protein; KPI, Kunitz protease inhibitor; BSA, bovine serum albumin.

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than 72 hr. Twenty-four hour [^{3}H]thymidine uptake under these conditions was typically less than 10% of control proliferating cells.

Isolation of Poly(A)$^+$ RNA. mRNA was prepared utilizing a proteinase K/SDS-based cell lysis followed by an oligo(dT)-cellulose separation (type 2, Collaborative Research) as previously described (13).

cDNA Library Construction. cDNA was synthesized from poly(A)$^+$ RNA according to the method of Gubler and Hoffman (14), using a commercially available cDNA synthesis kit (Boehringer Mannheim). Briefly, cDNA was synthesized by using poly(A)$^+$ RNA isolated from cultured human foreskin fibroblasts at passages 14 and 37. The cDNA·mRNA hybrid was treated with RNase H and DNA polymerase 1. Double-stranded cDNA was then treated with *Eco*RI methylase and *S*-adenosylmethionine to protect *Eco*RI cleavage sites. The cDNA was then blunt-ended with DNA polymerase I and *Eco*RI linkers (New England Biolabs) were ligated onto the ends. Digestion with *Eco*RI was performed, followed by Sepharose CL-4B (Sigma) chromatography to remove excess linkers. Double-stranded cDNA was pooled and ligated, with EcoRI-digested λgtII DNA and packaged *in vitro* (Amersham packaging kit).

Hybridization of Radiolabeled Probes with Proliferating, Quiescent, and Senescent IMR-90 mRNA. mRNA from proliferating and senescent fibroblasts (100 ng per slot) was slot-blotted onto nitrocellulose. Hybridizations were carried out with a β-amyloid cDNA fragment [base pairs (bp) 1795–2856 of the Kang *et al.* sequence (2)], radiolabeled with [^{32}P]dCTP by random priming (5×10^8 cpm/μg) in 50% (vol/vol) formamide at 42°C for 12 hr followed by high-stringency washes at 57°C. Control hybridizations were carried out with cDNA probes of sphingolipid activator protein (SAP) (15) and PG-40, the small dermatan sulfate/chondroitin sulfate proteoglycan (16). Oligonucleotide probes were constructed to the sequences flanking the Kunitz protease inhibitor (KPI) insert, which would hybridize to the insert-free APP 695 [bp 851–880 (2); probe 1]; to the KPI insert region [bp 902–927 (5); probe 2]; to an additional 19 amino acid sequence adjacent to the KPI insert [bp 1059–1084 (5); probe 3]; and to the junction region spanning the KPI sequence and the additional 19 amino acid sequence [bp 1016–1046 (5); probe 4]. These were 5′-end-radiolabeled by treatment with T4 polynucleotide kinase, using [γ-^{32}P]ATP (3000 Ci/mmol).

Peptide Synthesis and Immunizations. The peptide employed for this study was APP residues 556–576 (ANTENEVEPVDARPAADRGLY), where a tyrosine replaced threonine at residue 576 of the Kang *et al.* (2) sequence. The peptide was synthesized by using the Merrifield solid-phase method, and the crude product was purified by preparative high-performance liquid chromatography (17). Peptides were conjugated to the carrier protein bovine serum albumin (BSA) via tyrosine residues. Conjugated and unconjugated peptide fragments were utilized as antigen in dot-blot testing of antibodies, in ELISAs, and for blocking.

Antibodies. Rabbits were immunized with conjugated peptide emulsified in Freund's adjuvant and were boosted essentially as described (18). After at least three boosts, serum samples were affinity purified. To immobilize the immunizing peptide, 30 mg dissolved in 2 ml of 0.1 M NaOH was coupled to epoxy-activated Sepharose 6B (Pharmacia/LKB) for 16 hr at room temperature. Preparations and washing of the gel, including blocking of excess reactive groups, was done according to the manufacturer's recommendations. Serum samples (15 ml) were diluted with 1 vol of Dulbecco's phosphate-buffered saline (PBS), clarified by centrifugation, and passed over a 1-ml column. The column was washed with PBS and bound antibodies were eluted with 0.1 M glycine buffer, pH 2.8, containing 1 M KCl. Fractions containing the affinity-purified antibody were identified by binding to BSA-peptide conjugates in a dot-blot assay, pooled, and adjusted to a BSA concentration of 1 mg/ml. Aliquots were stored at −80°C, and once thawed they were kept at 4°C for use. Typically, use of 15 ml of serum led to 4 mg of affinity-purified antibody. A monoclonal antibody to peptide 604–613 was also used to confirm ELISA results, and was a kind gift from David Allsopp (Psychiatric Institute of Tokyo).

RESULTS

Characterization of cDNA Libraries. Plating of the resulting phage on *Escherichia coli* Y1088 in the presence of isopropyl β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) revealed a total of 2×10^5 and 2.5×10^5 plaque-forming units for the senescent and proliferating libraries (85% and 92% recombinants), respectively. *Eco*RI digestion revealed inserts with sizes ranging from 250 bp to 1.5 kilobases (kb) for both libraries. Dot-blot analysis of cDNA inserts with ^{32}P-labeled PG-40 and SAP cDNA probes showed equal representation of each within the two libraries.

Immunospecificity of Antibody 556–576. Immunospecificity of the precipitated bands was established by several criteria. Blocking of antibodies with specific peptides was carried out, and a preclearing step with preimmune serum was also performed. Apart from a band at 46 kDa, all the bands that antibody 556–576 immunoprecipitated from both culture medium and cell extract were not precipitated when specific peptide was added. This has been described in detail as part of another manuscript (19). Immunoprecipitation of all the bands by a monoclonal antibody to residues 604–613 could be blocked by specific peptide (not shown). This antibody was used to confirm ELISA results obtained with anti-556–576.

Expression of β-Amyloid in Early Proliferating and Senescent Foreskin Fibroblast cDNA Libraries. Slot-blot analysis of hybridization of the cDNA libraries from proliferating and senescent human foreskin fibroblasts with ^{32}P-labeled APP cDNA revealed strikingly more APP cDNA represented in the senescent fibroblast library than in the proliferating fibroblast library (data not shown). Since the abundance of any cDNA in a library may be altered by a variety of factors, a direct comparison of mRNA levels in human fibroblast cell line IMR-90 was undertaken. Hybridization of mRNAs in a slot-blot (100 ng of mRNA per slot) with ^{32}P-labeled APP cDNA revealed dramatically more amyloid message in senescent fibroblasts than in early proliferating fibroblasts (Fig. 1). Equal amounts of mRNA for comparison were ensured both by A_{260} readings and by parallel lanes probed with two cDNAs, for SAP-1 and PG-40, both of whose expression is cell-cycle independent. Densitometric analysis of autoradiographs from five separate experiments revealed a greater than 17-fold mean increase in intensity of signal from senescent fibroblasts, $P < 0.001$.

mRNA Expression of APP 695, 751, and 770 in Proliferating, Quiescent, and Senescent IMR-90 Fibroblasts. Since three alternatively spliced APP transcripts have been shown to be present in humans, coding for proteins that consist of 695 (APP 695), 751 (APP 751), and 770 (APP 770) amino acids, hybridizations were performed to assess the difference in expression of these mRNAs in proliferating, senescent, and quiescent fibroblasts. In slot-blots of mRNA, a significantly greater signal from senescent than from either proliferating or quiescent samples was found (18- to 40-fold increase shown by densitometric analysis, $P < 0.001$ for probes 1, 2, and 4; $P < 0.015$ for probe 3) (Fig. 2). Thus, the previous finding of increased APP in senescent cells was a sum of the separate increase in all three alternative APP forms. A comparison of hybridization of all four oligonucleotide probes to quiescent and proliferating mRNA analyzed by densitometry showed higher values for quiescent fibroblasts with every probe. The

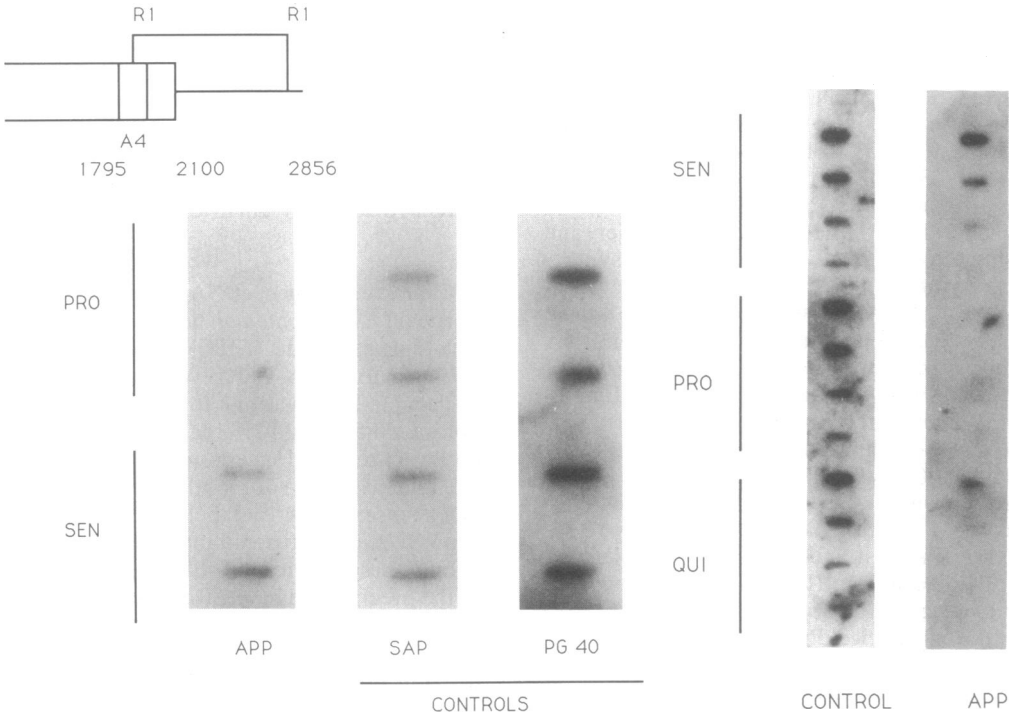

FIG. 1. (*Left*) Hybridization of mRNA from proliferating (PRO) and senescent (SEN) IMR-90 fibroblasts with APP cDNA. mRNA (100 ng per slot) was blotted in duplicate onto nitrocellulose and hybridized with random-primed ^{32}P-labeled APP cDNA (bp 1795–2856 of the Kang *et al.* sequence (2), schematically shown on the top left-hand corner, with R1 indicating *Eco*RI sites) (lane 1), SAP cDNA (1-kb *Eco*RI 3′ fragment) (lane 2) (15), and 1.8-kb full-length cDNA for proteoglycan PG-40 (16) (lane 3). The mean densitometric value for signal with APP cDNA was 0.23 for PRO and 4.0 for SEN mRNA; the difference was significant at $P < 0.001$. (*Right*) Serial dilutions of mRNA from proliferating (PRO), quiescent (QUI), and senescent (SEN) IMR-90 fibroblasts. mRNA was slot-blotted onto nitrocellulose in decreasing amounts (100, 33, 10, and 3 ng from top to bottom) from the fibroblasts and hybridized with ^{32}P-labeled SAP (control) and APP cDNA.

differences with probes 1 and 2 were statistically significant at $P < 0.17$ and 0.08, respectively, $n = 8$.

Expression of APP in Senescent, Quiescent, and Proliferating IMR-90 Fibroblasts. To investigate whether the differences in mRNA observed were reflected at the protein level, ELISAs were performed on extracts of fibroblasts that were proliferating, senescent, quiescent, and quiescent repleted with serum for over 48 hr, using antibodies to a synthetic peptide of the APP sequence common to all three forms (amino acids 556–576) (2). The results showed significantly more APP in senescent than in proliferating fibroblasts ($P < 0.05$, $n = 6$) (Fig. 3). Furthermore, quiescent fibroblasts also expressed significantly more APP than proliferating fibroblasts, but with serum repletion these same cells expressed APP levels that were not significantly different from levels found in the proliferating cells. When fibroblast culture

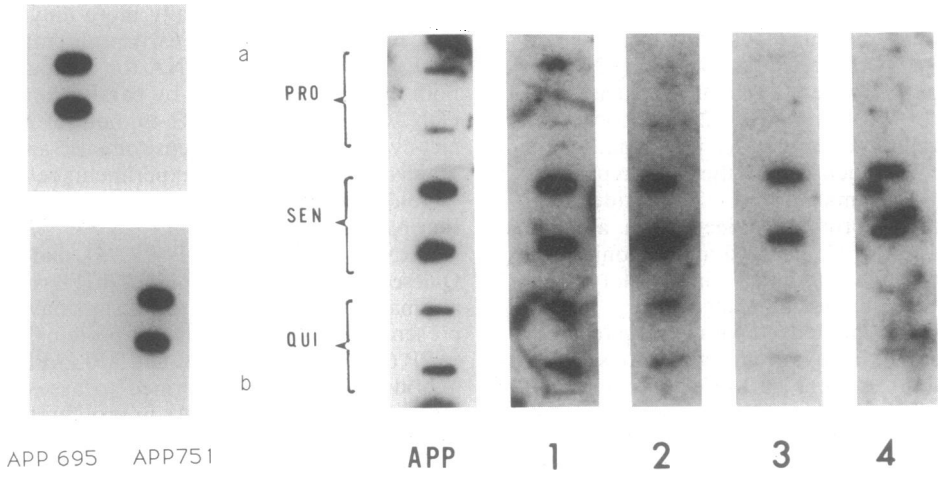

FIG. 2. (*Left*) Specificity of probes. Full-length cDNA (150 ng per slot) from APP 695 (non-insert-bearing) and APP 751 (insert-bearing) was blotted onto nitrocellulose in duplicate. (*a*) Hybridization with a ^{32}P-labeled oligonucleotide probe synthesized to bp 851–880 of APP 695 (probe 1). (*b*) Hybridization with a ^{32}P-labeled oligonucleotide probe synthesized to bp 902–927 of the Kunitz insert region (probe 2). (*Right*) Hybridization of mRNA from proliferating (PRO), senescent (SEN), and quiescent (QUI) IMR-90 fibroblasts with oligonucleotide probes specific to APP 695, APP 751 and APP 770. mRNA (100 ng per slot) was hybridized with APP cDNA (lane APP) and oligonucleotide probes 1–4 (lanes 1–4, respectively), where probe 1 was synthesized to bp 851–880 of the insert-free APP 695, probe 2 was synthesized to bp 902–927 of the Kunitz insert region, probe 3 was synthesized to bp 1059–1084 of the additional 19 amino acids adjacent to the Kunitz insert, and probe 4 was synthesized to bp 1016–1046, representing a region that would form a junction between the Kunitz insert and the additional 19 amino acids.

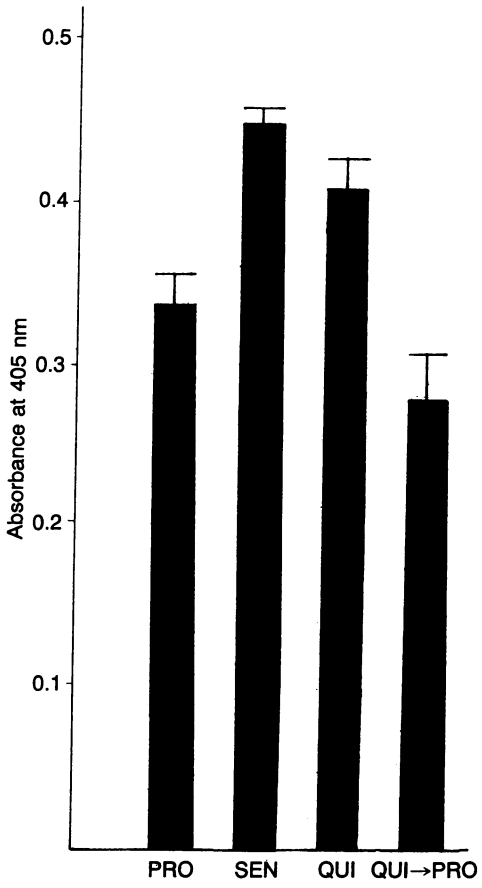

FIG. 3. Absorbance at 405 nm of IMR-90 fibroblast extracts with antibodies to APP residues 556–576. Fibroblasts were sonicated in water and total cell extract was used for ELISAs. One microgram of protein was plated and allowed to react with antibodies. Peroxidase-conjugated goat anti-rabbit IgG was added as the secondary antibody and product of the peroxidase reaction was measured by absorbance at 405 nm. PRO, SEN, QUI, and QUI → PRO represent the mean A_{405} of 5 samples from fibroblasts that were proliferating, senescent, quiescent, and quiescent replenished with serum, respectively. Bars represent SEM.

medium was examined, an increase of the same approximate order as found in cell extracts was found in secreted APP from quiescent and senescent fibroblasts over proliferating cells. Since antibodies to peptide 556–576 recognize a non-specific 46-kDa band, the results were confirmed on ELISAs with a monoclonal antibody to APP peptide residues 604–613. This is an immunospecific antibody that does not recognize the 46-kDa band. Parallel findings were observed, with an increase with quiescence and senescence in IMR-90 fibroblasts of the same approximate order (1.75-fold), and a decrease to levels observed for proliferating fibroblasts following repletion of serum.

DISCUSSION

The findings presented in this paper suggest the possibility that β-amyloid deposition, believed to be a very early change in AD and Down syndrome brain, may at least in part be due to a more generalized increase in synthesis of the precursor protein with age. This suggests that β-amyloid deposition may not require preceding neuronal injury. It also suggests that abnormalities previously reported in the fibroblasts of normal aged people and AD patients (20, 21) could relate in part to progressive deposition of this protein. These findings are consistent with a recent observation of elevated β-amyloid in the skin and intestine of AD patients and three normal aged controls, each over the age of 77 years (22). It should be pointed out that while our results show a dramatic increase in mRNA in senescent IMR-90 fibroblasts (approximately 17-fold), the increase in protein expression is of a much lower order (approximately 1.5-fold). The increase of secreted protein is also of the same low order. Further experiments are necessary to determine if there are significant differences in the rates of synthesis and degradation of the protein in senescent versus proliferating cells and whether this is altered in AD.

While the present findings offer the possibility for use of APP as a marker for cellular senescence, the question of a direct causal role in growth regulation remains unresolved. The tempting assumption that accumulation of APP leads to cell-cycle arrest, the hallmark of senescence, is too simplistic. In fact, a dichotomy in evidence is emerging from various reports and has contributed towards greater complexity. In recent reports, overexpression of β-amyloid by PC 12 cells led to decreased survival (23), degeneration, and cell death (24); it has recently been reported that increased APP mRNA accompanies the confluent state in epithelial cells (25). These observations are somewhat difficult to reconcile with a report that fibroblasts harboring an antisense construct to APP mRNA grew poorly and could be salvaged by the addition of either parent cell-conditioned medium or purified APP (26), and another report in which APP 751 and 770 were found to be mitogenic for Swiss 3T3 cells (27). In addition, a variety of cell types which overexpress β-amyloid are capable of propagating in culture (28), and peptide ligands homologous to the first 28 and 42 residues of β-amyloid have demonstrated trophic effects upon hippocampal neurons (29, 30). The latter is concordant with the observation that extracts from AD brain show more trophic effect upon cortical cells in culture than do extracts from normal brain (31).

The recent identification of APP containing the KPI domain as protease nexin II (PN-II) has expanded the speculation on its function (32). PN-II complexes with epidermal growth factor binding protein, and this may potentially influence a variety of cellular processes. In addition, it binds to the γ subunit of nerve growth factor (NGF) and may bind the glycosaminoglycans heparin and heparan sulfate (33). Heparin suppresses cell proliferation (34) and it is suggested that this is mediated through protein kinase C, whose suppression inhibits the induction of both c-*fos* and c-*myc*.

Our present findings demonstrate elevation of APP in two separate states of cell-cycle arrest. Quiescent and senescent states involve arrest at different points prior to the S phase, and while the former is inducible and reversible, senescence is not inducible and is permanent under normal culture conditions (35, 36). That these two divergent states overlap in their increased production of APP decreases the probability that this is an epiphenomenal event and supports a growth-inhibitory function.

While amyloid accumulation is a well-recognized hallmark of pathology in AD, a more modest accumulation occurs in the normal aging brain. Our findings demonstrate a direct relationship of increased amyloid mRNA synthesis with cellular aging. It is possible that a neuronal manifestation of this increase with age contributes in part to the pathogenesis of AD.

Note Added in Proof. In a recent report (37), amino acids 25–35 of the amyloid β protein were found to be neurotrophic to undifferentiated neurons at low concentrations and neurotoxic to mature neurons at high concentrations. Our findings demonstrate that the cell normally produces a situation which would then be potentially toxic, as a function of age.

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