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Increased Production of 4 kDa Amyloid β Peptide in Serum Deprived Human Primary Neuron Cultures: Possible Involvement of Apoptosis

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The etiology of the amyloid β peptide in sporadic Alzheimer's disease (AD) is not known. Amyloid β peptide (Aβ), a proteolytic product of the amyloid precursor protein (APP), is deposited in the senile plaques and cerebrovascular tissues of individuals with either sporadic or familial AD (FAD). Increased Aβ production from mutant APPs in FAD fosters the hypothesis that overexpression of Aβ plays a primary role in the pathogenesis of AD. The absence of APP mutations in sporadic AD which displays identical pathological features than FAD such as synapse and neuronal loss, senile plaques and neurofibrillary tangles, suggests other causes for overexpression and/or deposition of Aβ. To investigate the effect of neuronal death on APP metabolism and Aβ secretion, human primary neuron cultures were induced to undergo apoptosis by serum deprivation. Serum deprived neurons display shrunken and rounded morphology, contain condensed chromatine and fragmented DNA, which are characteristic of apoptosis. In serum deprived neurons, metabolism of APP through the nonamyloidogenic secretory pathway is decreased to 20% from 40% in control cultures whereas 4kDa Aβ is increased three- to fourfold. The results suggest that human neurons undergoing apoptosis generate excess Aβ and indicates a possible mechanism for increased Aβ in the absence of APP mutations.

[Key words: amyloid β peptide (Aβ), amyloid precursor protein metabolism, neuronal death, apoptosis, programmed cell death, human primary neuron cultures]

Initial manifestations of memory loss, cognitive deficits and behavioral problems reveal neuronal distress in individuals with AD. Alterations of neuronal cytoskeleton proteins in neurofibrillary tangles (Hanger et al., 1991), dystrophic neurites in senile plaques (Benes et al., 1991), loss of synapse in the brain (Hamos et al., 1989), and neuronal loss confirm this neuronal deterioration (Davies and Maloney 1976; Whitehouse et al., 1982; Terry et al., 1981). Despite considerable progress in AD research, the cause of neuronal distress and death is not clear. Chromosomes 14, 19, and 21, are genetically linked to FAD (Hyslop 1987; Schellenberg et al., 1992; Nechiporuk et al., 1993). The role of APP in FAD linked to chromosome 21 is highly supported by the identification of APP mutations and remains today our strongest link to understanding some of the molecular mechanisms of AD.

Abundant amounts of the 40–43 amino acid Aβ (4 kDa Aβ), arising from the metabolism of APP, a transmembrane glycoprotein, are deposited in the senile plaques and cerebrovascular tissues of AD brains (Glenner and Wong, 1984; Shoji et al., 1992; Haass et al., 1992; Busciglio et al., 1993). The differentially spliced APP_{695} form is the most highly expressed isoform of APP in brain and primary neuron cultures (Goedert, 1987; LeBlanc et al., 1991). Metabolism of APP occurs through at least three pathways. The secretory pathway involves an α-secretase cleavage of APP within the Aβ domain thereby preventing the formation of 4 kDa Aβ, and generates a large secreted N-terminal peptide (sAPP) and a ~10 kDa cell-associated C-terminal peptide which can be further processed into a secreted nonamyloidogenic 3 kDa Aβ peptide (Esch et al., 1990; Bhasin et al., 1991; Wang et al., 1991; Busciglio et al., 1993; Haass et al., 1993). The endosomal-lysosomal system yields a series of five cellular APP C-terminal peptides in human brain, the largest and potentially amyloidogenic containing the full Aβ domain (Estus et al., 1992; Goldc ct al., 1992; Hâass et al., 1992; Cheung et al., 1994). A third pathway, involving endocytosis, generates secreted 4 kDa Aβ mostly composed of 40 amino acids ($Aβ_{1-40}$) although lower levels of variants ranging from −6 to 42 or 43 amino acids exist ($Aβ_{1-42/43}$) (Seubert et al., 1992; Shoji et al., 1992; Haass et al., 1992; Koo and Squazzo 1994; LeBlanc and Gambetti, 1994). APP mutations at codon 717 (Chartier-Harlin et al., 1991; Goate et al., 1991; Murell et al., 1991; Van Duijn et al., 1991), and codon 670/671 in FAD (Mullan et al., 1992; Hendricks et al., 1992) as well as codon 692 in hereditary cerebral hemorrhage with amyloidosis of the Dutch type (HCHWAD) (Levy et al., 1990), strongly support a primary role for APP in the pathogenesis of AD. Both APP670/671 and APP692 mutations produce excess $Aβ_{1-40}$ *in vitro* and *in vivo* (Cai et al., 1993; Citron et al., 1992, 1994; Haass et al., 1994; Nakamura et al., 1994). The APP717 mutations increase the level of $Aβ_{1-42/43}$ which, because of its higher hydrophobicity, may promote amyloid deposition (Jarrett et al., 1993; Suzuki et al.,

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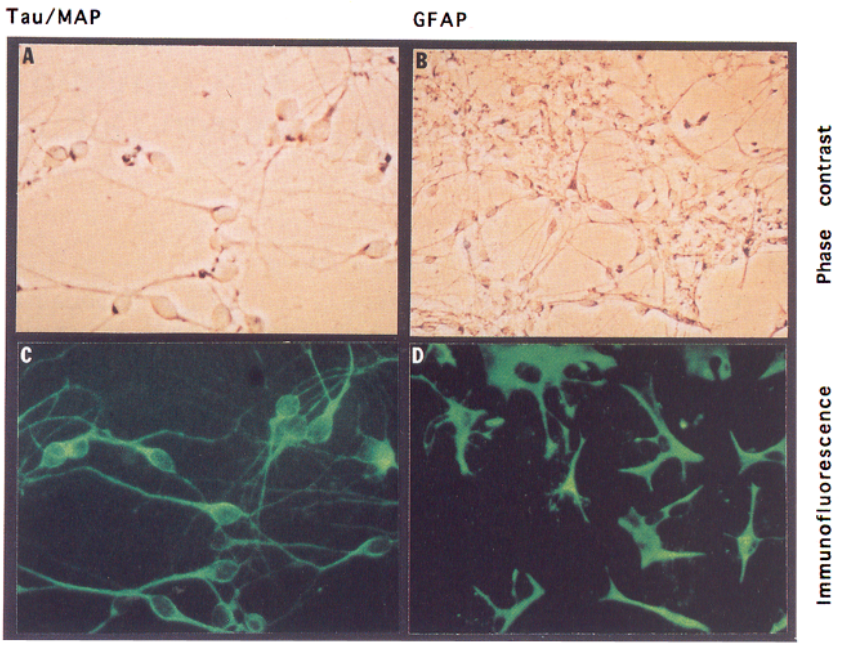
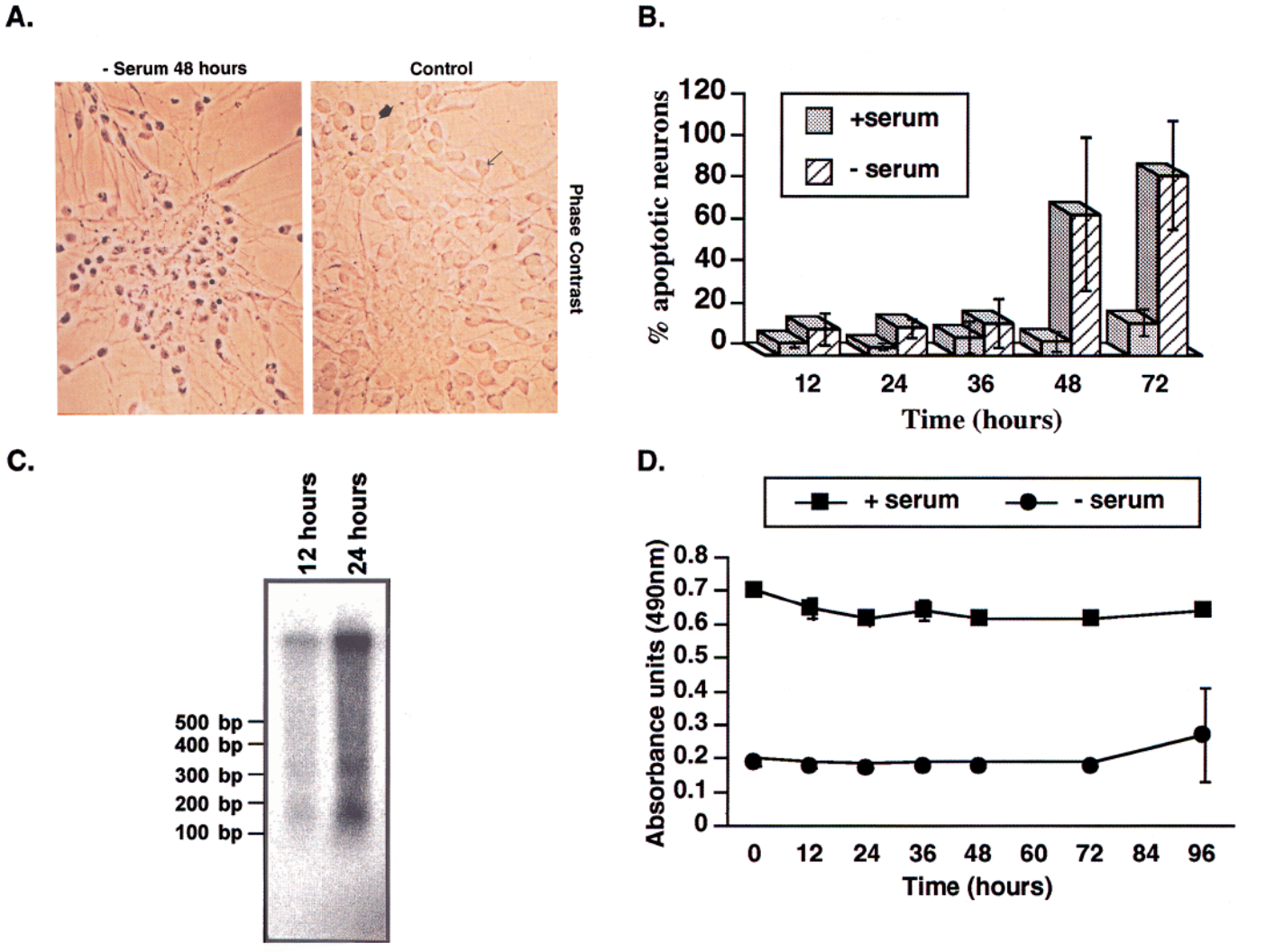


Figure 1. Immunocytochemical characterization of human foetal primary neuron cultures. Phase contrast and immunofluorescence micrography at high magnification of tau (*A, C*) and lower magnification of GFAP (*B, D*) immunostaining in human primary neuron cultures.



1994; Tamaoka et al., 1994). Overexpression of Aβ from APP717 mutant constructs in transgenic mice results in Aβ deposits with specific characteristics of senile plaque amyloid (Games et al., 1995). Thus, overexpression of Aβ appears to be an important factor in Aβ deposition in individuals carrying APP mutation.

The small amount of FAD individuals carrying APP mutations (Tanzi et al., 1992) questions the etiology of Aβ in sporadic AD. Sporadic and FAD have similar pathology (Karlinsky et al., 1992; Lantos et al., 1992). Despite the information gleaned from the APP mutations, it is still not clear whether Aβ deposition precedes neuritic problems (Martin et al., 1994). Since Aβ induces apoptosis of rodent primary foetal hippocampal neurons and pancreatic islet cells (Yankner et al., 1990; Forloni et al., 1993; Loo et al., 1993; Lorenzo et al., 1994), it is proposed that neuronal loss in AD could be a consequence of apoptosis (Su et al., 1994; Thompson 1994). Apoptotic cell death entails a genetically programmed series of events which results in cell rounding, shrinkage and membrane blebbing, cytoskeletal reorganization, chromatine condensation and DNA fragmentation while retaining plasma membrane integrity and precluding an inflammatory response (Thompson, 1994; Tomei and Cope, 1991, 1994). Since neuronal dysfunction and eventually cell death is common to sporadic and FAD, this study investigates APP metabolism in serum deprived human primary neurons which display the apoptotic phenotype. The results show that challenged neuron cultures generate three times more 4 kDa Aβ than healthy neurons while metabolism of APP through the non-amyloidogenic secretory pathway decreases twofold. The results show that increased Aβ can occur in neuron cultures deprived of essential survival factors and may provide a source for Aβ deposition in the absence of APP mutations.

Materials and Methods

Primary neuron cultures

Neurons. The brain tissue from 14–18 week old human foetus was obtained according to the guidelines of the Medical Research Council of Canada, and was approved by McGill University Institutional Review Board. The isolation of neurons is a modification of methods published (Mattson and Rychlik, 1990; LeBlanc et al., 1991) and was set up in association with Dr. Cinthya Goodyer, Dr. Jack Antel, and Manon Blain from McGill University. Essentially, the cerebral tissue is minced in serum free neuronal media (MEM in Earle's balanced salt containing 0.225% sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1% dextrose, 1× antibiotic Pen-Strep; all products bought from GIBCO-BRL, Ontario) and dissociated by trypsin treatment. The cells are plated at 3×10^6 cells/ml of 5% decomplemented serum containing media on poly-lysine coated dishes. After 4, 7, and 10 d in culture, 1 mM of the antimitotic agent, 5′ fluoro-2′-deoxyuridine (FdU: Sigma, St-Louis, MO), is added in fresh media (Martin et al., 1990).

Astrocytes. The astrocytes were purified by differential shaking as described by LeBlanc et al. (1991).

Characterization of cell types in the cultures

Immunofluorescence was carried out to determine the cellular composition of the FdU treated primary neuronal and astrocytic cultures after 12 DIC. Neuron-specific antibodies included monoclonals anti-tau (tau-2 clone), anti-microtubule associated protein (MAP2; clone AP20), and anti-neurofilament 68 (clone NR4) obtained from Sigma, St-Louis, MO, monoclonal anti-neurofilament SMI33 (Sternberger, Inc.) and polyclonal anti-bovine tau/MAP (kind gift from Hemant Paudel, McGill U, Montreal). Astrocytes were recognized with anti-glial fibrillary acidic protein (GFAP; Sigma, St. Louis, MO), microglia by low density lipoprotein (LDL)-diI incorporation (Pitas et al., 1981) (Biomedical Technologies Inc., MA), oligodendrocytes with anti-galactocerebroside (GalCer), and fibroblasts with fibronectin (Sigma, St. Louis, MO). Typically, cells were fixed in acid/alcohol for cytoskeletal markers or 4% paraformaldehyde for membrane markers and incubated with antibodies for 2 hr to overnight. Immunoreactivity is detected with rhodamine or fluoresceinated secondary antibodies.

Apoptosis of neuron cultures

Apoptosis was achieved by serum deprivation for various times, thereby removing essential growth factors to the FdU treated neurons.

Staining of the condensed chromatine. Apoptotic nuclei were visualized with the DNA stain, propidium iodide. Serum deprived and control serum treated neuron cultures were permeabilized and fixed in acid-alcohol before staining in 0.1 μg/ml propidium iodide (Sigma, St. Louis, MO) for 20 min in PBS. Apoptotic and normal nuclear DNA were detected by epifluorescence using a 40× objective. Propidium iodide homogeneously stains well-rounded nuclei in normal cells whereas clumped and broken chromatine is observed in irregularly shaped nuclei in apoptotic neurons. Approximately 500 neurons were scored per time point in three independent cell cultures.

LDH assay of neuronal media. The LDH was detected in the culture media from serum treated and deprived cultures using the Cytotox 96 calorimetric test as described by the manufacturer (Promega, Madison, WI).

DNA ladder. Cells were lysed in 0.4% Triton X-100 in TE (10 mM Tris pH 8.0 and 1 mM EDTA) and treated 30 min at 37°C with 100 μg/ml of RNase A followed by 100 μg/ml proteinase K at 56°C overnight. The DNA was phenol extracted and end labeled as described (Rosl 1992).

Metabolic labeling and assessing APP metabolism

Neurons were incubated in 250–500 μCi/ml translation grade ^{35}S-methionine (NEN Dupont, Canada) in serum and methionine free DMEM containing 1 mM sodium pyruvate and 2 mM glutamine after an initial incubation in methionine-free media for an hour. To assess APP synthesis neurons were pulsed for 30, 60, and 120 min. To assess secretion of sAPP and degradation of APP, neurons were labeled for 60 min and chased in a 3000-fold excess cold methionine for 30, 60, 120, 240, and 360 min. Cells were labeled 5 hr for the detection of full length APP, C-terminal fragments and Aβ. The cells were lysed in NP-40 detergent lysis buffer and immunoprecipitations were carried out in RIPA buffer (Lane, 1989) Immunoprecipitations of APP and APP fragments were conducted with the following antibodies: anti-N against human $sAPP_{770}$ (kind gift from Barry Greenberg, Cephalon, Inc., PA), anti-C_{21}, monoclonal 6E10 and 4G8 against epitope 1–17 and 17–24 of Aβ, respectively (Kim et al., 1990a,b) and anti-I antisera against amino acids 649-664 of APP_{695} (kind gift from D. Selkoe, Harvard Medical School). Competition of anti-C_{21} and anti-Aβ immunoprecipitations was carried out with 1 μg C_{21} and $A\beta_{1-40}$ peptides. Immunoprecipitates were electrophoresed on a three layer Tris Tricine polyacrylamide gel (16.5%, 10%, 4%) (Schagger and Von Jagow, 1987) then fixed, dried and exposed to x-ray film X-OMAT AR and phosphorimaging screen.

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Figure 2. Apoptosis of human primary neuron cultures by serum deprivation. *A*, Phase contrast of control and serum deprived neurons at 48 hr treatment. Note that the large nucleated cells (*arrowhead*) are astrocytes while smaller nucleated cells are neurons (*arrow*) (also confirmed by double staining with anti-tau and GFAP antibodies; not shown). The apoptotic neurons are darker and shrunken on phase contrast. *B*, Percentage of apoptotic neurons determined by propidium iodide staining. The results represent the average and SD (*vertical line* in column) of three independent experiments. The total number of cells in serum deprived cultures was the same as sister control cultures until 48 hr, but then decreased to 90%, 70%, and 30%, respectively at 48, 72, and 96 hr. *D*, LDH release during serum deprivation was measured at the various time points of serum deprivation in the media of the culture. Each point represents the average of three experiments. *Vertical lines* indicate the standard deviation. *C*, DNA ladder at 12 and 24 hr of serum deprivation. DNA 100 bp markers are indicated on the side.

Quantitation and statistical analysis of phosphorimaging data

Quantitation of the immunoprecipitated fragments was done by phosphorimaging (Molecular Dynamics). Relative amounts of sAPP produced from serum deprived and serum treated cultures were calculated as a percentage of full length cellular APPs immunoprecipitated with anti-N. Relative amounts of Aβ produced from serum deprived and serum treated cultures were calculated as a percentage of full length secreted and cellular APPs immunoprecipitated with anti-N. The number of pixels obtained from quantitative phosphorimaging were corrected for the content in methionine of the measured APP or APP fragment and also corrected for the volume immunoprecipitated from the cellular lysate or secreted proteins. The amount of C-terminal fragments was calculated as a percentage of full length APP immunoprecipitated with anti-C_{21}. Absolute levels were not calculated because of the possible variations in the rate of ^{35}-S methionine incorporation, amino acid pool size and number of cells synthesizing the APPs in serum deprived compared to control neuron cultures (Wiesner and Zak, 1991). Rate of synthesis was calculated as the accumulation of APP with time. Rate of degradation was calculated during the exponential decay of cellular APP in the chase period ($T_{1/2} = \ln 2/K_g$; $K_g = (dP/dT)/P$). The significance of results from P serum deprived and serum treated neurons within a same experiment (identical cultures), were evaluated statistically by a paired two-tailed t test. Significance of results from independent experiments was determined by an unpaired two-tailed t test. Each set of data is the average of at least three independent experiments.

Results

To understand APP metabolism in healthy and serum deprived neurons, highly purified human primary neuron cultures were obtained from foetal brain tissue between 14 and 16 weeks of development. Human primary neuron cultures are ideal for these studies since Aβ deposits are fairly restricted to the brain of primates and bigger animals and is absent from the conventional rodent models. Treatment of mixed primary cultures with 1 mM fluorodeoxyuridine (FdU) generates neuronal cultures containing approximately 90% neurons by 7 d of treatment. The neurons are well differentiated and establish healthy networks of neurites under these conditions as shown under phase contrast and by tau immunostaining (Fig. 1*A–C*). Neurons were also immunostained with tau, MAP2 and neurofilament monoclonal antibody (not shown). GFAP-positive astrocytes (10.5% of the cells; SD of 3) remaining after FdU treatment are always in close association with the neurons (Fig. 1*B,D*). Few microglia, fibroblasts, or oligodendrocytes (<0.5%) were detected by LDL-DiI incorporation, anti-fibronectin and anti-galactocerebroside antibodies, respectively (not shown).

Serum deprivation was used to remove essential growth factors to the neurons and therefore initiate the apoptotic mechanism of cellular death. Cell morphology, chromatine condensation and DNA fragmentation were assessed at various times after serum deprivation. Phase contrast micrographs of serum deprived neurons show obvious shrinkage and rounding of the neuronal cell body, a typical feature of apoptotic cells, (Tomei and Cope, 1991), as well as loss of neuritic integrity manifested by neurite bubbling, breakage and retraction (Fig. 2*A*). In contrast, serum treated neurons display larger cell bodies and entertain a healthy neuritic network. Chromatine condensation was detected by irregularly shaped dense nuclear masses brightly stained by propidium iodide as opposed to homogeneous staining of the normal nuclear DNA. Double staining with a polyclonal tau/MAP antibody revealed that only neurons undergo apoptosis, not astrocytes (not shown). The percentage of apoptotic neurons in serum deprived cultures at various time points was calculated by counting the number of condensed chromatine, tau/MAP positive neuronal cells as a percent of total neurons in at least three independent experiments. The percentage of apoptotic neurons is 14% after 12 hr of serum deprivation and increases slightly between 12 and 36 hr. Between 36 and 48 hr of serum deprivation, a sharp increase to 68% of apoptotic neurons is observed. At 48 hr, approximately 10% of the cells have floated off the attachment matrix and this value may be an underestimation of the actual numbers of apoptotic neurons. By 72 hr of serum deprivation few healthy neurons remain (<10%) (Fig. 2*B*). Preliminary studies to determine the commitment time of the neurons to apoptosis indicates that reversal of apoptosis can be achieved before 12 hr of serum deprivation by adding serum to the culture but not after 12 hr. The variability in numbers of apoptotic neurons between isolated cultures as denoted by the high standard deviation (Fig. 2*B*) is consistent with that observed in other primary neuron culture systems (Edwards and Tolkovsky, 1994). To determine the presence of DNA fragmentation, DNA was extracted from 12 and 24 hr serum deprived neurons. Because of the limitation in neuron numbers for the experiments, end labeling with ^{32}P-dCTP allowed visualization of DNA fragments in expected sizes of 180 bp and multiples thereof (Wyllie, 1980) (Fig. 2*C*). Therefore, identification of rounded, shrunken neurons containing condensed nuclear chromatine and the presence of DNA fragments in the neuronal cultures confirms the apoptotic nature of the neurons (Fig. 2*C*). To evaluate the possibility that serum deprivation causes cell death by necrosis in these same cultures, LDH release was measured in the serum deprived neuron culture media (Fig. 2*D*). LDH levels remained stable for up to 72 hr of serum deprivation increasing slightly between 72 and 96 hr. In control neuronal cultures, LDH levels remained constant although much higher than in serum deprived cultures because of the presence of LDH in serum which turns out to be very useful as a positive control for the LDH assay. These results indicate that little necrotic cell death occurs between 12 and 72 hr of serum deprivation. We are presently investigating in more details, the molecular mechanisms of apoptotic cell death in human neurons with this model.

To capture APP synthesis before abundant neuronal cell death occurs but in cells committed to apoptosis, the 12 hr time point of serum deprivation was chosen for the following experiments on APP synthesis, degradation, and metabolism. To compare APP isoforms and detect major shifts in quantitative expression of APP in serum deprived compared to serum treated neurons, cultures treated for 12 hr were metabolically labeled for 5 hr and the proteins immunoprecipitated with antibodies directed against various epitopes of APP (Fig. 3*A*). Metabolic labeling of cells requires serum free conditions which include one hour starvation of the cells in methionine/serum free media before labelling. Therefore, in this experiment, the control serum treated cells were 6 hr without serum whereas serum deprived cells were 18 hr without serum. The full length APP analyzed in this figure is that accumulated within the cells during the 5 hr labeling period following the 1 hr methionine starvation period. In normal cultures, anti-C_{21} and anti-N antiserum immunoprecipitate three major proteins of 95–125 kDa (Fig. 3*B*). Anti-C_{21} immunoprecipitated proteins were completely competed out with C_{21} peptide (C21+). These likely represent full length immature and mature APPs. The size is consistent with APP_{695}, the major APP isoform in neurons. To check for the possible presence of APP-like proteins (APLPs) which do not contain the Aβ domain (Wasco et al., 1993), immunoprecipitation was carried out with the 4G8 antibody which recognizes amino acids 17–24 of the Aβ domain. The same three major APPs were immunoprecipitated with the 4G8 antibody. Similarly, the anti-I antisera

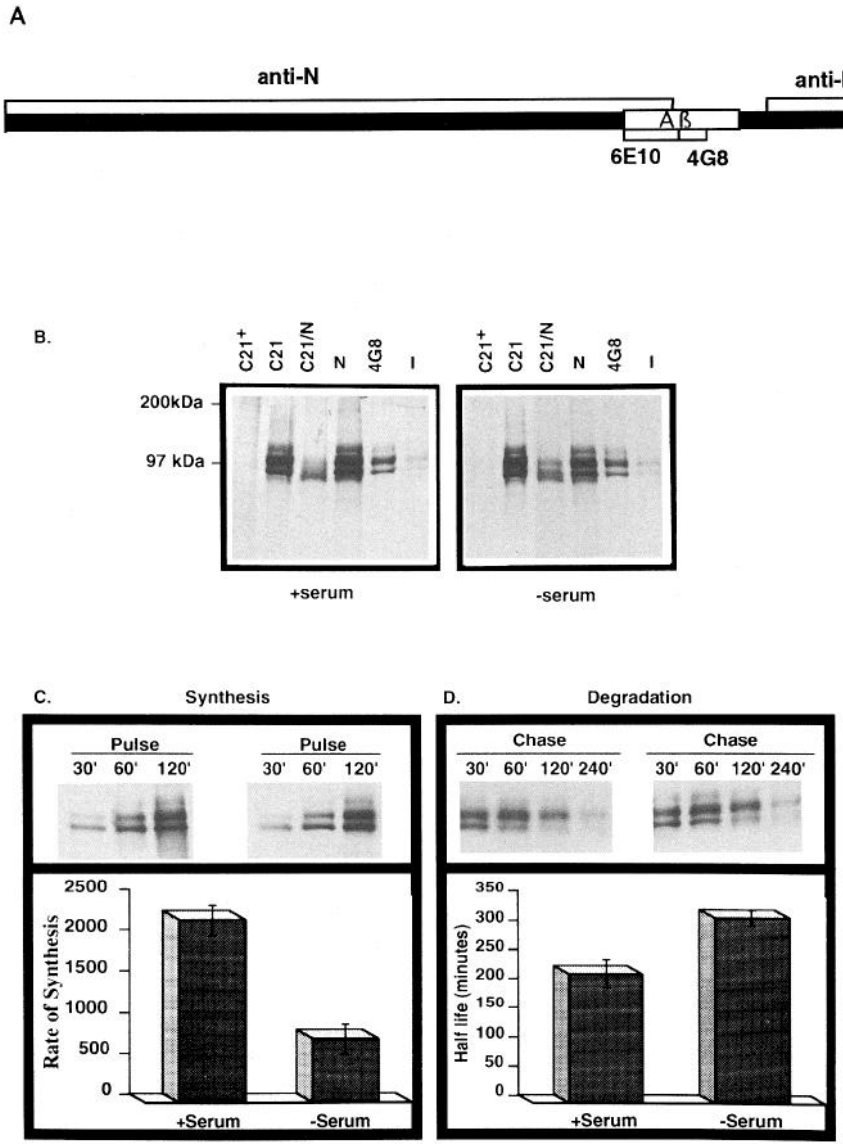

Figure 3. Comparison of the expression of APPs in serum deprived and control neuron cultures. *A,* Schematic representation of APP_{695} and epitopes detected by the antisera anti-N (against sAPP), anti-C_{21} (against amino acids 675–695), anti-I (amino acids 649–664) and monoclonals 6E10 (against amino acids 597–613 of APP or amino acids 1–17 of Aβ) and 4G8 (against amino acids 613–620 of APP or amino acids 17–24 of Aβ). The transmembrane domain spans amino acids 625–648 and the α-secretase cleavage occurs between amino acids 16–17 of Aβ. *B,* Autoradiogram showing immunoprecipitated ^{35}S-methionine pulse labelled full length APPs with anti-C_{21} (*C21*), anti-N (*N*), 4G8 and anti-I (*I*) from control (*+serum*) and serum deprived (*−serum*) neuron cultures. Competition of anti-C_{21} immunoprecipitation with C_{21} peptide (*C21+*) is shown in the first lane. *C,* Autoradiogram and graphic representation of the rate of synthesis of full length APPs in serum deprived and control neural cultures. Standard deviation is represented by the vertical line in the columns. *D,* Autoradiogram and bar graph representation of the rate of degradation of full length APPs in serum deprived and control neural cultures.

against amino acids 649–664 of APP immunoprecipitates these same three proteins (lane I). Therefore, the three major proteins immunoprecipitated with anti-N, anti-C, 4G8 and anti-I are APPs. A fourth APP protein migrating below immature full-length APP is obvious in the anti-N but not anti-C_{21} immunoprecipitation. To further characterize this protein, APLPs and APPs were first immunoprecipitated with anti-C_{21} antisera and the remaining proteins reimmunoprecipitated with anti-N antisera (lane C21/N). A considerable amount of the lower MW APP was immunoprecipitated. This protein was not immunoprecipitated with 4G8, anti-I (Fig. 3*B*) or 6E10 (not shown) and likely represents the truncated N-terminal APP fragment resulting from β-secretase cleavage of APP (Seubert et al., 1993). The APPs in serum deprived neurons did not differ qualitatively from those of control serum treated neurons.

To examine the synthesis and degradation of APPs in serum treated and serum deprived cultures, pulse chase experiments were carried out (Fig. 3*C*). After metabolic labeling of the culture for 30, 60, and 120 min, the rate of APP synthesis was assessed by phosphorimaging quantitation of immunoprecipitated full length APPs at these time points. An average of three independent experiments shows that the rate of APP synthesis decreases threefold (2.98 ± 0.5) in serum deprived neurons. Since no notable difference is observed in the steady state of newly synthesized APPs in serum deprived and control neuron cultures (Fig. 3*B*), the APP degradation was assessed by ^{35}S-methionine labeling for 60 min followed by a chase at various time points in cold methionine containing media. The APP half-life, assessed during the exponential decay of the APP during the chase, was 317 ± 14 min in serum deprived neurons but only 223 ± 25 min in control neurons (Fig. 3*D*). Therefore, the longer half-life in serum deprived neurons should compensate partially for the decreased rate of APP synthesis observed.

To determine if one particular APP metabolic pathway is affected in serum deprived neuron primary cultures, the amount of sAPP, C-terminal fragments and Aβ peptides were assessed as markers of the secretory, lysosomal–endosomal and 4 kDa Aβ-producing pathways, respectively. Metabolism of APP through the secretory pathway was determined from a pulse chase experimental paradigm. Neurons deprived of serum for 12 hr and control serum treated neuron cultures were both starved in serum and methionine free media for 1 hr before labeling

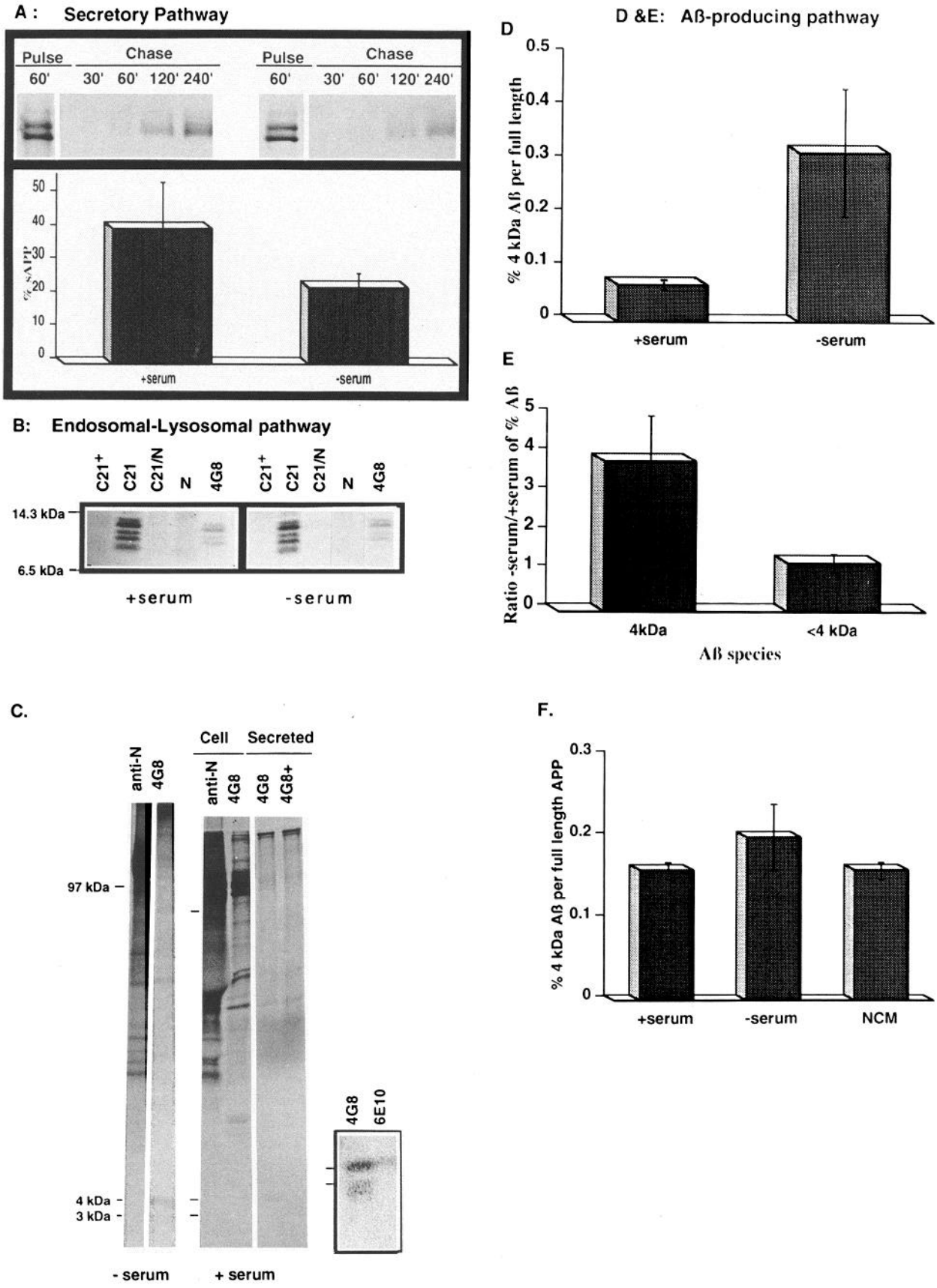

Figure 4. Metabolism of APP through the secretory, endosomal–lysosomal, and Aβ-producing pathways in control and serum deprived neurons. *A,* Secretory pathway: autoradiogram and bar graph showing secretion of sAPP in a pulse chase experiment. Time of pulse and chase are indicated in minutes. The graph represents phosphorimaging quantitation of sAPP immunoprecipitated with anti-N at 4 hr of chase as a percentage of the cellular APP immunoprecipitated with anti-N at 0 min of chase (60 min pulse). *Vertical lines* in the columns indicate SD. *B,* Endosomal–

with ^{35}-S methionine for 1 hr. After 1 hr of labeling, the proteins were chased with methionine containing media. During the pulse chase experiment, the amount of sAPP from serum deprived neurons after 4 hr of chase was only 20% of the newly synthesized APP compared to 40% in normal neurons (Fig. 4*A*). Thus, decreased metabolism of APPs through the non-amyloidogenic secretory pathway leaves 20% more APP to be metabolized through either of the two amyloidogenic pathways.

Metabolism of APP through the endosomal–lysosomal pathway was assessed in neurons labelled for 5 hr. Five APP C-terminal fragments were immunoprecipitated with the anti-C_{21} antisera and fully competed by the addition of C_{21} peptide (C21+) during the immunoprecipitation (Fig. 4*B*). The four larger fragments were also immunoprecipitated with the 4G8 monoclonal antisera indicating that the fragments contain at least part of the Aβ domain. Only the largest C-terminal fragment was immunoprecipitated with 6E10 indicating that the top C-terminal fragment contains the entire Aβ domain (not shown). Phosphorimaging quantitation shows that the second and fourth largest C-terminal fragments (C2 and C4) represent 0.52% and 0.48% of full length APP immunoprecipitated with anti-C_{21} and C5, C1, and C3 represent 0.42%, 0.32% and 0.29%, respectively (Table 1). The amount of C-terminal fragments in serum deprived neurons were not significantly different from control cultures. Therefore, serum deprived neurons do not produce excess potentially amyloidogenic C-terminal fragments.

To assess Aβ production, neurons serum deprived for 12 hr and controls were labeled 5 hr. Full length APP and Aβ were immunoprecipitated with anti-N and 4G8. The monoclonal 4G8 antibody immunoprecipitates three Aβ peptides from the secreted proteins of neuron cultures (Fig. 4*C*). These peptides were fully competed by addition of synthetic Aβ peptide (4G8+) in the immunoprecipitation. The higher MW and most abundant Aβ peptide likely represents 4 kDa Aβ since it is also immunoprecipitated with 6E10 which recognizes amino acids 1–17 of Aβ (Fig. 4*C*). The two lower bands which are not immunoprecipitated with 6E10, likely represent the non-amyloidogenic 3 kDa Aβs (Haass et al., 1993). The amount of 4 kDa Aβ generated in 5 hr of ^{35}S-methionine labeling appeared to be higher in neurons serum deprived for 12 hr before labeling (Fig. 4*C*). Immunoprecipitation of cellular APP holoprotein with 4G8 confirms the integrity of the antibody in this immunoprecipitation. Since the rate of translation of newly synthesized APP is lower in serum deprived neurons than in controls, the amount of Aβ was calculated as a percentage of full length cellular APP immunoprecipitated with anti-N after 5 hr labeling. Phosphorimaging quantitation of five independent experiments shows a three- to fourfold increase in 4 kDa Aβ production in serum deprived cultures (Fig. 4*D*). Similar results were obtained when Aβ was measured as a percentage of APP immunoprecipitated with anti-C_{21} (not shown). To assess the specificity of the increased production of 4 kDa Aβ, the production of the 3 kDa Aβs was also measured as a proportion of full length APP. The amount of the 3 kDa Aβs referred to as <4kDa Aβs are equivalent in serum deprived and normal cultures (Fig. 4*E*). Therefore, metabolism of newly synthesized APP through the 4 kDa Aβ-producing pathway is increased three fold in serum deprived neural cultures.

Table 1. Quantitation of C-terminal fragments

C-Terminal fragment	Percentage C-terminal fragment per full length	
	+ Serum	− Serum
C1	0.32 ± 0.01	0.33 ± 0.21
C2	0.48 ± 0.10	0.48 ± 0.09
C3	0.29 ± 0.04	0.28 ± 0.07
C4	0.52 ± 0.18	0.56 ± 0.33
C5	0.42 ± 0.08	0.48 ± 0.24

Values represent mean ± SD.

Since 10% of the neuronal cultures are astrocytes, the possibility that serum deprived neurons may signal astrocytes to overproduce Aβ or that serum deprived astrocytes generate excess Aβ was tested. Treatment of astrocytes for 12 hr with serum deprived neuron conditioned media of 12 hr failed to induce Aβ production in normal astrocytes (Fig. 4*F*). Thus, it is unlikely that apoptotic neurons mediate increased Aβ production from astrocytes through secreted factors. Astrocyte cultures of over 99% purity were submitted to serum deprivation for 12 hr and radiolabeled for 5 hr in a manner identical to the neuron radiolabeling. These astrocytes did not generate more 4 kDa Aβ than normal astrocytes (Fig. 4*F*). Therefore, the contaminating astrocytes of the neuronal cultures do not generate the threefold increase in 4 kDa Aβ observed during serum deprivation.

Discussion

In this study, APP metabolism in healthy and serum deprived human primary neuron cultures was investigated. A threefold increase in the production of 4 kDa Aβ in human primary neuron cultures submitted to serum deprivation for 12 hr is shown. The increased Aβ production is accompanied by decreased metabolism of the APP through the non-amyloidogenic secretory pathway while metabolism through the endosomal lysosomal pathway remains constant. Furthermore, the neurons submitted to serum deprivation undergo apoptosis thereby suggesting the possible influence of a dying neuron on overproduction of Aβ.

APP metabolism in human primary neuron cultures

Many lines of investigations indicate that APP metabolism is implicated in the pathogenesis of AD. First, the APP gene is

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lysosomal pathway: Autoradiogram showing the five C-terminal APP fragments immunoprecipitated with anti-C_{21} antisera (*C21*) and 4G8 and competed with peptide (*C21+*). The specificity of the antibodies is also indicated by the lack of immunoprecipitation with anti-N (*N* and *C21/N*). *C*, Autoradiogram showing immunoprecipitated cellular APP with anti-N and secreted Aβ with 4G8 from total secreted proteins of serum treated and serum deprived neurons after a 5 hr ^{35}S-methionine labeling period. In addition, 4G8 immunoprecipitation of cellular APP is shown in the +serum experiment. Aβ immunoprecipitation with 4G8 is completely competed with the addition of synthetic Aβ peptide (*4G8+*). The *right-hand side panel* shows control immunoprecipitation of Aβ peptides with 6E10. *D*, Aβ-producing pathway: graph showing the relative amount of Aβ generated from full length APP in control and serum deprived neuron cultures. *Vertical lines* represent the SD. Statistical significance between the two cultures was $p = 0.01$. *E*, Graphic representation of the ratio of 4 kDa and 3 kDa Aβs in serum deprived versus control neurons. *F*, Graphic representation of the relative amount of Aβ generated from APP holoprotein in pure astrocyte cultures submitted to serum deprivation (*−serum*) or 12 hr serum deprived neuron conditioned media for 12 hr (*NCM*). Bars represent variance of the mean.

localized to chromosome 21 which has been linked to FAD (Hyslop 1987; Patterson et al., 1988). Second, the proteolytic product of APP, the Aβ, is abundant in two main lesions of AD brains; the senile plaque and cerebrovascular amyloid deposits (Glenner and Wong, 1984; Masters et al., 1985). Third, mutations of APP associated with FAD generate excess Aβ (Citron et al., 1992, 1994; Cai et al., 1993; Suzuki et al., 1994; Tamaoka et al., 1994). Fourth, trisomy 21 individuals have increased APP expression and develop AD pathology with age (Wisniewski et al., 1985; Tanzi et al., 1987). Fifth, transgenic mice overexpressing mutant APP717 develop Aβ deposits, synaptic loss, neuritic plaques, and gliosis (Games et al., 1995). Despite considerable research on the three pathways of APP metabolism; the secretory, the endosomal lysosomal, and the 4 kDa Aβ producing pathways, little is known on APP metabolism in the human neuron. Since naturally occurring AD pathogenesis is fairly limited to primate brain where APP is highly expressed particularly in neurons (Goedert, 1987; Benowitz et al., 1989; LeBlanc et al., 1991), the present study in human primary neuron cultures addresses an important aspect to understand APP metabolism in humans and can offer a more direct correlation with AD. The results show selective expression of APP_{695} in human neurons. The 40% secretion of newly synthesized APP in normal neurons agrees with levels detected in human CSF and brain (Palmert et al., 1989, Pasternack et al., 1992). The five C-terminal fragments described in human brain (C1 to C5) (Estus et al., 1992) and arising from the endosomal–lysosomal pathway (Golde et al., 1992) (Haass et al., 1992) represent a significant fraction of the APP fragments in neuron cells. The immunoreactivity of the four larger C-terminal fragments agree with the sequence obtained from APP_{695}-transfected neuroblastoma cells, the largest only (C1) being capable of further processing to 4 kDa Aβ (Cheung et al., 1994). However, whereas equivalent levels of all five fragments were reported in human brain, in neuron cultures, an order of decreasing abundance from C4, C2, C5, C1, and C3 is clear. The relative abundance of these C-terminal fragments in human neurons suggest that either the metabolism of APP through the endosomal–lysosomal pathway is prominent in human neurons or the C-terminal fragments have a longer half-life. The amyloidogenic nature of human neuron APP metabolism is further supported by greater 4 kDa Aβ than 3 kDa Aβ levels as previously observed (Busciglio et al., 1993).

Effect of serum deprivation on APP metabolism in human primary neuron cultures

APP metabolic studies in primary human neuron cultures deprived of serum for 12 hr agree with the hypothesis that decreased APP metabolism through the nonamyloidogenic secretory pathway provides more APP for the two amyloidogenic pathways. As APP secretion is decreased twofold in serum deprived neurons, 4 kDa Aβ increases threefold compared to control cultures. Serum deprivation of the primary neuronal cultures also has an effect on APP synthesis and degradation. APP synthesis is decreased 3-fold while APP half-life increases by 1.5-fold. One may wonder whether the absolute increase in 4 kDa Aβ is significant since de novo APP synthesis is decreased. However, it is likely that the unlabeled pool of APP holoprotein in serum deprived and control cultures will be metabolized the same way as the labeled pool. Since the pools of unlabeled APP should be identical at the beginning of the experiment, increased Aβ from the unlabeled APP pool should also occur in serum deprived neurons compared to control.

Is there a link between neuronal apoptosis, increased 4 kDa Aβ production and decreased sAPP?

Apoptotic primary neurons have been extensively described in NGF deprived rat sympathetic cervical ganglion cells (Edwards et al., 1991; Martin et al., 1992; Edwards and Tolkovsky 1994; Estus et al., 1994). The neurons undergo initial decreased protein synthesis, cell shrinkage and rounding, membrane blebbing and the appearance of condensed chromatine denotes a commitment of the neurons to apoptosis (Martin et al., 1992; Deckwerth and Johnson 1993; Edwards and Tolkovsky, 1994). Although the human primary neuron culture has to be further defined, apoptosis definitely occurs in serum deprived cultures which also shows neurons with cell rounding and shrinkage, definite condensed nuclear chromatine and fragmented DNA which appears as early as 12 hr of serum deprivation (Fig. 2). Since serum deprived astrocytes fail to undergo apoptosis and secrete normal levels of 4 kDa Aβ, it is likely that the altered production of Aβ in serum deprived neurons is related to neurons undergoing apoptosis.

The reason for decreased secretion of APP in serum deprived neuronal cultures is not clear. Increased secretion of APP is regulated by protein kinase C activation either through direct activation by phorbol esters (Buxbaum et al., 1990; Caporaso et al., 1992; Gillespie et al., 1992; Slack et al., 1993) or through stimulation of receptors linked to protein kinase C (Nitsch et al., 1992; Buxbaum et al., 1993). Therefore, decreased sAPP in serum deprived neurons may result from the removal of protein kinase C activating factors normally present in serum. However, while repression of protein kinase C activity inhibits receptor activated APP secretion (Nitsch et al., 1992), it also activates apoptosis (Walker et al., 1993; Jacobson et al., 1994). Thus, decreased secretion of sAPP could be a consequence of apoptosis.

Possible implications for sporadic AD

Increased 4 kDa Aβ production in serum deprived neuron cultures supports a novel hypothesis explaining Aβ deposits in the brain of sporadic AD individuals. Focally increased Aβ from a dying neuron could engender further neurodegeneration of neighboring neurons. The lesser frequency of neurons dying in a younger versus an aging brain could explain the later manifestation of the disease in sporadic AD. In contrast, increased Aβ either as the result of APP gene mutations or increased APP gene expression could be the initiator of apoptosis in FAD.

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