Neuronal cell death, neurofibrillary tangles, and amyloid β peptide (Aβ) deposition depict Alzheimer’s disease (AD) pathology, but neuronal loss correlates best with dementia. We have shown that increased production of Aβ is a consequence of neuronal apoptosis, suggesting that apoptosis activates proteases involved in amyloid precursor protein (APP) processing. Here, we investigate key effectors of cell death, caspases, in human neuronal apoptosis and APP processing. We find that caspase-6 is activated and responsible for neuronal apoptosis by serum deprivation. Caspase-6 activity precedes the time of commitment to neuronal apoptosis by 10 h, indicating possible activity without subsequent apoptosis. Inhibition of caspase-6 activity prevents serum deprivation-mediated increase of Aβ. Caspase-6 directly cleaves APP at the C terminus and generates a C-terminal fragment of 3 kDa (Capp3) and an Aβ-containing 6.5-kDa fragment, Capp6.5, that increases in serum-deprived neurons. A pulse-chase experiment reveals a precursor-product relationship between Capp6.5, intracellular Aβ, and secreted Aβ, indicating a potential alternate amyloidogenic pathway. Caspase-6 proenzyme is present in adult human brain tissue, and the p10 active caspase-6 fragment is detected in AD brain tissue. These results indicate a possible alternate pathway for APP amyloidogenic processing in human neurons and a potential implication for this pathway in the neuronal demise of AD.

Neuronal loss distinguishes Alzheimer’s disease (AD) from normal aging and correlates best with cognitive decline in AD individuals (Ref. 1, and reviewed in Ref. 2). In mild cases of AD, there is already a 50% loss of neurons in the entorhinal cortex, which forms the connections necessary for memory and learning between the hippocampus and the neocortex (3). Neuronal loss in AD is accompanied by the deposition of amyloid β peptide (Aβ) in senile plaques and cerebrovascular tissue and the presence of neurofibrillary tangles. Generally, Aβ deposition is considered important in AD. Increased production of an Aβ of 40 (Aβ1–40) or 42 (Aβ1–42) amino acids in length, which arises through proteolytic processing of the amyloid precursor protein (APP), is common to all familial forms of AD whether caused by mutations of APP, presenilin 1, or presenilin 2 genes (reviewed in Ref. 4). The etiology of the most common sporadic form of the disease, which includes approximately 90% of AD cases, remains unknown. Whereas the pathology of sporadic cases of AD is identical to that of familial AD, the reason for increased Aβ in sporadic AD is unclear. We have previously shown that human primary neuron cultures committed to apoptosis produce 2–4-fold more Aβ than healthy neurons (5). Therefore, in sporadic AD, initiation of a neuronal cell death program may contribute significantly to the increased production of Aβ. It is also possible that neuronal apoptosis contributes to increased Aβ in familial AD cases because overexpression of APP, and mutations of APP or presenilin, induce neuronal cell apoptosis or vulnerability (6–10). Because Aβ is neurotoxic and induces apoptosis, the neuronal apoptosis-mediated increase in Aβ could trigger a cascade of events, leading to further initiation of neuronal cell death (11–14).

The progressive nature of neuronal cell dysfunction and death in AD individuals is consistent with an apoptotic mechanism of neuronal cell death (15). However, due to the continuous clearance of apoptotic bodies in live tissues, progressive neuronal apoptosis will unlikely yield high numbers of detectable apoptotic neurons at any time after the onset of disease (16). In addition, the obligate use of post-mortem brain tissue only allows evaluation of the end point of the disease and not necessarily the process by which neuronal cell death has occurred. Despite these problems in the detection of apoptotic cells in tissues, there is some evidence that apoptosis occurs in AD brains. Although terminal deoxynucleotidyl transferase dUTP end labeling (TUNEL) staining, which identifies 3’-ends of DNA strands, a phenomenon resulting from DNA fragmentation in apoptotic cells, yields variable results in AD brains (17–19), alterations in gene expression reflect an apoptotic state in AD neurons. Transcriptional factors c-Jun and c-Fos, which play a key role in neuronal apoptosis (20, 21), increase in the brains of AD patients as compared with age-matched controls (22, 23). Both Jun and Fos co-localize with neurofibrillary tangle marker, PHF-1, in some neurons. SGP-2 (clusterin, apo J), a gene that is highly expressed in cells undergoing apopto-
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E. P. Anver, C. E. Copeland, J. B. Wood, L. P. Bigsby, K. Chang, M. P. DeStefano, M. J. Liotta, Organization and Structure of the Canadian Tissue Brain Bank, 1995.

EXPERIMENTAL PROCEDURES

Cell Cultures—Neurons were cultured as described previously (5, 14, 47). Briefly, brains were dissociated in trypsin, treated with deoxyribonuclease I, filtered through 130 and 70 μm nylon mesh, and plated on poly-lysine coated tissue culture flasks or multwell plates at a density of 3 × 10^5 cells/ml. Neurons elaborate an extensive neuritic network within 3 days of plating and do not show any signs of neurodegeneration for approximately 4 weeks.

Determination of Neuronal Apoptosis—Neurons plated on acar coverslips were serum-deprived at 12 h in the absence or presence of various concentrations of caspase inhibitors Z-VAAD-fmk, BOC-Asp(OMe)-fmk (BOC), Z-DEVD-fmk, and Z-IETD-fmk for 24, 48, 72, and 96 h. Neurons were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X 100 in 0.75 mM sodium citrate, and stained for apoptotic neurons by TUNEL using the Cell death Kit I (Roche Molecular Biochemicals) as described by the manufacturer. Cells were counterstained with 100 ng/ml propidium iodide for 20 min. The number of TUNEL-positive neurons was counted over approximately 100 cells in five areas of each coverslip (minimum of 500 cells per sample). The percentage of apoptotic neurons was determined by calculating the number of TUNEL-positive neurons (green fluorescence) over the total amount of neurons (red fluorescence by propidium iodide). The IC50 was determined as the concentration of Z-DEVD-fmk inhibiting 50% of apoptosis at each time point. Duplicates of each experiment were done and the experiment was repeated on three independent cultures. Data represent the mean ± S.E.

Determination of Caspase Activity—Protein from neuron cultures or AD brain tissue (see description below) were extracted in cell lysis buffer (50 mM HEPEs, pH 7.4, 1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA with 0.05% phenylmethylsulfonyl fluoride, 0.1 μg/ml pepstatin A, 1 μg/ml N-p-tosyl-L-lysine chloromethyl ketone (TLCK), and 0.5 μg/ml leupeptin as protease inhibitors; all protease inhibitors from ICN, Montreal, Quebec, Canada) on ice. Insoluble proteins were removed by centrifugation. Samples were maintained at −80 °C until use. Caspase activities were determined as the concentration of Z-DEVD-fmk inhibiting 50% of protease activity. For the pulse-chase experiment, neurons were labeled for 5 h as described above. After the pulse-chase experiment, neurons were labeled for 5 h as described above.
Inhibition of Caspase Activation Prevents Serum Deprivation-Mediated Increase in Aβ Production—We have previously observed a 2–4-fold increase in secreted Aβ in apoptotic human neurons (5). To determine whether the apoptosis-dependent proteolytic caspases are responsible for the increased production of Aβ in serum-deprived neurons (5), APP metabolism was assessed in serum-deprived neurons in the presence or absence of a caspase-3-type inhibitor, Z-DEVD-fmk, shown to be involved in neuronal cell death (37). Commitment of neurons to apoptosis by serum deprivation induced the production of Aβ by over 2-fold and reduced secretion of the nonamyloidogenic α-secretase-clipped APP (sAPP) by 50%, whereas cellular APP levels remained normal (Fig. 1, A and B). Serum deprivation of neurons in the presence of 10 μM Z-DEVD-fmk abolished the increased production of Aβ and decreased the amount of Aβ produced by 50% compared with untreated neurons. Low levels of intracellular Aβ paralleled the amount of secreted Aβ (not shown). Z-DEVD-fmk did not inhibit the reduced secretion of sAPP. The addition of Z-DEVD-fmk considerably reduced the level of apoptosis to 10% or less, compared with 40% in serum-deprived neurons (Fig. 1C). The most effective concentration at 10 μM Z-DEVD-fmk limited apoptosis to approximately 5% of the culture. An IC_{50} of 0.1 μM was maintained between 24 and 96 h of serum deprivation. These results show that caspases are involved in the metabolism of APP into Aβ but not in the alternate α-secretase pathway. The inhibition of apoptosis by Z-DEVD-fmk coupled with the reduced amount of Aβ clearly indicates a crucial role for caspases in the production of Aβ and human neuronal cell death.

Caspase-6 Activity in Neuronal Cell Death and APP Metabolism—Caspase inhibitors are usually used at ~10 μM to inhibit cell death in cultures but only at 10 μM did caspase-6 activity correlate with neuronal death (39). We therefore determined whether caspase-6 is involved in the metabolism of APP into Aβ. In contrast to caspase-3 and caspase-6, caspase-7 did not cleave full-length APP (Fig. 4A). In contrast, both caspase-7 and caspase-8 reduced the fraction of: 95–100 kDa (Fig. 4B). Caspase-3 and caspase-6 did not cleave full-length APP (Fig. 4B). In contrast, both caspase-7 and caspase-8 reduced the amount of N-terminally detected APP and abrogated immunodetection of the C terminal region of APP. The cleaved N-terminal fragments of APP are clearly detected between 19 and 28 kDa in the caspase-7 cleaved protein extracts. Specific caspase inhibitors (Ac-DEVD-CHO for caspase-7 and Ac-IETD-CHO for caspase-8) eliminated cleavage of APP. The C-terminal fragments (CTFs) generated by caspase-7 and -8 were not detected by Western blot, possibly indicating small fragments of less than 15 kDa.

The lack of caspase-3 and caspase-6 activity on neuronal APP_{695} is surprising because excellent caspase-3 or -6 sites are present in the APP_{695} sequence (Fig. 4A) and caspase inhibitors Z-DEVD-fmk and Z-IETD-fmk prevent serum deprivation-mediated increase Aβ in neuron cultures. These results suggest that either (1) these sites are not available for caspase cleavage in native APP_{695}, (2) the suspected effect of caspase-6 on APP metabolism is indirect, (3) endogenous caspase inhibitors present in the neuronal extracts prevent recombinant caspase-3 or -6 cleavage of APP_{695}, or (4) APP is specifically protected
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against caspase-3 and caspase-6 cleavage. In these same neuronal extracts, tau was cleaved by caspase-3 and caspase-6, and Ac-DEVD-CHO and AC-IETD-CHO inhibited cleavage by caspase-3 and -6, respectively (Fig. 4C). Therefore, these results eliminated the possibility of natural strong inhibitors of caspase-3 or -6 in neuronal protein extracts and suggested that APP was somehow protected against caspase-3 and caspase-6 cleavage.

To verify whether neuronal APP<sub>695</sub> can be cleaved by caspase-3 and caspase-6 in absence of other neuronal proteins and to clearly identify APP cleavage products by caspasas, we repeated the cleavage of APP by recombinant caspasas using immunoprecipitated metabolically labeled neuronal APP<sub>695</sub> (Fig. 4E). Anti-C<sub>21</sub> immunoprecipitates full-length immature and mature APP<sub>695</sub> and CTFs between 6.5 and 12 kDa as described previously (5, 47). Caspase-3 and caspase-6 effectively cleaved the immunoprecipitated APP<sub>695</sub>, confirming the presence of APP specific caspase inhibitors in neurons. Caspase-3, -6, and -7 generated a variety of fragments that differed with each caspase (Fig. 4E). Biological techniques are planned to confirm the identity of each fragment. For the purpose of this study, we focused on potential C-terminal fragments containing Aβ to determine whether caspases could directly affect the production of Aβ. Closer evaluation of the sequence of APP<sub>695</sub> revealed two interesting potential caspase sites flanking the Aβ sequence (Fig. 4D). One site, at 66<sup>1</sup>VEVD<sup>664</sup>, would release 31 amino acids from the C-terminal end. The other site, at 591VKMD<sup>594</sup>, encompasses the first amino acid of the Aβ peptide and would further release a 70-amino acid fragment containing Aβ, except for the first amino acid (Fig. 4A). A fragment of around 3 kDa (named Capp3, for “caspase-generated APP fragment of 3 kDa”) is generated by caspase-3, -6, and -7. In addition, caspase-6 created a fragment of 6.5 kDa (Capp6.5), which could account for the additional expected caspase cleavage at 591VKMD<sup>594</sup>. Due to the promiscuity of the caspases and their ability to cleave most caspase sites at high concentration, we confirmed that Capp6.5 and Capp3 were generated with low amounts of caspase-6 recombinant enzyme (Fig. 4F). APP<sub>695</sub> was mostly degraded at high concentrations of caspase, indicating cleavage at many caspase sites in APP<sub>695</sub>. At lower concentrations, APP<sub>695</sub> generated only Capp3 and Capp6.5, resulting in high molecular weight truncated APP<sub>695</sub>. Epitope mapping confirmed the suspected identity of Capp3 and Capp6.5. Capp3 remained attached to the protein A-agarase beads used to immunoprecipitate APP<sub>695</sub>, consistent with it being generated at the C-terminal caspase site (Fig. 4G, retained). Furthermore, Capp3 and Capp6.5 were absent in caspase cleavage of secreted APP, consistent with their expected position at the C terminus of APP (not shown). Capp6.5 released by caspases from the beads contained the 4G8 (Aβ17–24) and anti-1 (anti-APP<sub>649</sub>–664; kind gift from D. Selkoe) but not the anti-C<sub>21</sub> epitope, which is compatible with the suspected fragment generated from cleavage at the caspase 591VKMD<sup>594</sup> and 66<sup>1</sup>VEVD<sup>664</sup> site. Together, these results indicate that caspase-6 can generate a potentially amyloidogenic fragment, Capp6.5, but cannot directly produce 4-kDa Aβ.

Capp6.5 Precedes Aβ Formation in Neurons—The presence of intracellular Capp6.5 was confirmed in 4G8 immunoprecipitates of metabolically labeled neurons. Capp6.5 increased in serum-deprived neurons similar to Aβ (Fig. 5A). A pulse-chase experiment confirmed that Capp6.5 is a likely precursor to 4-kDa Aβ (Fig. 5B). Full-length APP decreases by 20, 40, and 80% at 1, 2, and 4 h of chase under these conditions (50). Capp6.5 increased in the first 1 h of chase. Intracellular 4-kDa Aβ increased only slightly for up to 2 h of chase. Capp6.5 and intracellular Aβ decreased sharply after 2 h and decrease to nondetectable levels by 4 h of chase. Secreted 4-kDa Aβ increased slowly for up to 4 h of chase and then levels off between
4 and 7 h. The results indicate a rapid progression from Capp6.5 to intracellular 4-kDa Aβ to secreted 4-kDa Aβ. The secreted Aβ surpasses the amount of intracellular Aβ, indicating that either the intracellular Aβ is transient and quickly secreted or that two paths for Aβ production exist. A well-characterized pathway for Aβ production is through the endosomal-lysosomal pathway and APP CTFs (51). We expect that a portion of the Aβ results from APP CTFs, as they are abundant in human neurons (5, 47, 50). However, APP CTFs do not increase in apoptotic neurons (5), indicating that the overproduction of Aβ in serum-deprived neurons does not arise from the endosomal-lysosomal pathway. We propose that increased Aβ in serum-deprived neurons arises through the Capp6.5, which lacks the C-terminal portion of APP but contains Aβ.

**Caspase Expression in Human Primary Neurons**—To confirm the pattern of caspase activity observed by fluorometric assays, we also examined the profile of caspase-3, -6, -8, and -9 proteins in neurons. Only caspase-3, caspase-6, and caspase-9 immunoreactivity were detected in neurons.

The level of caspase-3 in serum-deprived primary cultures of human neurons was measured in proteins extracted at 0, 1.5, 3.0, 6, 12, 24, 48, and 72 h of untreated or serum-deprived neurons in the absence or presence of caspase inhibitor, Z-DEVD-fmk (Fig. 6A). High levels of caspase-3 proenzyme were evident in serum-deprived and nontreated neurons. However, few proteolytic active components of caspase-3 (at 29 and 17 kDa) were present relative to the amount of proenzyme. Although it is possible that the active fragments of caspase-3 undergo rapid turnover in neurons, the concomitant lack of caspase-3 activity (Fig. 2A) suggests that high levels of caspase-3 activity are never reached during apoptosis of these human neurons.

Caspase-6 expression has never been reported in neurons. Monoclonal antibody to caspase-6 p10 specifically recognizes six bands of 10, 28, 32, 36, 49, and 64 kDa (Fig. 6B, top left panel). These are not present in the absence of primary antibody (right panel), but are recognized by an additional polyclonal antiserum to p10 (Stressgen) and competed with the immunogen peptide (not shown). The 10 kDa band represents active p10 in recombinant caspase-6. The 32 and 28 kDa bands represent procaspase-6 and D-pro-arm caspase-6. Procaspase-6 is more abundant than D-pro-arm caspase-6 in adult tissue, whereas D-pro-arm caspase-6 is more abundant than procaspase-6 in fetal brain and cultured neurons. However, the 36 kDa band is present only in fetal brain and neurons, suggesting that this is a posttranslationally modified form of procaspase-6. The amount of p28, p32, and p36 procaspase-6 decreased in serum-deprived neurons despite equal amounts of β-actin (Fig. 6B, bottom left panel), as expected from activation of caspase-6. The higher molecular mass bands at 49 and 64 kDa likely represent dimers of p28 and p32. To confirm caspase-6 activity, we assessed cleavage of PARP. Fig. 6C shows the expected fragments of ~75 and 45 kDa of caspase-6 cleaved PARP rather than the 85- and 35-kDa fragments generated by caspase-3 (52). Together, these results indicate that caspase-6 but not caspase-3 is activated in serum-deprived neurons. Like caspase-3, caspase-9 is expressed in neurons and fetal and adult human brains but not activated (Fig. 6D).
Caspase Expression in Normal and AD Brain Tissue—To determine whether caspase-3 or -6 cleavage of APP may be involved in altered APP metabolism in human adult brain, we examined the expression level and activity of caspases in low post-mortem interval AD and non-AD control brain tissue. Similar to our observations in cultured human neurons, caspase-3 is abundant in human brain tissue in the frontal, temporal, parietal and cerebellar cortices (Fig. 7A). The level of caspase-3 appears higher in AD but is not really increased relative to the amount of β-actin. In contrast, synaptophysin levels decrease in the AD tissue compared with control brain tissue as expected (53). Despite high levels of caspase-3 proenzyme, few proteolytic fragments of caspase-3 are present in human brains. There is only a 28 kDa band that may represent caspase-3 lacking its pro-arm and possibly a small amount of p17 in the temporal cortex tissue of the AD case.

Caspase-6 proenzyme of 32 kDa is detectable in both non-AD and AD brain tissue (Fig. 7B). In contrast to primary neuron cultures or fetal brains, we detected equivalent or higher amounts of the p32 procaspase-6 than p28 caspase-6 lacking the pro-arm (Δprocaspase-6) in adult brain and both p28 and p32 decrease slightly in AD. The p10 proteolytically cleaved active caspase-6 is present in either frontal, temporal, parietal, or cerebellar areas of the AD brain. In addition to p10, we observed a 13-kDa fragment. The additional fragment is likely to be the result of alternative cleavage at amino acids 179 and 193 during activation of caspase-6 (54). The analyses of adult brain tissue will have to be repeated on many other cases to ascertain reproducibility of the levels of caspase and active fragments in AD and non-AD brain. However, although these approaches need to be done.
FIG. 4. APP is directly cleaved by caspases. A, schematic diagram of potential caspase-3-like and caspase-6 sites in APP<sub>695</sub>. B, Western blot detection of neuronal APP<sub>695</sub> cleaved by recombinant caspases in total protein extracts of primary neurons in the absence or presence of caspase inhibitor Ac-DEVD-CHO for caspase-3 and caspase-7, and Ac-IETD-CHO for caspase-6 and caspase-8. C, Western blot analysis of neuronal tau cleaved by recombinant caspases in total protein extracts of primary neurons in the absence or presence of specific caspase inhibitors. D,
secreted Aβ radiography of metabolically labeled immunoprecipitated cellular and secreted Aβ fragments. A, autoradiogram showing increased Capp6.5 in serum-deprived neurons in parallel to increased levels of intracellular Aβ and secreted Aβ. B, pulse-chase of immunoprecipitated Capp6.5, intracellular Aβ, and secreted Aβ. The levels of radioactivity were measured with a PhosphoImager (Molecular Dynamics).

DISCUSSION

Caspase inhibitors hold promise as neuronal apoptosis inhibitors and may eventually prove useful in the treatment of some conditions where neuronal cell death is the primary problem. The success of this therapy is hard to predict in progressive neurodegenerative diseases and is highly dependent on the complete understanding of the molecular mechanisms of neuronal cell death. As mentioned in the Introduction, caspase activation can be both cell type- and insult-dependent. Because human neurons are one of the unique neuronal cell types, with an expected long life span of 80–100 years, a complete understanding of the underlying events of neuronal apoptosis is essential for the evolution of therapies against apoptosis. We have established human primary neuron cultures to assess clearly the unique features of neuronal apoptosis in terminally differentiated and long-lived neurons. In the present study, we show that 1) caspase-6 is implicated in human neuronal apoptosis, 2) caspase activation considerably precedes the time of commitment of neurons to apoptosis, 3) caspase-6 activity is involved in amyloidogenic processing of APP, 4) proteins in normal neurons prevent caspase-3 and -6 cleavage of APP, and 5) caspase-6 proenzyme is detected in human adult brain tissue and the active p10 fragment is detected in AD brains. These results are the first to implicate caspase-6 in human neuronal cell death and suggest a potential role for caspase-6 in amyloidogenic processing of the amyloid precursor protein. These data bring forth the hypothesis that initiation of programmed cell death through caspase activation may not necessarily commit human neurons to immediate cell death but can cause dysfunction of neurons through altered proteolytic processing of key neuronal proteins.

**Caspase-6 Is Involved in Human Neuronal Cell Death—**
Caspase-6 is involved in apoptosis of primary cultures of human neurons. Group III caspase activity (caspase-6, -8, or -9) increases and caspase-6 protein decreases in serum-deprived neurons indicating activation of this caspase. Caspase-6-specific PARP cleavage pattern (52) confirms the activation of caspase-6. Group III caspase inhibitors prevent neuronal cell death by serum deprivation despite group I caspase activity. Therefore, our results indicate that caspase-6 can act as an effector caspase in these primary cultures of human CNS neurons.

We failed to detect group II caspase activity in neurons by either fluorometric (caspase-2, -3, -7, or -10) or Western blot analysis of caspase-3. The results are surprising, because caspase-3 is important for developmentally regulated neuronal cell death in vivo (37, 38), and in various neuronal apoptosis models (37, 44–46). Caspase-3 proenzyme is abundant in normal and apoptotic human primary neuron cultures. The exact reason for the absence of active fragments in these neurons is not clear but we do see a considerable increase in Bcl-2 levels in serum-deprived neurons,7 which raises the possibility that Bcl-2 prevents caspase-3 activation in these neurons as previously observed (55).

Together, these results indicate that caspase-6 is most important for apoptosis of serum-deprived primary cultures of human neurons. To our knowledge, caspase-6 expression and activity has not been shown in neuronal cell types, and these results raise an alternate possibility for the control of apoptosis in neurons.

**Involvement of Caspases in APP Metabolism: Caspase Inhibitors Prevent Serum Deprivation-mediated Increase in Aβ**
We have previously shown that serum deprivation-induced apoptosis of human neurons increases APP metabolism through the Aβ-producing pathway while decreasing that of the secretory pathway (5). These results indicated that proteases involved in APP metabolism are activated during active apoptosis in neurons. Initially, we found that the increase in Aβ previously observed in serum-deprived neurons was inhibited with group II inhibitor, Z-DEVD-fmk, indicating the involvement of caspase-3-type of activity. During our studies, Barnes et al. (56) also showed caspase-3 cleavage of APP in motorneurons. However, direct measurement of caspase activity by fluorogenic assay revealed the absence of group II caspase activity with time of serum deprivation and the potential for Z-DEVD-fmk to inhibit group III caspases in primary cultures of human neurons. Similar to Z-DEVD-fmk, the group III caspase substrate, Z-IETD-fmk, inhibits the increase in Aβ. These results indicate that caspase-6, which is most active on Ac-IETD-AMC

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fluorogenic substrate under our conditions and is active in serum-deprived neurons, is likely to be responsible for increased Aβ production in serum-deprived neurons. The role of caspase-6 is also supported by recent findings that group III caspase inhibitor, IETD, prevents APP cleavage in staurosporin induced cell death of COS-7 transfected cells (57).

In contrast, caspase inhibitors do not repress the reduction of sAPP release, indicating that serum deprivation down-regulates the α-secretase pathway of APP metabolism by a mechanism independent of apoptosis. These results once again show that the pathways of APP metabolism in human neurons are segregated so that the reduction of APP metabolism through one pathway does not necessarily increase metabolism through the other pathways (58).

**Recombinant Caspases Cleave APP695 and Neurons Contain Natural Inhibitors of APP Cleavage by Caspase-3 and Caspase-6**—We also show direct cleavage of APP with commercially available recombinant active caspase-3, -6, -7, and -8. The other caspases are not available at this time. The results show that these four caspases cleave APP at the N and C termini of APP. However, caspase-3 and caspase-6 cleavage of APP are inhibited in normal human neuronal protein extracts. Because tau protein is cleaved in these assays, we conclude that natural inhibitors of APP cleavage by caspase-3 and -6 but not caspase-7 or -8 exist in normal neurons. We suspect that these may be the APP-binding proteins Fe65 and X11, which bind to the C-terminal region of APP (59–61).

**Caspase-6 Generates a Potentially Amyloidogenic Fragment, Capp6.5; Possible Alternate Pathway for the Production of Aβ**—We identified a novel APP C-terminally truncated caspase-generated 6.5-kDa fragment, Capp6.5, which is a potential precursor for 4-kDa Aβ. Capp6.5 increases in serum-deprived neurons and degradation of Capp6.5 in a pulse-chase paradigm precedes the appearance of 4-kDa Aβ. The increase in Aβ in serum-deprived neurons is unlikely to be from the endosomal-lysosomal pathway because CTFs of APP do not increase in serum-deprived neurons (5). Therefore, serum deprivation-mediated increased Aβ likely arises from Capp6.5. The C-terminal of APP is oriented toward the cytosol either in transport vesicles or on the plasma membrane. Therefore, activation of cytosolic caspase-6 would initially clip the 3-kDa fragment at the 661VEVD664 site resulting in the loss of the NPTY receptor-mediated endocytotic signal and preventing the processing of C-terminally clipped APP through the endosomal-lysosomal pathway. It is possible that the C-terminally truncated APP traffics through a different metabolic route to produce Capp6.5 and eventually generate 4-kDa Aβ through access of β- and γ-secretase. We are not yet sure whether the N-terminal Aβ site is cleaved by caspases or β-secretase. Mass spectroscopic analysis on AD brain tissue or cultured cells reveals that the most abundant species is Aβ1–40 (62). However, Aβ peptides starting at alanine-2 instead of aspartic acid-1 in AD brain tissue have been observed in AD tissues (63) and therefore could have been generated by caspase-6. Moreover, the Swedish mutation replacing DKMD to DNLD

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**FIG. 6. Western blot analysis of caspases.** A. Caspase-3 levels in untreated neurons (+) or serum-deprived neurons in the absence (−) or presence (I) of 10 μM Z-DEVD-fmk caspase inhibitor. Each lane contains 10 μg of total protein and immunoreactivity was detected by chemiluminescence. B. caspase-6 in adult AD and non-AD frontal cortex, untreated (neurons + serum) or serum-deprived (neurons − serum) primary cultures of human neurons and fetal brain immunodetected with a monoclonal antibody to caspase-6 active fragment (top left panel) or β-actin (bottom left panel). R-Csp-6 represents 20 ng of recombinant caspase-6. The right panel is an identical copy of the top left panel probed without primary antibody. C. Western blot of PARP in Jurkat cell nuclear extract control (PARP) and untreated (neurons + serum) or serum-deprived (neurons − serum) neurons for 24 h. D. Caspase-9 immunoreactivity in neurons and brain tissue was as described in B.
increases caspase-6 cleavage in vitro (64). Therefore, there is a distinct possibility that C-terminally clipped APP is made accessible to cytosolic caspases to generate N-terminally cleaved Aβ.

Caspase Expression and Active Fragments in Adult Human Non-AD and AD Brains—Despite the potential problems that plague the detection of active enzymes in post-mortem tissues, we were able to confirm the presence of caspase-6 proenzyme and active proenzyme fragments in brain tissue obtained post-mortem from non-AD and AD patients. There is also evidence of caspase-6 mRNA in AD tissue (65). However, the lack of caspase-3 active fragment in these brains is unexpected because previous reports show caspase-3-like generated actin fragments in AD brains (34). We have detected cleaved 32-kDa actin in serum-deprived neurons and find that recombinant caspase-6 and caspase-7 cleave neuronal β-actin in vitro suggesting that caspase-6 could also generate 32-kDa β-actin in brains. Using the specific CM1 antisera produced by Idun Pharmaceuticals, Kevin Roth has failed to detect active caspase-3 in AD plaques or tangles,3 consistent with our findings that caspase-3 is not active. Therefore, it does not appear the human neurons in primary cultures undergo a type of caspase activation that is different than in vivo. On the other hand, one has to interpret the data using post-mortem tissue with caution. The use of post-mortem tissue generates a number of problems in the analysis of enzymatic activity that can potentially be affected by drugs taken by patients, duration of disease, length of agonal state, collection of tissue at the end point of the disease, amount of neuronal loss, post-mortem interval before autopsy, interval for freezing the tissue, and stability with time of freezing. At this time, the data that we present should be interpreted simply to suggest that caspase-6 is present in adult brains and that it could alter APP proteolytic processing in AD brains. However, the presence of caspase-6 proenzyme and proteolytically generated active enzyme fragment indicate a distinct possibility for a role for caspase-6 in both APP metabolism and neuronal cell dysfunction, if not death.

Caspase Activity May Not Always Be Accompanied by Apoptosis—The first peak of activity of caspase-6 increases within 1.5 h of serum deprivation, indicating a rapid response that precedes by 10 h the commitment time point of these neurons to serum deprivation-mediated apoptosis (5). These results indicate that there can be caspase activity in neurons uncommitted to apoptosis and that this caspase activity could be responsible for aberrant processing of many proteins over a lengthy period of time. Regulation of caspase activity and its effect on neuronal cell death will need to be clarified, but these results raise an interesting and logical possibility to explain aberrant proteolytic processing in human aging neurons without necessarily the presence of cell death. In fact, some neurons, although visibly sick, as indicated by the presence of neurofibrillary tangles, are not dead and may survive 15–20 years (66). Therefore, a lengthy process of neuronal cell death in adult brain may give neurons the opportunity to produce enough Aβ to explain the observed increased levels in AD brain (67). Our present results showing activation of caspase-6 in neurons that are not yet committed to neuronal apoptosis and in areas of AD brains that lack neuronal cell loss support this hypothesis.

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FIG. 7. Western blot analysis of caspases in AD and normal brain tissue. A, Western blot ECL analysis of β-actin, caspase-3, and synaptophysin in protein extracts of frontal (F), temporal (T), and parietal (P) cortex and cerebellum (C) tissue (50 μg/ lane). B, Western blot analysis of caspase-6 in the same AD and non-AD tissues used in A.
Caspase-6 in Neuronal Apoptosis, APP Metabolism, and AD