Cell death induced by a caspase-cleaved transmembrane fragment of the Alzheimer amyloid precursor protein

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Abstract

The Alzheimer amyloid precursor protein (APP) is a transmembrane protein whose abnormal processing is associated with the pathogenesis of Alzheimer's disease. Activated caspases cleave APP and generate its carboxyl-terminally truncated fragment (APP Δ C31). We have previously reported that overexpression of wild-type APP induces caspase-3 activation and apoptosis in postmitotic neurons. We now report that APP Δ C31 potentially plays pathophysiological roles in neuronal death. Adenovirus-mediated overexpression of wild-type APP695 induced activation of caspase-3 and accumulation of APP Δ C31 in postmitotic neurons derived from human NT2 embryonal carcinoma cells, whereas an APP mutant lacking the A β (1–20) region induced neither caspase-3 activation nor APP Δ C31 generation. Inhibition of caspase-3 suppressed the generation of APP Δ C31 in APP-overexpressing neurons. Forced expression of APP Δ C31 induced apoptotic changes of neurons and non-neuronal cells, but failed to activate caspase-3. The cytotoxicity of APP Δ C31 was also dependent on the A β (1–20) region. These results suggest that accumulation of wild-type APP activates neuronal caspase-3 to generate APP Δ C31 that mediates caspase-3-independent cell death.

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Abbreviations: APP, amyloid precursor protein; AD, Alzheimer's disease; A β , β /A4; MAP2, microtubule-associated protein 2; β -Gal, β -galactosidase; EthD-1, ethidium homodimer-1; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling; Z-DEVD-FMK, carbobenzoxy-Asp-Glu-Val-Asp-fluoromethylketone; Z-DEVD-AFC, carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin; PCR, polymerase chain reaction; FCS, fetal calf serum

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by massive amounts of neuronal death associated with histopathological hallmarks such as extracellular deposition of amyloid fibrils and intracellular accumulation of neurofibrillary tangles. The principal constituent of the amyloid fibrils is β /A4 (A β)¹ which is generated by processing of the amyloid precursor protein (APP). APP and A β have been considered as key molecules involved in the pathogenesis of AD.^{2–5} APP695, an APP isoform lacking the protease inhibitor domain, is abundantly expressed in postmitotic neurons during early periods of their differentiation.⁶ However, physiological and pathological roles of APP in neurons remain largely unknown. Earlier studies using APP-deficient cells suggest that cell-associated APP enhances neuronal survival and exerts neuroprotective functions.^{7,8} In contrast, we have previously reported that overexpression of wild-type APP695 induced death of postmitotic neurons derived from murine embryonal carcinoma P19 cells.⁶ Furthermore, viral vector-mediated overexpression of wild-type APP induces apoptosis-like death of postmitotic neurons both *in vivo* and *in vitro*.^{9–12} In APP-accumulating neurons *in vitro*, caspase-3 is activated in a cleavage-dependent manner, and a caspase-3 inhibitor significantly reduces the severity of degeneration exhibited by APP-overexpressing neurons.¹¹ These observations suggest that unphysiological levels of wild-type APP induce neuronal apoptosis in a caspase-dependent manner.

Caspases have a regulatory role in programmed death of chick spinal motoneurons,¹³ in which APP expression is upregulated during the course of cell death.¹⁴ Neurodegenerative insults induce cytoplasmic cleavage of endogenous APP by caspases and generate an APP fragment that lacks the carboxyl (C)-terminal 31-residue region (APP Δ C31).¹⁵ Caspase-6, which is activated in apoptotic neurons under serum-deprived conditions, cleaves APP directly at the cytoplasmic domain and generates A β -containing fragments.¹⁶ These findings suggest a close link between APP and the caspase-mediated death machinery.

In this study, we demonstrate that APP Δ C31 *per se* exerts cytotoxic effects on human postmitotic neurons and its precursor stem cells. Immunocytochemistry using end-specific antibodies against caspase-cleaved APP fragments revealed that wild-type APP undergoes proteolytic processing into APP Δ C31 and APP665–695 (APP-C31) when overexpressed in postmitotic neurons. We also show that APP Δ C31 induces nuclear changes indicative of apoptosis in postmitotic neurons and non-neuronal cells but fails to activate caspase-3. These findings raise the possibility that this caspase-cleaved form of APP has its own role in the modulation of neuronal death.

Results

Activation of caspase-3 and generation of APP Δ C31 in neurons by serum deprivation

We raised end-specific antibodies against activated caspase-3 subunits (ACP3) and the C-terminus of APP Δ C31 (SAC) to detect intraneuronal caspase-3 activation and APP Δ C31 generation. It has been reported that neurodegenerative insults activate neuronal caspases that cleave endogenous APP into APP Δ C31.¹⁵ We used postmitotic neurons under serum-deprived conditions to test the specificities and sensitivities of these antibodies (Figure 1). Differentiated neurons, which were identified by staining for the neuronal marker microtubule-associated protein 2 (MAP2), showed apoptotic features such as nuclear fragmentation and condensation by serum deprivation for 96 h. Immunocytochemistry using these antibodies revealed that these apoptotic neurons contained both activated caspase-3 and APP Δ C31. In contrast, MAP2-positive neurons had neither activated caspase-3 nor APP Δ C31 under serum-supplemented conditions. These data suggest that neuronal caspases are activated by serum-deprivation and cleave endogenous APP at the cytoplasmic domain, and that the end-specific antibodies ACP3 and SAC properly recognize activated caspase-3 subunits and the C-terminus of APP Δ C31, respectively.

Neuronal caspase-3-activation by accumulation of wild-type APP is dependent on the A β domain

We infected recombinant adenoviruses expressing wild-type APP and its mutant lacking the A β (1–20) region (APP Δ A β 20) into neurally differentiated NT2 neurons (Figure 2A). APP-accumulating neurons had condensed, fragmented nuclei and active caspase-3 subunits 48 h after infection, whereas a few neurons overexpressing APP Δ A β 20 or β -galactosidase (β -Gal) were positively stained for activated caspase-3. Quantification of caspase-3-immunopositive neurons revealed that 40% of neurons accumulating wild-type APP contained activated caspase-3 at 48 h (Figure 2B). Under these conditions, the population of caspase-3-immunopositive neurons among APP Δ A β 20-accumulating neurons was similar to that of β -Gal-accumulating neurons (~10%). When dead neurons were quantified using ethidium homodimer-1 (EthD-1), a fluorescent dye excluded by viable cells, 36% of neurons accumulating wild-type APP were found to be dead at 72 h (Figure 2C). In contrast, the population of dead neurons among APP Δ A β 20-accumulating neurons was ~6%, a level similar to that of β -Gal-accumulating neurons. These results suggest that the A β (1–20) region of APP is required for activation of neuronal caspase-3.

Cleavage of APP at the cytoplasmic site in neurons overexpressing wild-type APP

Because the cytoplasmic region of wild-type APP₆₉₅ contains the caspase cleavage site (VEVD), overexpressed full-length APP can be cleaved by caspases into the 664-residue amino (N)-terminal fragment (APP Δ C31) and the 31-residue C-

Figure 1 Caspase-3 activation and generation of the caspase-cleaved fragment APP Δ C31 within neurons induced by serum deprivation. Neurally differentiated NT2 cells (NT2 neurons) were cultured for 96 h in the presence (S+) or absence (S-) of 10% fetal calf serum. Cells were triply labeled for MAP2 (MAP2), chromosomal DNA (Hoechst), and activated caspase-3 subunits (ACP3) [or APP Δ C31 (SAC)]. MAP2-immunopositive S- neurons with apoptotic nuclei (arrows) are intensely immunostained with ACP3 and SAC, whereas control MAP2-immunopositive S+ neurons are hardly stained with these antibodies (arrowheads). Scale bar, 50 μ m

terminal fragment (APP-C31). Using antibodies against the C-terminal end of APP Δ C31 (SAC) and the N-terminal end of APP-C31 (ACT1), we examined whether APP Δ C31 and APP-C31 are actually generated in postmitotic neurons overexpressing wild-type APP. APP-overexpressing NT2 neurons were stained for the N-terminus of APP with antibody P2-1 and chromosomal DNA with Hoechst 33342 (Figure 3A). Most of wild-type APP-accumulating neurons had shrunken and fragmented nuclei. These apoptotic neurons were immunopositive for both APP Δ C31 and APP-C31 at 72 h as judged by staining with SAC and ACT1, respectively. In contrast, APP Δ C31 and APP-C31 were hardly detected in APP Δ A β 20-accumulating neurons or APP-accumulating non-neuronal cells in the mixed cultures.

To examine whether caspase-3 is involved in the cleavage of APP at the cytoplasmic site, we treated APP-overexpressing neurons with the cell permeable caspase-3 inhibitor carbobenzoxy-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-FMK) and examined the generation of APP Δ C31 and APP-C31 with the end-specific antibodies SAC and ACT1 (Figure 3B). In the absence of the inhibitor, 44% and 29% of APP-accumulating neurons were found to be immunopositive for SAC and ACT1 immunoreactivities, respectively. In the presence of the inhibitor (150 μ M), the proportions of APP Δ C31- and APP-C31-accumulating neurons were significantly decreased to 18% and 13%, respectively. The inhibitor had no appreciable effects on the proportions of SAC- and ACT1-immunopositive neurons that overexpressed APP Δ A β 20. These results suggest that activated caspase-3 caused by intracellular accumulation

Figure 2 Caspase-3 activation in neurons accumulating wild-type APP and the mutant lacking the A β (1–20) domain. **(A)** Immunocytochemistry for APP, chromosomal DNA, and activated caspase-3 subunits. NT2 neurons were infected with AxCALacZ (β -Gal), AxCAYAP (WT), or AxCAP Δ A β 20 ($\Delta\beta$ 20), fixed 48 h later, and triply stained for the N-terminus of APP (P2-1) (or β -Gal), chromosomal DNA (Hoechst), and activated caspase-3 subunits (ACP3). Some neurons accumulating wild-type APP are strongly immunostained with ACP3 (arrows), with which neurons accumulating APP Δ A β 20 and β -Gal are hardly labeled (arrowheads). Scale bar, 50 μ m. **(B)** Quantification of activated caspase-3-immunopositive neurons. NT2 neurons were infected with each recombinant adenovirus and fixed 48 h later for staining with ACP3. ACP3-immunopositive cells among APP (or β -Gal)-immunopositive neurons were counted (mean $\pm$ s.e.m., $n=3$; ≥ 200 cells per each group). * $P<0.05$, significantly different from the values of cells accumulating β -Gal or $\Delta\beta$ 20. **(C)** Quantification of neuron death. NT2 neurons were infected with each adenovirus, labeled with EthD-1 72 h later, and immunostained for APP or β -Gal. EthD-1-positive cells among APP (or β -Gal)-immunopositive neurons were counted (mean $\pm$ s.e.m., $n=4$; ≥ 100 cells per each group). * $P<0.05$, significantly different from the values of cells accumulating β -Gal or $\Delta\beta$ 20.

of wild-type APP substantially cleaves APP at the cytoplasmic site.

Western blot analysis revealed that wild-type APP and mutant proteins were abundantly expressed as analyzed using antibodies against the C-terminus of APP (AC1) (Figure 4A) and the N-terminus (AN2) (Figure 4B, upper panel). Neurons overexpressing wild-type APP contained a 96 kDa SAC-immunoreactive fragment, which was detected in APP Δ C31-overexpressing neurons (Figure 4B, lower panel). The amount of APP Δ C31 in neurons overexpressing wild-type APP was similar to that in neurons overexpressing APP Δ C31, suggesting that a considerable amount of wild-type APP is processed into APP Δ C31. In contrast, APP Δ C31 was undetected in neurons overexpressing

Figure 3 Generation of the caspase-cleaved fragments in NT2 neurons overexpressing wild-type APP. **(A)** Immunocytochemistry for APP Δ C31 and APP-C31. NT2 neurons were fixed 72 h after infection with AxCAYAP (WT) or AxCAP Δ A β 20 ($\Delta\beta$ 20), and stained for the N-terminus of APP (P2-1), chromosomal DNA (Hoechst), and the C-terminus of APP Δ C31 (SAC) [or the N-terminus of APP-C31 (ACT1)]. Most of wild-type APP-accumulating neurons with shrunken and fragmented nuclei contain SAC- and ACT1-immunoreactivities (arrows), but non-neuronal cells infected with AxCAYAP and neurons infected with AxCAP Δ A β 20 are hardly labeled with SAC and ACT1 (arrowheads). Scale bar, 50 μ m. **(B)** Quantification of APP Δ C31- and APP-C31-immunopositive neurons treated with a caspase-3 inhibitor. NT2 neurons were cultured in the presence of the caspase-3 inhibitor Z-DEVD-FMK after infection with AxCAYAP (WT) or AxCAP Δ A β 20 ($\Delta\beta$ 20). Cells were fixed at 72 h and stained for the N-terminus of APP (P2-1) and the C-terminus of APP Δ C31 (SAC) or the N-terminus of APP-C31 (ACT1). SAC- and ACT1-immunopositive neurons among P2-1-positive neurons were counted (mean $\pm$ s.e.m., $n=3$; ≥ 50 cells per each group). * $P<0.05$, significantly different from the values of cells cultured in the absence of Z-DEVD-FMK.

APP Δ A β 20 or β -Gal. These results, together with the data shown in Figures 2 and 3, suggest that overexpression of wild-type APP activates neuronal caspases that eventually cleave APP at the cytoplasmic site.

We have previously shown that a C-terminal 100 amino acid fragment of APP (APP-C100), which encompasses the A β domain and the C31 region, forms amyloid fibril-like structures in cDNA-transfected COS cells.¹⁷ We then examined whether full-length APP self-associates when accumulated on the membrane. Cell surface proteins in

NT2 neurons infected with recombinant adenoviruses expressing β -Gal, wild-type APP, APP Δ A β 20 and APP Δ C31 were treated with a membrane-impermeable cross-linker and analyzed by Western blotting using the antibody against the N-terminus of APP (AN2) (Figure 4C). APP immunoreactive bands were detected at $\sim$ 110 kDa and $>$ 220 kDa. The $>$ 220 kDa bands, which may correspond to APP cross-linked with other membrane proteins, were undetected in the absence of the cross-linker (data not shown). Only the neurons overexpressing wild-type APP contained a distinct $\sim$ 220 kDa APP immunoreactive band, whose size corresponds to that of the APP homodimer. These results suggest that the transmembrane form of APP tend to self-associate when concentrated on the membrane.

Caspase-3-independent neuronal death induced by APP Δ C31

Because APP Δ C31 was generated in neurons overexpressing wild-type APP, we then examined whether APP Δ C31 *per se* affects neuronal viability. We constructed the adenovirus vector expressing APP Δ C31 (AxCAP Δ C31) and infected it into NT2 neurons (Figure 5). A number of APP Δ C31-expressing neurons underwent death as analyzed by EthD-1 retention test (top panels). These degenerated neurons had nuclei stained positively by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) (middle panels). Intriguingly, APP Δ C31-accumulating neurons carrying apoptotic nuclei contained little or no activated caspase-3-immunoreactivity (bottom panels). These results suggest that APP Δ C31 induces neuronal apoptosis in a caspase-3-independent manner.

Quantification of neuron death by the EthD-1 retention assay revealed that 28% of APP Δ C31-expressing neurons were dead. Under the same conditions, β -Gal and wild-type APP caused neuron death at 7% and 33%, respectively (Figure 6A). We then measured caspase-3-like protease activity in neurons overexpressing wild-type APP, APP Δ C31, APP Δ A β 20, or β -Gal using the fluorogenic substrate carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Z-DEVD-AFC) (Figure 6B). In agreement with the immunocytochemical data shown in Figures 2A and 5, caspase-3-like activities in APP Δ C31, APP Δ A β 20, and β -Gal-overexpressing neurons remained low during the 96-h incubation period, whereas the activity in wild-type APP-overexpressing neurons increased and reached a peak at 48 h.

Cytotoxicity of APP Δ C31 is not specific to postmitotic neurons

We have previously shown that caspase-3-mediated apoptosis by wild-type APP is specific to postmitotic neurons.¹¹ The present study has shown that APP Δ C31 induced caspase-3-independent neuronal death. These findings suggested that APP Δ C31 induces apoptosis in a manner distinct from that of wild-type APP. When NT2 embryonal carcinoma stem cells (NT2S) and differentiated neurons (NT2N) were infected with the adenovirus vector expressing APP Δ C31, some of the

Figure 4 Western blot analysis of APP, APP Δ A β 20 and APP Δ C31. NT2 neurons were infected with AxCALacZ (β -Gal), AxCAYAP (WT), AxCAP Δ A β 20 (Δ A β 20), or AxCAP Δ C31 (Δ C31). Cell lysates were prepared 48 h after infection, and proteins were analyzed by Western blotting. (A) The APP C-terminus immunoreactivity (AC1). (B) APP N-terminus immunoreactivity (AN2; upper panel), and the C-terminal end of APP Δ C31 (SAC; lower panel). (C) A cross-linking experiment for cell surface proteins. Infected neurons were incubated with the cross-linker bis (sulfosuccinimidyl) suberate, and proteins in cell lysates were analyzed by Western blotting using antibody AN2. Molecular weight markers are indicated at the left (in kilodaltons) (A, C). Arrows, molecular sizes of reference bands in kilodaltons

stem cells and neurons showed apoptotic changes and EthD-1 retention (Figure 7). In contrast, most of the stem cells overexpressing wild-type APP were morphologically intact and showed little or no EthD-1 retention.

We then quantified the cytotoxic effects of β -Gal, APP Δ C31, wild-type APP, and APP Δ A β 20 on NT2 stem cells and transformed cell lines (Figure 8A). Only APP Δ C31 caused a significant increase in the dead population of non-neuronal cells such as NT2 stem cells, African green monkey kidney COS-1 cells, human glioma Bu17 cells, and human cervical carcinoma HeLa cells. The cytotoxic effects of wild-type APP and APP Δ A β 20 were hardly detected in these non-neuronal cells as compared with the values of the negative control of β -Gal ($P > 0.05$). In contrast, the cytotoxic effect of wild-type APP was seen only in NT2 neurons. These results indicate that APP Δ C31, unlike wild-type APP, is cytotoxic to non-neuronal cells as well as to postmitotic neurons.

Figure 5 Caspase-3-independent neuronal death induced by APP Δ C31. NT2 neurons were infected with AxCAP Δ C31, fixed 72 h later, and analyzed for cell death (top panels) and DNA fragmentation (middle panels). Cells were labeled for the N-terminus of APP (P2-1), chromosomal DNA (Hoechst), and EthD-1 (EthD-1) (or TUNEL). Some of the APP Δ C31-accumulating neurons showing nuclear abnormalities are positively labeled with EthD-1 and TUNEL (arrows). Cells were also fixed 48 h after infection with AxCAP Δ C31 and stained for activated caspase-3 subunits (ACP3) (bottom panels). APP Δ C31-accumulating neurons have condensed nuclei, severe membrane blebbing, and dystrophic neurites, but are negative for activated caspase-3 (arrowheads). Scale bar, 50 μ m for top and middle panels; 25 μ m for bottom panels

Figure 6 Lack of caspase-3-activation in neuron death induced by APP Δ C31. (A) Quantification of neuron death. NT2 neurons were infected with AxCALacZ (β -Gal), AxCAP Δ C31 (Δ C31), or AxCAYAP (WT). Cells were fixed 72 h later for EthD-1 retention assay. EthD-1-positive cells among APP- or β -Gal-positive neurons were counted (mean $\pm$ s.e.m., $n=4$; ≥ 100 cells per each group). * $P<0.05$, significantly different from the value of β -Gal-accumulating cells. (B) Caspase-3-like protease activity in infected NT2 neurons. NT2 neurons were infected with AxCAYAP (WT), AxCAP Δ C31 (Δ C31), AxCAP Δ A β 20 (Δ A β 20), or AxCALacZ (β -Gal), and caspase-3-like protease activity in the cell lysate was measured using the fluorogenic substrate Z-DEVD-AFC (mean $\pm$ s.e.m., $n=3$)

In order to examine whether the A β (1–20) domain in APP Δ C31 is responsible for its cytotoxicity, we constructed a vector expressing an APP mutant lacking both the A β (1–20) and C-terminal 31-residue regions (termed APP Δ A β 20/C31) and transfected it into COS-1 cells. The EthD-1 retention assay revealed that 32% of APP Δ C31-expressing cells were dead (Figure 8B). In this assay system, the

Figure 7 APP Δ C31-induced death of NT2 stem cells. NT2 stem cells (NT2S) and differentiated NT2 neurons (NT2N) were infected with AxCAP Δ C31 (Δ C31) or AxCAYAP (WT), fixed 72 h later, and triply labeled for the N-terminus of APP (P2-1), chromosomal DNA (Hoechst), and cell viability (EthD-1). APP Δ C31 is toxic to both NT2 stem cells and NT2 neurons (arrows), but only neurons undergo death by wild-type APP (arrowheads). Scale bar, 50 μ m

cytotoxic level of APP Δ A β 20/C31 was similar to those ($\sim 15\%$ dead cells) of wild-type APP, β -Gal and APP- Δ A β 20, which showed only the non-specific viral cytotoxicity. These findings suggest that the A β (1–20) region of APP Δ C31 is indispensable for its cytotoxicity.

Discussion

Caspase-3-independent apoptosis induced by APP Δ C31

Recent studies have demonstrated that APP is cleaved by caspases at Asp 664.^{14–16,18–20} The present immunocytochemical study has shown that apoptotic neurons under serum-deprived conditions contained activated caspase-3 subunits and APP Δ C31 (Figure 1). Furthermore, both APP Δ C31 and APP-C31 were detected in neurons overexpressing wild-type APP (Figure 3A). Generation of these fragments were significantly suppressed by treatment with caspase-3 inhibitor (Figure 3B). These findings suggest that overexpression of wild-type APP causes activation of caspases that subsequently cleave APP at the cytoplasmic Asp664 site (Figures 2A,B and 6B). Intriguingly, APP Δ C31 *per se* caused apoptosis of neurons in a caspase-3-independent manner (Figures 5 and 6). We also found that overexpression of APP Δ C31 caused death of non-neuronal cells including proliferative neural precursor cells and transformed cell lines originating from non-neuronal tissues (Figures 7 and 8A), indicating that the cytotoxic effects of APP Δ C31 are independent of cell species and their differentiation states. APP-C31, which is the counterpart of APP Δ C31 generated by caspase cleavage, has recently been reported to act as a cytotoxic peptide in cells treated with

Figure 8 Quantification of APP Δ C31-induced cytotoxicity. **(A)** APP Δ C31-induced cell death of NT2 cells and transformed cell lines. NT2 stem cells (NT2S), NT2 neurons (NT2N), COS-1 (COS), Bu17 glioma (Bu17), and HeLa cervical carcinoma (HeLa) cells were infected with AxCALacZ (β -Gal), AxCAP Δ C31 (Δ C31), AxCAYAP (WT), or AxCAP Δ A β 20 (Δ A β 20). Cells were fixed 72 h (for NT2S and NT2N cells) and 48 h (for COS, Bu17 and HeLa cells) after infection and double labeled with P2-1 (or β -Gal) and EthD-1. EthD-1-positive cells among P2-1 (or β -Gal)-positive cells were counted (mean $\pm$ s.e.m., $n=3$; ≥ 100 cells per each group). * $P < 0.05$, significantly different from the values of cells accumulating β -Gal. **(B)** Absence of cytotoxicity of the double-deletion mutant APP Δ A β 20/C31. COS-1 cells were transfected with pcDNA3.1 vectors carrying LacZ (β -Gal), wild-type APP (WT), APP Δ A β 20 (Δ A β 20), APP Δ C31 (Δ C31), and APP Δ A β 20/C31 (Δ A β 20/C31) cDNAs. Cells were fixed 72 h after transfection and double labeled with P2-1 (or β -Gal) and EthD-1. EthD-1-positive cells among P2-1 (or β -Gal)-positive cells were counted (mean $\pm$ s.e.m., $n=3$; ≥ 200 cells per each group). * $P < 0.05$, significantly different from the values of β -Gal control

apoptosis-inducing agents.²⁰ Thus, it is inferred that proteolytic processing of APP by caspases concomitantly generates APP Δ C31 and APP-C31 that exert their effects on cell death.

The molecular mechanisms whereby APP Δ C31 causes apoptotic cell death remain to be elucidated. Activated caspase-3 was undetected in neurons overexpressing APP Δ C31 (Figures 5 and 6B). Furthermore, we were unable to detect the activation of other caspases such as caspases-7, -6, -8, and -9 or upregulation of the endoplasmic reticulum stress marker proteins such as Grp78 and GADD153 (I Nishimura and K Yoshikawa, unpublished observations). The APP Δ C31-induced cytotoxicity has previously been assessed using mouse neuroblastoma cells treated with the apoptosis-inducing compounds staurosporine and tamoxifen.²⁰

Figure 9 A schematic model of the roles of APP and APP Δ C31 in neuronal apoptosis. See Discussion for details

APP Δ C31 had no cytotoxic effects on the neuroblastoma cells treated with staurosporine. However, they also reported that APP Δ C31 significantly reduced the cell viability when treated with tamoxifen. We have found that rodent cells require higher titers of APP-expressing adenoviruses than primate cells to undergo similar changes. We thus used primate cell lines infected with lower titers of adenovirus to demonstrate the cytotoxic effects of APP and APP Δ C31 in the present study. Thus we infer that cytotoxicity of APP Δ C31 is dependent on various factors such as cell types, APP Δ C31 expression levels, and presence of other cytotoxic agents.

In APP-overexpressing neurons, APP and subsequently generated APP Δ C31 would show additive or cooperative toxicity because they both have cytotoxic effects. However, we found that the neurotoxic potency of wild-type APP was similar to that of APP Δ C31 (Figure 6A). We used adenovirus titers that show a dead population of $\sim 30\%$ among total infected cells, because higher titers exert non-specific toxicity as generally observed in the adenovirus gene transfer system. Under these conditions, each infected neuron responds to APP with either death or survival. Thus, the fate of each APP-accumulating neuron cannot be changed even when cytotoxic APP Δ C31 is generated within the same cell. We speculate that this is the reason why wild-type APP (plus endogenously generated APP Δ C31) and APP Δ C31 showed similar cytotoxic potencies.

Some intracellular proteins have been reported to gain pathophysiological functions when cleaved by caspases. For example, Acinus and transcription factor NRF2 are cleaved by caspases and their cleaved products function as effectors for apoptosis.^{21,22} Furthermore, tau, a microtubule-associated protein whose abnormal phosphorylation is thought to cause formation of neurofibrillary tangles seen in AD, is cleaved by caspase-3 *in vitro*, and overexpression of the caspase-cleaved N-terminal fragment of tau induces apoptosis of NT2 stem cells and COS cells.^{23,24} These

findings, together with our present findings, raise the possibility that caspase-cleaved products of endogenous APP and tau, whose posttranslational modifications are believed to be involved in the pathogenesis of AD, directly induce neuronal death.

Neuronal specificity of caspase-mediated apoptosis induced by wild-type APP

Overexpression of wild-type APP causes caspase-3-mediated apoptosis of postmitotic neurons but not of non-neuronal cells,¹¹ suggesting that a neuron-specific machinery is involved in APP-induced caspase-3 activation. It has been demonstrated that two adaptor proteins, mammalian Disabled and FE65, which are primarily expressed in neurons, interact with the cytoplasmic tail of APP that contains the adaptor protein-binding motif NPTY.²⁵ This suggests that APP acts as a neuronal surface receptor that mediates intracellular signal transduction. Thus, APP-binding adaptor proteins such as Disabled and FE65 may contribute to the neuronal specificity of APP-induced caspase-3 activation. This idea is supported by the present findings that APP Δ C31, which lacks the adaptor-binding C31 region, failed to activate caspase-3 (Figures 5 and 6B). Another possibility is that neuron-specific ion channels or neurotransmitter receptors are involved in APP-induced neuronal apoptosis. We have previously reported that wild-type APP increases glutamate-induced Ca²⁺ influx into neuronal cytoplasm.²⁶ Furthermore, we have recently found that APP causes elevation of intracellular Ca²⁺ levels in postmitotic neurons prior to caspase-3 activation (manuscript in preparation). These findings suggest that Ca²⁺ channels or molecules regulating Ca²⁺ channel activities are involved in caspase-3 activation caused by wild-type APP.

Transgenic mice carrying APP mutants such as APP (Val717Phe)²⁷ and APP695 (Lys670Asn/Met671Leu)²⁸ have been reported to show neuropathological changes accompanied by extracellular A β depositions. However, no overt neuronal loss is detected in the brain regions in which A β is extensively deposited in APP (Val717Phe) transgenic mice,²⁹ suggesting that extracellular A β deposits *per se* are not toxic to neurons. The APP mutants in the transgenic mice *in vivo* may not be accumulated to toxic levels within neurons, whereas adenovirus-mediated overexpression of wild-type APP *in vivo* and *in vitro* induces a rapid elevation of APP levels sufficient for neuronal caspase-3 activation.^{10,11}

Self-association of APP may be responsible for caspase-3 activation

It is noteworthy that the APP mutant lacking the A β (1–20) domain (APP Δ A β 20), like APP Δ C31, induced no neuronal caspase-3 activation (Figures 2A,B and 6B). These findings suggest that both the A β domain and the cytoplasmic C31 region are indispensable for caspase-3 activation. We have previously reported that a C-terminal 100 amino acid fragment of APP, termed APP-C100, forms amyloid fibril-like structures in cDNA-transfected COS cells.¹⁷ APP-C100 which encompasses the A β and cytoplasmic C31 regions, is neurotoxic *in vitro* and *in vivo*.⁵ Thus, we speculate the self-associating nature of the C-terminal region of APP is responsible for the

caspase-3 activation and neuronal death. The cross-linking experiment revealed that wild-type APP-overexpressing neurons contained the ~220 kDa APP-immunoreactive band (Figure 4C), suggesting that wild-type APP, which comprises intact APP-C100 region, is prone to self-associate to form homodimer when accumulated on the membrane. It has recently been reported that antibodies against subsequences of the extracellular APP domain induce neuronal apoptosis.^{30,31} A monoclonal antibody (22C11) against the extracellular APP66–81 region induces caspase-3 activation and apoptosis in neurons, suggesting that the antibody facilitates APP dimerization and activates the caspase pathway.³⁰ Furthermore, we confirmed that antibody 22C11 induced caspase-3 activation and apoptosis in NT2 neurons within 24 h (I Nishimura and K Yoshikawa, unpublished observations). These findings together suggest that self-association of APP on the cell surface triggers the caspase cascade in neuronal cytoplasm.

Pathophysiological implications of APP-induced neuronal death

Based on the present findings, we schematized our hypothesis for APP-induced events operative in postmitotic neurons (Figure 9). Earlier studies have revealed that pathological stimuli and trophic factor deprivation upregulate endogenous APP levels.^{14,32,33} Furthermore, histopathological studies have revealed that neurons in AD brain contain abnormally dense APP-immunoreactive materials.^{34–36} Thus, neurons may have a propensity to accumulate APP in response to various insults ranging from trophic factor deprivation to neurotoxic stimuli. This phenomenon can be a compensatory response of stressed neurons to protect themselves against degeneration because cell-associated APP possesses neuroprotective functions.^{7,8} When the amounts of APP accumulations in affected neurons reach abnormally high levels, APP may in turn start to self-associate and activate caspases. Neuron-specific machinery including intracellular signal transduction molecules, ion channels, or neurotransmitter receptors may be involved in caspase activation. The caspase-cleaved transmembrane fragment APP Δ C31 may facilitate neuronal death in cooperation with other caspase-cleavable death effectors. The present findings that APP Δ A β 20 and APP Δ A β 20/C31, both of which lack the A β (1–20) region, failed to induce appreciable cell death lead to the speculation that the A β (1–20) region is indispensable for apoptosis by both caspase-dependent and -independent pathways. It might be of particular interest to examine whether such events are actually operative in neurons that are eliminated during nervous system development and under neurodegenerative conditions such as AD.

Materials and Methods

Recombinant adenoviruses

Recombinant adenoviruses expressing wild-type APP and its mutants were constructed as previously.^{10,11} Sequences encoding APP mutants were synthesized by polymerase chain reaction

(PCR)-mediated template modification. PCR was performed using primers carrying mutated sequences and restriction site (*Bgl*II, *Sal*I) at the ends and human full-length APP695 cDNA as a template.^{6,37} To construct cDNA of A β (1–20)-lacking APP (APP Δ A β 20), a PCR product lacking A β (1–20) region was inserted between *Bgl*II and *Sal*I sites of APP695 cDNA. To construct APP Δ C31, a sequence corresponding APP (amino acids 576–664) was amplified by PCR with a primer that carries two stop codons at the 3' end and inserted in APP cDNA. APP Δ A β 20 and APP Δ C31 DNAs were fully sequenced, blunt-ended, and subcloned at the *Swa*I site of pAxCawt. Cosmid DNA was cotransfected with the EcoT221-digested DNA-terminal protein complex of Ad5-dIX into 293 cells to generate recombinant viruses by homologous recombination. Adenovirus vectors expressing wild-type APP, APP Δ A β 20, and APP Δ C31 were designated as AxCAYAP, AxCAP Δ A β 20, and AxCAP Δ C31, respectively. Adenovirus expressing β -galactosidase (β -Gal) (AxCALacZ) was provided by Dr. I Saito (University of Tokyo, Tokyo, Japan). Propagation, and titration of recombinant adenoviruses were performed as described previously.^{10,11} Experiments using recombinant adenoviruses were approved by the Recombinant DNA Committee of the Osaka University and performed according to the institutional guidelines.

Adenovirus infection into cultured cells

Human embryonal carcinoma cells of NTera2/cl.D1 (NT2) cell line³⁸ (Stratagene, La Jolla, CA, USA) were cultured and neurally differentiated as reported previously.^{11,39} Briefly, NT2 cells were treated with 10 μ M all-trans retinoic acid (Sigma, St. Louis, MO, USA) for 30 days and subcultured at $\sim 3.0 \times 10^5$ cells per 35 mm dish at 37°C and 5% CO₂ in the medium Opti-MEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) containing 1 μ M cytosine arabinoside (Sigma) to obtain enriched postmitotic neurons. Serum-deprived neuronal cultures were obtained by incubating neurons in the absence of FCS for 96 h. COS-1 cells, human Bu17 glioma cells, and HeLa cells were cultured in the medium Dulbecco's modified Eagle medium supplemented with 10% FCS. NT2 neurons and other cells were exposed to recombinant adenovirus vectors at 2×10^7 pfu/ml culture medium for 12 h (multiplicity of infection 25–50) and then incubated in a virus-free fresh medium up to 96 h.¹¹

Antibodies

End-specific antibodies against activated caspase-3 p20/p17 subunits (ACP3), APP Δ C31 (SAC), and APP-C31 (ACT1) were raised in rabbits against synthetic peptides corresponding to the caspase-3 cleavage site,⁴⁰ APP 658–664 of APP695,¹⁵ APP 665–670 of APP695, respectively. Immunoglobulins G (IgGs) of SAC and ACT1 were depleted of IgG recognizing a bridging peptide corresponding to APP (amino acids 657–670) that spans the caspase cleavage site by affinity chromatography (ProtOn Kit1, Multiple Peptide Systems, San Diego, CA, USA). End-specific reactivities of the antibodies ACP3, SAC, and ACT1 were confirmed by ELISA titration, Western blotting analysis, and immunocytochemistry. Other antibodies used are: a mouse monoclonal antibody against MAP2 (Chemicon, Temecula, CA, USA); a rabbit polyclonal antibody against β -Gal (Cappel, Aurora, OH, USA); a mouse monoclonal antibody P2-1 against the N-terminus of APP⁴¹ (a gift from Dr. Van Nostrand); a rabbit polyclonal antibody against APP (amino acids 671–695) (AC1);⁶ a rabbit polyclonal antibody against APP (amino acids 18–38) (AN2).

Immunocytochemistry

NT2 neurons and other cells grown on coverslips were fixed 48–72 h after adenovirus infections with 4% formaldehyde in phosphate-buffered saline (PBS) (pH 7.4) at 4°C for 20 min and permeabilized with acetone at –20°C for 20 min. Then, fixed cells were incubated with primary antibodies in PBS containing 0.05% Tween 20 at 4°C overnight. The cells were then incubated at room temperature for 1 h with anti-rabbit and anti-mouse IgGs conjugated with fluorescein isothiocyanate (FITC) or rhodamine (Cappel). For chromosomal DNA staining, cells were incubated with 5 μ M Hoechst 33342 (Sigma) in PBS at room temperature for 10 min. Nuclear DNA fragmentation was analyzed by the TUNEL method.⁴² combined with immunostaining for APP with P2-1. TUNEL reactivity and APP were visualized with Texas Red and FITC, respectively. For caspase-3 inhibitor protection analysis, NT2 neurons were preincubated for 1 h with DMSO vehicle or Z-DEVD-FMK (Caspase-3 Inhibitor II; Calbiochem, La Jolla, CA, USA), infected with adenoviruses, and cultured for 72 h. After fixation, NT2 neurons were immunostained for APP Δ C31 with SAC and for APP-C31 with ACT1. Immunoreactive images were observed by fluorescence microscopy (BX50-34-FLAD1, Olympus, Tokyo, Japan).

Cell death analysis

NT2 neurons and other cells were infected with adenoviruses, and 48 h or 72 h later the cells were incubated with 10 μ M EthD-1 (Molecular Probes, Eugene, OR, USA) in PBS at 37°C for 30 min. After fixation, the cells were incubated with the antibody P2-1 against the N-terminus of APP and anti- β -Gal antibody, and subsequently incubated with FITC-conjugated anti-mouse and anti-rabbit IgGs, respectively. EthD-1-positive cells among 100–200 APP- and β -Gal-positive cells were counted by choosing five random fields per each dish and expressed as a percentage. Statistical significance was tested using Student's *t*-test.

Cross-linking of cell surface proteins

NT2 neurons were infected with adenoviruses. The cells were washed 24 h later with a buffer containing 137 mM NaCl, 5.3 mM KCl, 170 μ M Na₂HPO₄, 220 μ M KH₂PO₄, 10 mM HEPES, (pH 7.3), 33 mM glucose, and 44 mM sucrose and incubated with 1 mg/ml bis (sulfosuccinimidyl) suberate (Pierce, Rockford, IL, USA) in the buffer for 30 min at 37°C. After cross-linking, neurons were harvested, and their lysates were analyzed by Western blot analysis.

Western blot analysis

NT2 neurons infected with adenovirus were dislodged by pipetting the medium up and down with a Pasteur pipette several times. Most of the non-neuronal cells remained attached after this treatment. Detached neurons were then collected by centrifugation at 120 $\times g$ for 5 min. Enriched neurons were resuspended in the buffer containing 10 mM Tris-HCl, pH 7.4, 100 μ M phenylmethanesulfonyl fluoride, 10 μ M pepstatin, 10 μ M leupeptin, and 2 μ g/ml aprotinin. The homogenates were centrifuged at 15 000 $\times g$ for 30 min, and proteins (2 μ g per lane) in the pellets (membrane fraction) were separated by 7.5% SDS-polyacrylamide gel electrophoresis. For analysis of cross-linked cell extracts, 2 μ g proteins were separated by 6% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), and blotted with AC1, AN2, and SAC. The membrane was incubated with peroxidase-labeled anti-rabbit IgG, and the signals were detected with chemiluminescence reagents (Renaissance Plus; NEN, Boston, MA, USA).

Measurement of caspase-3-like protease activity

Adenovirus-infected NT2 neurons were collected and incubated on ice for 10 min in a cell lysis buffer for caspase activity assay (MBL, Nagoya, Japan). Lysates were centrifuged at $15\,000 \times g$ for 10 min at 4°C , and the supernatant (10 μg protein) was used for caspase-3-like protease assay. Caspase-3-like activity was measured by cleavage of the fluorogenic substrate Z-DEVD-AFC using a kit (FluorAce Apopain kit; Bio-Rad, Hercules, CA, USA) and a spectrofluorometer (Versa-Fluor; Bio-Rad). One unit was defined as the amount of enzyme required to cleave 1 pmol AFC per 60 min incubation at 37°C .

COS-1 cytotoxic assay

cDNA encoding an APP mutant lacking both the A β (1–20) and the C-terminal 31-residue regions (APP Δ A β 20/C31) was constructed by PCR using primers for APP Δ C31 and APP Δ A β 20 cDNAs as described above. β -Gal, wild-type APP, APP Δ A β 20, APP Δ C31, and APP Δ A β 20/C31 cDNAs were subcloned into pcDNA3.1(+) carrying the cytomegalovirus promoter (Invitrogen, San Diego, CA, USA). Vectors expressing APPs and β -Gal (1 μg per assay) were introduced into COS-1 cells using Lipofectamine Plus (Gibco, Gaithersburg, MD, USA), and cell death was analyzed at 72 h as above.

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