Selective cytotoxicity of intracellular amyloid β peptide₁₋₄₂ through p53 and Bax in cultured primary human neurons

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Extracellular amyloid β peptides (Aβs) have long been thought to be a primary cause of Alzheimer’s disease (AD). Now, detection of intracellular neuronal Aβ₁₋₄₂ accumulation before extracellular Aβ deposits questions the relevance of intracellular peptides in AD. In the present study, we directly address whether intracellular Aβ is toxic to human neurons. Microinjections of Aβ₁₋₄₂ peptide or a cDNA-expressing cytosolic Aβ₁₋₄₂ rapidly induces cell death of primary human neurons. In contrast, Aβ₁₋₄₀, Aβ₄₀₋₁, or Aβ₂₋₁ peptides, and cDNAs expressing cytosolic Aβ₁₋₄₀ or secreted Aβ₁₋₄₂ and Aβ₁₋₄₀ are not toxic. As little as a 1-pM concentration or 1500 molecules/cell of Aβ₁₋₄₂ peptides is neurotoxic. The nonfibrillized and fibrillized Aβ₁₋₄₂ peptides are equally toxic. In contrast, Aβ₁₋₄₂ peptides are not toxic to human primary astrocytes, neuronal, and nonneuronal cell lines. Inhibition of de novo protein synthesis protects against Aβ₁₋₄₂ toxicity, indicating that programmed cell death is involved. Bcl-2, Bax-neutralizing antibodies, cDNA expression of a p53R27H dominant negative mutant, and caspase inhibitors prevent Aβ₁₋₄₂-mediated human neuronal cell death. Taken together, our data directly demonstrate that intracellular Aβ₁₋₄₂ is selectively cytotoxic to human neurons through the p53–Bax cell death pathway.

Introduction

Alzheimer’s disease (AD)* is a progressive neurodegenerative disease of the central nervous system characterized by the presence of extracellular senile plaques, intraneuronal neurofibrillary tangles (NFTs), and the loss of synapses and neurons. The amyloid β peptide (Aβ) is a major component of the extracellular senile plaque. Aβ naturally arises from the metabolic processing of the amyloid precursor protein (APP) in the ER, the Golgi apparatus, or the endosomal–lysosomal pathway, and most is normally secreted as a 40– (Aβ₁₋₄₀) or 42– (Aβ₁₋₄₂) amino acid peptide (Martin et al., 1995; Chyung et al., 1997; Tienari et al., 1997; Lee et al., 1998; Morishima-Kawashima and Ihara, 1998; Greenfield et al., 1999; Perez et al., 1999). However, an NH₂-terminally truncated form of Aβ₁₋₄₂ (Aβ₄₂) accumulates in the ER in aging cell cultures (Greenfield et al., 1999; Yang et al., 1999). Recently, the presence of intracellular Aβ₄₂ has been detected in the brains of individuals with AD or Down’s syndrome, and in APP transgenic mice and aging monkeys. In AD brains, the intracellular Aβ₄₂ accumulates as aggregates or granules in the cytoplasm of neurons (Gouras et al., 2000; D’Andrea et al., 2001). The accumulation of Aβ precedes the appearance of NFTs and senile plaques and is observed in regions affected early in AD: the hippocampus and entorhinal cortex. The intracellular Aβ does not appear to be fibrillar, as it is not stained by Bielchowsky silver stain, Thioflavin S, or Congo red, nor does it require formic acid treatment for immunostaining (Gouras et al., 2000). In Down’s syndrome brains, accumulation of intracellular Aβ₁₋₂₈ and Aβ₁₋₄₀ precedes Aβ₄₂, and these intracellular Aβ accumulations precede the appearance of diffuse plaques, senile core plaques, and NFT formation (Gyure et al., 2001). In aging...
Intracellular Aβ neurotoxicity in primary human neurons. (A) Fluorescent photomicrographs of microinjected neurons. Neurons were microinjected with the peptides in DTR and incubated 24 h before staining with TUNEL for cell death or Hoechst for nuclear stain. (B) Aged Aβ 1–40, Aβ 1–42, Aβ 42–1, and Aβ 40–1 peptides (10 nM) were microinjected into the cytosol of human neurons and cell death was measured by TUNEL at 1, 2, 4, and 16 d after injection. Two-way ANOVAs (df = 4; df = 3) followed by Sheffé’s test were performed to determine the statistical significance between Aβ-injected and control DTR-injected neurons. *, P < 0.01. (C) Various doses of Aβ 1–42 and Aβ 40–1 were injected into human neurons and cell death was determined by TUNEL staining at 2, 4, or 16 d after injection. Two-way ANOVAs (df = 2; df = 29) followed by Sheffé’s test were performed to determine the statistical significance. *, P < 0.01. (D) Human neurons were exposed to 10 μM extracellular Aβ 1–40, Aβ 1–42, and Aβ 42–1 for 24 h and stained with propidium iodide to reveal cellular nuclei and TUNEL to reveal cell death. (E) Cell death in neurons 24 h after microinjection with pCep4 episomal cDNA constructs expressing cytosolic Aβ 1–40 and Aβ 1–42 (cAβ) or secreted Aβ 1–40 and Aβ 1–42 (sAβ). One-way ANOVA (df = 5) followed by Sheffé’s test determined a statistically significant difference between the Cep4β construct alone and Aβ 1–42 peptide or Cep4β-cAβ 1–42 expression construct. *, P < 0.01. For B–E, the data represent the mean ± SEM of three independent experiments.

monkeys, nonfibrillar neuronal and nonneuronal intracellular Aβ precede the deposition of extracellular and fibrillar Aβ (Martin et al., 1994). Furthermore, the accumulation of intracellular Aβ also precedes plaque formation in mutant APP/presenilin (PS)-1 transgenic mice (Wirths et al., 2001), accumulates in the APPV717F mutation where synaptic loss...
precedes extracellular Aβ deposition (Masliah et al., 1996; Hsia et al., 1999; Li et al., 1999), and is associated with neuronal loss in PS-1 transgenic mice in the absence of extracellular Aβ deposition. Although extensive research has been conducted on the relevance of extracellular Aβ deposits in AD (Selkoe, 1998), the biological significance of the accumulation of intracellular Aβ is not known. The early appearance of the intracellular Aβ42 suggests that it could be the initial cause of neuronal dysfunction and neuronal loss in AD. To determine if intracellular Aβ may be detrimental to neurons, we microinjected Aβ1-40, Aβ1-42, and control reverse peptides Aβ40-1 and Aβ42-1, or cDNAs expressing cytosolic or secreted Aβ1-40 and Aβ1-42 in primary cultures of human neurons, neuronal, and nonneuronal cell lines. Aβ1-42 was selectively toxic to human neurons. Microinjections of the Aβ1-42 peptide in the presence of transcriptional and translational inhibitors showed that the cytotoxicity of Aβ1-42 required de novo protein synthesis. Because of the known role of the p53 transcriptionally regulated proapoptotic Bax protein in neuronal cell death (Xiang et al., 1998), we assessed the effect of the antiapoptotic Bcl-2 protein and Bax neutralizing antibodies on Aβ1-42-mediated neurotoxicity. Both eliminated toxicity. Furthermore, the p53R27H dominant negative mutant, which prevents transactivation of Bax expression (Aurelio et al., 2000), eliminated Aβ1-42 toxicity. Lastly, caspase-6, but not caspase-3-type inhibitors, prevented Aβ1-42 toxicity. We conclude that Aβ1-42 is selectively toxic to human neurons through activation of the p53 and Bax proapoptotic pathway.

Results

Intracellular Aβ1-42, but not Aβ1-40, Aβ42-1, or Aβ40-1, is neurotoxic to human neurons

To directly determine whether intracellular Aβ is toxic to human neurons, we microinjected aged Aβ1-40, Aβ1-42, or the reverse control peptide Aβ40-1 into the cytoplasm of primary cultured human neurons. Aβ1-42 induces significant cell death in 60% of microinjected neurons within 24 h of injection (Fig. 1, A and B). Cell death further increases to 78 and 90% at 48 and 96 h after injection. However, Aβ1-40, the control reverse peptides, Aβ40-1, Aβ42-1, or the fluorescent marker dye Dextran Texas red (DTR), do not affect cell viability for up to 4 d (Fig. 1 B). After 16 d of injection, only Aβ1-40 induces 50% cell death. Because we inject 25 pl/shot of a 10-nM concentration of peptide, the above results indicate that 0.25 × 10^-18 moles of Aβ1-42 delivered into the neuron cytosol is sufficient to induce rapid neuronal death. A 100-fold dilution in the amount injected still induces 50% cell death in these neurons (Fig. 1 C). Using confocal microscopy, we estimated the volume of these human neurons at 4.97 ± 0.8 nl (n = 3). The nuclei occupy >50% of the cell, thus the cytosolic area is ~2.5 nl. Therefore, the actual toxic concentration of injected Aβ1-42 is 0.25 × 10^-18 to 0.25 × 10^-20 moles/2.5 nl, which equals 10^-10 to 10^-12 M, or 1 to 100 pM. These neurons do not undergo cell death even with 10 μM of extracellular Aβ1-42, Aβ1-40, or Aβ40-1, a concentration known to induce cell death in a variety of neuronal cell lines (Paradis et al., 1996; Klein et al., 2001). To test if this particular batch of peptide might be neurotoxic, we treated the neurons with 10 μM of these peptides for 24 h. Neither extracellular aged Aβ1-40, Aβ1-42, or Aβ40-1 is toxic to these neurons after 24 h of treatment (Fig. 1 D). Therefore, the toxicity of intracellular Aβ1-42 is at least 100,000 times greater than extracellular Aβ. These results indicate that an infinitesimal amount of intracellular Aβ1-42 is detrimental to human neurons. Calculation of the number of molecules of Aβ1-42 injected in neurons based on the Avogadro number shows maximal toxicity with 150,555 molecules and 50% toxicity with 1505.5 molecules/neuron. The level of toxic Aβ1-42 is probably at least 10,000-fold lower than the amount of immunologically detectable intracellular Aβ1-42 in AD neurons. However, because neurons in the brain are bathed in extracellular milieu that promotes their survival, the in vivo neurons may resist higher concentrations of intracellular Aβ1-42 than the neurons in culture. Finally, to confirm the toxicity of naturally produced intracellular Aβ peptides, neurons were microinjected with cDNA constructs expressing cytosolic or secreted Aβ1-40 and Aβ1-42. As observed with the synthetic Aβ1-42 peptide, only the cytosolically expressed Aβ1-42 was toxic, whereas secreted Aβ1-42 or cytosolic or secreted Aβ1-40 did not induce cell death in neurons (Fig. 1 E).

Nonfibrillized Aβ1-42 is neurotoxic

Because the fibrillar form of Aβ is commonly seen in the senile plaques in AD brains and has long been proposed to be more toxic than soluble Aβ (Pike et al., 1993), we examined the toxicity of both fibrillized and nonfibrillized Aβ peptides. Transmission electron microscopy on the preparation of Aβ confirms the fibrillar and nonfibrillar nature of the Aβ preparation (Fig. 2 A). The nonfibrillized Aβ1-42 peptides show well defined globular as well as diffuse aggregate morphology. Similar well-defined globular structures are also seen in the nonfibrillized Aβ1-40 preparation, but are much less abundant than in the Aβ1-42 preparation. The fibrillized Aβ1-42 peptide shows a heterogeneous mixture of fibrils of various sizes, protofibrils, and globular structures. The fibrils in the Aβ1-40 preparation were less heterogeneous and consisted of thick fibrillar aggregates and thin aligned fibrils. The large Aβ1-40 fibrils appear not to pass through the micropipette, and thus were unlikely to have been injected in neurons (unpublished data). However, some of the smaller fibrils from the Aβ1-42 and Aβ40-1 preparation did pass through the pipette. Both fibrillized and nonfibrillized preparations of Aβ1-42 induce 50–90% neuronal cell death between 24–96 h after injection (Fig. 2 B). In contrast, neither fibrillized nor nonfibrillized Aβ1-40 cause significant cell death. Previously, it was shown that dimers/oligomers of Aβ are toxic to neurons when applied in the extracellular milieu (Walsh et al., 1999). Western blot analysis of the nonfibrillized and fibrillized Aβ peptides shows that the Aβ1-42 peptide forms aggregates with the expected size of Aβ dimers, trimers, and oligomers. In contrast, the nonfibrillized and fibrillized Aβ1-40 peptides show a 6.5-kD aggregate and with longer exposure, a smear of higher MW oligomers unpublished data. Taken together, these data show that the fibrils are not required for toxicity and suggest that either the monomeric Aβ1-42 is significantly more toxic than the monomeric Aβ1-40 or the dimers, trimers, and oligomers of the nonfibrillized and fibrillized Aβ1-42 peptide may be the toxic forms of the Aβ peptides.
Intracellular \( \text{A}\beta_{1-42} \) toxicity appears selective to human neurons

Because neurons specifically undergo cell death in AD, we explored the specificity of intracellular \( \text{A}\beta \) toxicity in primary cultured human astrocytes. The volume of these cells is 10 times that of human neurons, therefore, we injected 100 nM of \( \text{A}\beta_{1-42} \) into the cytosol of astrocytes to keep the same concentration of injected \( \text{A}\beta \). In human neurons, cell death is easily observed in the DTR-microinjected cells by TUNEL and condensed nuclear DNA (Fig. 3 A). In contrast, microinjected astrocytes do not show any sign of condensed chromatin by Hoechst staining, nor positive nuclear TUNEL staining (Fig. 3 A). The faint green fluorescence detected in these cells is the result of bleed-through from the
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Similarly, microinjections of the Aβ1–42 peptide into human neuroblastoma, La-N-1 and M17 cell lines, teratocarcinoma NT2 cells, mice NIH 3T3 fibroblasts, and BHK cells fail to induce cell death. Quantitative analysis on 600 microinjected cells from three independent experiments confirms that among the cell lines tested, only human primary neurons are susceptible to the intracellular Aβ1–42 toxicity (Fig. 3 B). Whether this toxicity occurs in vivo in adult human neurons remains to be determined. However, the in vivo intracellular toxicity of Aβ1–42 occurs in transgenic animals (LaFerla et al., 1995). Taken together, our data demonstrates that intracellular Aβ1–42 is selectively toxic to these human neurons.

Aβ1–42 neurotoxicity requires do novo protein synthesis

To address the underlying molecular mechanism of intracellular Aβ toxicity, we microinjected Aβ1–42 into neurons and incubated the cells in the presence or absence of the transcriptional inhibitor, actinomycin D, and the translational inhibitor, cycloheximide, for 24 h. Both cycloheximide and actinomycin D efficiently block Aβ1–42-induced neuronal death (Fig. 4). These results indicate that de novo protein synthesis is necessary for intracellular Aβ toxicity.

Bax is responsible for intracellular Aβ toxicity

Because the proapoptotic Bax protein can induce rapid human neuronal cell death through overexpression (Bounhar et al., 2001), we suspected that Bax could be involved in Aβ1–42-mediated cell death. Therefore, we microinjected a human cDNA expression construct of Bcl-2, the major anti-Bax protein, with the Aβ1–42 peptide (Fig. 5 A). The microinjection of the Bcl-2 construct completely eliminates Aβ1–42-mediated neurotoxicity as observed before against Bax (Bounhar et al., 2001). In contrast, coinjection of an APP cDNA construct does not alter Aβ1–42-mediated cell death.

To confirm that Bax is involved, we tested whether antibodies against human Bax could neutralize Bax-mediated killing. We found that two monoclonal antibodies, 6A7 and 2D2, and the polyclonal antisera N-20, but not an anti-APP
antibody, rabbit serum nor a mouse IgG, could neutralize the pro-apoptotic properties of Bax (Fig. 5 B). Similarly, these anti-Bax antibodies completely neutralized the A\textsubscript{β}\textsubscript{1–42}-mediated neurotoxicity. In contrast, none of these antibodies had any effect on recombinant active caspase-6–mediated cell death. Therefore, these experiments show that anti-Bax antibodies specifically inhibit Bax-mediated or A\textsubscript{β}\textsubscript{1–42}-mediated cell death and indicate that A\textsubscript{β}\textsubscript{1–42} induces cell death through Bax activation. Together, these results implicate Bax in A\textsubscript{β}\textsubscript{1–42}-mediated neuronal cell death.

p53 is involved in intracellular A\textsubscript{β}\textsubscript{1–42}-mediated neurotoxicity

Bax is transcriptionally regulated by p53; therefore, we tested whether p53 activation was involved in intracellular A\textsubscript{β}\textsubscript{1–42}-mediated neurotoxicity. We chose to use the p53	extsuperscript{R273H} dominant negative (p53\textsuperscript{DN}) mutant because it effectively inhibits p53 transcriptional activation of Bax (Aurelio et al., 2000). Whereas the expression of p53 wild-type (WT) or p53\textsuperscript{DN} does not induce neuronal apoptosis in absence or presence of A\textsubscript{β}\textsubscript{1–42}, the p53\textsuperscript{DN}, but not the p53 WT, effectively inhibits A\textsubscript{β}\textsubscript{1–42}-mediated cell death and indicate that A\textsubscript{β}\textsubscript{1–42} induces cell death through Bax activation. Together, these results implicate Bax in A\textsubscript{β}\textsubscript{1–42}-mediated neuronal cell death.
Caspase inhibitors prevent Aβ1–42-mediated cell death
Previously, we have shown that serum deprivation induces caspase-6-, but not caspase-3-mediated cell death in human neurons (LeBlanc et al., 1999). To assess the role of caspases in Aβ1–42-mediated neurotoxicity, we incubated Aβ1–42-injected neurons in the presence or absence of various caspase inhibitors. The results show that the pan caspase inhibitor, Z-VAD-fmk, the caspase-6-like inhibitor, Z-VEID-fmk, and the caspase-8-like inhibitor, Z-IETD-fmk, completely prevent Aβ1–42-induced neuronal cell death (Fig. 7). In contrast, the caspase-1-like inhibitor, Z-YVAD-fmk and the caspase-3-like inhibitor, Z-DEVD-fmk, do not inhibit Aβ1–42-induced cell death. Therefore, the data show that caspase-6- or caspase-8-like activities regulate Aβ1–42-mediated neuronal apoptosis.

Discussion
The accumulation of intraneuronal Aβ42 before the appearance of senile plaques and NFT in AD suggests the relevance of intraneuronal Aβ42 in neurons (Gouras et al., 2000; D’Andrea et al., 2001). In the present manuscript, we show that (a) very low levels of intracellular Aβ1–42, but not Aβ1–40, Aβ2–1, or Aβ6–1, are selectively cytotoxic to human neurons; (b) nonfibrillized Aβ1–42 peptide is as toxic as the fibrillized peptide and toxicity may be attributable to the formation of Aβ1–42 oligomers; and (c) Aβ1–42 toxicity is mediated through the p53 and Bax cell death pathway. Therefore, our findings support the hypothesis that intracellular accumulation of Aβ1–42 may be an initial cause of neuronal dysfunction and loss in AD. Intracellular accumulation of Aβ1–42 preceding other pathological lesions is observed not only in AD (Gouras et al., 2000; D’Andrea et al., 2001), but also in cells or transgenic mice expressing AD-associated PS-1 mutations, APP mutations, or double APP/PS-1 mutations (Masliah et al., 1996; Sudoh et al., 1998; Xia et al., 1998; Chui et al., 1999; Petanceska et al., 2000; Wirths et al., 2001) and in aging monkeys (Martin et al., 1994). Some of these animal models have been shown to present behavioral abnormalities or neurodegeneration before the appearance of extracellular plaques (Chui et al., 1999; Hsia et al., 1999). In addition, despite most studies showing that extracellular Aβ deposition in senile plaques does not correlate well with the severity of AD (Terry, 1999), analysis of total Aβ levels in protein extracts from brains of well-ascertained cognitively impaired patients reveals an elevation of both Aβ40 and Aβ42 levels with increasing cognitive deficits (Naslund et al., 2000). In the frontal cortex, the increased Aβ levels precede significant NFT pathology. Therefore, these data suggest that intracellular Aβ may be involved in cognitive decline. Because a positive correlation exists between cognitive decline and loss of synaptophysin or neurons, our results indicate that the intracellular Aβ could be the initial insult leading to neuronal dysfunction or death in AD (Masliah et al., 1989, 2001; Gomez-Isla et al., 1996).

The in vivo cytotoxicity of intracellular Aβ1–42 is supported by the results of LaFerla and colleagues (1995) who showed that neurofilament L promoter-directed expression of cytotoxic Aβ1–42 results in neuronal cell death in mice (LaFerla et al., 1996). Additionally, our experiments show that levels of Aβ1–42 likely to be more physiological can rapidly induce cell death and that this toxicity is restricted to a nonfibrillar form of Aβ1–42 peptide.

The extreme toxicity of intracellular Aβ1–42 raises some important questions. First, can Aβ1–42, which is made through the secretory pathway, access the cytosol? Normally, it should not. However, there are three possible modes of entry of Aβ1–42 into the cytosol. Insoluble Aβ42 in the ER could access the cytosol through the ER quality control system where misfolded proteins are reverse translocated to the cytosol, ubiquitinated, and degraded through the proteosome system (Lippincott-Schwartz et al., 1988; Bonifacino et al., 1990; Bonifacino and Lippincott-Schwartz, 1991; Greenfield et al., 1999). In aging, the degradation of the Aβ1–42 could be compromised by a reduction of proteosomal activity thus resulting in intracellular Aβ1–42 neurotoxicity (Merker et al., 2001). Second, newly synthesized Aβ1–42 could be directed to endosomes/lysosomes either from the trans-Golgi or by endocytosis of secreted peptide. In this situation, the Aβ1–42 would have to be released in the cytosol through breakdown of membrane or passive diffusion of the peptide from endosomes/lysosomes into the cytosol. Interestingly, it has been shown that Aβ1–42, but not Aβ1–40, increases lysosomal membrane permeability possibly resulting in leakage of the Aβ1–42 in the cytosol (Yang et al., 1998). Third, the secreted Aβ1–42 could passively diffuse back into the cytosol through the plasma membrane. Many have reported the neuronal toxicity of the extracellular Aβ peptides on primary and cell line systems (for review see Klein et al., 2001). In contrast to these findings, we were unable to find toxicity of the extracellular Aβ1–42 or Aβ1–40 in our cultures of human neurons despite high concentrations of the peptides. However, we have pre-
viously shown that the amyloid peptides render neurons vulnerable to a secondary insult (Paradis et al., 1996). Others have also observed this effect in human neuronal cultures (Mattson et al., 1992). Possibly, the secondary stress increases Aβ receptors or endocytosis or alters the plasma membrane permeability resulting in the diffusion of the extracellular Aβ1–42 peptide inside the cell. However, this latter possibility is unlikely to be a primary cause of AD, as intracellular Aβ1–42 accumulation precedes senile plaques but nevertheless could contribute to a secondary round of cell death after the deposition of extracellular amyloid.

The selective toxicity of Aβ1–42 compared with Aβ1–40 poses another interesting problem: the extracellular Aβ peptide toxicity has been attributed to the fibrillar, protofibrillar, or aggregating properties of Aβ (for review see Klein et al., 2001). For intracellular Aβ1–42 toxicity, there exists three possibilities. First, it could be that the extra two amino acids on Aβ1–42 are responsible for inducing toxicity. Second, as has previously been suggested, amyloid fibrils could be the toxic molecules. However, both less toxic Aβ1–40 and extremely toxic Aβ1–43 form significant amounts of fibrils and nonfibrillized Aβ1–42 is still very toxic. Third, the oligomers observed by Western blot analysis in the nonfibrillized and fibrillized Aβ1–42 could be responsible for cell death (Walsh et al., 1999). Furthermore, it remains a possibility that the forms observed by electron microscopy and by Western blot analysis in the nonfibrillized and fibrillized Aβ1–42 could be extremely toxic Aβ1–42 compared with Aβ1–40 and APPV717G mutant (Yan et al., 1999). Whether ERAB is involved in cytosolic Aβ1–42 toxicity remains to be determined but since ERAB is localized to mitochondria and ER, it is unlikely to have access to the injected Aβ1–42 unless mitochondrial ERAB is released into the cytosol during apoptosis as are many other mitochondrial factors.

It is well known that Bax activates caspsases by promoting the release of mitochondrial cytochrome c, which forms an apoptosome with a number of other factors including caspsases (Adrain and Martin, 2001). The caspase-9 has specifically been shown to be activated by cytochrome c and further activate caspase-3. Our result showing that the caspase-6 and -8, but not the caspase-3 inhibitors, prevent Aβ1–42 toxicity is surprising. The lack of involvement of caspase-3 is likely due to the fact that these neurons contain high levels of inhibitor of apoptosis proteins known to inhibit caspase-3 (Zhang and LeBlanc; unpublished data). These results are consistent with our previous observation that caspase-6, but not caspase-3, is activated in serum-deprived primary human neurons and that only recombinant active caspase-6 induces apoptosis of human primary neurons (LeBlanc et al., 1999; Zhang et al., 2000). Because little is known of caspase-6 regulation, much more work will be required to understand how caspase-6 is activated in the presence of intracellular Aβ1–42.

Uncovering the acute human neuronal toxicity of intracellular Aβ1–42 questions the validity of the currently developed therapies against extracellular amyloid in AD. If intracellular Aβ1–42 precedes extracellular amyloid deposits, then anti-amyloid therapies need to be cell permeable. Otherwise, they may have little effect on the prevention or early treatment of AD but could be beneficial to prevent further damage incurred by the extracellular amyloid. However, if the intracellular Aβ1–42 is a consequence of extracellular amyloid deposits, then these therapies would presumably be advantageous to AD patients. Attempts directed toward the inhibition of the secretases responsible for the production of Aβ1–42 should also have a favorable impact on the disease assuming that it is only Aβ1–42 that is the problem in AD and not an underlying general problem with the secretory pathway resulting in the misfolding and cytosolic accumulation of other insoluble proteins. Our study indicates that in addition to the antiamyloid strategies, antineuronal cell death therapies against p53, Bax, or caspsases could be extremely valuable in preventing neuronal loss in AD.

Materials and methods

cDNA clones

Human Bcl-2 cDNA was obtained from Dr. Walter Nishioka (Vical, Inc.). Bax cDNA was amplified from a human neuron cDNA library (Bounhar et al., 2001). APP695 cDNA in pCepB was obtained from Steve Younkin (Mayo Clinic, Jacksonville, FL). The cDNAs were cloned into pCepB (Invitrogen). The p53 wild-type and p53E239del dominant negative cDNAs were produced by Arnold Levine, and were cloned in the pCMV-NEO vector (Hinds et al., 1990). These cDNAs were purified through GlassMAX™ (GIBCO BRL) and diluted at 30 ng/μl in PBS before the microinjections.

Primers were designed to amplify secreted and cytosolic Aβ1–42 from APP695. These primers amplify the entire Aβ1–42 that is the problem in AD and not an additional methionine ATG codon at the 5′ end and a stop codon at the 3′ end to ensure translation. Cytosolic Aβ was amplified with the Aβ1–40 primers: forward primer 5′-TCA CTC GAG AAT GGA TGC AGA ATT CCG ACA T-3′ (contains a built-in 5′ XhoI site) and Aβ1–42 reverse primer 5′-ATG
GAT CCT TAC GCT AGA ACA CCA CGG AA-3' (has a 3' BamH1 site) or A\(_{1-40}\) reverse primer 5'-TCG ATC CCT AGT CAA CAC CCC GCA CCA CCA TG-3' (has a 3' BamH1 site). Secreted A\(_\beta\) was made by linking the APP signal peptide (SP) to the A\(_\beta\) sequence. The SP was amplified with APP-S1 forward primer, 5'-TTA CTC GAG ATG CTG CCC GGT TGG TTA GCA-3' (contains a 3' XhoI site) and APP-SP2 reverse primer, 5'-GGA ATT CTG CAT CAC GCG CCC GCC GCG CTT C-3' (contains a 3' EcoRI site). A ligation between EcoRI-cleaved PCR-amplified A\(_\beta\) coding sequence and the EcoRI-cleaved SP sequence was reamplified with the A\(_{1-40}\)-for-ward primer and APP-SP2 reverse primers. The PCR-amplified SP and A\(_\beta\) sequence were cloned into the prokaryotic pBSKII and eukaryotic episomal pCep4 vectors through the XhoI/BamH1 restriction sites. All clones were restriction mapped and sequenced before use.

Neutralizing B antibodies
Monoclonal anti-Bax 6A7 (amino acids 12–24; Pharmingen) and 2D2 (amino acids 3–16; Trevigen), polyclonal anti-Bax N-20 (Santa Cruz Biotechnology, Inc.), monoclonal anti-APP 22C11 (Roche), mouse IgG, or monoclonal anti-Bax 6A7 (amino acids 12–24; PharMingen) and 2D2 (amino acids 3–16; Trevigen) were diluted at 50 μg/ml rabbit sera were diluted at 50 μg/ml and 5% decomplemented FBS. The cells were plated at a density of 3 \times 10^6 cells/ml on poly-L-lysine–coated ACLAR™ (Cat. No., 33C; thickness, 0.5 mm; Allied Chemical) coverslips. Neuron cultures were treated successively through 130 m cell strainers (Becton Dickinson), and centrifuged at 5,000 g for 20 min at room temperature and permeabilized in 0.1% Triton X-100, 2 mM i-glutamine, 0.1% dextrose, antibiotic Pen-Strep (all from Gibco BRL), and 5% decomplemented FBS. The cells were plated at a density of 3 \times 10^5 cells/ml on poly-L-lysine–coated ACLAR™ (Cat. No., 33C; thickness, 0.5 mm; Allied Chemical) coverslips. Neuron cultures were treated successfully three times with 1 mM fluorodeoxyuridine (GIBCO BRL) at feeding, and subsequently every week to prevent proliferation of dividing cells. In general, the neurons attach to the coverslips within 24 h and develop dense neuritic networks within 3 d. The cultures contain 90–95% neurons and 5–10% astrocytes (LeBlanc, 1995). Microinjections or treatments were performed 10 d after plating for neurons and astrocytes.

Human neuroblastoma M17 cells were obtained from Dr. J. Biedler (American Type Culture Collection) and human teratocarcinoma NT2 (Stratagene) and neuroblastoma LA-N-1 cells, a gift from Dr. L. Culp (Case Western Reserve University, Cleveland, OH), were cultured on ACLAR™ coverslips at 10^7 cells/ml in DMEM (GIBCO BRL) containing 10% FBS. BHK cells, a gift from William Bowers (University of Rochester, Rochester, NY), and the mouse NIH3T3 fibroblasts, a gift from Dr. Stephen Richard (McGill University, Montréal, Québec, Canada) were grown in DME and 10% FBS.

A\(_\beta\) peptides
Initially for Fig. 1, A\(_\beta\) peptides (Bachem) were dissolved in sterile distilled water at 25 μM and incubated at 37°C for 5 d. The peptides’ stock solutions were frozen and diluted in PBS immediately before microinjection. Thereafter, nonfibrillar A\(_\beta\) peptides (American Peptide Co.) were disaggregated at 25 μM in 5 mM Tris buffer pH 7.4, an aliquot diluted to 0.25 μM and immediately frozen at −20°C in aliquots of 50 μl. The remaining 25 μM A\(_\beta\) was incubated at 37°C in continuous mixing by inversion to fibrillize the peptides. After incubation, the samples were removed, vortexed, sonicated twice for 1 min in a bath type sonica-tor (ELMA GmbH & Co. KG), and frozen at −20°C in 50 μl aliquots. Each aliquot was used once to avoid possible effects of freeze and thaw cycles.

Electron microscopy
A 3-μl aliquot of A\(_\beta\) peptide was placed on freshly cleaved mica plates (BioForce Laboratory, Inc.). The specimens were dried and subsequently transferred to a Balzers High-Vacuum Freeze-Etch Unit (model 301) under a 1.3 \times 10^{-4} Pa vacuum. The specimens were shadowed with platinum (BAL-TEC EM-Technology and Application, NH) at a 30° angle and coated with a carbon film platinum (BAL-TEC EM-Technology and Application). The replicas were detached from the mica by flotation in deionized water and transferred onto a 300-μm-grid mesh (Canemco, Inc.). The grids were examined with a Joel 200FX transmission electron microscope (Joel) at 21,000× magnification.

**Western blot analysis of A\(_\beta\) peptides**
Nonfibrillar and fibrillar forms of A\(_{1-40}\) and A\(_{1-42}\) (5 μg) were added to sample buffer and electrophoresed on a triple layer (4%, 10%, 16.5%) Tris-Tricine gel at 50 V for 1 h followed by 70 V for 16 h (Schagger and Von Jagow, 1987). The proteins were transferred to Immobilon-P PVDF Membrane (Millipore) at 200 miliamps for 2 h. The membrane was blocked with 5% nonfat milk in Tris buffered saline with 0.1% Tween 20 (TBST) at room temperature for 1 h, incubated with a 1/100 dilution of the anti-A\(_{1-40}\) antibody 6E10 (Signet) and detected by chemiluminescence.

**Microinjection**
Thin-walled Borosilicate glass capillaries (OD 1.0 mm, ID 0.5 mm) were microinjected with a Flaming/Brown Micropipette Puller (P-87; Sutter) to obtain injection needles with a tip diameter of ~0.5 μm. Microinjections were performed in the cytosol of each cell using the Eppendorf Microjector 5246 and Burleigh Micromanipulator MIS-5000. Human neurons were injected with 25 pl/shot at an injection pressure of 100 hPa, a compensation pressure of 50 hPa, and an injection time of 0.1 s. Human astrocytes, M17, NT2, LA-N-1, BHK, and NIH 3T3 cells were injected with 8 pl/shot at an injection pressure of 50 hPa, a compensation pressure of 30 hPa and an injection time of 0.1 s. The diluted peptides were injected at the indicated concentrations with 100 μg/ml DTR (MW: 3000; Molecular Probes) as a fluorescent marker to recognize the injected cells. Approximately 90% neurons and NT2 cells, and 50% astrocytes, M17, LA-N-1, BHK, and NIH 3T3 cells survive the injections for at least 16 d.

**Measurement of neuronal apoptosis**
Cells were fixed in freshly prepared 4% paraformaldehyde/4% sucrose for 20 min at room temperature and permeabilized in 0.1% Triton X-100, 0.1% sodium citrate on ice for 2 min. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed using the In situ Cell Death Detection Kit I as described by the manufacturer (Roche). The percentage of cell death was determined as the ratio of the number of DTR-TUNEL–double positive cells over the total number of DTR-positive cells.

Hoechst staining was used to recognize cell nuclei and detect apoptotic nuclear condensation and fragmentation. Hoechst dye (Intergen) was dissolved in sterile distilled water at 200 μg/ml and diluted 500 times in PBS immediately before staining. After the incubation for TUNEL staining, cells were washed three times for 10 min each in PBS, treated with the diluted Hoechst dye for 15 min at room temperature (in the dark), washed three times for 10 min each in PBS, washed once in water for 5 min, and mounted with Immunon™ mounting medium (Shandon) onto glass slides to be observed under the fluorescence microscope.

**Treatment with caspase inhibitors, cycloheximide, and actinomycin D**
Caspase pan inhibitor, Z-Valine-Alanine-Aspartic acid-fluoromethylketone (Z-VAD-fmk) (Biomol), caspase-1 inhibitor, Z-Tyr-Aspartic acid-Valine-Aspartic acid-fmk (Z-YVAD-fmk), caspase-6 inhibitor, Z-Valine-Glu-tamic acid-Histidine-Aspartic acid-fmk (Z-DEVD-fmk), and caspase-8 inhibitor, Z-Isoleucine-Glu-tamic acid-Threonine-Aspartic acid-fmk (Z-IETD-fmk) (Sigma-Alrich), were dissolved in 100% DMSO (Sigma-Aldrich) at 20 μM and diluted at 5 μM into culture media immediately before use. Stock solutions of 5 mg/ml cycloheximide (Sigma-Aldrich) and 200 μM actinomycin D (Sigma-Aldrich) were made in sterile distilled water and diluted to 5 μg/ml for cycloheximide and 5 μM for actinomycin D in the culture media immediately before use. The media was changed every 48 h.

**Statistical evaluation**
One-way or two-way analyses of variance (ANOVA) with post hoc tests (Statview 5.01) determined the statistical significance of the difference between treatments. The Dunnett’s test was applied when comparing each group with one certain group (control), for example, comparison between different treatment groups vs. untreated group. The Sheffé’s test was applied when comparing between every other group, for example, compari-

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son between each treatment group. A P value of <0.05 was taken as the criteria for statistical significance.

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